ASIP Journal CME Program

American Journal of Pathology, Vol. 168, No. 3, March 2006
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DOI: 10.2353/ajpath.2006.050375

*Immunopathology and Infectious Diseases*

# Nerve Damage in *Mycobacterium ulcerans*-Infected Mice

## *Probable Cause of Painlessness in Buruli Ulcer*

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**Buruli ulcer is an emerging chronic painless skin disease found in the tropics and caused by *Mycobacterium ulcerans*; however, it remains unknown why the large and deep ulcers associated with this disease remain painless. To answer this question, we examined the pathology of BALB/c mice inoculated in the footpads with *M. ulcerans* African strain 97-107. On days 54 to 70 after inoculation, extensive dermal ulcers, subcutaneous edema, and numerous acid-fast bacilli were noted at the inoculate region. Nerve invasion occurred in the perineurium and extended to the endoneurium, and some nerve bundles were swollen and massively invaded by acid-fast bacilli. However, Schwann cell invasion, a characteristic of leprosy, was not observed. Vacuolar degeneration of myelin-forming Schwann cells was noted in some nerves which may be induced by mycolactone, a toxic lipid produced by *M. ulcerans*. Polymerase chain reaction analysis of microdissected nerve tissue sections showed positive amplification of *M. ulcerans*-specific genomic sequences but not of *Mycobacterium leprae*-specific sequences. Behavioral tests showed decrease of pain until edematous stage, but markedly ulcerated animals showed ordinary response against stimulation. Our study suggests that the painlessness of the disease may be partly due to intraneural invasion of bacilli. Further studies of nerve invasion in clinical samples are urgently needed.** ***(Am J Pathol 2006, 168:805–811; DOI: 10.2353/ajpath.2006.050375)***

Buruli ulcer is an emerging chronic skin disease found in the tropics and caused by *Mycobacterium ulcerans*.[1,2] *M. ulcerans* is known to produce a unique toxic lipid mycolactone.[3] The disease was first reported in Australia[4] in 1948, and is mostly observed in tropical and subtropical areas of West Africa and Australia. Large, necrotizing, and relatively painless deep-skin ulcers are formed mainly in the extremities, often resulting in severe deformities, and evoke significant socioeconomic problems.[1]

The reason for painlessness has not been clarified, but recent immunohistochemical studies have suggested that phenolic glycolipid-I (PGL-I), a potential adhesin for Schwann cells[5] that is also known as a *Mycobacterium leprae*-specific membranous antigen, is present in the ulcerative lesion of Buruli ulcer.[6] Inspired by this study, we hypothesized that not only *M. leprae* but also *M. ulcerans* may invade peripheral nerve tissue, and hence, we conducted a morphological surveillance of *M. ulcerans*-infected mice, with a special focus on nerve damage.

## *Materials and Methods*

### M. ulcerans *Infection*

Twenty-five microliters of bacterial suspension (colony forming units = $3.3 \times 10^4$) of *M. ulcerans* strain 97-107 (isolated from a Buruli ulcer patient in Africa, provided by Prof. Francoise Portaels [Institute of Tropical Medicine, Antwerp, Belgium]) cultured at 32°C in 7H9 medium was inoculated into the bilateral footpads of female BALB/c mice aged 5 weeks old. Local swelling and redness were

Supported by grants from the U.S.-Japan Cooperative Medical Science Program and an International Cooperation research grant from the International Medical Centre (Japan).

Accepted for publication November 16, 2005.

Supplementary data are available at *http://ajp.amjpathol.org*.

