論 文 要 旨

Mycobacterium leprae in Neurons of the Medulla Oblongata and Spinal Cord in Leprosy

（ハンセン病剖検例における延髄と脊髄の神経細胞内のらい菌の証明）

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Introduction and Purpose

Leprosy is a chronic, infectious, neurodegenerative human disease caused by *Mycobacterium leprae* and is still a major global health problem. Neurotropism and involvement of *M. leprae* in the peripheral nervous system (PNS) have been commonly reported as a cause of neuropathy, but less attention has been paid to central nervous system (CNS) damage in patients with leprosy, despite several reports of this phenomenon. Furthermore, the human brain and spinal cord have been considered to be free from bacilli in leprosy. In leprosy, although nerve damage and paralysis are most often associated with an attack of acute or sub-acute neuritis as part of the reaction episode, often nerve trunks become paralyzed quietly without such manifestations; a condition known as quiet nerve paralysis (silent neuropathy).

To date, the exact etiology and pathogenesis of motor paralysis in leprosy have not been identified. However, degeneration of motor neurons occurs in patients with motor neuron diseases such as amyotrophic lateral sclerosis, and in this study we looked for similar nerve damage in leprosy.

Materials and Methods

Autopsy cases of cured lepromatous leprosy (n=67) from the National Hansen Disease Hospital Hoshizuka-Keiaien, Kagoshima, Japan and autopsy cases of non-leprosy patients (n=15) as control cases from Kagoshima University Hospital were selected for the study. Autopsy records and the patients' clinical charts were reviewed and searched for the clinical information.

All H & E stained sections were reviewed. For cases with obvious morphological changes (vacuolar changes of neurons) in both the medulla oblongata (MO) and spinal cord (SC) (n=19), cases without any morphological changes (n=8) and control cases (n=10 for MO and SC, and n=5 for SC and dorsal root ganglia {DRG}), the following experiments were performed: sections of the MO and SC were cut into slides of 4-μm thickness for hematoxylin and eosin (H & E) staining, Fite-Faraco acid-fast staining, immunohistochemistry for *M. leprae*-specific anti-phenolic glycolipid-1 (PGL-I) and Anti-BCG, a TUNEL assay, and serial sections of 10-μm thickness were mounted on membranes for laser-captured micro-dissection. Additionally, specimens from DRG taken from lepromatous leprosy cases (n=27/67) and those of control cases (n=5) were examined for similar study.

DNA extraction was performed from micro-dissected tissue. A nested PCR targeting the *M. leprae*-specific repetitive sequence (RLEP) was performed yielding a 129-bp outer product and a 99-bp nested product. The following oligonucleotides primers were used: LP-1, 5'-TGCATGTCATGGCCTTGAGG-3'; LP-2, 5'-CACCGATACCAGCGGCAGAA-3'; LP-3, 5'-TGAGGTGTCGGCGTGGTC-3'; and LP-4, 5'-CAGAAATGGTGCAAGGGA-3'. Gel electrophoresis was performed and DNA was visualized by ethidium bromide and ultraviolet light. Nucleotide sequencing of amplified DNA was performed using an

automated sequencer.

Results

Majority of the patients showed evidence of neurological complications such as bending of the fingers, shortening of the extremities, lagopthalmos and/ or blindness. These features were recorded according to WHO disability grading. 97% (65/67) had grade 2 disability, and 3% (2/67) were free from disability (grade 0). No clinical evidence of bulbar palsy was noted.

Of the 67 cases of leprosy, 44 (67%) had vacuolar changes of motor neurons either in medulla oblongata (nucleus ambiguus, hypoglossal nucleus) or spinal cord. The neurons were swollen and showed foamy changes; although the normal shape was preserved for some neurons, the whole cytoplasm of others has been replaced by tiny vacuoles. No acid fast bacilli were identified by Fite staining, but PGL-I and BCG immunostaining were positive in vacuolated areas. Most cases showed strong staining intensity and PGL-I staining showed an intracytoplasmic vacuolar and granular pattern. PCR revealed *M. leprae*-specific genomic DNA in 18/19 of cases (95%) with vacuolated changes and 5/8 (63%) without vacuolated changes. DNA sequencing result demonstrated more than 99% homology with M. *leprae* specific genomic sequences. In DRG 21/27 cases of lepromatous leprosy showed vacuolated neurons and 22/27 were positive for PGL-I. Endothelial cells of 2 lepromatous leprosy cases also showed PGL-I positive vacuolative changes. All above findings were negative in control cases. TUNEL staining did not show significant increase of apoptosis in the neurons. The PCR positivity had a significant correlation with PGL-I immunostaining ($P<0.05$). Presence of vacuolar changes in the spinal cord was correlated with hands and feet deformity grades ($P=0.04$). Neuronal loss was evaluated by counting the number of spinal anterior horn neurons from leprosy cases and age matched control cases. Cervical and lumbar cords were counted separately, (cervical cord of leprosy (n=22), 56.3 ± 30.8; cervical cord of control (n=6), 48.0 ± 27.6; lumbar cord of leprosy (n=22), 64.5 ± 28.9; lumbar cord of control (n=10), 71.1 ± 28.9). There was no statistically significant neuronal loss between leprosy and control cases. (cervical, $P= 0.67$; lumbar, $P= 0.24$).

Discussion and Conclusion

Our immunohistochemical data show the presence of mycobacterial antigens in neurons of the central nervous system in cases of leprosy. PGL-I is a phenolic-diacylphthiocerol triglycerides and forms a loose capsule around the bacillus and is known to be specific to *M. leprae*. Treated patients still frequently harbor a significant level of antibodies to PGL-I, and detectable by immunohistochemistry in tissue sections from leprosy specimens. All our cases were long-term cured leprosy cases (disease-free for more than ten years and slit skin smear-negative), and therefore only dead organisms were thought to be present in CNS. This may explain the lack of acid fastness, although conversion to the non-acid fast, cell wall-deficient dormant form found in *M. tuberculosis* may also have occurred, and the negative results in Fite staining are in accordance with this. Highly sensitive methenamine silver was performed in CNS specimens of lepromatous leprosy cases and motor neurons were not stained by this method, and only doubtful staining of fragmented bacilli was identified in endothelial cells which were also positive for PGL-I.

The presence of *M.leprae*-specific genomic DNA was further confirmed by nested PCR. In addition to the histopathological and immunohistochemical findings, the DNA analysis confirms the persistence of *M. leprae* in the CNS even after long-term clinical cure; this is the first demonstration of this phenomenon. The route of entry of *M. leprae* into motor neurons is not yet proven. Neural spread of bacilli may occour via motor nerve axon to motor neuron or anterograde spread via sensory nerve axon to motor neuron. Hematogenous spread should also need to be considered. In conclusion, our results confirms that there is clear morphological change in motor neurons of lepromatous leprosy patients associated with PGL-I and mycobacterial DNA. This study provides significant additional evidence to indicate that *M. leprae* is present in the CNS in a subset of patients. Further investigation is required to correlate this finding to motor dysfunction and silent neuropathy in leprosy.

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