Animal Model

Localization of *Mycobacterium leprae* to Endothelial Cells of Epineurial and Perineurial Blood Vessels and Lymphatics

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Infection of peripheral nerve by *Mycobacterium leprae*, the histopathological hallmark of leprosy, is a major factor in this disease, but the route and mechanisms by which bacilli localize to peripheral nerve are unknown. Experimentally infected armadillos have recently been recognized as a model of lepromatous neuritis; the major site of early accumulation of *M. leprae* is epineurial. To determine the epineurial cells involved, 1-cm segments of 44 nerves from armadillos were screened for acid-fast bacilli and thin sections were examined ultrastructurally. Of 596 blocks containing nerve, 36% contained acid-fast bacilli. Overall, *M. leprae* were found in endothelial cells in 40% of epineurial blood vessels and 75% of lymphatics, and in 25% of vessels intraneurally. Comparison of epineurial and endoneurial findings suggested that colonization of epineurial vessels preceded endoneurial infection. Such colonization of epineurial nutrient vessels may greatly increase the risk of endoneurial *M. leprae* bacteremia, and also enhance the risk of ischemia following even mild increases in inflammation or mechanical stress. These findings also raise the possibility that early, specific mechanisms in the localization of *M. leprae* to peripheral nerve may involve adhesion events between *M. leprae* (or *M. leprae*-parasitized macrophages) and the endothelial cells of the vasa nervorum. (Am J Pathol 1999, 154:1611–1620)

The histopathological hallmark of leprosy is the infection of peripheral nerves by *Mycobacterium leprae*, the only bacterial pathogen with this unique tropism.¹ The result-

ing injury to peripheral nerves causes the hypesthesia of cutaneous lesions and the distal anesthesia and paralysis which are major clinical features of leprosy.² These neuropathic changes, in turn, are responsible for the deformities that elicit most of the stigma and opprobrium that are the social hallmarks of this disease.³

The multiplex mononeuropathy of leprosy usually affects the facial, ulnar, radial, or peroneal nerves.^{2,4} The remarkable full-length dissections and histopathological examination of peripheral nerves at autopsy performed a century ago demonstrated that the distal anesthesia and motor deficits in leprosy are due to peripheral neuropathy, rather than a central lesion.^{5,6} Those studies also revealed an ascending degeneration of nerves, together with interstitial inflammation and perineurial thickening, greatest near the cutaneous lesion and declining proximally.

The mechanisms of nerve injury in leprosy are very poorly understood, largely because, due to the lack of an animal model, investigations have depended entirely on studies of biopsies of human nerves. In well-established disease, perineurial inflammation of cutaneous nerves is a cardinal feature in skin biopsies and has also been a consistent finding in nerve biopsies.⁷ In advanced endoneurial lesions macrophages and Schwann cells are infected, the latter more heavily,⁸ and this is ultimately associated with demyelination and decreased conduction velocity.^{9,10} Attempts to examine early lesions in nerve biopsies have demonstrated perineurial mononuclear cell infiltrates, subperineurial edema, and small numbers of acid-fast organisms.¹¹ Such studies have necessarily been limited almost entirely to small biopsies of the radial cutaneous and sural nerves (which cause minimal sequelae even when these are not major sites of clinical neuropathy). The medical and ethical limitations on nerve biopsy have thus limited the ability to obtain information about established nerve lesions, so it has not

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Table 1. Nerves Examined and Percentage of Sections Containing Acid-Fast Bacilli

Armadillo	Number of nerves	Sections positive for AFB	Total sections	% Positive
5F-11	4	1	29	3.4
F6-7	8	5	109	4.6
N1201	8	17	91	18.7
N1200	8	90	116	77.6
5A22	4	6	38	15.8
N252	4	17	64	26.6
M10	4	28	76	36.8
D135	4	49	73	67.1
Total	44	213	596	35.7

AFB, acid-fast bacilli.

been possible to examine materials that can address the unique mechanisms of localization of *M. leprae* to peripheral nerve.