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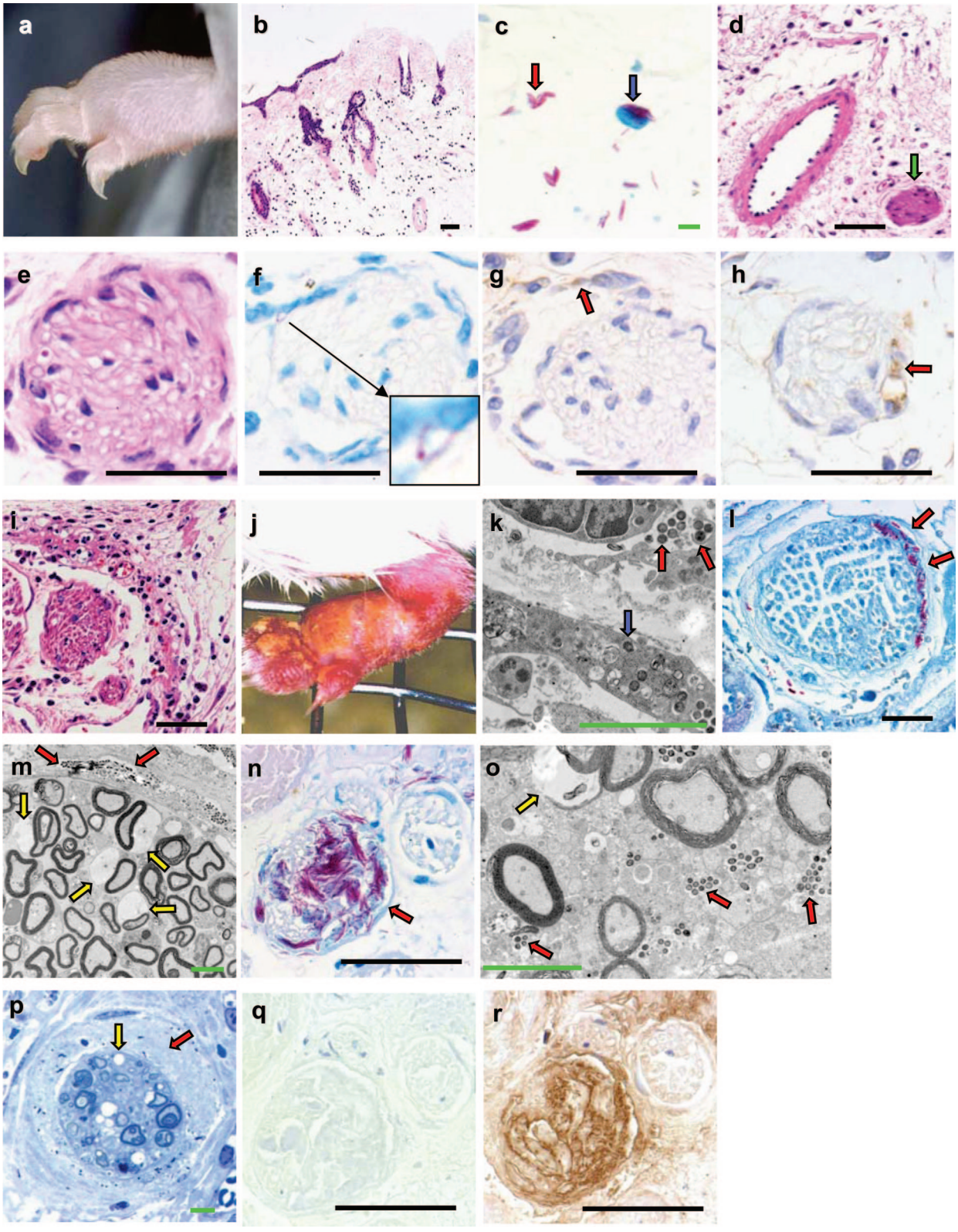
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observed on day 33 after inoculation, and sequential histopathological examinations were performed. After 57 days, all mice had died. One mouse inoculated at the age of 9 weeks survived for 70 days. Care and treatment of animals followed the regulations of the Animal Care and Use Committee of the National Institute of Infectious Diseases, Japan.

