multibacillary cases. Although there has been little standard monitoring of clinical outcomes and relapse rates, accurate diagnosis of relapse requires clinical, bacteriological and histopathological evidence.[70]

Rifampicin is an effective bactericidal agent against *M. leprae*. Within a few days of administrating a single 600-mg dose to multibacillary patients, the bacilli are no longer viable when inoculated into mouse footpads.[71] DDS is bacteriostatic or weakly bactericidal against *M. leprae* and was the mainstay leprosy treatment for many years until widespread resistant strains appeared. CLF binds preferentially to mycobacterial DNA and exerts a slow bactericidal effect on *M. leprae* by inhibiting mycobacterial growth. Skin discoloration ranging from red to black, is one of the most troublesome side-effects of CLF, although the pigmentation fades slowly in most cases after withdrawal. A characteristic ichthyosis is also some times evident. Other effective chemotherapeutic agents against *M. leprae* include ofloxacin (OFLX), minocycline (MINO), levofloxacin (LVFX), sparfloxacin (SPFX), moxifloxacin (MFLX) and clarithromycin (CAM).[72]

As with most chemotherapies, drug-resistant strains are becoming a problem in leprosy, which is a potential threat to the success of current leprosy control efforts. Dapsone resistance is associated with missense mutations in the *folP1* gene encoding dihydropteroate synthase.[73,74] Resistance to RFP is induced by a mutation in *rpoB*, which encodes DNA-dependent RNA polymerase subunit-b.[75] PCR analysis can provide a simple assessment for possible susceptibility to these drugs.[73,74]

LEPRA REACTIONS

Lepra reactions (or reactional states) are acute inflammatory complications that occur in treated or untreated leprosy and often present as medical emergencies. There are two major clinical types of lepra reactions that affect 30–50% of all leprosy patients.[76–78] Severe inflammation associated with these reactions results in nerve injury accompanied by subsequent loss of sensation, paralysis and deformity. The different types of reactions appear to have different underlying immunological mechanisms; however, the factors that initiate them are unknown.

Reversal reactions (type 1 reactions) manifest as erythema and edema of dermal lesions and tender peripheral nerves with rapid loss of nerve function. It generally occurs during the first several months of treatment, and occasionally after MDT is completed.[79,80] Treatment is aimed at controlling acute inflammation, easing pain.

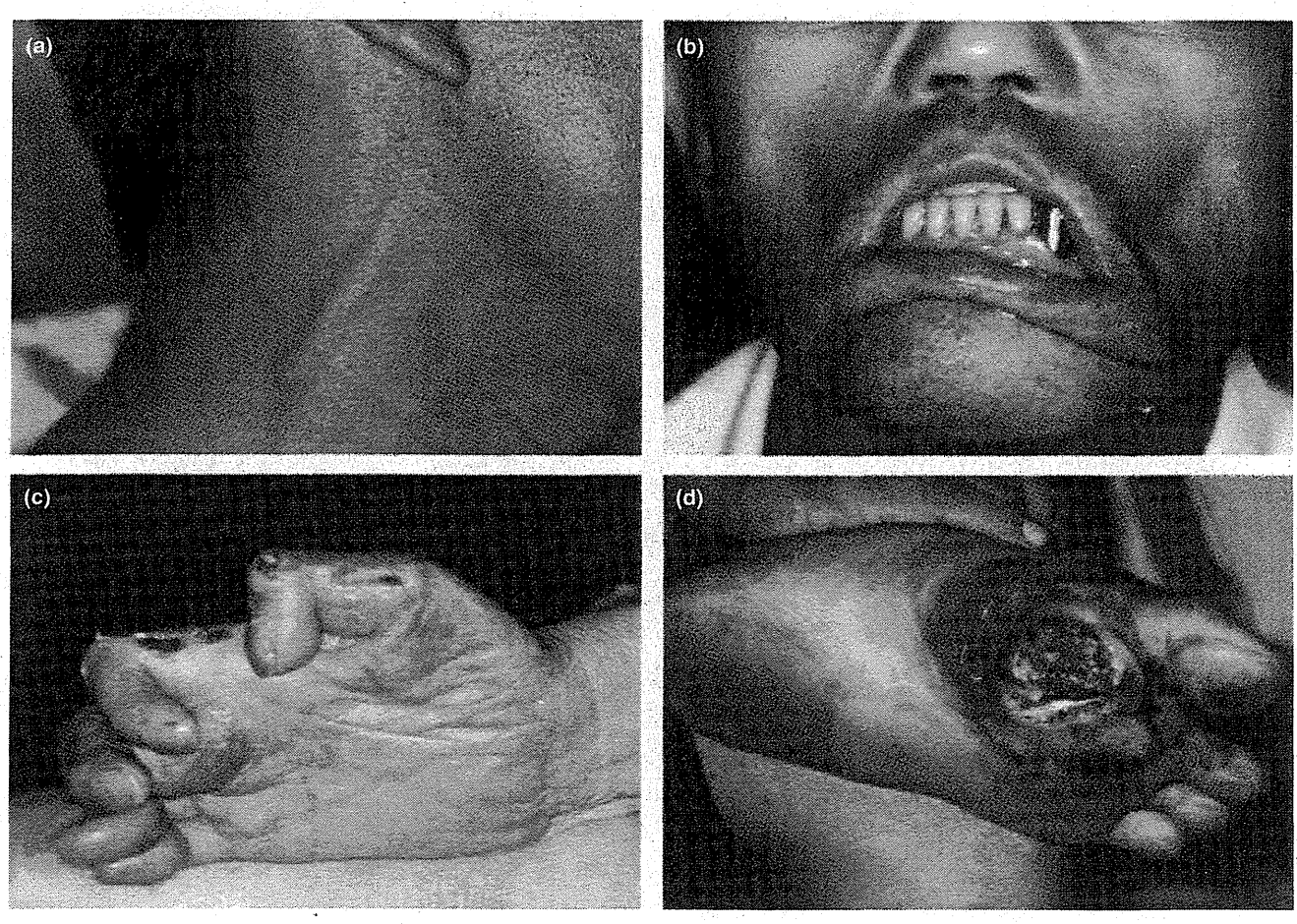

Figure 5. Leprosy with peripheral nerve damage. Swelling of the great auricular nerve (a), facial nerve paralysis (b), dropped wrist, clawed fingers with stiff joints due to ulnar and median nerve damage (c), and foot ulceration due to loss of sensation (d).

reversing nerve and eye damage, and reassuring the patient. Standard courses of corticosteroids have been used to treat patients for several weeks to months. Erythema nodosum leprosum (ENL or type 2 reactions) occurs in lepromatous and borderline lepromatous patients with higher bacterial loads in their lesions.[81] ENL can begin during the first or second year of treatment. Patients are febrile with skin nodules accompanied by iritis, neuritis, lymphadenitis, orchiitis, bone pain, dactylitis, arthritis, and proteinuria that is difficult to treat.[82] CLF has an anti-inflammatory effect on ENL, and thalidomide is better than steroids in controlling ENL, although thalidomide is not available in many countries because of its teratogenic effects.[83] The use of monoclonal antibodies or inhibitors of TNF-α, as used in rheumatoid arthritis, Crohn's disease and psoriasis, seems to be a logical choice for treatment, but more evidence is needed.[84]

DISABILITY AND STIGMA

Leprosy is a leading cause of permanent physical disability among communicable diseases. The disease and its associated deformities have been responsible for social stigmatization and discrimination against patients and their families in many societies. If unchecked, the disease gradually spreads over the entire body, attacks the soft tissue of the nose and throat, impairs vision and damages the nervous system. The morbidity and disability associated with leprosy are secondary to nerve damage (Fig. 5). Ultimately, the extremities become deformed and paralyzed, and may fall off after repeated but unperceived injuries. Therefore, timely diagnosis and treatment of the patient, before nerve damage has occurred, is extremely important in preventing disabilities. Management of lepra reactions and neuritis is also effective in preventing or minimizing the development of further disabilities.

The occurrence of leprosy in families has led to the misinterpretation that the disease is hereditary. The progressive symptoms and sometimes lethal secondary infections probably led to the assumption that patients are beyond medical support and that death is inevitable. In many societies, public stigmatization and exclusion coexist, and in some countries, the stigma is promoted by legislation against leprosy patients.[85] The accumulation of misnomers and misunderstandings have triggered unreasonable reactions in people, which have been difficult to overcome.

Self-awareness is crucial if the patient is to minimize damage. Treatment and/or surgical management, including reconstructions, should be provided for ulcers, and it is important that the patient understand the need for daily self-care and inspection for trauma.[86,87] Protective footwear and other tools are available to help patients improve their abilities and quality of life.[88] Community-based rehabilitation programs and other socioeconomic rehabilitation are required to support patients and families.[89]

CONCLUSIONS AND FUTURE PERSPECTIVES

Leprosy has affected humans for millennia. However, the MDT regimen recommended by the WHO has had a significant impact in reducing the global burden of leprosy, and research activities have led to increased knowledge of *M. leprae* genomic structure and host responses. Health-care workers and researchers should continue to support the intensive implementation of the elimination strategy and address issues related to the detection of *M. leprae*-infected individuals as a matter of urgency. Sustained quality patient care that is equitably distributed, affordable and easily accessible is still needed. A goal of the WHO is to bring institutional and management changes that strengthen the operational capacity of leprosy control programs. Improvement is needed in efforts to provide appropriate information to societies, dermatologists and patients. *M. leprae* is a very unique microorganism. It is expected that basic research for leprosy can be sustained.

REFERENCES

1 WHO. Global leprosy situation, 2010. *Wkly Epidemiol Rec* 2010; **85**: 337–348.
2 Saikawa K. The effect of rapid socio-economic development on the frequency of leprosy in a population. *Lepr Rev* 1981; **52** (Suppl 1): 167–175.
3 Cole ST, Eiglmeier K, Parkhill J *et al.* Massive gene decay in the leprosy bacillus. *Nature* 2001; **409**: 1007–1011.
4 Brosch R, Gordon SV, Eiglmeier K, Garnier T, Cole ST. Comparative genomics of the leprosy and tubercle bacilli. *Res Microbiol* 2000; **151**: 135–142.
5 Harrison PM, Gerstein M. Studying genomes through the aeons: protein families, pseudogenes and proteome evolution. *J Mol Biol* 2002; **318**: 1155–1174.
6 Liu Y, Harrison PM, Kunin V, Gerstein M. Comprehensive analysis of pseudogenes in prokaryotes: widespread gene decay and failure of putative horizontally transferred genes. *Genome Biol* 2004; **5**: R64.
7 Nakata N, Matsuoka M, Kashiwabara Y, Okada N, Sasakawa C. Nucleotide sequence of the *Mycobacterium leprae katG* region. *J Bacteriol* 1997; **179**: 3053–3057.
8 Madan Babu M. Did the loss of sigma factors initiate pseudogene accumulation in *M. leprae*? *Trends Microbiol* 2003; **11**: 59–61.
9 Cole ST, Supply P, Honoré N. Repetitive sequences in *Mycobacterium leprae* and their impact on genome plasticity. *Lepr Rev* 2001; **72**: 449–461.
10 Lawrence JG, Hendrix RW, Casjens S. Where are the pseudogenes in bacterial genomes? *Trends Microbiol* 2001; **9**: 535–540.
11 Vissa VD, Brennan PJ. The genome of *Mycobacterium leprae*: a minimal mycobacterial gene set. *Genome Biol* 2001; **2**: REVIEWS1023.
12 Eiglmeier K, Parkhill J, Honore N *et al.* The decaying genome of *Mycobacterium leprae*. *Lepr Rev* 2001; **72**: 387–398.
13 Akama T, Suzuki K, Tanigawa K *et al.* Whole-genome tiling array analysis of *Mycobacterium leprae* RNA reveals high expression of pseudogenes and noncoding regions. *J Bacteriol* 2009; **191**: 3321–3327.
14 Akama T, Tanigawa K, Kawashima A, Wu H, Ishii N, Suzuki K. Analysis of *Mycobacterium leprae* gene expression using DNA microarray. *Microb Pathog* 2010; **49**: 181–185.
15 Nakamura K, Akama T, Bang PD *et al.* Detection of RNA expression from pseudogenes and non-coding genomic regions of *Mycobacterium leprae*. *Microb Pathog* 2009; **47**: 183–187.
16 Suzuki K, Nakata N, Bang PD, Ishii N, Makino M. High-level expression of pseudogenes in *Mycobacterium leprae*. *FEMS Microbiol Lett* 2006; **259**: 208–214.
17 Monot M, Honore N, Baliere C *et al.* Are variable-number tandem repeats appropriate for genotyping *Mycobacterium leprae*? *J Clin Microbiol* 2008; **46**: 2291–2297.
18 Monot M, Honore N, Garnier T *et al.* On the origin of leprosy. *Science* 2005; **308**: 1040–1042.
19 Monot M, Honore N, Garnier T *et al.* Comparative genomic and phylogeographic analysis of *Mycobacterium leprae*. *Nat Genet* 2009; **41**: 1282–1289.