Experimental infection of nine-banded armadillos (*Dasypus novemcinctus*) with *M. leprae* is now well recognized as a model of lepromatous disease.^{12–14} Brief reports have described infection of peripheral nerves in these animals,^{15,16} but this phenomenon has not been examined in detail. We have recently observed that the *M. leprae*-infected armadillo develops an extensive lepromatous neuritis very similar to that of human lepromatous leprosy, with respect to both histopathological features and distribution, and constitutes a true animal model of nerve involvement in this disease.¹⁷ Unlike previous attempts to develop animal models by inoculating *M. leprae* directly into nerves or inducing nerve localization by means of trauma,^{18,19} no effort is made in the armadillo to direct the organisms to nerves. Instead, this model recapitulates the unique natural localization of *M. leprae* to nontraumatized peripheral nerves.

The initial observations of this experimental lepromatous neuropathy indicated that in any segment of involved nerve the intensity of *M. leprae* infection was greater in the tissues on the surface of nerves than in the endoneurial compartment.⁷ The suggestion that colonization of the epineurial surface tissues might therefore play an important role in the pathogenesis of nerve injury in leprosy prompted us to examine in further detail the sites of localization of *M. leprae* in another set of experimentally infected armadillos, to determine the types of cells and epineurial structures that are infected.

Materials and Methods

Animals

Eight adult nine-banded armadillos from a colony maintained in special facilities at the Research Branch, GWL

Hansen's Disease Center, were inoculated intravenously with $3\text{--}4 \times 10^8$ *M. leprae* as described previously.^{17,20} Bacilli were freshly obtained from other experimentally infected armadillos or from nude mice. After 12–18 months, when widespread dissemination of the infection had developed,²⁰ animals were anesthetized and sacrificed by exsanguination and the liver and spleen were removed.

Nerve Fixation and Processing

At the time of sacrifice, the distal one-half to two-thirds of major peripheral nerve trunks in each extremity were dissected and placed in cold 0.1 mol/L sodium cacodylate buffer, pH 7.3, containing 1.25% glutaraldehyde and 2.0% formaldehyde (fixative). While immersed in fixative, each nerve was divided into 1-cm lengths from which cross-sectional and longitudinal blocks were prepared. The tissue was postfixed in 1% osmium, dehydrated, and embedded in Spurr resin (Electron Microscopy Sciences, Ft. Washington, PA) and polymerized overnight at 70°C.

Semithin (1.5- μm) sections were cut on a diamond knife using a Model 2128 ultramicrotome (LKB, Deerfield, IL), stained for acid fast-bacilli,²¹ and screened by light microscopy to identify blocks containing acid-fast organisms. For cross-sections of nerves the selected blocks were trimmed directly and ultrathin sections prepared using a diamond knife. To examine ultrastructurally more than one portion of larger blocks (particularly longitudinal ones), additional 1.5- μm sections were cut, stained with 1% Toluidine blue in 1% sodium borate buffer, and individually mounted on blank Spurr blocks using dental bond (Prime & Bond, Densply Caulk, Inc., Milford, DE) polymerized with blue light.²² These remounted blocks were trimmed to appropriate size, each retaining a different portion of the original semithin section, and ultrathin sections were prepared.

Ultrathin (90- to 100-nm) sections were collected on 200-mesh copper grids and stained with uranyl acetate and lead citrate in an automatic stainer (2126 Ultrastainer, LKB). Specimens were examined with a Philips 410 electron microscope and photographed using EM 4489 film (Kodak, Rochester, NY). Epineurial blood vessels and lymphatics in each cross-section were examined and the number with and without *M. leprae* in their endothelial cells was recorded. Similar determination was made of the number of infected and uninfected endoneurial blood vessels in cross-sections. Some composite images for publication (specifically identified in figure legends) were prepared from digital files of photographs scanned and joined using Adobe Photoshop 4.0 software and printed on a Kodak 8650 dye-sublimation printer.

Figure 1. Epineurial thickening with *M. leprae* infection of interstitial macrophages and lymphatic and vascular endothelial cells. **A:** Inflammatory thickening of the epineurium is seen at the top and bottom of this 1.5- μm cross-section of the right superficial peroneal nerve. (Toluidine blue stain; scale bar, 100 μm). **B:** Ultrastructural examination of the enclosed area in **A** revealed heavily infected interstitial macrophages (M) heavily infected with *M. leprae* (arrows), as well as infection of vascular endothelial cells (box) and adjacent lymphatics (L). The fibrous layers of the epineurium (EP) are slightly thickened and a small number of bacilli are also seen within them. Mononuclear leukocytes (MNC) are also seen just beneath the epineurium. (Digital photomontage; scale bar, 5 μm). **C:** Enlargement of the enclosed area in **B** reveals the typical ultrastructural appearance of *M. leprae* with its clear zone, lying within a vascular endothelial cell. Scale bar, 1 μm .