### *Histology and Immunohistochemistry*

Twelve animals in total (day 33, $n = 2$; day 39, $n = 2$; day 40, $n = 2$; day 42, $n = 1$; day 49, $n = 1$; day 54, $n = 3$; day 57, $n = 1$) were examined. After induction of deep anesthesia, perfusion fixation was performed using 10% formalin. Hind limbs and general organs were embedded in paraffin, cut into 4-$\mu$m sections, and histopathologically examined by hematoxylin and eosin (H&E) and Fite-Faraco acid-fast staining. In selected cases in which nerve invasion was observed, immunohistochemistry was performed using anti-Bacillus Calmette-Guérin (BCG) polyclonal antibody (B0124; DakoCytomation, Glostrup, Denmark) and anti-PGL-I monoclonal antibody specific for a trisaccharide of *M. leprae* (1-21 and DZ2C11).[7] Immunohistochemical staining was achieved by the immunoperoxidase method using the ABC complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA).

### *Electron Microscopy*

Tissues from three animals (day 51, $n = 2$; day 70 inoculated at the age of 9 weeks, $n = 1$) were examined by electron microscopy after deep anesthesia with intraabdominal injection of pentobarbital, perfusion by heparinized saline from left cardiac ventricle with drainage from right atrial auricle for short period, and perfusion fixation using 2% glutaraldehyde in phosphate buffer. Hind limbs were postfixed with osmium tetroxide and embedded in Epon. Toluidine blue-stained 1-$\mu$m sections were screened, and nerve bundles were ultrathin-sectioned and stained with uranium and lead for electron microscopy.

### *Detection of Bacterial Genomic DNA in Tissue Sections*

DNA was extracted from paraffin-embedded tissue sections of samples from day 54, using a DNeasy Tissue System (Qiagen Inc., Valencia, CA). As positive controls, DNA extracted from cultured *M. ulcerans* (strain 97-107) and nude mouse-propagated *M. leprae* (strain Thai-53) were used. An *M. ulcerans*-specific 97-bp fragment of genomic DNA was amplified by polymerase chain reaction (PCR) using a newly designed forward primer 5′-TCGGCGACAGCGAGTTGACC-3′ (PGP3.5) and a previously reported reverse primer 5′-CTGCGTGGTG-CTTTACGCGC-3′ (PGP4).[8] Forty cycles of PCR were performed with Ex-*Taq* polymerase Hot Start (Takara, Tokyo, Japan), using an annealing temperature of 62°C. An *M. leprae*-specific 122-bp fragment was amplified using primers 5′-TTGAGCCCAGCGAGGACATC-3′ (rpoT1) and 5′-TTCGCCATCCTCGGTTTCAC-3′ (rpoT2).[9] Forty cycles of PCR were performed, using an annealing temperature of 58°C. UV-laser microdissection of acid-fast bacilli-invaded nerve bundles from paraffin sections was performed using a Nikon AU2000 (Nikon, Tokyo, Japan), which was followed by *M. ulcerans*-specific PCR.

### *Sensory Test of Footpads*

To examine whether the lesions are painless or not, a behavioral test was performed. Nociceptive reflex was quantified using von Frey filaments. One animal was put in a plastic cage with punched metal floor, and mechanical force to the dorsum of the footpads of the mouse from the bottom was measured using monofilaments having different size and pressure (Touch-Test Sensory Evaluator Instruments; North Coast Medical, Inc., Morgan Hill, CA). Each animal was stimulated three times with filaments having the pressures of 0.02, 0.04, 0.07, 0.4, 1, 4, and 10 $\times$ *g*, when the animal was not grooming or standing. If an animal raised the stimulated foot, it was counted as positive response. Data of animals were summed up in each group. We compared sex-matched control mice without inoculation (aged 10 weeks old, $n = 11$), mice inoculated with *M. ulcerans* strain 97-107 and examined