20 Han XY, Seo YH, Sizer KC *et al.* A new *Mycobacterium* species causing diffuse lepromatous leprosy. *Am J Clin Pathol* 2008; **130**: 856–864.
21 Matsuo E. Leprosy as a challenge to science on the ability to decode its enigma. A hypothesis on how to respond. *Jpn J Infect Dis* 2005; **58**: 59–64.
22 Alford PL, Lee DR, Binhazim AA, Hubbard GB, Matherne CM. Naturally acquired leprosy in two wild-born chimpanzees. *Lab Anim Sci* 1996; **46**: 341–346.
23 Gormus BJ, Wolf RH, Baskin GB *et al.* A second sooty mangabey monkey with naturally acquired leprosy: first reported possible monkey-to-monkey transmission. *Int J Lepr Other Mycobact Dis* 1988; **56**: 61–65.
24 Hubbard GB, Lee DR, Eichberg JW. Diseases and pathology of chimpanzees at the southwest foundation for biomedical research. *Am J Primatol* 1991; **24**: 273–282.
25 Hubbard GB, Lee DR, Eichberg JW, Gormus BJ, Xu K, Meyers WM. Spontaneous leprosy in a chimpanzee (Pan troglodytes). *Vet Pathol* 1991; **28**: 546–548.
26 Leininger JR, Donham KJ, Meyers WM. Leprosy in a chimpanzee. Postmortem lesions. *Int J Lepr Other Mycobact Dis* 1980; **48**: 414–421.
27 Leininger JR, Donham KJ, Rubino MJ. Leprosy in a chimpanzee. Morphology of the skin lesions and characterization of the organism. *Vet Pathol* 1978; **15**: 339–346.
28 Meyers WM, Gormus BJ, Walsh GP, Baskin GB, Hubbard GB. Naturally acquired and experimental leprosy in nonhuman primates. *Am J Trop Med Hyg* 1991; **44**: 24–27.
29 Meyers WM, Walsh GP, Brown HL *et al.* Leprosy in a mangabey monkey – naturally acquired infection. *Int J Lepr Other Mycobact Dis* 1985; **53**: 1–14.
30 Suzuki K, Udono T, Fujisawa M, Tanigawa K, Idani G, Ishii N. Infection during infancy and long incubation period of leprosy suggested in a case of a chimpanzee used for medical research. *J Clin Microbiol* 2010; **48**: 3432–3434.
31 Truman R. Leprosy in wild armadillos. *Lepr Rev* 2005; **76**: 198–208.
32 Walsh GP, Meyers WM, Binford CH, Gerone PJ, Wolf RH, Leininger JR. Leprosy – a zoonosis. *Lepr Rev* 1981; **52** (Suppl 1): 77–83.
33 Truman RW, Singh P, Sharma R *et al.* Probable zoonotic leprosy in the southern United States. *N Engl J Med* 2011; **364**: 1626–1633.
34 Davey TF, Rees RJ. The nasal discharge in leprosy: clinical and bacteriological aspects. *Lepr Rev* 1974; **45**: 121–134.
35 McDougall AC, Rees RJ, Weddell AG, Kanan MW. The histopathology of lepromatous leprosy in the nose. *J Pathol* 1975; **115**: 215–226.
36 Job CK, Jayakumar J, Kearney M, Gillis TP. Transmission of leprosy: a study of skin and nasal secretions of household contacts of leprosy patients using PCR. *Am J Trop Med Hyg* 2008; **78**: 518–521.
37 Krutzik SR, Ochoa MT, Sieling PA *et al.* Activation and regulation of Toll-like receptors 2 and 1 in human leprosy. *Nat Med* 2003; **9**: 525–532.
38 Tanigawa K, Suzuki K, Kimura H *et al.* Tryptophan aspartate-containing coat protein (CORO1A) suppresses Toll-like receptor signalling in *Mycobacterium leprae* infection. *Clin Exp Immunol* 2009; **156**: 495–501.
39 Ferrari G, Langen H, Naito M, Pieters J. A coat protein on phagosomes involved in the intracellular survival of mycobacteria. *Cell* 1999; **97**: 435–447.
40 Suzuki K, Takeshita F, Nakata N, Ishii N, Makino M. Localization of CORO1A in the macrophages containing *Mycobacterium leprae*. *Acta Histochem Cytochem* 2006; **39**: 107–112.
41 Barker LP. *Mycobacterium leprae* interactions with the host cell: recent advances. *Indian J Med Res* 2006; **123**: 748–759.
42 Chan J, Fujiwara T, Brennan P *et al.* Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. *Proc Natl Acad Sci USA* 1989; **86**: 2453–2457.
43 Tanigawa K, Suzuki K, Nakamura K *et al.* Expression of adipose differentiation-related protein (ADRP) and perilipin in macrophages infected with *Mycobacterium leprae*. *FEMS Microbiol Lett* 2008; **289**: 72–79.
44 Mattos KA, Lara FA, Oliveira VG *et al.* Modulation of lipid droplets by *Mycobacterium leprae* in Schwann cells: a putative mechanism for host lipid acquisition and bacterial survival in phagosomes. *Cell Microbiol* 2011; **13**: 259–273.
45 Bleharski JR, Li H, Meinken C *et al.* Use of genetic profiling in leprosy to discriminate clinical forms of the disease. *Science* 2003; **301**: 1527–1530.
46 Mira MT, Alcais A, Nguyen VT *et al.* Susceptibility to leprosy is associated with PARK2 and PACRG. *Nature* 2004; **427**: 636–640.
47 Santos AR, Suffys PN, Vanderborght PR *et al.* Role of tumor necrosis factor-alpha and interleukin-10 promoter gene polymorphisms in leprosy. *J Infect Dis* 2002; **186**: 1687–1691.
48 Croft RP, Richardus JH, Nicholls PG, Smith WC. Nerve function impairment in leprosy: design, methodology, and intake status of a prospective cohort study of 2664 new leprosy cases in Bangladesh (The Bangladesh Acute Nerve Damage Study). *Lepr Rev* 1999; **70**: 140–159.
49 Mahajan PM, Jogaikar DG, Mehta JM. A study of pure neuritic leprosy: clinical experience. *Indian J Lepr* 1996; **68**: 137–141.
50 Suneetha S, Arunthathi S, Chandi S, Kurian N, Chacko CJ. Histological studies in primary neuritic leprosy: changes in the apparently normal skin. *Lepr Rev* 1998; **69**: 351–357.
51 Van Brakel WH, de Soldenhoff R, McDougall AC. The allocation of leprosy patients into paucibacillary and multibacillary groups for multidrug therapy, taking into account the number of body areas affected by skin, or skin and nerve lesions. *Lepr Rev* 1992; **63**: 231–246.
52 Ridley DS, Jopling WH. Classification of leprosy according to immunity. A five-group system. *Int J Lepr Other Mycobact Dis* 1966; **34**: 255–273.
53 Ishikawa A, Ishikawa S, Hirakawa M. Osteoporosis, bone turnover and hypogonadism in elderly men with treated leprosy. *Lepr Rev* 2001; **72**: 322–329.
54 Boldsen JL. Leprosy in the early medieval Lauchheim community. *Am J Phys Anthropol* 2008; **135**: 301–310.
55 Boldsen JL, Mollerup L. Outside St. Jorgen: leprosy in the medieval Danish city of Odense. *Am J Phys Anthropol* 2006; **130**: 344–351.
56 Aufderheide AC, Rodriguez-Martin C. *The Cambridge Encyclopedia of Human Paleopathology*. Cambridge University Press, Cambridge 1998.
57 Ortner DJ. *Identification of Pathological Conditions in Human Skeletal Remains*, 2nd edn. Academic Press, London 2003.
58 Roberts C, Manchester K. *The Archaeology of Disease*, 3rd edn. Cornell University Press, New York 2007.
59 Suzuki K, Takigawa W, Tanigawa K *et al.* Detection of *Mycobacterium leprae* DNA from archaeological skeletal remains in Japan using whole genome amplification and polymerase chain reaction. *PLoS ONE* 2010: **5**: e12422.
60 Courtright P, Daniel E, Sundarrao *et al.* Eye disease in multibacillary leprosy patients at the time of their leprosy diagnosis: findings from the Longitudinal Study of Ocular Leprosy (LOSOL) in India, the Philippines and Ethiopia. *Lepr Rev* 2002; **73**: 225–238.
61 WHO. *Expert Committee on Leprosy*. 7th report 1998: 1–13.
62 Report of the International Leprosy Association Technical Forum. Paris, France, 22–28 February 2002. *Int J Lepr Other Mycobact Dis* 2002; **70**: S1–S62.
63 Sugita Y, Koseki M, Narita M, Kim S, Ishii N, Nakajima H. Suitability of a skin-smear examination needle for leprosy screening by PCR. *Int J Lepr Other Mycobact Dis* 1992; **60**: 658–659.
64 Buhrer-Sekula S. PGL-I leprosy serology. *Rev Soc Bras Med Trop* 2008; **41** (Suppl 2): 3–5.
65 Kampirapap K. Assessment of subclinical leprosy infection through the measurement of PGL-1 antibody levels in residents of a former leprosy colony in Thailand. *Lepr Rev* 2008; **79**: 315–319.
66 Mwanatambwe M, Yajima M, Etuaful S *et al.* Phenolic glycolipid-1 (PGL-1) in Buruli ulcer lesions. First demonstration by immuno-histochemistry. *Int J Lepr Other Mycobact Dis* 2002; **70**: 201–205.
67 Burki T. Old problems still mar fight against ancient disease. *Lancet* 2009; **373**: 287–288.
68 Nicholls PG, Ross L, Smith WC. Promoting early detection in leprosy – a literature review to identify proven and potential interventions addressing patient-related delay. *Lepr Rev* 2006; **77**: 298–310.
69 WHO Technical Advisory Group on Leprosy Control. *Report of the Tenth Meeting of the WHO Technical Advisory Group on Leprosy Control*. World Health Organization Regional Office for South-East Asia, New Delhi 2009.
70 Kaimal S, Thappa DM. Relapse in leprosy. *Indian J Dermatol Venereol Leprol* 2009; **75**: 126–135.

71 Levy L, Moon N, Murray LP, O'Neill SM, Gustafson LE, Evans MJ. Studies of the mouse foot pad technic for cultivation of Mycobacterium leprae. 1. Fate of inoculated organisms. *Int J Lepr Other Mycobact Dis* 1974; **42**: 165–173.
72 Ishii N, Sugita Y, Sato I, Nakajima H. Sparfloxacin in the treatment of leprosy patients. *Int J Dermatol* 1997; **36**: 619–621.
73 Kai M, Matsuoka M, Nakata N *et al.* Diaminodiphenylsulfone resistance of *Mycobacterium leprae* due to mutations in the dihydropteroate synthase gene. *FEMS Microbiol Lett* 1999; **177**: 231–235.
74 Williams DL, Spring L, Harris E, Roche P, Gillis TP. Dihydropteroate synthase of *Mycobacterium leprae* and dapsone resistance. *Antimicrob Agents Chemother* 2000; **44**: 1530–1537.
75 Honore N, Cole ST. Molecular basis of rifampin resistance in *Mycobacterium leprae*. *Antimicrob Agents Chemother* 1993; **37**: 414–418.
76 Becx-Bleumink M, Berhe D. Occurrence of reactions, their diagnosis and management in leprosy patients treated with multidrug therapy; experience in the leprosy control program of the All Africa Leprosy and Rehabilitation Training Center (ALERT) in Ethiopia. *Int J Lepr Other Mycobact Dis* 1992; **60**: 173–184.
77 Kumar B, Dogra S, Kaur I. Epidemiological characteristics of leprosy reactions: 15 years experience from north India. *Int J Lepr Other Mycobact Dis* 2004; **72**: 125–133.
78 Scollard DM, Smith T, Bhoopat L, Theetranont C, Rangdaeng S, Morens DM. Epidemiologic characteristics of leprosy reactions. *Int J Lepr Other Mycobact Dis* 1994; **62**: 559–567.
79 Britton WJ. The management of leprosy reversal reactions. *Lepr Rev* 1998; **69**: 225–234.
80 Croft RP, Nicholls PG, Richardus JH, Smith WC. Incidence rates of acute nerve function impairment in leprosy: a prospective cohort analysis after 24 months (The Bangladesh Acute Nerve Damage Study). *Lepr Rev* 2000; **71**: 18–33.
81 Manandhar R, LeMaster JW, Roche PW. Risk factors for erythema nodosum leprosum. *Int J Lepr Other Mycobact Dis* 1999; **67**: 270–278.
82 Lockwood DN. The management of erythema nodosum leprosum: current and future options. *Lepr Rev* 1996; **67**: 253–259.
83 Jakeman P, Smith WC. Thalidomide in leprosy reaction. *Lancet* 1994; **343**: 432–433.
84 Moreira AL, Kaplan G, Villahermosa LG *et al.* Comparison of pentoxifylline, thalidomide and prednisone in the treatment of ENL. *Int J Lepr Other Mycobact Dis* 1998; **66**: 61–65.
85 Senior K. Stigma, chemoprophylaxis, and leprosy control. *Lancet Infect Dis* 2009; **9**: 10.
86 Kazen RO. Management of plantar ulcers in leprosy. *Lepr Rev* 1999; **70**: 63–69.
87 Cross H, Newcombe L. An intensive self care training programme reduces admissions for the treatment of plantar ulcers. *Lepr Rev* 2001; **72**: 276–284.
88 Seboka G, Saunderson P, Currie H. Footwear for farmers affected by leprosy. *Lepr Rev* 1998; **69**: 182–183.
89 Nicholls PG. Guidelines for social and economic rehabilitation. *Lepr Rev* 2000; **71**: 422–465.