Figure 2. Infection of epineurial blood vessels at sites of minimal *M. leprae* load. **A:** One leprosy bacillus (arrow) is seen in an endothelial cell of a medium-sized blood vessel near the epineurium, visible in the lower left corner. Scale bar, 5 μ m. At higher magnification (inset), the bacillus is clearly demonstrated in the endothelial cell. Scale bar, 0.5 μ m. **B:** The only organism in this section was a single bacillus (arrow) lying within a pericyte investing one of two small vessels on the surface of a nerve (scale bar, 3 μ m), clearly identified at higher magnification (inset scale bar, 0.5 μ m).

Statistical Analysis

Overall epineurial inflammation and bacillary load were assessed on a semiquantitative scale of 1+ to 3+ as follows: 1+, <10 bacilli or minimal mononuclear cell infiltration; 2+, 11–50 bacilli or moderate cellular infiltrate; 3+, >50 bacilli or heavy inflammation. Differences in the frequency of epineurial and endoneurial endothelial cell infection were then evaluated against this scale using a non-parametric χ^2 test, and correlation was tested using the Spearman rank correlation.

Results

A total of 44 separate nerves were examined from eight armadillos (Table 1). Mild focal thickening was observed grossly in many specimens. More than 600 blocks were screened, of which 596 contained peripheral nerve. Acid-fast bacilli were found by light microscopy in sections of 36% of these blocks and, of these, organisms were located in the epineurium in 86%. The extent of infection and inflammation of nerves varied greatly in different

animals, and in most instances the involvement was intermittent rather than continuous, with focal lesions separated by intervals of normal nerve.

Histopathological examination of semithin sections revealed mild to severe thickening of the epineurium and perineurium at many but not all foci of *M. leprae* infection, involving several components of the epineurium and its associated connective tissues (Figure 1A). Infection of vascular and lymphatic endothelial cells by *M. leprae* was a prominent finding on ultrastructural examination (Figure 1, B and C), typically involving only one or two organisms per endothelial cell, which might easily be overlooked on light microscopy. The interstitial inflammatory infiltrates on the epineurial surface were composed primarily of macrophages, many of which were infected and which contained the majority of the acid-fast organisms observed by light microscopy. The fibrous layers of the epineurium were thickened and fibroblasts were focally infected with *M. leprae*. Also frequently observed at sites of substantial inflammatory thickening were accumulations of mononuclear leukocytes just beneath the epineurium (Figure 1B).

At some of the sites of earliest infection bacilli were observed within endothelial cells of epineurial blood vessels, but there was little or no inflammatory thickening (Figure 2). At such sites, careful examination revealed little or no perivascular or interstitial inflammatory infiltrate and few bacilli in neighboring cells (Figure 2A). At other sites of early, minimal infection, *M. leprae* were observed in pericytes (Figure 2B). Among infected epineurial blood vessels, organisms were seen in small arteries and veins; no attempt was made in this study to determine the relative prevalence of infection among these.

At sites of heavier mycobacterial infection, mononuclear leukocytes were often observed forming a perivascular cuff around vessels in the epineurium or perineurium (Figure 3). Such cuffing consisted primarily of macrophages, infected and uninfected, which had accumulated around vessels with either infected or uninfected endothelial cells. Circulating infected monocytes were also occasionally observed within the lumen (Figure 3).

In addition to the consistent infection of vascular endothelial cells, lymphatics on the surface of the epineurium were often also distended with infected monocytes at sites of moderate to heavy infection (Figure 4A). Infection of lymphatic endothelial cells was prominent, and at such sites extracellular *M. leprae* were sometimes observed within the lymphatic lumen (Figure 4A). Sites of attachment of extracellular *M. leprae* to the lymphatic endothelium were noted (Figure 4B), as was attachment of infected monocytes to lymphatic endothelial cells (Figure 4C).