**Figure 1.** Skin lesion and nerve damage caused by *M. ulcerans*. **a:** Swelling of a mouse footpad without significant ulcer formation 33 days after *M. ulcerans* inoculation. **b:** H&E-stained section of skin showing epidermal erosion and massive edema of the dermis and subcutaneous tissue. **c:** Fite-Faraco staining showing solid acid-fast bacilli forming small clusters in the monocytes (**blue arrow**) and in the edematous stroma (**red arrow**). **d:** H&E-stained sections showing a small number of neutrophils and monocytes scattered in the edematous stroma. A nerve bundle (**green arrow**) is well preserved. **e–h:** Nerves on days 40 to 42 after inoculation of *M. ulcerans*. **e:** H&E-stained sections showing a well-preserved nerve bundle. **f:** A few acid-fast bacilli (**inset**) were located in the perineurium. **g:** Mycobacterial cross-reactive anti-BCG immunohistochemistry showing positive staining in the corresponding area (**red arrow**). **h:** Anti-BCG immunohistochemistry of another nerve bundle showing subperineurial localization of acid-fast bacillary antigen (**red arrow**). **i:** H&E sections showing aggregation of neutrophils near the nerves in small number of cases. **j:** Massive dermal swelling and ulceration in a mouse footpad 54 days after *M. ulcerans* inoculation. **k:** Electron micrograph of an edematous ulcer demonstrating cross sections of bacilli in the extracellular space (**red arrows**) and in the cytoplasm of a macrophage (**blue arrow**) (higher magnification picture available as Supplemental Figure 1 at *http://ajp.amjpathol.org*). **l:** Fite-Faraco staining showing massive perineurial and subperineurial invasion of acid-fast bacilli in a peripheral nerve bundle. **m:** Ultrastructure of a peripheral nerve, confirming perineurial and subperineurial invasion of bacilli (**red arrows**) and vacuolar change of Schwann cell cytoplasm (**yellow arrows**) (higher magnification picture available as Supplemental Figure 2 at *http://ajp.amjpathol.org*). **n:** Fite-Faraco staining showing massive invasion of acid-fast bacilli in a nerve bundle (**red arrow**). **o:** Ultrastructure showing massive invasion of bacilli in the endoneurium with degenerated nerve fibers (**yellow arrow**) (higher magnification picture available as Supplemental Figure 3 at *http://ajp.amjpathol.org*). The bacilli (**red arrows**) are in the extracellular space. **p:** Epon section (1 $\mu$m) showing marked subperineurial edema with degeneration of nerve fibers in a small nerve bundle (higher magnification picture available as Supplemental Figure 4 at *http://ajp.amjpathol.org*). **q:** Anti-PGL-I immunohistochemistry showing negative staining in the bacilli, performed on a serial section of **n**. **r:** Anti-BCG immunohistochemistry on a serial section showing positive staining in the bacilli within the nerve bundle. Scale bars = 5 $\mu$m (green) and 50 $\mu$m (black).

on day 52 after inoculation (day 52, $n = 10$), and mice inoculated with low-dose *M. ulcerans* and examined on day 94 after inoculation (day 94, $n = 5$). In this behavioral test, the bacilli grew slowly, and the lesions appeared about 1 week later than in the morphological studies.

## Results

### Progression of Disease in Mice

From day 33 to about week 7, swelling of inoculated footpads gradually increased and become ulcerated. Subcutaneous edema appeared in the hind legs, followed by anuria and cyanosis about 0.5 day before death. Gross and histological examination of naturally deceased mice showed liquefaction of subcutaneous adipose tissue with edema, ascites, and atrophy of spleen. Heart, lung, liver, pancreas, salivary gland, lymph node, kidney, brain, spinal cord, and bone marrow of footpads were well preserved. Acid-fast staining and anti-BCG immunohistochemistry were negative in these organs and spleen. A small number of animals showed generalized subcutaneous swelling, which we called "balloon mouse"; however, histology of these mice was identical with other mice. Granulomatous inflammation was not observed in any organs.