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Essential role of hormone-sensitive lipase (HSL) in the maintenance of lipid storage in *Mycobacterium leprae*-infected macrophages

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ABSTRACT

Mycobacterium leprae (M. leprae), the causative agent of leprosy, parasitizes within the foamy or enlarged phagosome of macrophages where rich lipids accumulate. Although the mechanisms for lipid accumulation in the phagosome have been clarified, it is still unclear how such large amounts of lipids escape degradation. To further explore underlying mechanisms involved in lipid catabolism in *M. leprae*-infected host cells, we examined the expression of hormone-sensitive lipase (HSL), a key enzyme in fatty acid mobilization and lipolysis, in human macrophage THP-1 cells. We found that infection by live *M. leprae* significantly suppressed HSL expression levels. This suppression was not observed with dead *M. leprae* or latex beads. Macrophage activation by peptidoglycan (PGN), the ligand for toll-like receptor 2 (TLR2), increased HSL expression; however, live *M. leprae* suppressed this increase. HSL expression was abolished in the slit-skin smear specimens from patients with lepromatous and borderline leprosy. In addition, the recovery of HSL expression was observed in patients who experienced a lepra reaction, which is a cell-mediated, delayed-type hypersensitivity immune response, or in patients who were successfully treated with multi-drug therapy. These results suggest that *M. leprae* suppresses lipid degradation through inhibition of HSL expression, and that the monitoring of HSL mRNA levels in slit-skin smear specimens may be a useful indicator of patient prognosis.

1. Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* (*M. leprae*). Although its prevalence has declined over the last several decades due to the introduction of multi-drug therapy (MDT), leprosy still remains a major public health problem in many developing countries. In 2009, 244,796 new cases were registered worldwide [1]. *M. leprae* is a typical intracellular pathogen that parasitizes tissue macrophages (histiocytes) and Schwann cells of the peripheral nerves of the dermis. In 1966 Ridley and Jopling used clinical, histological and immunological criteria to classify leprosy patients across the spectrum, and suggested five member groups: Tuberculoid (TT), Borderline Tuberculoid (BT), Borderline (BB), Borderline Lepromatous (BL) and Lepromatous (LL) [2]. Lepromatous leprosy is a stable condition (patient status does not shift from these polar positions), while borderline lepromatous leprosy is immunologically unstable. Lepromatous leprosy is characterized by widespread skin lesions that form due to an impaired cellular immune response. The lesions consist of numerous bacilli that live in the foamy or enlarged lipid-filled phagosome within macrophages. Although lipid-laden macrophages are observed in other mycobacterial infections, including tuberculosis [3,4], the amount of lipid and the number of infected macrophages are most prominent in cases of lepromatous leprosy.

The PAT protein family is named after perilipin, adipophilin/adipose differentiation-related protein (ADRP) and the tail-interacting protein of 47 kDa (TIP47). Members of the PAT family are responsible for lipid transportation and lipid droplet formation in a variety of tissues and cultured cell lines, including adipocytes [5–8]. We previously reported that ADRP and perilipin play important roles in lipid accumulation in *M. leprae*-infected macrophages [9]. ADRP and perilipin localized to the phagosomal membrane of histiocytes, which contained numerous *M. leprae*, in the skin lesions of patients with lepromatous leprosy. *M. leprae* infection increased mRNA and protein expression of ADRP and perilipin in cultured human THP-1 monocytes. The results suggested that ADRP and perilipin contribute to the creation of

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a lipid-rich environment that is favorable for *M. leprae* parasitization and survival in the host.

However, accumulated lipids are supposed to undergo degradation and reutilization by cells over time. In fact, fatty acids mobilized from stored triacylglycerols (TAG) are a major energy source in humans. Mobilization occurs through the consecutive action of three lipases: adipose triglyceride lipase (ATGL), monoacylglycerol lipase (MGL) and hormone-sensitive lipase (HSL) [10]. Among these, HSL was the first enzyme identified in the induction of lipo-catabolic action initiated by hormones and is the predominant lipase effector of catecholamine-stimulated lipolysis in adipocytes [11]. Therefore, ADRP/perilipin and HSL have opposing functions, *i.e.* lipid accumulation vs. its degradation. In addition to adipocytes, HSL is expressed in the cytoplasm of macrophages, pancreatic β cells, skeletal muscle cells, steroid producing cells, the intestine, and spleen [10]. HSL serine residues are phosphorylated by enzymes such as protein kinase A (PKA), 5′ AMP-activated protein kinase (AMPK) and mitogen-activated protein kinase (MAPK) to regulate the process of hormone-induced lipolysis [11].

To date, the molecular mechanism(s) that allows the phagosome of *M. leprae*-infected macrophages to escape lipolytic activities is not known. In this study, we investigate the expression and phosphorylation of HSL in *M. leprae*-infected cultured macrophages. We also examine clinical samples from leprosy patients and explore the impact of *M. leprae* on lipid metabolism in infected host cells.

2. Materials and methods

2.1. M. leprae isolation and cell culture

Hypertensive nude rats (SHR/NCrj-*rnu*), in which the Thai53 strain of *M. leprae* was actively grown [12,13], were kindly provided by Dr. Y. Yogi of the Leprosy Research Center, National Institute of Infectious Diseases, Japan. *M. leprae* was isolated as previously described [14,15]. The human premonocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC; Manassas, VA). The cells were cultured in 10 cm tissue culture dishes in RPMI medium supplemented with 10% charcoal-treated fetal bovine serum (FBS), 2% non-essential amino acids and 50 mg/ml penicillin/streptomycin at 37 °C in 5% CO_2 [9,16]. Typically, 3×10^7 bacilli were added to 3×10^6 THP-1 cells, for a multiplicity of infection (MOI) of 10. Peptidoglycan (PGN) and lipopolysaccharide (LPS) were purchased from Sigma (St Louis, MO) and added at final concentrations of 2 μg/ml and 1 μg/ml, respectively. TLR2 antibody (sc-21759; Santa Cruz Biotechnology, Santa Cruz, CA) was used at a final concentration of 5 μg/ml.

2.2. Immunohistochemistry and lipid staining

THP-1 cells were grown on glass coverslips in 24-well plates for 24 h before the culture medium was exchanged with RPMI 1640 containing *M. leprae*. Control and *M. leprae*-infected THP-1 cells were fixed in 10% paraformaldehyde for 10 min. They were then washed with Dulbecco's phosphate buffered saline (DPBS) containing 0.4% Triton-X 100 (DPBST), incubated with anti-HSL antibody (Cell Signaling Technology, Danvers, MA) diluted to 1:100 for 24 h at 4 °C and washed again with DPBST. The signal was detected using peroxidase-labeled streptavidin-biotin (LSAB2 Kit; DAKO, Carpinteria, CA) and 3,3-diaminobenzidine tetrahydrochloride (DAB) [9]. Cells were counterstained with methylene blue. Lipid staining was performed with oil red O (Muto Pure Chemicals, Tokyo, Japan) for 10 min, and counterstained with hematoxylin for another 5 min.

2.3. RNA preparation and RT-PCR

RNA from cultured cells was prepared using RNeasy Mini Kits (Qiagen Inc., Valencia, CA) as described previously [9,16]. RNA preparation from slit-skin smear samples was performed as described [9]. Briefly, stainless steel blades (Feather Safety Razor Co., LTD, Osaka, Japan) used to obtain slit-skin smear specimens were rinsed in 1 ml of sterile 70% ethanol, then the tube was and centrifuged at 20,000 × g for 1 min at 4 °C. After removing the supernatant, RNA was purified with the same protocol used for cultured cells. RNA was eluted in 20 μl of elution buffer and treated with 0.1 U/μl of DNase I (TaKaRa Bio, Kyoto, Japan) at 37 °C for 60 min in order to degrade any contaminating genomic DNA. RNA concentration and purity were assessed using a NanoVue spectrophotometer (GE Healthcare, Little Chalfont, UK). Total RNA from each sample was reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) [9]. The following primers were used to amplify specific cDNAs: HSL: 5′-CTCCTCATGGCTCAACTCCTTCC-3′ (forward) and 5′-AGGGGTTCTTGACTATGGGTG-3′ (reverse); ADRP: 5′-TGTGGAGAAGACCAAGTCTGTG-3′ (forward) and 5′-GCTTCTGAACCAGATCAAATCC-3′ (reverse); and actin: 5′-AGCCATGTACGTAGCCATCC-3′ (forward) and 5′-TGTGGTGGTGAAGCTGTAGC-3′ (reverse). Touchdown PCR was performed using a PCR thermal cycler DICE (TaKaRa Bio) as previously described [9]. The products were analyzed by 2% agarose gel electrophoresis.

Slit-skin smear samples from leprosy patients were used according to the guidelines approved by the National Institute of Infectious Diseases, Tokyo, Japan.

2.4. Protein preparation and Western blot analysis

Cellular protein was extracted and analyzed as previously described [9,17]. Briefly, cells were lysed in a lysis buffer containing 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 0.1% NP40, 20% glycerol, and protease inhibitor cocktail (Complete Mini, Roche, Indianapolis, IN) for 1 h. After centrifugation, the supernatant was transferred and 10 μg of protein was used for analysis. Samples were heated in SDS sample loading buffer at 95 °C for 5 min and loaded on a polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA). The membrane was washed with PBST (PBS with 0.1% Tween 20), blocked in blocking buffer (PBST containing 5% nonfat milk) overnight, and then incubated with either anti-HSL, anti-phospho-HSL (Ser^{563}) or anti-phospho-HSL (Ser^{565}) antibody (Cell Signaling Technology, 1:2000 dilution). After washing with PBST, the membrane was incubated for 1 h with biotinylated donkey anti-rabbit antibody (GE Healthcare, 1:2000 dilution) and streptavidin-HRP (GE Healthcare, 1:10,000 dilution) according to the manufacturer's protocol. The signal was developed using ECL Plus Reagent (GE Healthcare).

