Pathological diagnosis of leprosy in developing countries

Masamichi GOTO*, Thida Aung, Shinichi KITAJIMA

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In the developing countries where leprosy is prevalent, diagnosis of leprosy is made from clinical signs and symptoms. However, when difficult and doubtful cases increase after the advance of leprosy control programs, definitive diagnosis of leprosy by histopathology become necessary. This report describes our experience of technical support to re-establish histopathology service and introduction of immunohistochemistry in the leprosy referral center of Myanmar, and we discuss the ideal way of international technical support. This activity was performed as a part of leprosy control and basic health services project of Japan International Cooperation Agency (JICA) since 2000 to 2005.

*Corresponding author :
Department of Human Pathology, Field of Oncology
Kagoshima University Graduate School of Medical and Dental Sciences
8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
Phone: +81-99-275-5270 FAX: +81-99-265-7235
E-mail: masagoto@m2.kufm.kagoshima-u.ac.jp

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Immunopathology and Infectious Diseases

Nerve Damage in *Mycobacterium ulcerans*-Infected Mice

Probable Cause of Painlessness in Buruli Ulcer

Masamichi Goto,* Kazue Nakanaga,† Thida Aung,* Tomofumi Hamada,* Norishige Yamada,* Mitsuharu Nomoto,* Shinichi Kitajima,‡ Norihisa Ishii,† Suguru Yonezawa,* and Hajime Saito§

From the Department of Human Pathology, Field of Oncology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Leprosy Research Center,† National Institute of Infectious Diseases Tokyo; the Department of Pathology,‡ Kagoshima University Hospital, Kagoshima; and the Hiroshima Environment and Health Association,§ Hiroshima, Japan*

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 = 3.3×10^4) 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

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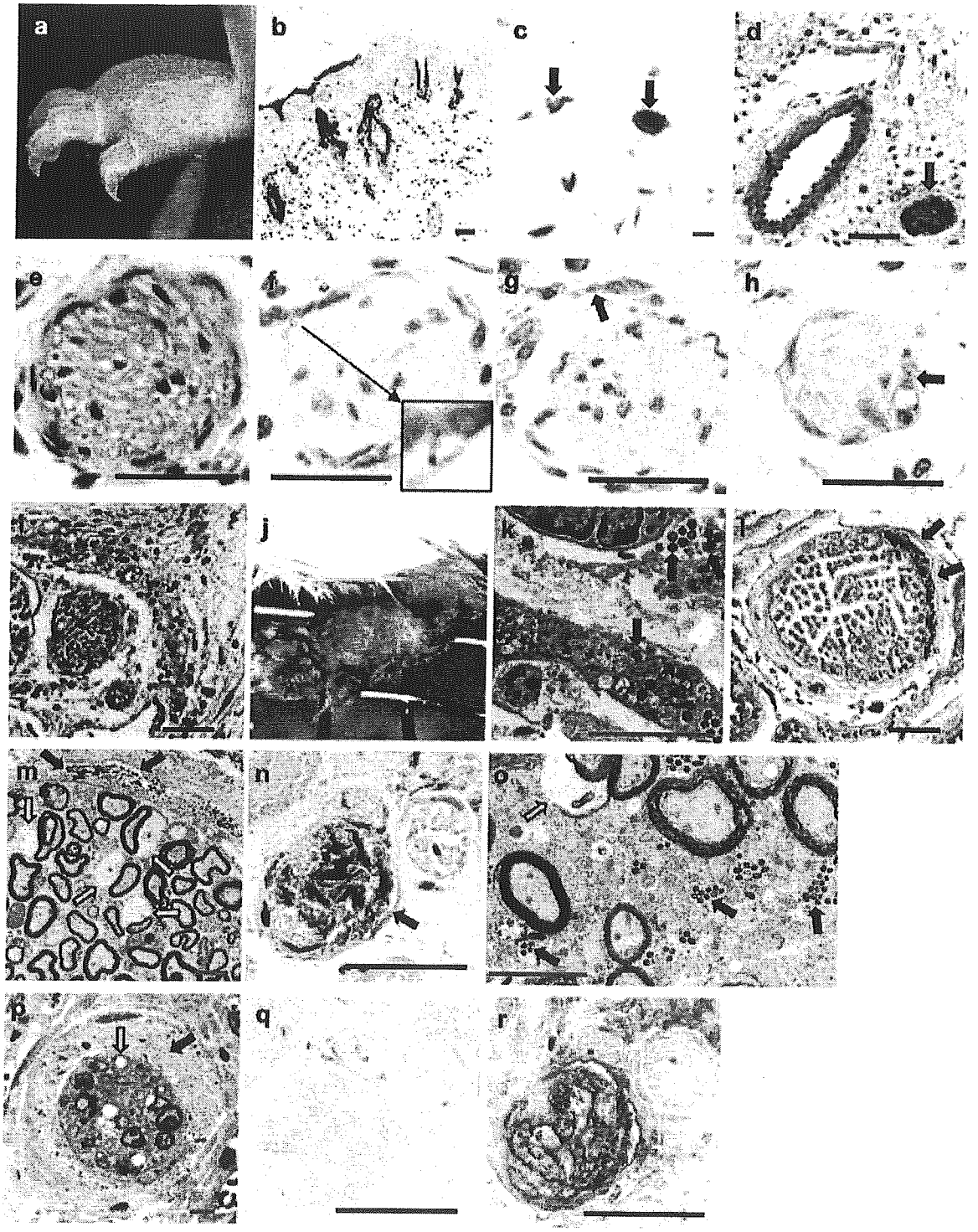
Address reprint requests to Masamichi Goto, M.D., Ph.D., Department of Human Pathology, Field of Oncology, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan. E-mail: masagoto@m2.kufm.kagoshima-u.ac.jp.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observed 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-μm 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-μm 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 × 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 μm) 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 μm (green) and 50 μm (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-μm 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-

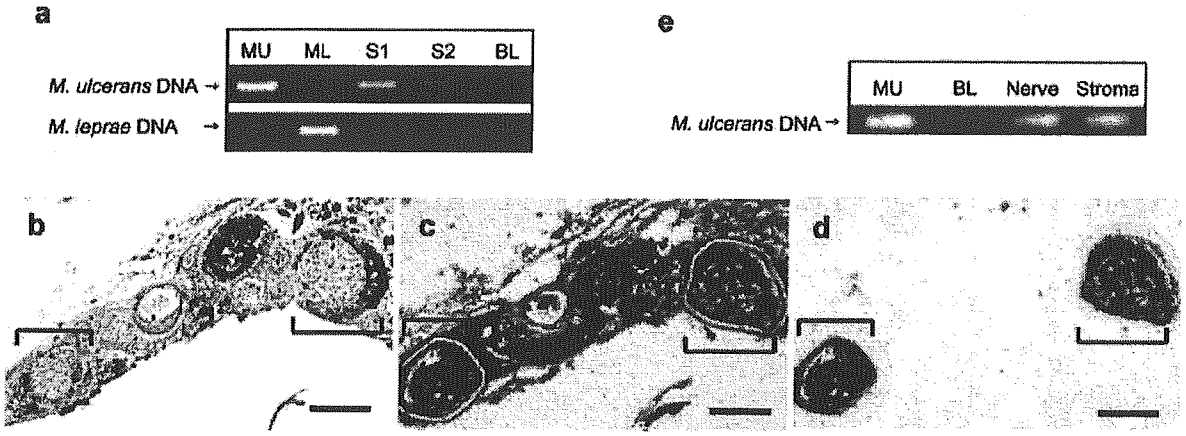

Figure 2. Detection of *M. ulcerans* and *M. leprae* DNA from tissue sections by PCR. **a:** The **top lane** shows positive amplification of *M. ulcerans*-specific genomic sequences in a positive control (DNA extracted directly from *M. ulcerans* culture, MU) and two histological sections on days 42 (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.