When the percentage of infected vessels was stratified according to a semiquantitative assessment of epineurial inflammation and bacillary load, infection of epineurial endothelial cells was observed in 17% of vascular and 24% of lymphatic vessels in foci of lighter infection (Table 2). The frequency of infection increased to 50% and 82%, respectively, in foci of high-intensity inflammation and infection. In endoneurial vessels, the frequency of endothelial cell infection was lower in all groups but also

Figure 3. Perivascular cuffing by infected and uninfected macrophages. **A:** Macrophages, several of which are infected by *M. leprae* (arrows), completely encircle this blood vessel close to the epineurium. No bacilli are seen in the endothelial cells. Scale bar, 5 μ m. **B:** A similar cuff of mononuclear cells is observed around a blood vessel in epineurial connective tissue. *M. leprae* (arrows) are seen in most of these perivascular cells and are also present within a circulating monocyte in the lumen. Scale bar, 5 μ m.

increased from 10% to 39% in low and high intensity sites of inflammation, respectively. The difference between groups was significant in all cases ($P = 0.001-0.002$) (Table 2), and increases in endoneurial endothelial cell infection followed increases in epineurial endothelial cell infection in all groups. No statistical correlation could be demonstrated between the epineurial and endoneurial increases according to the semiquantitative scale used, however, due to unexpectedly low numbers of infected endothelial cells in the 2+ group (Table 2).

Within endothelial cells, *M. leprae* were usually found within membrane-bound vacuoles (Figure 5A). In this study, such vacuoles were usually small or tight vacuoles, in which the organisms were surrounded by a thin, clear zone typical of this organism (Figure 5A, inset). In some instances, however, bacilli appeared to lie free within the endothelial cell cytoplasm, without evidence of a vacuolar membrane (Figure 5B).

Morphological evidence of activation was often observed in infected endothelial cells (Figure 6A), including thickening of the cell and narrowing of the lumen. At other sites, apparent activation was observed in uninfected endothelial cells surrounded by infected macrophages (Figure 6B). In some instances, activation of infected endothelial cells in one vessel was observed without apparent change in the endothelium of adjacent vessels in the epineurial connective tissue (Figure 6C).

Discussion

This report presents the first detailed study of peripheral nerves during the height of active lepromatous neuritis in an animal model, which has offered a unique opportunity to examine some aspects of this major infectious peripheral neuropathy that are not otherwise accessible. Our examination has focused on the epineurial and perineurial coverings of major peripheral nerve trunks, based on information from a previous study which indicated that the surface of the nerve is a major site of bacterial accumulation.¹⁷ The current findings indicate that 1) although a majority of the epineurial organisms are located within extravascular interstitial macrophages, the endothelial cells of epineurial lymphatics and blood vessels are heavily colonized by *M. leprae*; 2) endothelial cells of endoneurial blood vessels also carry a substantial bacterial load, even in early foci of infection; and 3) as the overall bacterial load around the nerve increases, the bacterial load of both epineurial and endoneurial endothelial cells also increases, thus placing the endoneurial compartment directly at risk of infection.

The experimental findings in this study bring together several histopathological elements of leprosy that have been well documented previously but have not been integrated into our understanding of the pathogenesis of neuritis in leprosy: perineurial proliferation and infection, infection of lymphatic endothelial cells, and infection of vascular endothelial cells.

Infection of vascular endothelial cells was noted in the original descriptions of Hansen and Looft in 1895.²³ This has been repeatedly noted in skin biopsies²⁴⁻²⁷ and has

Figure 4. Involvement of epineurial lymphatics by *M. leprae*, heavily infected site. **A:** A large number of intraluminal bacilli are seen within the lumina of lymphatic vessels (L). Many of these are adherent to endothelial cells and appear to be free, not intracellular. No bacilli are seen in the endothelium of the large blood vessel at the upper end of the photo (V) but a perivascular cuff of mononuclear cells is present. Digital photomontage; scale bar, 2 μ m. **B:** Enlargement of the area enclosed in **A** shows extracellular *M. leprae* in the lumen of a lymphatic vessel, adherent to the endothelium. Scale bar, 0.5 μ m. **C:** A circulating monocyte infected with *M. leprae* (arrows) has attached to the luminal surface of an endothelial cell. Scale bar, 1 μ m.