3. Results

3.1. HSL expression is suppressed in macrophages infected with M. leprae

To confirm the possible relationship between lipid accumulation and HSL expression in macrophage, we infected *M. leprae* in THP-1 cells and performed oil red O staining and HSL and ADRP immunostaining. Lipid droplets were not evident in control THP-1 cells (Fig. 1A), but accumulation was clearly demonstrated in cells 24 h after *M. leprae* infection (Fig. 1B). ADRP expression, which contributes to lipid intake, was not evident in control cells, but was significantly increased following *M. leprae* infection as previously reported (Fig. 1C and D, respectively) [9]. Conversely, HSL expression

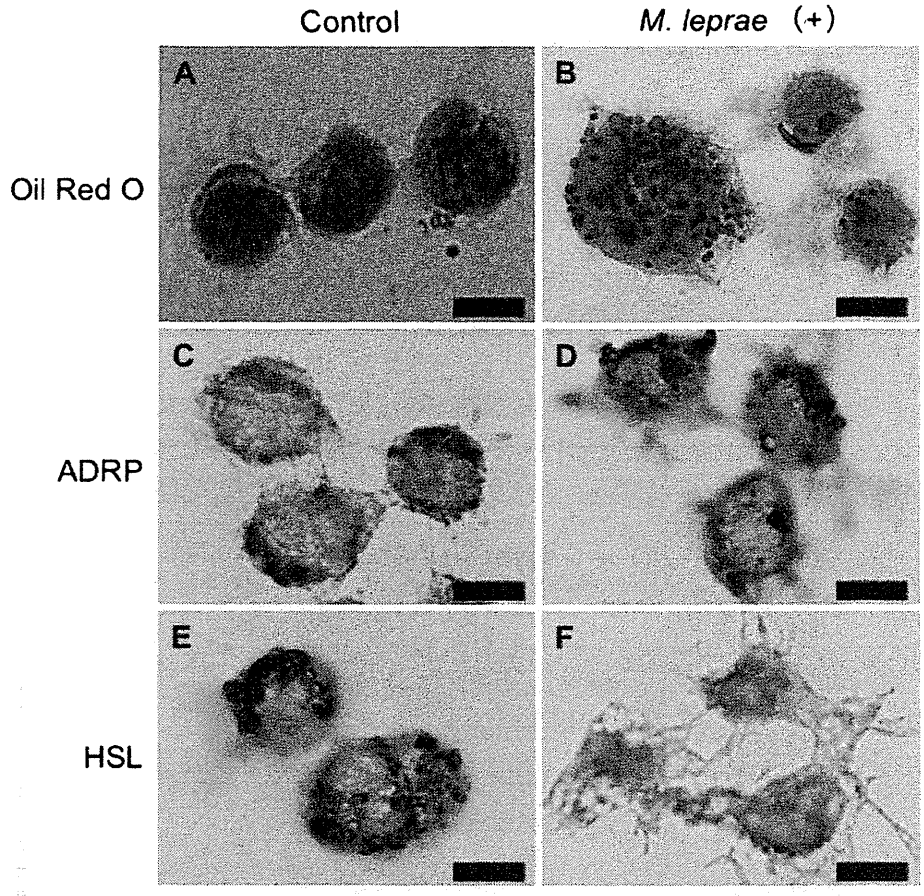

Fig. 1. HSL expression in macrophages is reduced by *M. leprae* infection. THP-1 cells grown on glass coverslips in 24-well plates for 24 h were used as a control or were infected with *M. leprae* for 24 h, then subjected to oil red O staining (A and B), ADRP immunostaining (C and D) or HSL immunostaining (E and F). Bars = 10 μm.

was clearly visible in the control THP-1 cells before infection, but it was significantly reduced by 24 h after *M. leprae* infection (Fig. 1E and F). Overall, oil red O staining was detected in 14.8% (9/61) of control THP-1 cells, whereas the percentage of *M. leprae*-infected cells increased to 91.7% (89/97). Similarly, only 16.9% (12/71) of cells were weakly immunostained with ADRP, but 88.2% (90/102) were strongly stained following infection. HSL-positive cells were observed in 80.3% (53/66) of control cells, but in only 7.2% (6/83) following *M. leprae* infection. These results suggested that the lipolytic pathway is constitutively activated in the control THP-1 cells, as evidenced by strong HSL staining; however, it was significantly suppressed by *M. leprae* infection, which in turn would reduce lipolysis in infected cells and maintain cellular lipids.

3.2. Only live *M. leprae* suppresses HSL expression

We next evaluated changes in HSL mRNA and protein levels in THP-1 cells following *M. leprae* infection. Reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that HSL mRNA levels were significantly decreased 6 h after *M. leprae* infection (Fig. 2A, left panel). HSL protein levels, as assessed by Western blot analysis, were also decreased by 6 h after infection (Fig. 2B, left panel). In both cases ADRP levels were increased as previously shown [9].

In order to clarify whether the observed decrease of HSL was specific to viable *M. leprae* or non-specific to phagocytosis, we compared the effects of live *M. leprae* with dead (heat-killed) *M. leprae* or latex beads on HSL expression. The mRNA and protein levels of HSL were transiently decreased at 6 h following exposure to dead *M. leprae* and latex beads, but they had mostly recovered to the original levels by 48 h (Fig. 2A and B, middle and right panels). In contrast, ADRP levels were transiently increased by dead *M. leprae* or latex beads at 6 h, but had returned to the original levels in 48 h. The transient effects of dead bacilli and the sustained effects of live bacilli on HSL suppression are similar to the effects of dead and live bacilli on the phagosomal localization of CORO1A [16,18]. Together, these results suggest that the phagocytosis of certain particles will transiently, but not permanently, decrease HSL expression; however, only live *M. leprae* was capable of maintaining the suppression of HSL expression (Fig. 2A and B).

Phosphorylation of HSL on Ser^{563} by PKA and Ser^{565} by 5′-AMP-activated protein kinase (AMPK) is required for the translocation and the functional activity of HSL [19,20]. Therefore, these serine residues were evaluated to determine if they are dephosphorylated following *M. leprae* infection. Western blot analysis using phosphorylation-specific antibodies revealed a rapid decrease in phosphorylation of HSL at both Ser^{563} and Ser^{565} 1 h after *M. leprae* infection (Fig. 2C). This result further confirmed that *M. leprae* infection not only reduces HSL expression, but potentially abrogates its function as well.

3.3. Innate immune activation increases HSL expression, but *M. leprae* infection reverses

The induction of innate immunity by activation of toll-like receptors (TLRs) modulates the expression of host proteins and

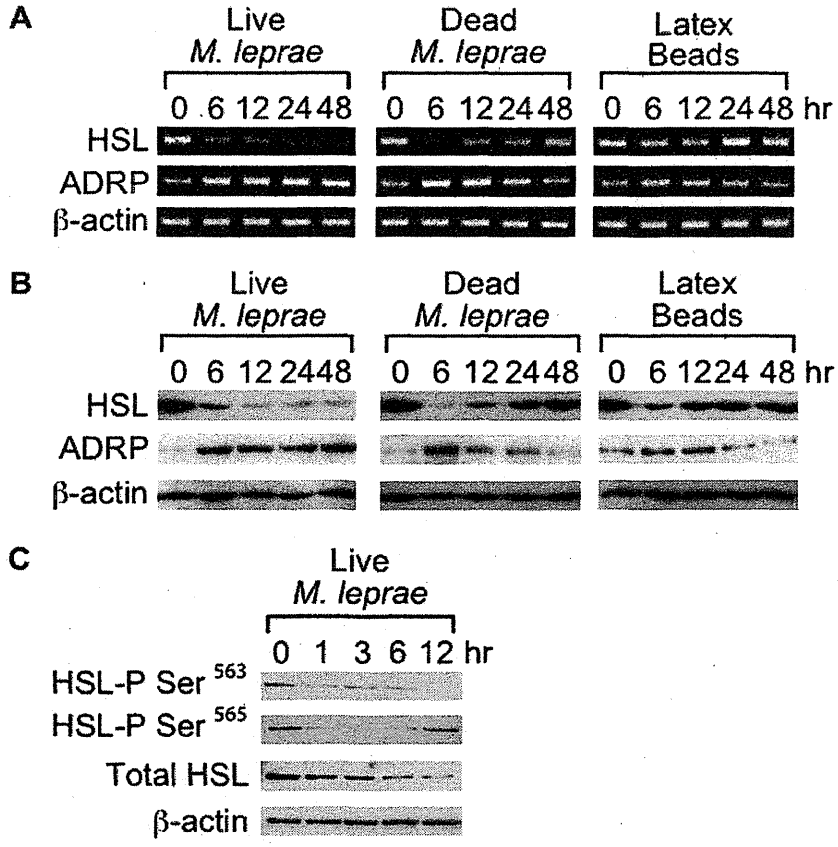

Fig. 2. Only live *M. leprae* persistently reduces HSL mRNA and protein levels in infected cells. THP-1 cells were cultured in a six-well plate and infected with live *M. leprae*, heat killed (80 °C for 30 min) *M. leprae* or latex beads. After incubating for the indicated period of time, total RNA and total cellular protein were purified and RT-PCR analysis (A) and Western blot analysis (B) for HSL, ADRP and β-actin were performed. Phosphorylation of HSL on Ser^{563} and Ser^{565} was evaluated by Western blot analysis in *M. leprae*-infected cells using specific antibodies (C).

contributes to host defense against *M. leprae* [9,16,17,21]. Therefore, the possible effect of PGN, a ligand for TLR2, on HSL expression levels was examined. When PGN was added to the culture medium of THP-1 cells, HSL mRNA expression was increased at 6 h and high levels of expression were maintained up to 24 h (Fig. 3A). HSL protein levels were also increased and high levels were still evident even 48 h after treatment (Fig. 3B). Since infection of live *M. leprae* significantly suppressed HSL expression (Fig. 2), we evaluated the possible effect of *M. leprae* infection on the effect of PGN. When *M. leprae* was added with PGN, the increase of PGN-induced HSL mRNA and protein levels observed at 6 h was abolished (Fig. 3C and D vs. Fig. 3A and B, respectively). Instead, HSL mRNA and protein levels had decreased by 48 h (Fig. 3C and D).

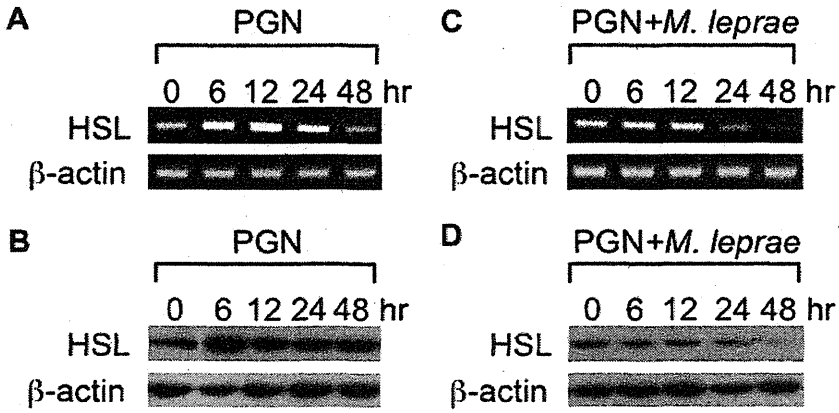

Fig. 3. *M. leprae* inhibits the ability of PGN to induce the expression of HSL. THP-1 cells were cultured in a six-well plate and treated with PGN (A and B), PGN plus *M. leprae* (C and D). After incubating for the indicated time, total RNA and total cellular protein for each experiment were purified and RT-PCR analysis (A and C) and Western blot analysis (B and D) for HSL and β-actin were performed.

TLR2 neutralizing antibody was able to suppress the PGN-mediated induction of HSL mRNA and protein levels (Fig. 4C vs. A and D vs. B, respectively), suggesting that the action of PGN is mediated through TLR2. Although activation of TLR4 by LPS enhanced HSL expression, *M. leprae* infection abolished LPS-induced HSL mRNA and protein levels (Fig. 4G vs. E and H vs. F, respectively). These results suggest that lipolysis is activated by TLR activation. However, infection of live *M. leprae* inhibits the TLR-mediated increase of HSL expression despite the fact that PGN is actually a component of the *M. leprae* cell wall. Therefore, *M. leprae* could potentially activate a hitherto unrecognized TLR-independent pathway that results in inhibition of TLR-mediated HSL activation in order to prevent the degradation of lipids in infected phagosomes.

3.4. Appearance of HSL mRNA in slit-skin smears correlates with clinical course of leprosy

All of the *in vitro* studies described herein indicated that *M. leprae* infection decreases HSL expression, which may correlate with the maintenance of a lipid-rich environment within the phagosome. To examine HSL expression in the skin lesions of leprosy patients, HSL mRNA levels were evaluated in slit-skin smear specimens by RT-PCR analysis. HSL mRNA was not detected in the five lepromatous leprosy (LL) patients nor in four out of seven borderline lepromatous leprosy (BL) patients, but was clearly detected in two of these patients (Fig. 5A, cases 8 and 12). Interestingly, these two cases, whose HSL mRNA levels were clearly detectable, exhibited a 'type 1 lepra reaction (or upgrading reaction)' after treatment (at one year for case 8 and three months for case 12), which is thought to be a cell-mediated, delayed-type of hypersensitivity immune response.

We also analyzed slit-skin smear samples from four patients who received MDT treatment, which consisted of diaphenylsulfone, clofazimine and rifampicin, as per WHO protocol. While HSL mRNA was not detected before treatment, HSL expression was induced (or recovered) after MDT treatment in all four cases (Fig. 5B). These results indicate that HSL expression is significantly suppressed following *M. leprae* infection in LL and BL patients; however, it reappeared in untreated patients who might have a potentially active immune response to *M. leprae* and in those whose bactericidal activity is enhanced by effective treatment.