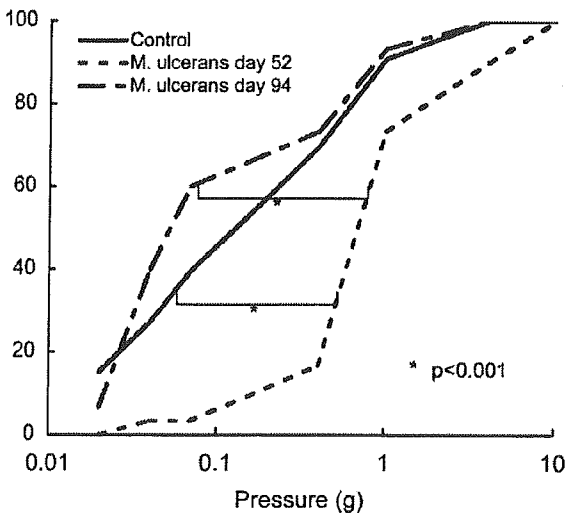

Figure 3. Sensory test of footpads. Mechanical force to the footpads of mice was measured using von Frey monofilaments of 0.02, 0.04, 0.07, 0.4, 1, 4, and 10 × *g*, when the animals are not grooming or standing. Data of animals were summed up in each group. There was decreased sensitivity ($P < 0.001$) 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

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ORIGINAL ARTICLE

Polymorphism of the 5′ flanking region of the IL-12 receptor β2 gene partially determines the clinical types of leprosy through impaired transcriptional activity

H Ohyama, K Ogata, K Takeuchi, M Namisato, Y Fukutomi, F Nishimura, H Naruishi, T Ohira, K Hashimoto, T Liu, M Suzuki, Y Uemura, S Matsushita

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See end of article for authors' affiliations

Correspondence to: Dr H Ohyama, First Department of Pathology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya 663-8501, Japan; ohyama@hyo-med.ac.jp

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Background: Individual differences in T cell responsiveness to interleukin 12 (IL-12), resulting from inherited factors, may be responsible for differences in the intensity of cell mediated immune (CMI) responses in patients with leprosy, a disease with a wide clinical spectrum.
Aim: Polymorphisms in the 5′ flanking region of the IL12RB2 gene were analysed to determine potential immunogenetic factors affecting CMI responses, using leprosy as a model.
Methods: Polymorphisms in the 5′ flanking region of IL12RB2 were examined using direct sequencing techniques, and allele frequencies between patients with lepromatous leprosy and patients with tuberculoid leprosy were compared. The effect of these single nucleotide polymorphisms (SNPs) on IL12RB2 expression was estimated using the dual luciferase reporter gene assay in Jurkat T cells.
Results: Several SNPs, including −1035A>G, −1023A>G, −650delG, and −465A>G, were detected within the 5′ flanking region of IL12RB2. The frequency of haplotype 1 (−1035A, −1023A, −650G, −464A) was high in the general Japanese population, but was significantly lower in lepromatous patients compared with tuberculoid patients and healthy controls. Reporter gene assays using Jurkat T cells revealed that all haplotypes carrying one or more SNP exhibited a lower transcriptional activity compared with haplotype 1.
Conclusion: SNPs within the 5′ flanking region of IL12RB2 affect the degree of expression of this gene and may be implicated in individual differences in CMI responsiveness to mycobacterial antigens, leading to lepromatous or tuberculoid leprosy.

The importance of the cell mediated immune (CMI) response is well established in the host defence to mycobacterial pathogens.[1 2] Leprosy, a chronic disease caused by infection with *Mycobacterium leprae*, shows a wide spectrum of clinical features.[3] Patients with tuberculoid type leprosy (T-lep) show a high CMI response to *M leprae*, with resistance to infection, whereas patients with lepromatous leprosy (L-lep) show a poor CMI response to the pathogen and have a progressive form of the disease. Although a leishmaniasis model using BALB/c and C57BL6 mice has improved our understanding of cellular and genetic control mechanisms for infectious diseases and allergy, no such models have yet been established in humans. We propose leprosy as an alternative model in humans.

> "Polymorphisms in the 5′ flanking region of IL12RB2 may affect the expression of the interleukin 12 receptor β2 chain, resulting in individual differences in the intensity of cell mediated immune responses to mycobacteria"

Interleukin 12 (IL-12) is secreted from macrophages and dendritic cells and is a potent inducer of interferon γ production by T helper type 1 (Th1) cells, which is in part dependent upon the degree of expression of the IL-12 receptor (IL-12R) on the cell surface.[4–6] IL-12R is composed of two protein subunits, referred to as the β1 and β2 chains, and expression of the β2 chain is a crucial determinant of Th1/Th2 balance, because STAT4 is activated through interaction with a tyrosine residue on the cytoplasmic domain of the IL-12R β2 subunit.[7–10] It has been shown that the expression of IL-12Rβ2 is greater in tuberculoid lesions than in lepromatous lesions, whereas the expression of IL-12Rβ1 is similar in both.[11] We hypothesise that the susceptibility to several diseases related to mycobacterial pathogens could be determined by the degree of expression of IL-12Rβ2, which might be regulated by genetic factors, including IL12RB2 polymorphism.

One hypothesis is that polymorphisms in the 5′ flanking region of IL12RB2 may affect the expression of IL-12Rβ2, resulting in individual differences in the intensity of CMI responses to mycobacteria. We examined single nucleotide polymorphisms (SNPs) within the 5′ flanking region of IL12RB2 as feasible markers to determine susceptibility to the disease and the effect of these SNPs on the transcription of IL-12Rβ2 molecules.

MATERIALS AND METHODS

Study population

Genomic DNA samples were collected from 176 Japanese patients with leprosy—130 with L-lep and 46 with T-lep—and 68 healthy Japanese donors. Patients were clinically diagnosed, according to the description of Ridley and Jopling, referring to results of the Mitsuda test and their sequelae.[3] Donors were recruited into our study under informed consent guidelines approved by the human ethical committee of Saitama Medical School, Japan.