Table 2. Number and Percentage of Vascular and Lymphatic Endothelial Cells Infected, According to Overall Degree of Inflammation and Bacillary Load

		Epineurial				Endoneurial	
		Epineurial Blood Vessels		Epineurial Lymphatics		Endoneurial BV	
	Score	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Total	1+	7	34	4	13	1	9
(%)		(17)		(24)		(10)	
	2+	31	65	32	16	2	30
(%)		(32)		(67)		(6)	
	3+	91	90	117	25	20	32
(%)		(50)		(82)		(39)	

1+, >10 bacilli or minimal mononuclear cell infiltration; 2+, 11–50 bacilli or moderate cellular infiltrate; 3+, <50 bacilli or heavy inflammation. BV, blood vessels.

been regarded as evidence of hematogenous dissemination of *M. leprae*. Several reports have emphasized the observation of *M. leprae* infection of endoneurial vascular endothelial cells,^{28,29} as well as those in the epineurium and perineurium.³⁰ The finding in this experimental model, however, of frequent, substantial endothelial cell infection in the epineurium and perineurium, with increasing frequency in endoneurial endothelium following increases in epineurial endothelium, provides the most direct evidence to date that *M. leprae* gain access to the endoneurial compartment via its blood supply. This is consistent with previous reports^{15,31,32} and supports the suggestion of Sabin² and others³³ of hematogenous spread to the endoneurial compartment. The perivascular cuffing of infected and uninfected macrophages, also noted previously,³¹ suggests emigration of these cells from nutrient epineurial vessels.

Involvement of lymphatic vessels has been less thoroughly examined in leprosy, although the thesis that *M. leprae* affect nerves via epineurial lymphatics has been advanced.^{33,34} Inflammation of dermal lymphatics has been reported to vary according to the patient's immunological type of leprosy, but fewer *M. leprae* were observed in lymphatics than in dermal blood vessels.³⁵ Although epineurial and perineurial inflammation are extensively discussed in a recent review of peripheral nerve pathology in leprosy, epineurial lymphatics are not mentioned.³⁶

The role of lymphatics may have been underestimated in this study, due to difficulty in identifying them clearly unless they were dilated with fluid or cells. Nevertheless, the finding of extensive infection of lymphatic endothelial cells indicates that this is at least a major reservoir of *M. leprae* on the surface of the nerve. The drainage of cutaneous lesions along epineurial lymphatics may partially explain the interstitial and epineurial inflammation described in full-length dissections at autopsy.^{5,6} The resulting focal interstitial accumulation of parasitized macrophages on the surface of the nerve provides an abundant source for infection of the adjacent vascular

plexus. In addition, bacilli and infected phagocytes may arrive at these vessels directly via the circulation. This explanation of the pathogenesis of nerve involvement in leprosy is contrary to a long-held opinion that *M. leprae* enter nerves at distal sites, where Schwann cells may be exposed, and travel centripetally within nerves through axons or Schwann cells.^{6,37,38}

Perineurial infection and inflammation have been recognized as characteristic features of cutaneous leprosy lesions since they were described in the earliest histopathological reports by Virchow³⁹ and Hansen and Looft.²³ These have generally been viewed as late consequences and of less import than the unique infection of Schwann cells by *M. leprae*, in the theory of pathogenesis noted above, which proposes that direct axonal or Schwann cell infection is the primary event. Thus, Sunderland⁴⁰ concluded that perineurial involvement occurs "only much later" than endoneurial infection and inflammation, and the presence of *M. leprae* in the perineurium and epineurium of cutaneous nerves in lepromatous leprosy has usually been interpreted as a breakout of bacilli⁴¹ "bursting out from a nerve bundle to perineurial macrophages."⁴²

Our observations suggest that *M. leprae* localize first to the epineurium and perineurium and subsequently infect Schwann cells. Previous studies of human nerves have interpreted the severity of lesions, ie, involvement of the perineurium, as evidence of the later occurrence of these lesions during the pathogenesis of neuritis, an issue very difficult to resolve in human clinical material without knowledge of *M. leprae*'s dose, route, and duration of infection. Varying degrees of resolution of perineurial infection may occur over the long course of this disease, whereas endoneurial infection may persist longer. Because Schwann cells are isolated behind the highly protective barrier of the perineurium, the only access to them in a normal nontraumatized nerve is via the blood vessels entering the endoneurial compartment. There is no clinical or experimental evidence that such trauma actually precedes the infection of peripheral nerves by *M. leprae*, although some mechanical and anatomical factors may