4. Discussion

In this report, we first demonstrated that *M. leprae* suppresses the expression of HSL mRNA and protein in infected macrophages. Only live *M. leprae* could sustain suppressed levels of HSL, although phagocytosis itself only transiently decreased HSL levels. This situation is quite similar to the induction of lipid droplet-associated proteins, ADRP and perilipin, in macrophages infected with *M. leprae* as previously reported [9]. In that study, only live *M. leprae* infection could induce and maintain high expression levels of ADRP and perilipin. The present study provides another mechanism by

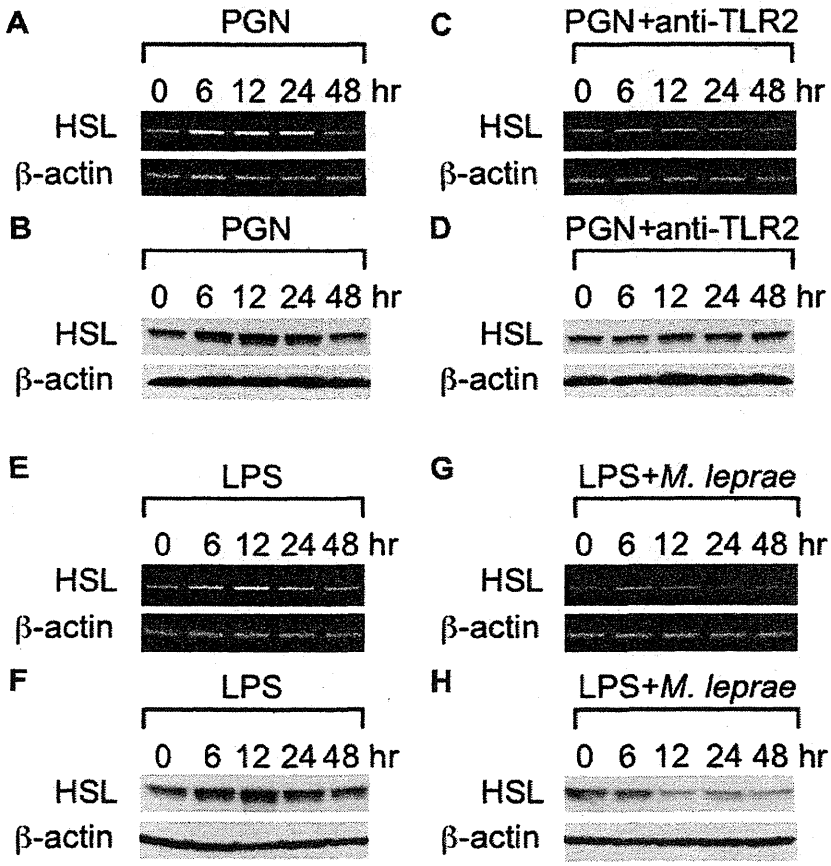

Fig. 4. TLR-mediated increase of HSL expression and its suppression by *M. leprae* infection. THP-1 cells were cultured in a six-well plate and treated with PGN (A and B), PGN plus TLR2 neutralizing antibody (C and D) or LPS (E and F) or LPS plus *M. leprae* (G and H). After incubating for the indicated time, mRNA expression or protein levels of HSL were evaluated by RT-PCR analysis (A, C, E and G) and Western blot analysis (B, D, F and H).

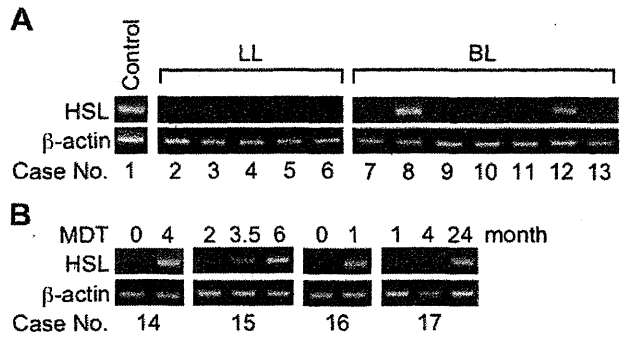

Fig. 5. Detection of HSL mRNA in slit-skin smear samples from leprosy patients. RNA was isolated from slit-skin smear specimens taken from five LL patients and seven BL patients for RT-PCR analysis (A). Cases 8 and 12 developed type 1 lepra reactions after MDT treatment. RNA isolated from four BL patients before and after MDT for the indicated period of time, was also used for RT-PCR analysis (B). The control sample was obtained from patients with skin granulomas where *M. leprae* was not found.

which *M. leprae* maintains host cell lipids, namely by suppressing their degradation.

Activation of the TLR signaling pathway by PGN increased HSL expression, indicating that activation of the innate immune response may induce lipid degradation that makes it difficult for *M. leprae* to survive within infected phagosomes. *M. leprae* infection not only suppressed HSL expression, but also inhibited the ability of PGN to increase HSL expression. We previously showed that PGN suppresses expression of ADRP and perilipin, and also significantly reduces expression of CORO1A, also known as tryptophan aspartate-containing coat protein (TACO), which contributes to the inhibition of lysosomal fusion and accounts for the survival of bacilli [9,16,17]. *M. leprae* infection invalidates all of these effects of PGN and thus maintains levels of CORO1A, ADRP and perilipin, and reduces HSL expression, thus ensuring a favorable phagosome environment for itself. These results showing that *M. leprae* and PGN, a cell wall component of *M. leprae*, have quite different effects are somewhat contradictory. It is plausible to speculate that some *M. leprae* components can activate a pathway that counteracts TLR signaling.

Suppression of HSL, in addition to the induction and phagosomal translocation of ADRP/perilipin and CORO1A, would reduce degradation of stored lipids, thereby maintaining the lipid-rich environment in the parasitized phagosome where *M. leprae* lives. *M. leprae* possesses only a small number of functional genes, which likely makes it difficult for the bacilli to survive without relying on host cell metabolism [14–16,22]. Therefore, *M. leprae* may regulate the expression of host genes that accumulate and maintain cellular lipids in order to utilize them as an essential nutrient for survival. Recent studies suggest that *Mycobacterium tuberculosis (M. tuberculosis)* persists within lipid-rich foamy phagosomes, while its translocation into the cytosol may relate to caseation and virulence [23–27]. Therefore, intracellular lifestyle and lipid requirements might differ substantially between *M. tuberculosis* and *M. leprae*, partly reflecting the massive gene decay in *M. leprae* [22]. Whether the small lipid droplets seen in *M. leprae*-infected cells (Fig. 1B) fuse with phagosomes containing *M. leprae* is still to be determined.

There was once debate over whether the lipids originate from *M. leprae* cell wall components or from the host [28,29]. It was suggested that lipids and fatty acids were important carbon sources for *M. leprae* in infected macrophages where the oxygen tension gradient is low [30]. It is now known that mycobacteria induce the accumulation of 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphorylcholine (PEIPC), a host-derived oxidized phospholipid, and is similar to the formation of foamy cells found in atherosclerotic lesions [31,32]. Of interest, *M. tuberculosis* has a large number of proteins involved in lipid metabolism, including at least one HSL family protein. However, *M. leprae* seems to have a small number of such genes, and no HSL-like genes were identified (http://genolist.pasteur.fr/Leproma/).

The lipid degradation process in adipocytes involves both HSL and perilipin [33]. Both are polyphosphorylated by protein kinase A (PKA), and phosphorylation of perilipin is required for the translocation of HSL from the cytosol to the surface of the lipid droplet, which is a critical step in the lipolytic reaction [34]. Furthermore, there is growing evidence that both perilipin and comparative gene identification-58 (CGI-58) protein act as scaffold proteins on lipid droplets in adipocytes [35]. We demonstrated that live *M. leprae* not only suppresses HSL protein expression, but that it also phosphorylates two serine residues, Ser^{563} and Ser^{565}, which are essential for its action. HSL expression is modulated by energy level changes in a variety of situations, such as obesity [36], type 2 diabetes mellitus [37] and in cultured adipocytes [38,39]. However, there have been no reports that pathogenic microorganisms have the ability to modulate HSL expression. Since *M. tuberculosis* and *Mycobacterium avium* utilize host lipids [40,41], our finding may highlight an important mechanism by which these bacteria interact and modify host gene expression. Phosphorylation of HSL occurs at multiple sites, including Ser^{563}, which is believed to be mutually exclusive with phosphorylation of HSL at the non-PKA site Ser^{565} [19,20]. Therefore, the observed decrease of HSL phosphorylation might be due to down-regulation of PKA or induction of a non-functional kinase following infection with *M. leprae*.

HSL expression was not detected in slit-skin smear samples from any of the LL patients and most of the BL patients examined in this study. Two BL patients who showed detectable levels of HSL mRNA developed a type 1 lepra (or reversal) reaction, which is thought to be a Th1-type cellular immune response and is characterized by an acute inflammatory reaction that causes worsening of skin lesions, neuritis and other systemic complications that occur in patients who are immunologically unstable [42]. Therefore, the increase in HSL expression detected in untreated BL patients is potentially activation of an immune reaction. The lepra reaction is one of the major problems faced by clinicians during treatment, and there is currently no method of predicting this critical side effect of treatment. Our observation suggests that detecting HSL mRNA in slit-skin smear samples from untreated patients could potentially be a reliable, convenient and minimally invasive procedure by which to predict possible occurrence of the type 1 lepra reaction. In addition, MDT treatment results in a rapid induction of HSL expression. Consequently, HSL expression levels after MDT treatment might also be a marker for treatment efficacy without the need for complicated tests to evaluate drug resistance of the bacilli. Although early diagnosis and appropriate treatment provide a complete cure, delays in diagnosis and treatment result in severe deformities and disabilities. Therefore, evaluation of HSL levels from slit-skin smear samples may be a simple and accurate method for clinical examination. In both cases, however, analysis of more clinical samples is needed to validate the clinical usefulness of this metric.

In conclusion, we have shown that *M. leprae* infection suppresses host HSL expression, which helps to retain the lipid-rich environment necessary for the survival of the pathogen within the phagosome. In addition, the measurement of HSL expression from slit-skin smears may be a useful diagnostic tool for patient prognosis.