Abbreviations: CMI, cell mediated immunity; IL-12, interleukin 12; IL-12R, interleukin 12 receptor; L-lep, lepromatous leprosy; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; Th, T helper; T-lep, tuberculoid type leprosy

Table 1 Allelic distribution in patients with leprosy

SNPs	Clinical form	Allele frequency	OR	RR	p Value
−1035A>G	L-lep	24.6%	3.97	3.24	<0.001
	T-lep	7.6%			
−1023A>G	L-lep	24.2%	2.95	2.48	<0.01
	T-lep	9.8%			
−650delG	L-lep	28.8%	3.74	2.95	<0.001
	T-lep	9.8%			
−464A>G	L-lep	23.1%	3.64	3.03	<0.01
	T-lep	7.6%			

Frequencies of four single nucleotide polymorphisms (SNPs) were compared between lepromatous (L-lep) and tuberculoid (T-lep) patients. The χ^2 test was used to compare differences in the distribution of clinical phenotypes and allele frequencies. The odds ratio (OR) and relative risk (RR) were also calculated by comparing the frequency of variant alleles between two clinical types of leprosy.

Analysis of polymorphism in the 5′ flanking region of IL12RB2

Genomic DNA was sequenced for polymorphisms in the 5′ flanking region of IL12RB2 using direct sequencing. Briefly, a fragment spanning −1247 to +55 of IL12RB2 was polymerase chain reaction (PCR) amplified and subsequently sequenced using the ABI 3730 DNA sequencer (PerkinElmer Life Sciences, Wellesley, Massachusetts, USA). The sequence data obtained were compared with the GenBank database (GenBank accession number AL389925) to determine the SNPs on IL12RB2. Numbers of base positions were defined as the distance from the start point of the reported cDNA sequence.[12] The haplotypes were determined by cloning the PCR products into the pGEM-T Easy plasmid (Promega, Madison, Wisconsin, USA) and sequencing.

Evaluation of transcriptional activity of the 5′ flanking region of IL12RB2

For transfection studies, the dual luciferase reporter gene assay system (Promega) was used with the IL12RB2–pGL3 plasmid constructs. The plasmids comprised a 1.3 kb NheI/HindIII digested PCR fragment of IL12RB2 ligated to the pGL3 basic vector (Promega). Jurkat T cells (1×10^7) were electroporated with 25 μg of plasmid DNA (IL12RB2–pGL3 constructs) and a control *Renilla* luciferase reporter plasmid (pRL-TK, 25 ng; Promega) using a Gene Pulser (Bio-Rad Laboratories, Hercules, California, USA), as described previously.[13] Cells were then cultured for 48 hours in the presence of antihuman CD3 monoclonal antibody (PharMingen, San Diego, California, USA), and antihuman CD28 monoclonal antibody (PharMingen) for the final 24 hour period. Cell lysates were prepared, and both firefly and *Renilla* luciferase activities were evaluated using a dual luminometer Fluoroskan Ascent FL (Thermo Electron Oy, Vantaa, Finland).

Table 2 Haplotypes of the 5′ flanking region of IL12RB2

Haplotype	−1035	−1023	−650	−464	Frequency (%)
1	A	A	G	A	41.2
2	G	G	del	G	32.4
3	A	A	del	A	11.8
4	G	G	G	A	8.3
5	A	A	del	G	5.9

Haplotypes were determined by sequencing 34 subcloned polymerase chain reaction amplified DNA fragments from 17 donors with heterozygous alleles in at least in one of the positions −1035, −1023, −650, and −464, but not from donors carrying homozygous alleles at all positions.

RESULTS

Analysis of polymorphism in the 5′ flanking region of IL12RB2

Twelve SNPs located within the 5′ flanking region of IL12RB2 (−1247 to +55), comprising −1047delT, −1035A>G, −1033T>C, −1023A>G, −650delG, −568A>C, −557T>C, −550T>C, −464A>G, −464A>C, −202T>C, and −188A>C, were identified. The surveillance study of 176 patients (130 with L-lep and 46 with T-lep) revealed significant differences in frequencies of SNPs between these patients groups; in particular: −1035A>G, −1023A>G, −650delG, and −464A>G (table 1).

We randomly selected 17 healthy donors heterozygous in at least one of the positions −1035, −1023, −650, and −464 to determine linkage disequilibrium. Five haplotypes were determined by sequencing 34 subcloned PCR amplified DNA fragments from the 17 heterozygous donors. Haplotype 1 consisted of −1035A, −1023A, −650G, and −464A, and haplotype 2 consisted of −1035G, −1023G, −650del, and −464G, and accounted for 73.6% of the haplotypes detected in our study (table 2).

Haplotype frequency was calculated based on the assumption that each group would be in accordance with the Hardy–Weinberg equilibrium because subjects were selected from the Japanese Wajin population of mainland Japan (Honshu). p Values were calculated using the StatView (SAS Institute, Cary, North Carolina, USA) statistical software program, by comparing the frequency of haplotype 1 in patients with L-lep leprosy, those with T-lep leprosy, and healthy controls. The frequency of haplotype 1 was significantly lower in patients with L-lep compared with those with T-lep and healthy donors (fig 1), suggesting that haplotype 1 might contribute to the intensity of CMI responses to mycobacteria.

Evaluation of transcriptional activity of the 5′ flanking region of IL12RB2

The transcriptional activity of each reporter construct was determined using the dual luciferase reporter gene assay. The transcriptional activity of haplotype 1 was significantly higher than haplotypes 2, 3, 4, and 5 (fig 2). Each experiment was performed using triplicate wells and was repeated four times. Because each SNP is a genetic factor potentially able to reduce the expression of IL-12Rβ2 molecules, a poor CMI response to mycobacteria may occur.

DISCUSSION

It was recently reported that the lack of IL-12Rβ1 expression caused by mutations in IL12RB1 resulted in human immunodeficiency, thereby demonstrating the essential role

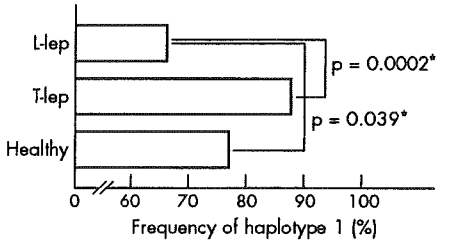

Figure 1 Frequency (%) of haplotype 1 in the three groups: patients with lepromatous leprosy (L-lep), patients with tuberculoid type leprosy (T-lep), and healthy controls. The haplotype frequency was calculated based on the assumption that each group would be in accordance with the Hardy–Weinberg equilibrium because subjects were selected from the Japanese Wajin population of mainland Japan (Honshu). p Values were calculated using the StatView statistical software program, by comparing the frequency of haplotype 1 in the L-lep leprosy, T-lep leprosy, and healthy control groups.