Figure 5. Within endothelial cells, *M. leprae* were found both within membrane-bound vacuoles and free in the cytoplasm. **A:** *M. leprae* were usually observed lying within a membrane-bound vacuole in endothelial cells, as demonstrated in this blood vessel. Scale bar, 2 μ m. **Inset:** Enlargement of bacillus and vacuolar membrane. **B:** In another section of the same nerve, however, intraendothelial *M. leprae* (arrows) appeared to lie free in the cytoplasm. Scale bar, 2 μ m. Although the typical clear zone is seen around the bacilli at greater magnification (inset), no membrane is seen surrounding the organisms. Note also the perivascular cuff of macrophages.

contribute. Therefore, mechanisms that do not directly involve the Schwann cell but may involve the neurovascular endothelium may play major roles in the tropism of *M. leprae* to peripheral nerve.

The high prevalence of *M. leprae* infection of epineurial and perineurial endothelial cells has several important implications for the pathogenesis of nerve injury in leprosy. First, by colonizing the vascular and lymphatic endothelial cells in the plexus of vessels on the epineurium, the nerve is placed at much greater risk of subsequent circulation of this organism through the blood vessels that branch off of the surface and enter the endoneurial compartment. This may partially explain the otherwise puzzling predilection of this pathogen for peripheral nerves, which comprise a very small percentage of total body mass. The resulting *M. leprae* bacteremia within endoneurial vasculature could conceivably be composed of extracellular *M. leprae*, of bacilli within mononuclear phagocytes, or both.

Secondly, the extensive perivascular colonization of the epineurium and infection of the endothelium itself greatly enhance the risk of ischemia of the underlying nerve following even a mild increase in inflammation, trauma, or mechanical stress. Such ischemia, either episodic and transient or chronic and persistent, may be a major factor in the overall development of neuropathy in this disease.^{40,43} This may apply even in instances where direct infection of the Schwann cell is minimal, and may partially account for the prompt relief of neuropathic symptoms after administration of corticosteroids, which probably alleviate epineurial inflammation and edema more rapidly than they may affect mechanisms by which *M. leprae* could cause direct injury to Schwann cells.

Finally, these findings raise previously unrecognized possibilities that an early, specific step in the localization of *M. leprae* to peripheral nerve may be mediated, at least in part, by adhesion events between *M. leprae* (or *M. leprae*-parasitized macrophages) and the endothelial cells of the vasa nervorum. Endothelial cells in many tissues are known to express specific molecular addressins responsible for the selective binding and retention of leukocytes with complementary ligands.⁴⁴ It is possible that endothelial cells associated with peripheral nerve have such a molecular addressin, which could facilitate the unique accumulation of *M. leprae* or *M. leprae*-infected macrophages in these vessels. If so, additional questions arise concerning both the afferent mechanisms of selective adhesion to epineurial endothelial cells and efferent mechanisms by which *M. leprae* may be released from these cells, allowing them to circulate through endoneurial blood vessels and reattach to endothelium there.

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Figure 6. Intracellular infection with *M. leprae* was sometimes associated with endothelial cell activation. **A:** Of the two epineurial blood vessels seen here, endothelial thickening and narrowing of the lumen, indicative of activation (ACT), are observed in endothelial cells infected with *M. leprae* in the vessel on the left (arrow), whereas uninfected endothelial cells in the vessel on the right have a resting appearance. A small but dense accumulation of mononuclear cells, some containing *M. leprae*, can be seen in the intraneural compartment between the epineurium (EP) and the myelin sheath of Schwann cells (S). (Digital montage of photos of two adjacent sections. Scale bar, 7.6 μm). **B:** Nuclear enlargement suggesting activation is observed in this blood vessel, which demonstrates both Weible-Palade-like organelles (white arrows) and a single intracellular leprosy bacillus (arrow) at this level. No mononuclear leukocytes were found near this blood vessel. Scale bar, 2 μm. **C:** In endothelial cells heavily infected with *M. leprae*, in a blood vessel adjacent to another nerve, the lumen is narrowed due to a marked increase in endothelial cytoplasmic thickness, and in nuclear size. The load of bacilli is greater than in B, but no mononuclear leukocytes were observed near this blood vessel. Scale bar, 2 μm.

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