Acknowledgments

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References

[1] Global leprosy situation. Wkly Epidemiol Rec 2010;85:337–48.
[2] Ridley DS, Jopling WH. Classification of leprosy according to immunity. a five-group system. Int J Lepr Other Mycobact Dis 1966;34:255–73.
[3] Cardona PJ, Llatjos R, Gordillo S, Diaz J, Ojanguren I, Ariza A, et al. Evolution of granulomas in lungs of mice infected aerogenically with *Mycobacterium tuberculosis*. Scand J Immunol 2000;52:156–63.
[4] Kondo E, Kanai K. Accumulation of cholesterol esters in macrophages incubated with mycobacteria in vitro. Jpn J Med Sci Biol 1976;29:123–37.
[5] Blanchette-Mackie EJ, Dwyer NK, Barber T, Coxey RA, Takeda T, Rondinone CM, et al. Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. J Lipid Res 1995;36:1211–26.
[6] Brasaemle DL, Barber T, Wolins NE, Serrero G, Blanchette-Mackie EJ, Londos C. Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. J Lipid Res 1997;38:2249–63.
[7] Miura S, Gan JW, Brzostowski J, Parisi MJ, Schultz CJ, Londos C, et al. Functional conservation for lipid storage droplet association among perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, Drosophila, and Dictyostelium. J Biol Chem 2002;277:32253–7.
[8] Wolins NE, Rubin B, Brasaemle DL. TIP47 associates with lipid droplets. J Biol Chem 2001;276:5101–8.
[9] Tanigawa K, Suzuki K, Nakamura K, Akama T, Kawashima A, Wu H, et al. Expression of adipose differentiation-related protein (ADRP) and perilipin in macrophages infected with *Mycobacterium leprae*. FEMS Microbiol Lett 2008; 289:72–9.
[10] Zimmermann R, Lass A, Haemmerle G, Zechner R. Fate of fat: the role of adipose triglyceride lipase in lipolysis. Biochim Biophys Acta 2009;1791: 494–500.
[11] Garton AJ, Yeaman SJ. Identification and role of the basal phosphorylation site on hormone-sensitive lipase. Eur J Biochem 1990;191:245–50.
[12] Yogi Y, Banba T, Kobayashi M, Katoh H, Jahan N, Endoh M, et al. Leprosy in hypertensive nude rats (SHR/NCrj-*rnu*). Int J Lepr Other Mycobact Dis 1999; 67:435–45.
[13] Yogi Y, Endoh M, Banba T, Kobayashi M, Katoh H, Suzuki K, et al. Susceptibility to *Mycobacterium leprae* of congenic hypertensive nude rat (SHR/NCrj-*rnu*) and production of cytokine from the resident peritoneal macrophages. Jpn J Lep 2002;71:39–45.
[14] Akama T, Suzuki K, Tanigawa K, Kawashima A, Wu H, Nakata N, et al. Whole-genome tiling array analysis of *Mycobacterium leprae* RNA reveals high expression of pseudogenes and noncoding regions. J Bacteriol 2009;191: 3321–7.
[15] Akama T, Tanigawa K, Kawashima A, Wu H, Ishii N, Suzuki K. Analysis of *Mycobacterium leprae* gene expression using DNA microarray. Microb Pathog 2010;49:181–5.
[16] Suzuki K, Takeshita F, Nakata N, Ishii N, Makino M. Localization of CORO1A in the macrophages containing *Mycobacterium leprae*. Acta Histochem Cytochem 2006;39:107–12.
[17] Tanigawa K, Suzuki K, Kimura H, Takeshita F, Wu H, Akama T, et al. Tryptophan aspartate-containing coat protein (CORO1A) suppresses Toll-like receptor signalling in *Mycobacterium leprae* infection. Clin Exp Immunol 2009;156:495–501.
[18] Ferrari G, Langen H, Naito M, Pieters J. A coat protein on phagosomes involved in the intracellular survival of mycobacteria. Cell 1999;97:435–47.
[19] Su CL, Sztalryd C, Contreras JA, Holm C, Kimmel AR, Londos C. Mutational analysis of the hormone-sensitive lipase translocation reaction in adipocytes. J Biol Chem 2003;278:43615–9.
[20] Anthonsen MW, Ronnstrand L, Wernstedt C, Degerman E, Holm C. Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro. J Biol Chem 1998;273:215–21.
[21] Krutzik SR, Ochoa MT, Sieling PA, Uematsu S, Ng YW, Legaspi A, et al. Activation and regulation of Toll-like receptors 2 and 1 in human leprosy. Nat Med 2003;9:525–32.
[22] Cole ST, Eiglmeier K, Parkhill J, James KD, Thomson NR, Wheeler PR, et al. Massive gene decay in the leprosy bacillus. Nature 2001;409:1007–11.
[23] Hunter RL, Jagannath C, Actor JK. Pathology of postprimary tuberculosis in humans and mice: contradiction of long-held beliefs. Tuberculosis 2007;87: 267–78.
[24] Peyron P, Vaubourgeix J, Poquet Y, Levillain F, Botanch C, Bardou F, et al. Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for *M. tuberculosis* persistence. PLoS Pathogens 2008; 4:e1000204.
[25] van der Wel N, Hava D, Houben D, Fluitsma D, van Zon M, Pierson J, et al. *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. Cell 2007;129:1287–98.
[26] Kim MJ, Wainwright HC, Locketz M, Bekker LG, Walther GB, Dittrich C, et al. Caseation of human tuberculosis granulomas correlates with elevated host lipid metabolism. EMBO Mol Med 2010;2:258–74.
[27] Welin A, Lerm M. Inside or outside the phagosome? the controversy of the intracellular localization of *Mycobacterium tuberculosis*. Tuberculosis; 2011.
[28] Sakurai I, Skinsnes OK. Lipids in leprosy. 2. histochemistry of lipids in human leprosy. Int J Lepr Other Mycobact Dis 1970;38:389–403.
[29] Ridley MJ, Ridley DS, De Beer FC, Pepys MB. C-reactive protein and apoB containing lipoproteins are associated with *Mycobacterium leprae* in lesions of human leprosy. Clin Exp Immunol 1984;56:545–52.
[30] Chan J, Fujiwara T, Brennan P, McNeil M, Turco SJ, Sibille JC, et al. Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. Proc Natl Acad Sci U S A 1989;86:2453–7.
[31] Cruz D, Watson AD, Miller CS, Montoya D, Ochoa MT, Sieling PA, et al. Host-derived oxidized phospholipids and HDL regulate innate immunity in human leprosy. J Clin Invest 2008;118:2917–28.
[32] Navab M, Ananthramaiah GM, Reddy ST, Van Lenten BJ, Ansell BJ, Fonarow GC, et al. The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL. J Lipid Res 2004;45:993–1007.
[33] Ogasawara J, Nomura S, Rahman N, Sakurai T, Kizaki T, Izawa T, et al. Hormone-sensitive lipase is critical mediators of acute exercise-induced regulation of lipolysis in rat adipocytes. Biochem Biophys Res Commun 2010;400:134–9.
[34] Sztalryd C, Xu G, Dorward H, Tansey JT, Contreras JA, Kimmel AR, et al. Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. J Cell Biol 2003;161:1093–103.
[35] Subramanian V, Rothenberg A, Gomez C, Cohen AW, Garcia A, Bhattacharyya S, et al. Perilipin A mediates the reversible binding of CGI-58 to lipid droplets in 3T3-L1 adipocytes. J Biol Chem 2004;279:42062–71.
[36] Large V, Reynisdottir S, Langin D, Fredby K, Klannemark M, Holm C, et al. Decreased expression and function of adipocyte hormone-sensitive lipase in subcutaneous fat cells of obese subjects. J Lipid Res 1999;40:2059–66.
[37] Jocken JW, Langin D, Smit E, Saris WH, Valle C, Hul GB, et al. Adipose triglyceride lipase and hormone-sensitive lipase protein expression is decreased in the obese insulin-resistant state. J Clin Endocrinol Metab 2007; 92:2292–9.
[38] Kim JY, Tillison K, Lee JH, Rearick DA, Smas CM. The adipose tissue triglyceride lipase ATGL/PNPLA2 is downregulated by insulin and TNF-alpha in 3T3-L1 adipocytes and is a target for transactivation by PPARgamma. Am J Physiol Endocrinol Metab 2006;291:E115–27.
[39] Kralisch S, Klein J, Lossner U, Bluher M, Paschke R, Stumvoll M, et al. Isoproterenol, TNFalpha, and insulin downregulate adipose triglyceride lipase in 3T3-L1 adipocytes. Mol Cell Endocrinol 2005;240:43–9.
[40] Sano K, Tomioka H, Sato K, Sano C, Kawauchi H, Cai S, et al. Interaction of antimycobacterial drugs with the anti-*Mycobacterium avium* complex effects of antimicrobial effectors, reactive oxygen intermediates, reactive nitrogen intermediates, and free fatty acids produced by macrophages. Antimicrob Agents Chemother 2004;48:2132–9.
[41] Brzostek A, Pawelczyk J, Rumijowska-Galewicz A, Dziadek B, Dziadek J. *Mycobacterium tuberculosis* is able to accumulate and utilize cholesterol. J Bacteriol 2009;191:6584–91.
[42] Sasaki S, Takeshita F, Okuda K, Ishii N. *Mycobacterium leprae* and leprosy: a compendium. Microbiol Immunol 2001;45:729–36.

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REVIEW ARTICLE

Buruli ulcer and current situation in Japan: A new emerging cutaneous *Mycobacterium* infection

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ABSTRACT

Buruli ulcer (BU) is a new emerging disease and the third most common chronic mycobacterial infection in humans, caused by *Mycobacterium ulcerans*. Approximately 5000 cases are reported annually from at least 33 countries around the globe, but more from the tropical nations. A total of 32 cases have been reported from Japan sporadically since 1980. None of the cases were related to international travel. Of the total reported, *M. ulcerans* ssp. *shinshuense*, a subspecies speculated to be domestic to Japan or in Asia, has been isolated from 23 cases. The mode of transmission and its incubation period remain unclear, despite several proposed hypotheses, including several vectors and cutaneous wound as port of entry for the pathogen. *M. ulcerans* invades the skin, subcutaneous tissue, fascia and eventually forms extensive ulceration. Smear, culture, histopathology and polymerase chain reaction are established diagnostic tools to identify *M. ulcerans*. Multiple antimicrobial therapy is a commonly used therapeutic method, but patients often need extensive debridement and, at times, skin grafting, especially when diagnosis is delayed. Thus, expanding a system for improved awareness and diagnosis in Japan and Asia is important, together with elucidating the candidate vector and the mode of transmission. Here, to establish a base for future progress in better understanding of this infectious disease, we reviewed the characteristics of the disease together with an update of reported cases in Japan.

Key words: **Buruli ulcer, *Mycobacterium ulcerans*, *Mycobacterium ulcerans* ssp. *shinshuense*, mycolactone, non-tuberculous mycobacteria.**

INTRODUCTION

Buruli ulcer (BU) is a necrotizing skin and soft tissue infection caused by *Mycobacterium ulcerans*, categorized as a non-tuberculous mycobacteria (NTM). It is the third most common mycobacterial infection after tuberculosis and Hansen's disease (leprosy), and cases have been reported from at least 33 countries with the incidence rate highest in sub-Saharan Africa.[1] Despite its image as a disease confined to tropical areas, in recent decades, reports have also been made from sub-tropical and non-tropical nations including Australia, China and Japan.[1] In Japan, a total of 32 cases have been reported sporadically since 1980. Interestingly, it is now evident that pathogens isolated from Japanese and Chinese cases slightly differ from those of other countries.

The World Health Organization (WHO)[2] includes BU as a neglected tropical disease (NTD) primarily due to its disabling and stigmatizing complications, and is working toward better diagnosis, treatment and prevention. Moreover, research is promoted, for there are various issues still remaining to be uncovered including its vector, mode of transmission and pathogenesis.[2] The objectives of this article are to: (i) review the current state of knowledge of Buruli ulcer; (ii) summarize the 32 cases reported in Japan; and (iii) propose future perspectives how these cases and diagnostic network in Japan may contribute to the better understanding and control of BU worldwide.

EPIDEMIOLOGY AND TRANSMISSION

The first report of BU dates back to 1897 when Sir Albert Cook described cases of chronic ulceration in Uganda. It took approximately half a century for it to be recognized as a mycobacterial skin infection; MacCallum (Australia) made the first definitive description of *M. ulcerans* in 1948.[3] The disease was named after Buruli County, Uganda, where the first large epidemic was investigated in 1961.[4]

Since the early 1980s, this infection has been rapidly re-emerging along with rapid environmental such as deforestation, eutrophication, dam construction, irrigation, farming, mining and habitat fragmentation.[5] Presently, the disease is reported from various

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parts of the world, at a rate of over 5000 new cases per year.[1,2] However, diversity in the incidence rate according to regions and lack of awareness prevents accurate sketching of the demographic of BU.

Most cases of BU are found in people living in or around aquatic environments (e.g. wetland, river, reservoir). Therefore, though its vector and mode of transmission are still unidentified, it is hypothesized that *M. ulcerans* is acquired through environmental contact. There are a number of reports that have detected *M. ulcerans* DNA from environmental samples including water filtrates, soil, fish, turtles, frogs, snails and various insects.[6–14] A recent published report by Lavender *et al.*[15] provided some insights into the potential for mosquitoes to be involved in the transmission of the disease by testing mosquitoes for *M. ulcerans* DNA in an endemic area of southeastern Australia. The study revealed the infection rate per 1000 mosquitoes to be 1.86 (1.48–2.32) with the highest rate obtained from the location with the highest prevalence of the disease.[15] However, these studies tested only for DNA, and this does not provide definite proof for it to be the reservoirs or vectors of *M. ulcerans*. Recently, three new cases of BU were found simultaneously from a family in Japan: a mother and her son and daughter. Close investigation of these kinds of cases may lead to further understanding of the epidemiology of the disease.

EPIDEMIOLOGY AND CURRENT SITUATION OF BU IN JAPAN

The first case of BU in Japan was reported by Mikoshiba *et al.*[16] in 1982. It was a case of a 19-year-old woman who presented a chronic and necrotic ulcer on her left elbow. The case was considered to be an endemic infection, because she lacked history of international travel. Tsukamura *et al.*[17] reported that the mycobacterium obtained from this ulcer showed a close resemblance to *M. ulcerans*, but with some differences. Later, with further research, he advocated this novel subspecies as "*M. ulcerans* ssp. *shinshuense*" in 1989.[18]

After a 21-year window period, the second case of BU was reported in 2003. Since then, there has been a steady increase in reported cases, summing up to a total of 32 as of October 2011 (Fig. 1). Amongst these cases, *M. ulcerans* ssp. *shinshuense*, a subspecies speculated to be domestic to Japan or in Asia, has been isolated from 23 cases. Of the total, 12 cases (37.5%) were male and 20 cases (62.5%) were female. A tendency was towards middle-aged adults in our cases (Fig. 1). Our age distribution differs from that of other countries. Quek *et al.*[19] reported that in southeastern Australia, there were more cases in patients over 60 years of age, while Debacker *et al.*[20] reported that the age distribution in Benin reached its peaks in the 10–14-year age group and amongst those older than 59 years.

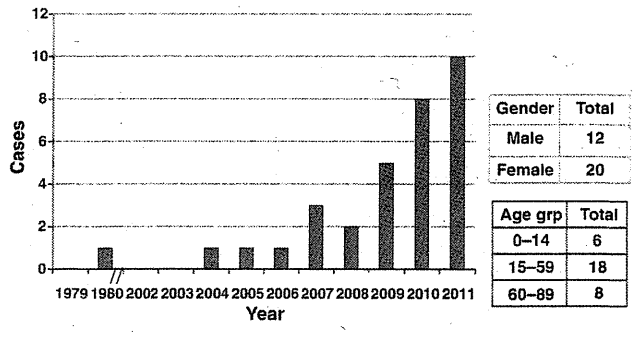

Figure 1. Buruli ulcer cases in Japan by year diagnosed.