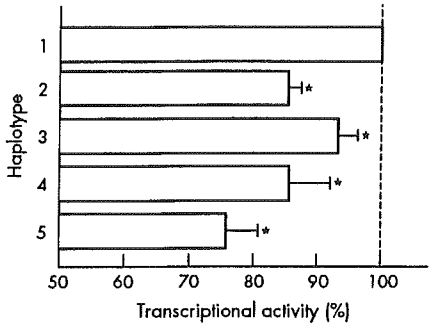

Figure 2 Basal promoter activity of reporter constructs containing −1247 to +55 of IL12RB2 harbouring each haplotype. The transcriptional activity of each haplotype is indicated by the percentage of relative luciferase units compared with the haplotype 1 construct. Each experiment was assayed using triplicate wells and was repeated four times and similar results were obtained. *$p < 0.05$; χ^2 test.

of IL-12 in resistance to infections caused by intracellular bacteria.[14–16] However, no differences in the expression of IL-12Rβ1 on T cells were detected between donors, including patients and healthy subjects (data not shown). It has been reported that IL-12Rβ2 is absent in freshly isolated peripheral blood mononuclear cells, whereas up to 72% of resting peripheral blood mononuclear cells from normal volunteers express IL-12Rβ1 molecules, and that IL-12Rβ2 is expressed selectively in Th1 cells but not in Th2 cells.[9 17] Moreover, because IL-12Rβ2 has tyrosine residues in the cytoplasmic domain that play a role in signal transduction, we hypothesised that IL-12Rβ2, but not IL-12Rβ1, could be important in explaining the low CMI responses induced by IL-12 in T cells from patients with leprosy.

> "Individual differences in the intensity of the cell mediated immune response to mycobacteria are probably regulated primarily by the degree of expression of IL-12Rβ2, rather than possible conformational changes"

Alternatively spliced mRNA with the absence of IL12RB2 exon 15 leads to the loss of induction of interferon γ production.[18] Similarly, we have previously found several coding SNPs of IL12RB2, but could not determine an effect on CMI response intensity or on alternatively spliced mRNA (data not shown). Epidemiological studies recently performed by two independent groups demonstrated that there was no influence of IL12RB2 coding SNPs on susceptibility to mycobacterial infection.[19 20] Therefore, individual differences in the intensity of the CMI response to mycobacteria are probably regulated primarily by the degree of expression of IL-12Rβ2, rather than possible conformational changes caused by the coding SNPs detected in the Japanese population. Bleharski *et al* showed differences in gene expression profiles according to the clinical type of leprosy,[21] and the IL-12R gene was included among those differentially expressed between patients with L-lep and T-lep.

The differences in transcriptional activity between haplotype 1 and other haplotypes are marginal, except for that between haplotypes 1 and 5. These findings suggest that haplotype 5 might be more closely associated with susceptibility to lepromatous leprosy than haplotypes 2, 3, and 4. To investigate this issue, it will be necessary to analyse the frequency of all haplotypes in the patient population. Moreover, the functional effects of SNPs −650 and −464 on the transcriptional mechanism should also be elucidated.

Take home messages

- Single nucleotide polymorphisms (SNPs) within the 5′ flanking region of the IL12RB2 gene could affect the expression of the interleukin 12 receptor β2 chain (IL-12Rβ2) and result in the individual differences in the intensity of cell mediated immune responses that lead to the lepromatous and tuberculoid types of leprosy
- Haplotype 5 appears to be more closely associated with susceptibility to lepromatous leprosy than haplotypes 2, 3, and 4, but further investigations are necessary
- These SNPs may also affect susceptibility to allergy because IL-12Rβ2 is involved in the allergic response

An SNP at position −464 of IL12RB2 was found to have high transcriptional activity compared with the wild-type allele, possibly because of disruption of a GATA site.[22] This appears to contradict our present data, although the effects of SNPs on the binding affinity with GATA-3 were not directly tested in this report. It was determined that the main target of GATA-3 is not IL-12Rβ2, but rather STAT4.[23] Further studies are needed to determine the precise molecular mechanism.

Taken together, we conclude that SNPs within the 5′ flanking region of IL12RB2 could affect the expression of IL-12Rβ2, thus causing individual differences in the intensity of CMI responses leading to the lepromatous and tuberculoid types of leprosy. It is probable that these SNPs also affect susceptibility to allergy, because IL-12Rβ2 is implicated in the Th1/Th2 balance in the allergic response. A study investigating this hypothesis is currently under way.

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Authors' affiliations

H Ohyama, K Hashimoto, T Liu, M Suzuki, Y Uemura, S Matsushita, Department of Allergy and Immunology, Saitama Medical School, Moroyama 350-0495, Japan
K Ogata, Life Science Laboratory, Shimadzu Corporation, Kyoto 604-8511, Japan
K Takeuchi, F Nishimura, H Naruishi, T Ohira, Department of Pathophysiology/Periodontal Science, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8525, Japan
M Namisato, National Sanatorium Kuryu-Rakusenen, Kusatsu 377-1711, Japan
Y Fukutomi, Leprosy Research Centre, National Institute of Infectious Disease, Higashi-murayama 189-0002, Japan

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ORIGINAL ARTICLE

Positional Effect of Amino Acid Replacement on Peptide Antigens for the Increased IFN-γ Production from CD4T Cells

Tianyi Liu[1], Hitoshi Kohsaka[2], Motoharu Suzuki[1], Rie Takagi[1], Kumiko Hashimoto[1], Yasushi Uemura[1], Hideki Ohyama[1] and Sho Matsushita[1]

ABSTRACT

Background: Based on the fact that site-specific amino acid replacement on peptide antigens stimulated T cell clones to produce increased amount of IFN-γ, we investigated this structure-function relationship, using various peptide analogues.

Methods: We used three human Th0 clones (BC20.7,BC33.5 and BC42.1) that express distinct TCRα and TCRβ chains, but recognize the same TCR ligand ; *i.e.*, the same framework of peptide antigen BCGa p84-100 in the context of DRB1*1405. These T cells were stimulated with various peptide analogues, followed by determination of proliferative responses and IFN-γ production.

Results: Replacement of Leu at peptide position 2 (P2) by amino acids which are less hydrophobic than the wild type (Val, Ala) or those with similar structural or neutral charge (Thr, Ser), induced increased IFN-γ production from T cells. This phenomenon was associated with structural features of TCR, especially the length of CDR3 region of TCRα. Amino acid replacement at the other positions did not induce increased IFN-γ production.

Conclusions: Amino acid substitution at P2 frequently induces increased IFN-γ production in a clone-specific manner, which is associated with the structure of CDR3 in TCRVα chains.