### Histology, Immunohistochemistry, and Electron Microscopy of Footpads

#### *Early Stage of the Disease*

On day 33 after inoculation of *M. ulcerans* (Figure 1, a–d), inoculated footpads became swollen, but dermal ulcers were absent (Figure 1a). However, dermal erosion (peeling off of epidermis) was observed histologically. Extensive edema of subcutaneous tissue was associated with loss of adipose tissue and infiltration of small numbers of neutrophils and monocytes in the edematous stroma (Figure 1b). Small clusters of long acid-fast bacilli were observed mainly in the monocytes and focally in the stroma (Figure 1c). These bacilli were arranged in a parallel pattern. Leukostasis was noted in the capillary lumen, and swelling of the endothelium with papillary projection (high endothelial venules) was observed in most blood vessels in the edematous stroma. Compared with ordinary dermal ulcers, the inflammatory reaction was slight, and granuloma formation was absent. Peripheral nerves were well preserved, even in the edematous lesion (Figure 1d).

#### *Middle Stage of the Disease*

On days 40 to 42 after inoculation of *M. ulcerans*, sharply demarcated dermal ulcers with fibrin exudation were observed at the ulcer base. Mild stromal edema and mild neutrophilic infiltration were also noted. A moderate number of acid-fast bacilli were present in the monocytes and edematous stroma, and granuloma formation was absent. Peripheral nerves were well preserved, but the perineurium of some nerve bundles showed the presence of acid-fast bacilli. As shown in Figure 1, e–h, H&E stainings showed no significant changes in the nerve bundles (Figure 1e), but careful examination of Fite-Faraco staining revealed a few acid-fast bacilli in the perineurium (Figure 1f), which was confirmed by anti-BCG immunohistochemistry (Figure 1, g and h). In the surrounding regions of the necrotic area, small clusters of neutrophils were observed in a few cases (Figure 1i), but there was no specific interaction between the inflammatory cells and damaged nerves, as observed in leprosy.

#### *Advanced Stage of the Disease*

On days 51 to 70 after inoculation of *M. ulcerans* (Figure 1, j–r), remarkable deep skin ulcers (Figure 1j) and extensive subcutaneous edema were associated with prominent venous dilatation and occasional fresh thrombosis. Small numbers of neutrophils had infiltrated the tissue, and fibrin exudates were moderate. Large numbers of elongated acid-fast bacilli formed clusters mainly in the edematous stroma and focally in the monocytes. Ultrastructurally, some bacilli were observed in the cytoplasm of macrophages, and others were present extracellularly in the stroma (Figure 1k). Most of the nerve bundles were well preserved, even in the extensive edema at the light microscopic level, but careful examination of 1-$\mu$m Epon sections and electron microscopy revealed vacuolar degeneration of the cytoplasm of myelin-forming Schwann cells in nerves of otherwise normal appearance (Figure 1m). Some nerve bundles were invaded by numerous acid-fast bacilli, beginning from the perineurium and subperineurial space (Figure 1, l and m), and the bacilli were observed extracellularly (Figure 1m). Other nerve bundles showed massive intraendoneurial invasion that evoked remarkable swelling, indicating loss of nerve function in the corresponding area (Figure 1n). The bacilli were observed in the extracellular space and were associated with massive destruction of nerve fibers, but invasion into the Schwann cell cytoplasm was not observed by electron microscopy (Figure 1o). In small nerve bundles, subperineurial edema with degeneration of nerve fibers was observed (Figure 1p). Immunohistochemistry performed on serial sections of Figure 1n using anti-PGL-I antibody was negative in these bacilli (Figure 1q), although mycobacterial cross-reactive anti-BCG was positive (Figure 1r).