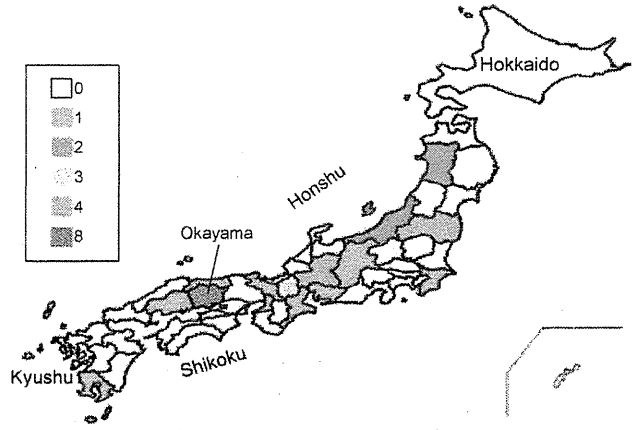

Figure 2. Distribution of Buruli ulcer cases in Japan: a total of 32 cases as of October 2011.

All but one case were reported from the main and largest island of Japan, Honshu (Fig. 2). More cases were found from the central western regions of Japan, especially from Okayama Prefecture where eight cases have been identified so far. This prefecture is facing the inland sea, Seto, and the climate is somewhat similar to the Mediterranean Sea, dry and moderate throughout the year. The adjacent prefecture of Hiroshima, also reports one case.

It is interesting to note that 25 cases (86.2%) were diagnosed during autumn and winter (Fig. 3). Interpretation of these statistics needs to be carefully assessed, for the incubation period of this infection is not known; however, it may be a clue to the seasonal inclination.

BACTERIOLOGY

Mycobacterium ulcerans is an NTM that may be cultured *in vitro* showing optimal but very slow growth at 28–34°C on the Löwenstein–Jensen (or Ogawa) medium for mycobacterial culture. This predilection for lower culture temperature explains the skin being its main foci of infection and its limited systemic dissemination. The colonies of *M. ulcerans* are usually yellowish, rough and have well-demarcated edges. The yellowish color may also be observed in the dark.[21] *M. ulcerans* produces a necrotizing immunosuppressive polyketide toxin, called mycolactone, that is responsible for its pathogenecity.[22] There are six structural variants to mycolactone: A, B, C, D, E and F. Most cases of BU are positive for mycolactone A/B (Fig. 4), while few cases present C or D.

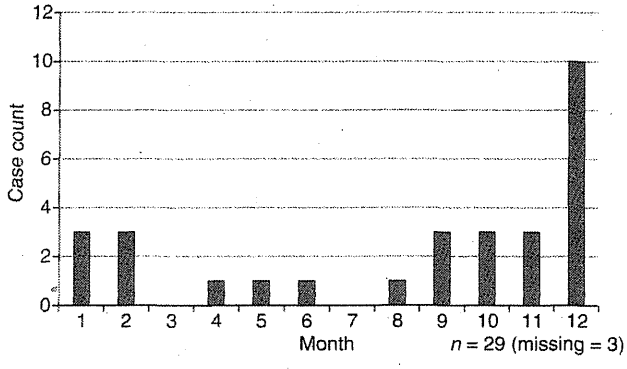

Figure 3. Month diagnosed with Buruli ulcer.

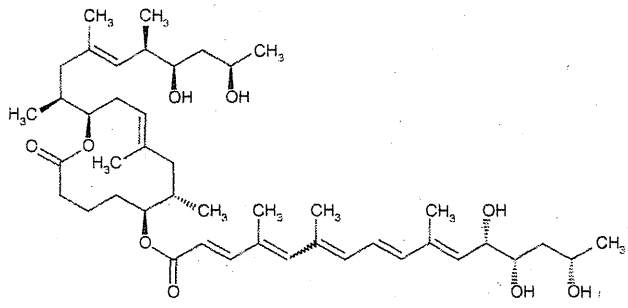

Figure 4. Mycolactone A/B.

PATHOGENESIS AND IMMUNOLOGY

Pathogenesis of *M. ulcerans* is closely related to the production of mycolactone. Mycolactone is a toxic lipid that is cytotoxic to fibroblasts, lipid cells, macrophages, and keratinocytes; inducing both apoptotic and necrotic changes in these cells. It is also known to suppress the local immune system.[22] These two major functions explain the extensive progression of the ulcer with relatively low inflammatory response, both clinically and histopathologically. It is also speculated that mycolactone damages the peripheral nerves, resulting in the ulcers being painless.[23]

CLINICAL MANIFESTATIONS

The common sites of the skin lesions are exposed parts of the body, particularly the extremities and the face. BU often starts as erythema or papule, which may resemble an insect bite (Fig. 5a). The lesion gradually develops into a painless nodule measuring a few centimeters in diameter (Fig. 5b). In a few days to several weeks, the papule or nodule perforates and forms an ulcer (Fig. 5c). The ulcer is often characterized by white or yellow necrotic tissue on the base, undermined borders and edematous surroundings. The lesion is not limited to a single focus, but when the ulcers are adjacent to each other, they may merge and form a massive ulcer. In rare cases, the ulcer invades the muscular layer.

Ulcers caused by *M. ulcerans* are often documented to be painless, unless secondary bacterial infection exists at the site. In

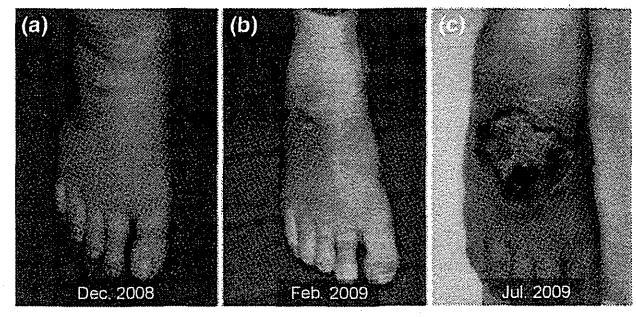

Figure 5. Clinical features. (a) Initial symptoms of Buruli ulcer. It often starts as erythema or papule. (b) The lesion gradually develops into painless nodule measuring few centimeters. The case in the photo is associated with redness and swelling. (c) In a few days to several weeks, the papule or nodule perforates and forms an ulcer. The ulcer is often characterized by white or yellow necrotic tissue on the base, undermined borders and edematous surroundings. Photos provided by Dr Tesshin Watanabe of Tottori University, Japan.

contrast, approximately half of the cases confirmed with *M. ulcerans* ssp. *shinshuense* in Japan are reported with pain. Swelling of the regional lymph node and fever are usually absent, and the host's general condition is often well. BU rarely causes direct death, but when not treated early, the disease often results in permanent functional disability. A massive ulcer that lies across the joint, without successful skin grafting and intensive rehabilitation, may leave contracture of the joint.

LABORATORY TESTS

Direct smear or stamp test

Direct smear specimens obtained from the ulcer or stamped biopsy specimens are magnified with Ziehl–Neelsen (Z-N) stain.

Culture test

Fresh skin biopsy, purulent discharge fluid and swab obtained from the surface of the ulcer are the options for specimen. Both liquid media and Löwenstein–Jensen (or Ogawa) medium are used, and cultured at 25°C and 32°C. *M. ulcerans* forms yellowish rough colonies. Because we have experienced a successful isolation at 11 months of culture, we recommend that culturing is continued for at least 6 months.

Polymerase chain reaction (PCR) and other molecular biological studies

Polymerase chain reaction is the best method for early diagnosis. It is performed on a fresh biopsy or previously obtained paraffin block, and targets the high-copy insertion sequence IS*2404* (Fig. 6).[24] A positive study will rule out *Mycobacterium marinum* or other non-*M. ulcerans* NTM. DNA–DNA hybridization is useful for culture-positive samples, but it cannot differentiate between *M. marinum* and *M. ulcerans*. Further, we perform 16S rRNA gene sequencing to separate *M. ulcerans* ssp. *shinshuense* from *M. ulcerans*.[24] Alternative methods include PCR targeting 174-kb plasmid pMUM001 and

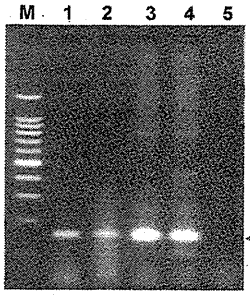

Figure 6. Detection of IS*2404* by polymerase chain reaction.

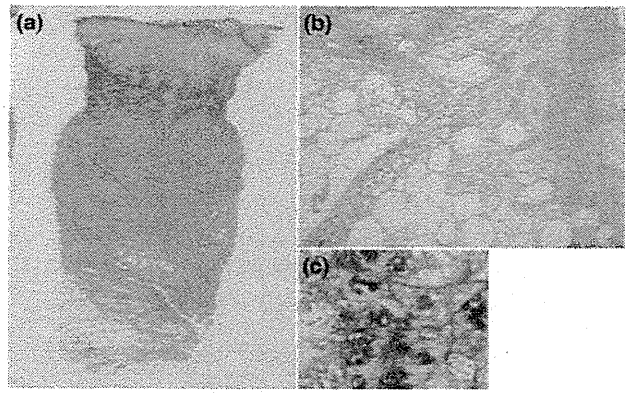

Figure 7. Histopathology. (a) Hematoxylin–eosin stain presents necrotic signs of the dermis, adipose tissue and occasional extension to the fascia. Granulomas or epithelioid cells are rare, as with caseous necrosis (original magnification ×40). (b) Infiltration of lymphocytes in the dermis and adipose tissue is relatively poor (original magnification ×200). (c) Ziehl–Neelsen stain often reveals the mycobacterium in the deep dermal layer to the adipose layer, which is often observed as clusters (original magnification ×400). Paraffin block provided by Dr Yoichi Kato of Okazaki City Hospital, Japan.

drug sensitivity tests, but are not yet common. These sophisticated tests can only be performed at equipped reference institutes that are highly experienced in molecular techniques.

Histopathology

The specimen is to be obtained from a nodule or the rim of the ulcer. Hematoxylin–eosin stain presents necrotic signs of the dermis, adipose tissue and at times extending to the fascia (Fig. 7a,b). Infiltration of lymphocytes in the dermis and adipose tissue is relatively poor, suggesting an immunosuppressant effect of the mycobacteria or the mycolactone. Granulomas or epithelioid cells are rare, as with caseous necrosis. Z-N stain often reveals the mycobacterium in the deep dermal layer to the adipose layer, which often are observed as clusters (Fig. 7c).

DIAGNOSIS

The diagnosis of BU is definitive if *M. ulcerans* is isolated from the ulcer presenting in the exposed parts of the body. Performing all

Table 1. Criteria to diagnose Buruli ulcer

1. Skin eruption accompanying ulcer (regardless of presence of pain)
2. Tissue necrosis with poor inflammatory cell infiltration evident by histopathology
3. Polymerase chain reaction amplification of IS*2404*
4. Detection of acid-fast bacilli in a smear specimen
5. Histopathological confirmation of acid-fast bacilli

The case is defined Buruli ulcer if it fulfills criteria 1, 2 and 3. Criteria 4 and/or 5 are needed to confirm diagnosis.

tests – smear, histopathology, culture and PCR – is essential for accuracy (Table 1). However, it is known that culture alone may take a very long time, and its success rate is low. 16S rRNA gene sequencing is recommended only if there is a necessity to precisely identify *M. ulcerans*, because it is very time-consuming and expensive. In Japan, laboratory tests for *M. ulcerans* and *M. ulcerans* ssp. *shinshuense* are performed at the Leprosy Research Center (LRC), a division within the National Institute of Infectious Diseases (Tokyo, Japan).

Differential diagnosis for BU includes: cutaneous tuberculosis, leprosy, leishmaniasis, myiasis, diabetic ulcer, necrobiosis lipoidica, pyoderma gangrenosum, pressure sore, malignant skin tumor and trauma.

TREATMENT

There is yet no established treatment regimen. Antimicrobial therapy is the standard treatment, but only a limited number of antimicrobials show high efficacy for *M. ulcerans*, and usually require surgical intervention due to the presence of mycolactone.