KEY WORDS

analogue peptide, complementarity determining region 3, interferon gamma, peptide antigens, T-cell antigen receptor

INTRODUCTION

Recent studies showed that T cell activation is not an all-or-none type of event ; rather, qualitative changes in T cell responses can be induced by amino acid substitutions by either MHC molecules or antigenic peptides, *i.e.*, TCR ligands. Flexibility in recognition results in T-cell activation in the absence of a proliferative response, which is designated by the following terminology as demonstrated in previous studies by our group and others : partial agonism,[1] TCR antagonism,[2] anergy,[3] survival[4] and cytokine-specific up-regulation.[5,6]

Amino acid residues on antigenic peptides have been roughly divided into two groups ; one that is important for binding to TCR (T cell epitope), and the other that is important for binding to MHC (MHC anchor). However, the crystal structure of the human class II HLA-DR1 complexed with the influenza peptide reported by Stern *et al.*[7] demonstrated that all the amino acid residues of the influenza virus peptide physically made contact with both HLA and TCR, with the exception of only one residue at the amino terminus which is buried deeply in the groove of class II, hence, there is no possibility for interaction with TCR.

[1]Department of Allergy and Immunology, Saitama Medical School, Moroyama and [2]Dapartment of Bioregulatory Medicine and Rheumatology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan.
Correspondence: Sho Matsushita, Department of Allergy and Immunology, Saitama Medical School, 38 Morohongo, Moroyama, Saitama 350-0495, Japan.
Email: shomat@saitama-med.ac.jp
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In our previous studies, single amino acid substitutions on a group I allergen in the *Cryptomeria japonica* -derived peptide resulted in a significant increase in IFN-γ production, with no remarkable changes either in proliferative response or IL-4 production.[5] In this study, by using various analogue peptide species, we stimulated three human Th0 clones that express distinct TCRα and TCRβ chains, but recognize the same TCR ligand, and tried to determine the structure-function relationship that leads to increased IFN-γ production from T cells.

METHODS

SYNTHESIS OF PEPTIDES

The wild-type BCGa p 84-100 (EEYLILSARDVLAVVSK) and its analogue were synthesized using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu Corp., Kyoto, Japan), and were purified by C18 reverse-phase HPLC (Millipore).

T CELL CLONES

BCGa p84-100-specific T cell lines were established as described.[8] Three human $CD4^+$ T cell clones (BC 20.7,BC33.5 and BC42.1) specific to BCGa p84-100+ DRA/DRB1*1405, yet bearing distinct TCRβ (BV13S3 ,BV6S1 and BV5S4, respectively ;)[8] established from PBMC of a BCG-primed healthy individual as described elsewhere,[4] were used throughout the study. T cells were fed 50 U/ml human rIL-2 and irradiated autologous PBMC prepulsed with the wild-type BCGa p84-100 on a weekly basis.

ASSESSMENT OF T-CELL RESPONSES

Antigen-induced proliferation of the T cell clones were assayed by culturing the T cells (3×10^4/well) in 96-well flat-bottomed culture plates in the presence of a peptide(s) and irradiated PBMC (1.5×10^5/well), using RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% heat-inactivated autologous plasma. For the proliferation assay, cells were cultured for 72 hr in the presence of 1 μCi/well of[^{3}H]thymidine during the final 16 hrs. Culture supernatants collected immediately before the addition of[^{3}H]thymidine were used to determine lymphokine concentrations, using hGM-CSF ELISA kits (R&D systems) and hIFN-γ ELISA kits (Otsuka,Tokyo,Japan).

DETERMINATION OF TCRA GENE USAGE BY T CELL CLONES

To determine TCRVA gene usage of the T cell clones that were cultured with irradiated autologous PBMC, RNA were extracted from the cell mixture, and converted to cDNA. TCRA variable region cDNA were amplified with anchored PCR as described previously for amplification of TCRA variable region cDNA.[9] A primary PCR was followed by two sequential nested PCR. TCRAC-specific primers used for primary PCR, nested PCR, and final PCR were CA4 (5'-CAG AAT CCT TAC TTT GTG AC), CA3 (5'-ATC GGT GAA TAG GCA GAC AG), and biotinylated CA5 (5'- CAC

Table 1 A panel of labeled TCRAV-specific oligonucleotide probes

AV gene	sequence	pool
AV4, 20	TGCTAAGACCACA/CCAGCC	A
AV11	TCTTCAGAGGGAGCTGTG	A
AV2	ATCCTTGAGAGTTTTACT	B
AV8a	CCATTCGAGCTGTATTTA	B
AV8b	GCATTCGAGCTTTATTTA	B
AV15	CATTTGCTGGATTTTCGT	B
AV17	GATCTTAGGAGCATCATT	B
AV21	TGGGGGCATCAGTGCTGA	B
AV3	GAGAAGAGGATCCTCAGG	C
AV5	ACTATTCTCCAGCATACT	C
AV10	CCGTGTCCATTCTTTGGA	C
AV13	GAGAGGAATACAAGTGGA	C
AV19	CAATTTTTGTTGGCTATT	C
AV24	AGCATCTGACGACCTTCT	C
AV25	TCCTTGAACATTTATTAA	C
AV26	CCTAGGGATATTGGGGTT	C
AV27	GAAAAAACTATACCATCT	C
AV29	CAGGCACCTTGTTGTGGC	C
AV32	ACTCATCACATCAATGTT	C
AV18	CTTTGGCAGCCCCATTAC	D
AV23	GAGACCCTCTTGGGCCTG	D
AV28	ACTAACTTTCGAAGCCTA	D
AV30	GGAGTGTGCATTCATAGT	D
AV7	GGAGGCACTA/GCAGGACAA	E
AV6	ACAGCTTCACTGTGGCTA	F
AV12	TGCCAGCCTGTTGAGGGC	F
AV14	GTGA/GTCTCCACCTGTCTT	F
AV1a	CTCCTGTTGCTCATACCA	G
AV1b	CTCCTGCTGCTCGTCCCA	G
AV1c	CTCCTGGAGCTTATCCCA	G
AV9	AAGCCCACCCTCATCTCA	G
AV16	GCCTCTGCACCCATCTCG	G
AV22	CTGATACTCTTACTGCTT	G
AV31	CCTCTCTGGACTTTCTAA	G

Oligomucleotide probes specific to TCRAV family genes. Degenerated probes were used to specify AV4 and AV20, AV7, and AV14 families. Three probes for AV1 family, and two probes for AV8 family were required to specify all members of each family. These probes were grouped into seven pools (pool A to G) depending on sequence similarity.

Table 2 TCRVA and TCRVB usage of BC clones

BC clone	TCRVA	TCRVB
20.7	(AV25S1)FCAGHNAG(AJ14S3)	(BV12S3)CASRQAGTAYE(BJ2S7)
33.5	(AV3S1)FCATERGQ(AJ13S2)	(BV6S1A1)CASSPTGTANT(BJ1S1)
42.1	(AV8S1A1)FCAASLDNY(AJ126)	(BV5S1A1)CASRRSTGE(BJ2S2)

TCRVA and VB usage are shown, with amino acid sequences in the N(D)N region.