### Detection of Bacterial Genomic DNA in Tissue Sections

To exclude the possibility that the nerve-invading bacilli in our study were *M. leprae* instead of *M. ulcerans*, PCR analyses were performed on paraffin sections of *M. ulcerans*-infected footpads. Because DNA is easily fragmented into short segments in formalin-fixed paraffin sections, relatively short sequences for PCR amplification were selected. PCR analysis showed positive amplification of *M. ulcerans*-specific genomic sequences, but not of *M. leprae*-specific sequences (Figure 2a), which con-

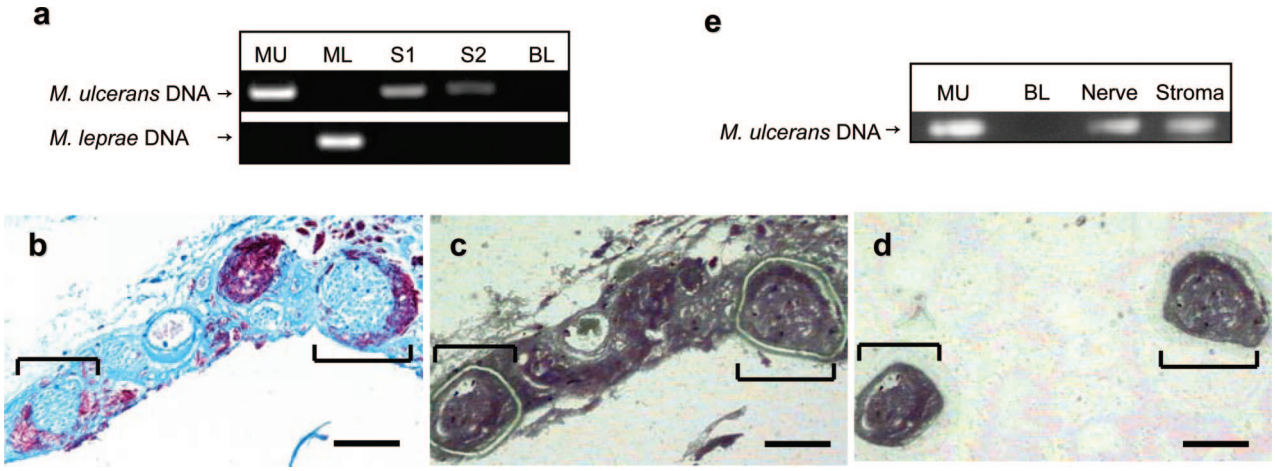


**Figure 2.** Detection of *M. ulcerans* and *M. leprae* DNA from tissue sections by PCR. **a:** The **top lane** shows positive amplification of *M. ulcerans*-specific genomic sequences in a positive control (DNA extracted directly from *M. ulcerans* culture, MU) and two histological sections on days 42 (S1) and 33 (S2) but no amplification in DNA extracted from *M. leprae* (ML) and water (BL). The **bottom lane** shows no amplification of *M. leprae*-specific genomic sequences in the histological samples. **b:** Fite-Faraco staining of a serially cut paraffin section next to microdissection showing invasion of acid-fast bacilli in two peripheral nerve bundles on day 57. **c:** An outline of the nerve bundles by laser beam. **d:** Excised nerve bundles invaded by acid-fast bacilli. **e:** Amplification of *M. ulcerans*-specific genomic sequences in the positive control (MU), water (BL), microdissected nerves (as shown in **d**), and microdissected extraneural tissue (stroma). DNA was amplified not only from the stroma but also from the nerve. Scale bars = 50 μm.

firmed the genomic specificity of *M. ulcerans* in the footpad infections. Furthermore, PCR of UV-laser microdissected nerve bundle tissues showed amplification of *M. ulcerans*-specific sequences (Figure 2, b–e).

### *Sensory Test of Footpads*

In the sensory test of footpads (Figure 3), there was decreased sensitivity ($P < 0.001$) against the stimuli in day-52 mice, when their footpads were moderately swollen and eroded. In the day-94 mice having more advanced lesions where marked swelling and ulceration are prominent, threshold of nociceptive reflex was not significantly different from the control.