TGG ATT TAG AGT CTC TC), respectively. A panel of labeled TCRAV-specific oligonucleotide probes (Table 1) were used to study TCRAV gene usage with PCR-ELISA.[10] First, seven pools of the AV-specific probes were hybridized with immobilized PCR products in microplates to find out positive wells. Then, the products were hybridized with individual AV probes in another set of plates to pin-point the AV genes predominantly used by the cDNA. To clone the entire variable region cDNA, cDNA were amplified with CA4 and reamplified with a nested primer, CA2 (5'-ACG CGT CGA CAC TGG ATT TAG AGT CTC TC). The products were subcloned into pBluscript II SK+ (Stratagene, La Jolla), and recombinant clones with the dominant VA gene were selected with dot blot DNA hybridization using corresponding VA-specific oligonucleotides. After sequence determination of these clones, dominant clones were selected as cDNA for the T cell clones.

RESULTS

TCRVA AND VB SEQUENCES

TCRVA and VB sequences of three T-cell clones BC 20.7, BC33.5 and BC42.1 are shown in Table 2. The N (D)N region sequences are shown as one-letter codes for amino acids, between V and J segments in parentheses. As described in our earlier studies, these T-cell clones recognize BCGa p84-100 (EEYLILSARD-VLAVVSK ; with first anchor underlined), in the context of HLA-DRB1*1405.[4] It is especially important to note that N(D)N region consists of 8 and 11 residues at TCRVA and VB of BC20.7 and BC33.5, respectively, whereas that of BC42.1 consists of 9 and 9 residues, repectively.

STIMULATORY ACTIVITIES OF BCGA P84-100-DERIVED ANALOG PEPTIDE L87V TO BC20.7

To evaluate the effects of single amino acid substitutions, proliferation and lymphokine production in response to analogue peptides were determined and findings were compared with those seen with the wild-type peptide. Most of the analogue peptides that stimulated BC clones showed a pattern of lymphokine production similar to that for the wild-type peptide (not shown). However, IFN-γ production of BC20.7 was increased in response to several analogue peptides at high concentration (16 μM), especially peptide L87V in which Leu is replaced by Val at the 87 th residue of the peptide BCGa p 84-100, whereas neither T cell proliferation nor production of other lymphokines, showed any remarkable change ; *i.e.*, only the production of IFN-γ was affected for recognition of the analog peptide L87V. As shown in Figure 1, to determine whether or not the change of IFN-γ production was due to differences in the HLA-peptide or TCR-TCR ligand avidity between L87V and the wild-type peptide, responses of BC20.7 to several different concentrations of L87V were compared with those of the wild-type peptide. In the range of concentrations from 0.016 μM up to 16 μM, IFN-γ production in response to L87V constantly exceeded that of the wild-type peptide. Moreover, the plateau level of L87 V-driven IFN-γ production was significantly higher. Mean IFN-γ production of BC20.7 for L87V increased significantly in comparison to the wild-type, whereas no statistical differences were noted in proliferative responses between R21K and the wild-type at a range of 0.16 μM to 16 μM. The IL-4 production of BC20.7 for each analogue peptide was proportional to the proliferative response to each peptide, at a range of 0.0016 to 16 μM (not shown). In contrast, production of GM-CSF gradually increased, in a dose-dependent manner throughout the range of 0.016 to 16 μM. These data indicate that the plateau responses and proliferation of IFN-γ are not due to saturation of the TCR ligand on the APC surface.

STIMULATORY ACTIVITIES OF BCGA P84-100-DERIVED ANALOGUES TO THREE BC CLONES

All three T-cell clones were stimulated with analogues at 16 μM, with replacements at P1 (=86Y) through P9 (=94V). Table 3 summarizes the results, regarding proliferative responses and IFN-γ production. P1 (=86Y) replaced by Ala (A) indicates a peptide species EEALILSARDVLAVVSK. Relative IFN-γ responses are shown, where IFN-γ production was divided by proliferation. P1 replaced by A gave values of 96/100/98, indicating that BC20.7,BC33.5 and BC 42.1 exhibited 96%, 100% and 98% responses respectively, as compared with the wild-type. Asterisks indicate peptide species that did not exert full agonistic activity ; *i.e.*, peptide stimulation even at 16 μM did not give a plateau response.

Most of analogues that exhibited full agonistic activity, stimulated IFN-γ production at levels roughly similar to the wild-type peptide, *i.e.*, at around 100%. However, it is important to note that L87T,L87S,L87A, and L87V significantly ($p<0.01$) induced increased levels of IFN-γ production of BC20.7 and BC33.5, but not of BC42.1. Such a clone-specific phenomenon was

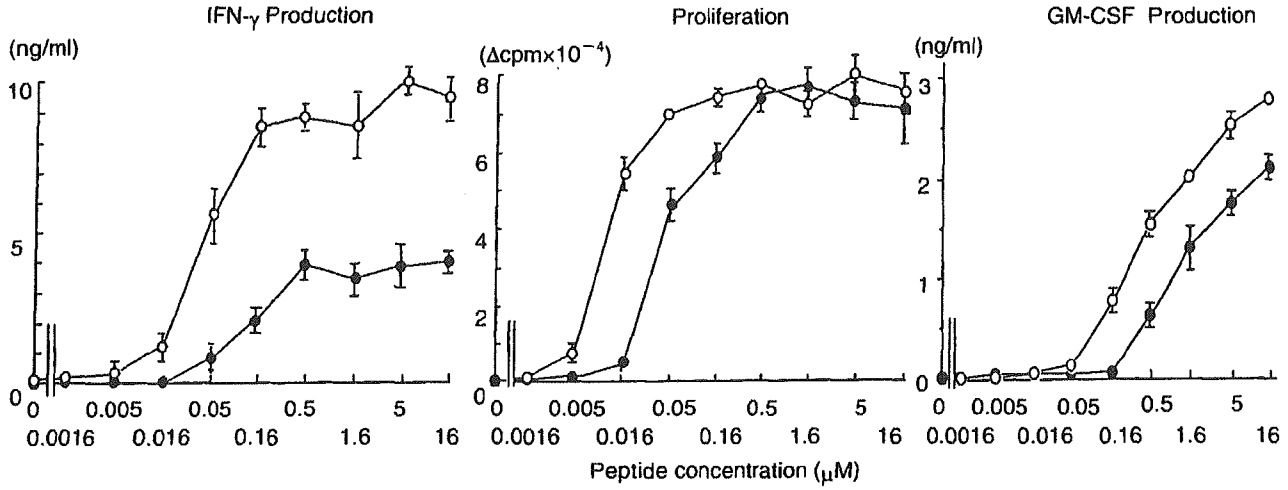

Fig. 1 IFN-γ production, GM-CSF production and proliferation of BC20.7 in recognition of either the wild-type peptide or L87V, at different concentrations. BC20.7 cells were cultured in triplicate with peptides and irradiated autologous PBMC, at the indicated concentrations. After 48-h incubation, supernatant fluids of triplicate cultures were collected. The remaining cells were pulsed with [^{3}H]-thymidine, harvested after 16h, and subjected to liquid scintillation counting. Closed circle, wild type peptide; open circle, L87V. Results are expressed as the geometric means ± standard error. IFN-γ production induced by L87V was significantly (p<0.01) higher than that induced by the wild-type peptide, at peptide concentrations ranging from 0.016 to 16 μM. On the other hand, plateau level of proliferation did not exhibit a significant difference, between 0.16 and 16 μM (p>0.05). GM-CSF production did not reach a plateau response even at 16 μM, without any statistical difference between L87V and the wild-type peptide, at 16 μM.

also observed when P5- and P8-substituted analogues were tested. Thus, S90E,S90G,S90M,D93Q,D93T and D93Y exhibited full agonism, in a clone-specific manner.