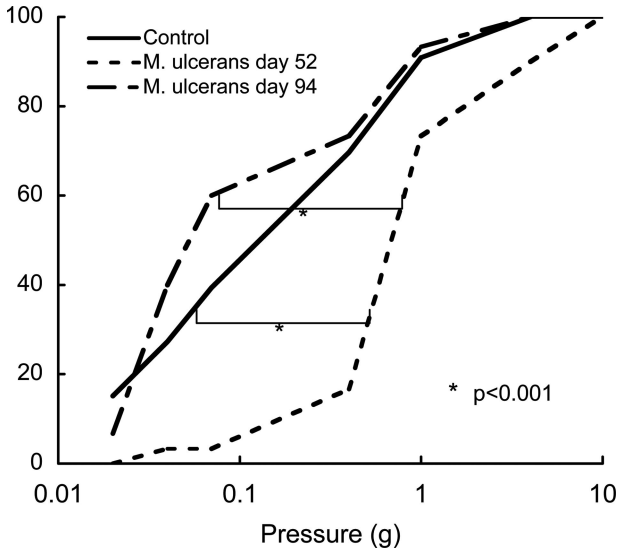


**Figure 3.** Sensory test of footpads. Mechanical force to the footpads of mice was measured using von Frey monofilaments of 0.02, 0.04, 0.07, 0.4, 1, 4, and 10 × *g*, when the animals are not grooming or standing. Data of animals were summed up in each group. There was decreased sensitivity ($P < 0.001$) in day-52 mice ($n = 10$) when their footpads were moderately swollen and eroded compared with sex-matched control mice ($n = 11$). In the day-94 mice ($n = 5$) with more advanced lesions with marked swelling and prominent ulceration, the threshold of nociceptive reflex was not significantly different from the control.

## *Discussion*

This is the first study demonstrating definite nerve damage after *M. ulcerans* infection. Direct invasion of the bacilli into the nerve fascicles beginning from perineurium and conspicuous vacuolar change of Schwann cells was confirmed in the local site of *M. ulcerans*-inoculated mice footpads.

Among the mycobacteria, *M. leprae* is known for its neurotropism and nerve damage. In tuberculosis, peripheral neuropathy is reported mostly as an adverse effect of chemotherapy with drugs such as isoniazid[10,11] and ethambutol.[12,13] Tuberculous granuloma is formed within peripheral nerves only on rare occasions.[14] In human Buruli ulcers, deep dermal ulcers, extensive necrosis of subcutaneous tissue with fat necrosis, and vasculitis have been reported; and granulomatous inflammation and pseudoepitheliomatous hyperplasia of the epidermis were also observed in healing lesions.[15] Concerning the neuropathology of Buruli ulcer, only mild degenerative changes associated with thickening of Schwann cell basal lamina and vacuolar changes of axons have been reported,[16] but direct nerve invasion by acid-fast bacilli has not been observed.

In studies of *M. ulcerans*-injected mice, only slight ulcer formation was reported, although extensive hind limb necrosis was evident.[17,18] There have been several studies on the effect of antimicrobial drugs in mice,[19–22] but nerve lesions have not been described in these experiments. In our mouse model, axons showed neither swelling nor vacuolar changes; instead, vacuolar changes were present in myelinated Schwann cells. A recent case presentation of "painless ulcers" in the *New England Jour-*

*nal of Medicine* was diagnosed as leishmania amastigotes infection, and Buruli ulcer was discussed as a differential diagnosis, but the mechanism underlying the painlessness was not discussed.[23] Our study is therefore the first to demonstrate that nerve bundles are damaged by numerous *M. ulcerans* in the mouse footpad injection model.

We could not confirm the previously reported presence of PGL-I antigen in *M. ulcerans* by immunohistochemistry.[6,16] PGL-I biosynthesis requires polyketide synthases (pks), glycosyltransferases, and methyltransferases. Of interest is the gene pks15/1 that is necessary for the synthesis of PGL. This gene has become a pseudogene in *M. ulcerans* (personal communication, Dr. Timothy Stinear, Monash University, Clayton, Australia), which supports our PGL-I negative result. For the glycosyltransferases and methyltransferases, genetic data for *M. ulcerans* were not available.