DISCUSSION

It is not very easy to identify TCR genes used by T cell clones, since they are usually cultured with irradiated autologous PBMC that includes polyclonal T cells. Random cloning of TCR cDNA derived from the cultured cells is minimally helpful in the identification, unless a large number of clones are examined. This problem was circumvented by the use of PCR-ELISA that was developed for TCRBV use,[9] and established in the present report for TCRVA usage. This technique allowed us to quantitate TCRV gene usage in the cDNA samples, and thus to identify the TCRV gene used by the T cell clones.

Three T-cell clones used in the present study recognize the same TCR ligand, as proven in our previous study. This is based on the fact that these clones recognize BCGa p84-100(^{84}EEYLILSARDVLAVVSK^{100}) in the context of DRB1*1405, and react to truncated peptides in a similar fashion.[11] Both BC20.7 and BC33.5 have 8 and 11 residues at N(D)N region of TCRVA and VB, respectively, whereas BC42.1 alone exhibits a different pattern, *i.e.*, 9 residues at N(D)N regions of TCRVA and VB. When peptide antigen is presented by class II MHC molecules, the N-terminal half of antigenic peptide is recognized mainly by CDR3 of TCRVA, whereas the C-terminal half is recoginized by CDR3 of TCRVB, which corresponds to N(D)N regions.[12] Interestingly, certain amino acid replacements on P2 induced increased IFN-γ production in BC20.7 and BC33.5 but not in BC42.1 cells, whereas those on P8 exhibited full agonism in BC 42.1 cells alone. It is thus likely that structural features of VACDR3 and VBCDR3 are responsible for specific responses induced by P2 and P8 analogues, respectively. Shuffling of N(D)N sequences between BC 42.1 and BC 20.7, or between BC 42.1 and BC 33.5 is underway to address this point.

Only L87T,L87S,L87A,and L87V induced IFN-γ enhancement. These arrangements are either smaller hydrophobic (A and V), or structurally similar neutral amino acids (T and S), indicating that close contact between P2 and TCRVA is taking place. Indeed, such a phenomenon is also seen in B-cell somatic hypermuation.[13] Thus, B-cell V region mutation in immunoglobulin heavy chain genes shows higher affinity than the germ-line sequence, usually associated with Gly, Ala, Val, Ser, Thr, or Cys, *i.e.*, small hydrophobic or small neutral residues. Apparently these mutations are not associated with static charges, but can affect either hydrogen bonding, van der Waar's force, or hydrophobic interactions.

In our previous studies using cedar pollen-derived peptides, T to V replacement on P2 also induced IFN-

Table 3 Increased IFN-γ production induced by peptide analogues

Replaced by	Relative IFN-γ response (BC20.7 / BC33.5 / BC42.1)								
	P1 =86Y	P2 =87L	P3 =88I	P4 =89L	P5 =90S	P6 =91A	P7 =92R	P8 =93D	P9 =94V
K	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	108/115/90	*/*/*	*/*/*
E	*/*/*	*/*/*	*/*/*	*/*/*	*/105/94	*/*/*	*/*/*	*/*/*	*/*/*
Q	*/*/*	88/79/90	*/*/*	92/79/81	*/*/*	*/*/*	*/*/*	*/*/87	*/*/*
N	*/*/*	105/97/95	*/*/*	88/92/97	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*
T	*/*/*	177/210/86	110/92/81	*/*/*	77/97/108	*/*/*	*/*/*	*/*/95	85/96/91
S	*/*/*	155/187/90	95/95/99	*/*/*	100/100/100	81/87/97	*/*/*	*/*/*	93/75/99
G	*/*/*	110/98/79	90/100/92	*/*/*	*/*/115	77/69/93	*/*/*	*/*/*	94/99/100
A	96/100/98	189/202/94	105/94/83	107/93/83	88/104/110	100/100/100	*/*/*	*/*/*	80/81/92
V	91/91/85	271/259/92	91/84/86	105/96/86	99/100/101	90/76/85	*/*/*	*/*/*	100/100/100
L	93/88/102	100/100/100	100/90/101	100/100/100	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*
Y	100/100/100	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/98	*/*/*
M	89/93/91	*/*/*	96/99/103	89/70/85	*/91/*	*/*/*	*/*/*	*/*/*	*/*/*
W	90/103/109	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*

Positions 1 – 9 (P1 – P9) of BCGa p84-100 (EEYLILSARDVLAVVSK; with P1 underlined), was replaced by indicated amino acids. T cells were stimulated with peptide species at 16 μM. To obtain relative IFN-γ response values, plateau responses of IFN-γ (pg/ml) were first divided by plateau responses of proliferation (cpm). Then, the following calculation was performed; relative IFN-γ responses=100×[IFN-γ/proliferation to analogues]/[IFN-γ/proliferation to the wild-type BCGa p84-100]. The denominator was 0.0533. *Peptide that did not induce fully agonistic proliferation.

γ enhancement, whereas proliferation remained the same. Therefore, although not generalized, mutual replacement on G, A, V, L, S, or T at P2, tends to induce IFN-γ-specific enhancement. Such observations also have been reported in another study with different peptide species.[14] In this sense, analogue-induced clonal anergy is often observed, especially when residue replacement is made on P7 or P8.[11] Moreover, truncation of the C-terminal moiety of antigenic peptides, in general, exhibit TCR antagonism.[15] In other words, if a rule that applies to altered polyclonal novel responses induced by peptide analogues is established, it will lead us to novel therapeutic interventions using peptide analogues. Our observations on P2 replacement which is associated with increased IFN-γ production are imperative to furthering our understanding.

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A Mutation at Codon 516 in the *rpoB* Gene of *Mycobacterium leprae* Confers Resistance to Rifampin[1]

Liangfen Zhang, Masako Namisato, and Masanori Matsuoka[2]

ABSTRACT

A missense mutation at codon 516 in the *rpoB* gene of *Mycobacterium leprae* conferring rifampin resistance was confirmed by the correlation between sequencing results and mouse footpad assay. The isolate was obtained from a relapsed lepromatous leprosy patient. This is the first report on the complete concordance between the mutation located at codon 516 in the *rpoB* gene and the corresponding resistance to rifampin in leprosy. The novel profile of mutation in the *rpoB* gene will contribute to the comprehensive understanding of rifampin resistant patterns and offer a useful tool for developing simple and rapid drug susceptibility testing approaches, which would promise more effective and successful control of leprosy.