Compared with the neuropathology of leprosy, our findings show a few similarities and many differences. Both in human leprosy and *M. ulcerans*-inoculated mice, nerve invasion starts from the perineurium.[24,25] In the nerves of lepromatous leprosy, *M. leprae* is observed in the cytoplasm of unmyelinated Schwann cells, myelinated Schwann cells, and intraneural macrophages but not in the extracellular space. In contrast, *M. ulcerans*-inoculated mice showed extracellular proliferation of bacilli in the endoneurium. In addition, vacuolar change of myelinated Schwann cells is an uncommon finding in leprosy.

*M. ulcerans* is known to produce a unique toxin, mycolactone,[3] that induces apoptosis in guinea pig lesions.[26] In this study, we did not observe nuclear pyknosis suggestive of apoptosis; however, the vacuolar degeneration of Schwann cells observed in this study could be caused by the cytotoxic effect of mycolactone.[27] It is also possible that mycolactone has an anesthetic effect causing paralysis, but studies supporting this concept have not been reported.

From the sensory test of footpads, two findings were obtained. First, there was decreased sensitivity ($P < 0.001$) against the stimuli in day-52 mice, when their footpads were moderately swollen and eroded. This suggests that pain sensation is decreased even though the lesion is inflamed, which may reflect the painlessness of the human ulcer. In addition, we should also consider the possibility that footpad swelling might have reduced the response to the stimuli by a mechanical insulator function. Second, the day-94 mice have more advanced lesions where marked swelling and ulceration are prominent and the threshold of nociceptive reflex was not significantly changed, suggesting that these animals recovered sensation or experienced pain from other factors such as secondary infection. We examined the electron micrographs again, but morphological signs of Schwann cell regeneration, such as thin myelin formation (remyalination), were not observed in this experiment. Further studies are necessary to define whether the ulcers are actually painless.

Our observation in the mouse infection model shows that only sparse inflammatory cell infiltration, which is compatible with most of the previous works in human and nine-banded armadillo[28] but different from the recent studies of Oliveira et al[29] and Coutanceau et al,[30] who demonstrated persistent inflammatory responses even in the advanced stage. We have carefully examined the surrounding regions of the necrotic area, but we could not find large numbers of recruiting neutrophils or monocytes, except for small clusters of neutrophils in three cases (days 39, 40, and 54) among 18 cases. It is not easy to interpret the difference of inflammatory cell infiltration, but it could be due to heterogeneity of mycolactones produced by various *M. ulcerans* strains[31] or different susceptibility of mouse strains against *M. ulcerans*. The bacilli were located both in monocytes and in extracellular space. In the early stage of the disease, when the number of bacilli is small, the bacilli were found primarily in the monocytes. In the more advanced stage, where numerous bacilli were present, extracellular bacilli were predominant.

Concerning the mortality after *M. ulcerans* injection, Krieg et al[32] showed that guinea pigs inoculated with *M. ulcerans* showed final healing after ulceration, and Read et al[18] showed that none of the inoculated guinea pigs died. On the contrary, Read et al[18] described that inoculation of mouse footpads with *M. ulcerans* resulted in progressive infection, leading to ulceration and eventual death. Also, in the recent study of Oliveira et al,[29] mice were sacrificed for ethical reasons after the emergence of ulceration, implying that the mouse infection was lethal. We tried to discern the direct cause of death in the mice, but we do not have any reasonable explanation yet. Our present study demonstrated nerve damage in *M. ulcerans* infection, suggesting that intraneural bacterial invasion may play an important role in the pathogenesis of "painlessness" of Buruli ulcer.

## Acknowledgments

DNA of *M. leprae* strain Thai-53 was a gift from M. Matsuoka (Leprosy Research Centre, National Institute of Infectious Diseases, Tokyo, Japan). We are grateful to T. Hatanaka, S. Yamauchi, Y. Nishimura, and Y. Arimura for technical assistance.

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