RÉSUMÉ

Une mutation faux-sens localisée au codon 516 du gène *rpoB* de *Mycobacterium leprae* a permis l'expression d'une résistance à la rifampicine, qui a été confirmée par une corrélation entre les résultats du séquençage et le test d'inoculation à la patte de souris. L'isolat a été obtenu à partir d'un patient souffrant de lèpre lépromateuse et qui a rechuté. Ceci est le premier article rapportant une concordance complète entre la mutation localisée au codon 516 du gène *rpoB* et une résistance à la rifampicine dans le contexte de la lèpre. Ce nouvel éventail de mutation du gène *rpoB* va contribuer à une compréhension plus complète des alternatives de résistance à la rifampicine. Il devrait offrir un outil utile au développement d'approches pour le test simple et rapide de la résistance à la rifampicine, qui devrait résulter en un contrôle plus efficace et réussi de la lèpre.

RESUMEN

Una mutación sin sentido en el codón 516 del gene *rpo B* de *Mycobacterium leprae*, que le confiere resistencia a la rifampina, fue confirmada por correlación de los resultados de la secuenciación y del ensayo en la almohadilla plantar del ratón. La cepa fue obtenida de un caso de recaída de lepra lepromatosa. Este es el primer reporte sobre la concordancia perfecta entre la mutación localizada en el codón 516 del gene *rpo B* y la resistencia a la rifampina en la lepra. El nuevo perfil de mutación en el gene *rpo B*, aparte de que ayudará a entender los patrones de resistencia a la rifampina, constituye una nueva herramienta para el desarrollo de métodos simples y rápidos para probar la susceptibilidad de la bacteria a la droga, lo cual seguramente contribuirá al control exitoso y efectivo de la lepra.

Rifampin is a key component of multidrug therapy (MDT) suggested by the World Health Organization (WHO) in the treatment of leprosy ([19]). Resistance to rifampin often gives rise to treatment failure, which subsequently threatens the effective control of leprosy ([1, 8]). However, resistance to rifampin has been constantly documented since 1976, by using standard mouse footpad assay ([2, 4, 12, 13, 15, 18]). For this reason, it is suggested that clinically suspected relapsed leprosy cases and those who exhibit an unsatisfactory response to antileprosy therapy should be subjected to drug susceptibility testing. Understanding of drug resistance is essential for the effec-

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[2] L. Zhang, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan; M. Namisato, National Leprosarium Kuriu-Rakusen-En, Gunnma, Japan; M. Matsuoka, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan.

Reprint requests to: Masanori Matsuoka, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1, Aobacho, Higashimurayama-shi, Tokyo, 189-0002, Japan.

tive treatment and control of leprosy. Therefore, simple and rapid methods for drug resistance testing are necessary. To establish such methods, solid basic data for the correlations between mutation and phenotype of drug resistance are required. In the present study, an isolate obtained from a relapsed Japanese leprosy patient was investigated for drug susceptibility testing by both genetic analysis and the standard mouse footpad method. It has been assumed that mutations which cause rifampin resistance in *Mycobacteium tuberculosis* are almost the same as in *M. leprae*. Although it has been confirmed that mutations at codon 513, 526, 531, and 533 in the *rpoB* gene of *Mycobacterium leprae* confer rifampin resistance, no substantial evidence shows whether a mutation at codon 516 relates to rifampin resistance or not. The goal of this report was to confirm the missense mutation at codon 516 in the *rpoB* gene conferring rifampin resistance.

MATERIALS AND METHODS

***M. leprae* isolate.** The isolate, named as Kusatsu-6, was detected in a skin biopsy sample obtained from a 75-yr-old Japanese lepromatous leprosy male patient. The patient had been treated with dapsone monotherapy for 18 yrs, and then with rifampin alone for 10 yrs before another 14 yrs monotherapy of dapsone. The patient relapsed and, because he was considered likely to have taken his medicine irregularly and had had long-term monotherapy, he was suspected of harboring drug-resistant *M. leprae*.

Drug susceptibility testing in the mouse footpad. The biopsy specimen was processed to recover *M. leprae* in the same manner as previously described ([12]). The initial bacillary suspension containing 1.0×10^6 in 0.05 ml of Hank's balanced salt solution (HBSS) was inoculated into the hind footpads of BALB/c-*nu/nu* mice since the viability of the bacilli in the material treated with antileprosy drugs was unknown. Approximately 12 months after inoculation, bacterial suspension for drug susceptibility testing was prepared from the nude mice footpads, which had shown bacillary multiplication. Drug susceptibility testing, for dapsone, rifampin, ofloxacin, sparfloxacin, clofazimine, and clarithromycin was performed in the same manner as previously presented ([11, 12, 13]). Additionally, 0.08% minocycline ([3]) was added to the drug group for susceptibility testing in the present study. Bacillary growth in the mice footpads was examined after treatment with each drug for 25 weeks ([17]).

Genetic analysis for mutation. Sequencing was conducted as previously reported ([11]). Briefly, the initial biopsy suspension of Kusatsu-6 was partially purified by differential centrifugation and the pellet was resuspended in 50 µl of lysis buffer consisting of Proteinase K and Tween 20, then incubated at 60°C for 18 hr, followed by snap freeze-heating (–84°C for 30 min and then 98°C for 10 min) to extract the genomic DNA and inactivate proteinase K.

Primers with the following sequences were used: folP F 5′GCT TCT CGT GCC GAA GCG CTC3′ and folP R 5′GCC ATC GCG GGA TCT GCT CGC CCA3′; rpoB F0 5′CAG GAC GTC GAG GCG ATC AC3′and rpoB R0 5′CAG CGG TCA AGT ATT CGA TC3′; gyrA FN 5′CAG GTG ACG GTT CTA TAC AG3′ and gyrA RN 5′TAC CCG GCG AAC CGA AAT TG3′. These amplimers target a 388-bp fragment of the *folP* gene, a 382-bp fragment of the *rpoB* gene and a 342-bp of the *gyrA* gene in *M. leprae*, which contain mutations corresponding to dapsone-, rifampin-, and quinolone-resistance, respectively. DNA was amplified by G mixture of FailSafe PCR System (EPICENTRE, Madison, WI. U.S.A.), the amplified product was verified by electrophoresis and recovered by using MinElute Gel Extraction Kit (QIAGEN, GmbH, Germany). The sequencing reaction was performed by the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer Applied Biosystems, Norwalk, CT, U.S.A.). Direct sequencing of the PCR products was performed with the ABI Prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Norwalk). Sequencing data was analyzed by the DNASIS program (Hitachi Software Engineering, Yokohama, Japan), as presented elsewhere ([9, 11, 14]). The DNA sequence was compared with that in the GenBank database.

RESULTS

Drug susceptibility in the mouse. Bacillary growth in mouse footpads administered