Commonly selected oral antimicrobial agents are the combination of two or three from the following: rifampicin (RFP) 450 mg/day, clarithromycin (CAM) 800 mg/day and levofloxacin (LVFX) 500 mg/day. Streptomycin (SM) 15 mg/kg per day via i.m. route can be adopted. The WHO recommend RFP and SM dual therapy for 8 weeks: a regimen widely used in the endemic countries at present.[25] Recently, Nienhuis *et al.*[26] conducted a trial of 4 weeks of RFP + CAM after 4 weeks of RFP + SM, and found no significant difference with the WHO recommendation. The significance of this study lies in the result that it presented the possibility of minimizing: (i) the duration needed for daily access to health facilities; and (ii) number of doses of i.m. injections which is a burden for many patients, particularly children. It also lessens the risk of acquiring other infectious diseases such as HIV/AIDS and hepatitis B. In Japan, we recommend the RFP + CAM + LVFX triple therapy, which has shown good outcome and compliance in our cases. This regimen consists only of oral antimicrobials, thus making it possible to completely overcome the shortfalls of i.m. injections. Our sensitivity test also supports this regimen, in which the three antimicrobials showed higher sensitivity to the mycobacterium compared to other choices.[27]

It is important to note that during antimicrobial therapy, new skin lesions may develop, a phenomenon known as "paradoxical

Table 2. Categories of lesions in Buruli ulcer[27]

Category I	A single lesion <5 cm in diameter. Most category I lesions may completely heal with antibiotic treatment
Category II	A single lesion between 5 and 15 cm in diameter. Some category II lesions may completely heal with antibiotic treatment
Category III	A single lesion >15 cm in diameter, multiple lesions, lesion(s) at critical sites (eye, breast, genitalia) and osteomyelitis. In addition to antibiotics, most category III lesions require surgery (excision, skin grafting or amputation in severe cases). However, multiple small lesions and lesions located at critical sites may heal with antibiotics alone

reactions". It is most likely, but still remains to be formally researched, that the decrease in the production of mycolactone due to the therapy enables the hosts' immune system to recover and leads to this phenomenon.[28]

The size of the ulcer is crucial in the determination of a therapeutic plan. The WHO categorizes clinical features into three stages in order to facilitate treatment selection and follow up (Table 2).[29] Category I is a single lesion of less than 5 cm in diameter; category II is a single lesion between 5 and 15 cm; and category III is a single lesion of more than 15 cm in diameter. Surgical intervention (debridement) including skin grafting is inevitable in cases not responding to antimicrobial therapy. The WHO does not endorse definitive indication of surgical treatment for ulcers in category I, but we suggest surgery of any ulcer larger than 1 cm in diameter, after completion of 4-week antimicrobial therapy to minimize bacterial colonization. We recommend that the excision is at least 2–5 cm away from the margin and deep enough to reach the fascia. If skin grafting is necessary, it should not be avoided. In either case, postoperative antimicrobial therapy should not be shorter than 4 weeks. If an ulcer of less than 1 cm in diameter does not respond to 2 weeks of antimicrobial therapy, we determine this as an indication of surgical intervention.

When the lesion extends above a joint and surgical intervention has been chosen, strict adherence to the rehabilitation schedule is imperative in order to prevent contracture and permanent functional disability.

PREVENTION/IMPLICATIONS FOR VACCINATION STRATEGIES

Despite the existent of contradictory reports, a few studies suggest the benefit of bacillus Calmette–Guérin (BCG) administration.[30–34] It leads to prevention of BU within 6–12 months post-administration, or if vaccinated in childhood, it may prevent aggravation into osteomyelitis.[32,33] BCG vaccine coverage in Japan between 2005 and 2007 was 96.6–98.7% (Control Program Support, The Research Institute Tuberculosis, Japan Anti-Tuberculosis Association). In our cases, history of BCG administration was not confirmed. However, none of the cases of BU in Japan extended into osteomyelitis. We cannot speculate if this is the result of the scheduled BCG administration in childhood, characteristics of *M. ulcerans* ssp. *shinsuense*, different living conditions, onset and timing of treatment, or simply by chance.

IMPLICATIONS OF THE JAPANESE CASES AND FUTURE PERSPECTIVES

Recently, we are experiencing an increase of newly reported cases of BU in Japan. Though there may be an actual rise in the endemicity of the disease itself, we believe that the cases of BU reported from Japan were limited until the present for several other reasons: (i) low awareness of the disease amongst the clinicians in Japan; and (ii) NTM, including *M. ulcerans*, are not infections designated by government ordinance, and so the Japanese Ministry of Health, Welfare and Labor does not mandate clinicians and laboratories to report or keep track of the case statistics. We have been conducting activities and developing an information network, thus increasing awareness and improving the diagnostic process. This effort, together with the fact that diagnosis is often made by the same clinician and facilities, led us to this realization. It is evident that BU already existed in Japan in the 1980s.[16] We speculate that there could have been cases treated with antimicrobials under the diagnosis of *M. marinum* or other bacterial infection. Moreover, considering the overlooked cases, there may be more cases waiting to be diagnosed and treated nationwide.

To the extent of our knowledge, the pathogen of BU in Japan and China is a different subspecies of *M. ulcerans*, distinctive from those from other countries. *M. ulcerans* ssp. *shinshuense* was isolated from the very first reported case in Japan.[17] It is not yet clear if this subspecies clinically acts in a different manner, other than some of its laboratory findings (Table 3). So far, dermatological characteristics, including nodule and ulcer forming, non-healing ulcer and the common need of surgical intervention, seem to be similar to the disease caused by the authentic *M. ulcerans* reported elsewhere. Pain seems to be more outstanding in Japanese cases, but our cases are yet too small to draw out any conclusion (Table 4). Interestingly, van der Werf *et al.*[35] mentioned that less subcutaneous tissue involvement was seen in Australian cases at the initial stage when compared to those in Africa.

Table 3. Bacteriological characteristics of *Mycobacterium ulcerans*

Culture temperature	28–34°C
Growth rate	4 weeks (slow grower)
Characteristic of colonies	Yellow, rough
Pigmentation in dark	Positive (yellow)
Urease activity	Negative (*M. u*), positive (*M. u* ssp. *s*)
Niacin accumulation	Negative
Toxin	Mycolactone
IS*2404* (PCR)	Positive
M. marinum in DDH†	Positive (misidentification)

M. u, *Mycobacterium ulcerans*; *M. u* ssp. *s*, *Mycobacterium ulcerans* ssp. *shinshuense*. †DNA–DNA hybridization using DDH Mycobacteria (Kyokuto Pharmaceuticals, Tokyo, Japan). PCR, polymerase chain reaction.

Table 4. Characteristics of cases reported in Japan

Known isolate	*Mycobacterium ulcerans* ssp. *shinshuense*
International traveling	None
Mode of transmission	Unknown, not clear with aquatic environment
Regional bias	Honshu Island (awareness of dermatologists unknown)
Seasonal bias	Autumn and winter (unclear incubation period)
Age	8–81 years
Male : female	3:5
Pain sensation	More outstanding in Japanese cases
Sensitivity against antibiotics	Sensitive
Affected regions	Extremities
Size of ulcer	Mainly <5 cm (category I)

These endemic cases reported outside the African and South-American continents, including those reported from Australia and Japan, possibly indicate a high likeliness of the presence of BU in other subtropical regions. In 2000, Faber *et al.*[36] reported one case of BU with a history of travel to China, the isolate of which was identified as *M. ulcerans* ssp. *shinshuense*. This important case suggests the possibility of existence of *M. ulcerans* ssp. *shinshuense* in Asian countries other than Japan. Hence, rapid awareness of this disease among clinicians is needed worldwide despite the countries' present status, in order for us to better understand this disease and for the better treatment of patients with persisting ulcer.

CONCLUSIONS

Buruli ulcer is a new emerging mycobacterial infection seen in many countries, yet not much is known about the disease including its epidemiology and bacteriology. Our 32 cases are unique in that they were all infected within Japan. There is a large possibility that there could be overlooked cases in Japan, and moreover, this leads us to hypothesize the possibility of hidden cases in other countries which have never experienced BU before. Thus, raising awareness and promoting further research is demanded in this field worldwide.

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REFERENCES

1 Global Buruli Ulcer Initiative (GBUI), World Health Organization. *Buruli ulcer endemic countries [Internet]*. 2011 [cited 2011 Nov 11]. Available from: http://www.who.int/buruli/country/en/index.html.
2 World Health Organization. *First WHO Report on Neglected Tropical Diseases: Working to Overcome the Global Impact of Neglected Tropical Diseases*. Geneva, Switzerland: World Health Organization, 2010.
3 MacCallum P, Tolhurst JC, Buckle G, Sissons HA. A new mycobacterial infection in man. *J Pathol Bacteriol* 1948; **60**: 92–122.
4 Clancey JK, Dodge OG, Lunn HF, Oduori ML. Mycobacterial skin ulcers in Uganda. *Lancet* 1961; **2**: 951–954.
5 Merritt RW, Walker ED, Small PL *et al.* Ecology and transmission of Buruli ulcer disease: a systematic review. *PLoS Negl Trop Dis* 2010; **4**: e911. doi: 10.1371/journal.pntd.0000911
6 Portaels F, Chemlal K, Elsen P *et al. Mycobacterium ulcerans* in wild animals. *Rev Sci Tech* 2001; **20**: 252–264.
7 Marsollier L, Sévérin T, Aubry J *et al.* Aquatic snails, passive hosts of *Mycobacterium ulcerans*. *Appl Environ Microbiol* 2004; **70**: 6296–6298.
8 Marsollier L, Robert R, Aubry J *et al.* Aquatic insects as a vector for *Mycobacterium ulcerans*. *Appl Environ Microbiol* 2002; **68**: 4623–4628.
9 Eddyani M, Ofori-Adjei D, Teugels G *et al.* Potential role for fish in transmission of *Mycobacterium ulcerans* disease (Buruli ulcer): an environmental study. *Appl Environ Microbiol* 2004; **70**: 5679–5681.
10 Portaels F, Elsen P, Guimaraes-Peres A, Fonteyne P, Meyers WN. Insects in the transmission of *Mycobacterium ulcerans* infection. *Lancet* 1999; **353**: 986.
11 Portaels F, Meyers WM, Ablordey A *et al.* First cultivation and characterization of *Mycobacterium ulcerans* from the environment. *PLoS Negl Trop Dis* 2008; **2**: e178.
12 Kotlowski R, Martin A, Ablordey A, Chemlal K, Fonteyne PA, Portaels F. One-tube cell lysis and DNA extraction procedure for PCR-based detection of *Mycobacterium ulcerans* in aquatic insects, molluscs and fish. *J Med Microbiol* 2004; **53**: 927–933.
13 Trott KA, Stacy BA, Lifland BD *et al.* Characterization of a *Mycobacterium ulcerans*-like infection in a colony of African tropical clawed frogs (*Xenopus tropicalis*). *Comp Med* 2004; **54**: 309–317.
14 Sakaguchi K, Iima H, Hirayama K *et al.* Mycobacterium ulcerans Infection in an Indian Flap-shelled Turtle (*Lissemys punctata punctata*). *J Vet Med Sci* 2011; **73**: 1217–1220.
15 Lavender CJ, Fyfe JA, Azuolas J *et al.* Risk of Buruli ulcer and detection of *Mycobacterium ulcerans* in Mosquitos in Southeastern Australia. *PLoS Negl Trop Dis* 2011; **9**: e1305.
16 Mikoshiba H, Shindo Y, Matsumoto H, Mochizuki M, Tsukamura M. A case of typical mycobacteriosis due to *Mycobacterium ulcerans*-like organism. *Jpn J Dermatol* 1982; **92**: 557–565.
17 Tsukamura M, Mikoshiba H. A new mycobacterium which caused skin infection. *Microbiol Immunol* 1982; **26** (10): 951–955.
18 Tsukamura M, Kaneda K, Imaeda T, Mikoshiba H. A taxonomic study on a mycobacterium which caused skin ulcer in a Japanese girl and resembled *Mycobacterium ulcerans*. *Jpn J Tubercul Chest Dis* 1989; **64**: 691–697.
19 Quek TY, Athan E, Henry MJ *et al.* Risk factors for *Mycobacterium ulcerans* infection, southeastern Australia. *Emerg Infect Dis* 2007; **13** (11): 1661–1666.
20 Debacker M, Aguiar J, Steunou C *et al. Mycobacterium ulcerans* disease: role of age and gender in incidence and morbidity. *Trop Med Int Health* 2004; **9** (12): 1297–1304.
21 Portaels F, Johnson P, Meyers WM. *Buruli Ulcer: Diagnosis of Mycobacterium Ulcerans Disease (WHO/CDS/CPE/GBUI/2001.4)*. Geneva, Switzerland: World Health Organization, 2001.
22 Silva MT, Portaels F, Pedrosa J. Pathogenetic mechanisms of the intracellular parasite *Mycobacterium ulcerans* leading to Buruli ulcer. *Lancet Infect Dis* 2009; **9**: 699–710.
23 En J, Goto M, Nakanaga K *et al.* Mycolactone is responsible for the painlessness of *Mycobacatenum ulcerans* infection (Buruli ulcer) in a murine study. *Infect Immun* 2008; **76**: 2002–2007.

24 Nakanaga K, Ishii N, Suzuki K *et al.* "*Mycobacterium ulcerans* subsp. *shinshuense*" isolated from a skin ulcer lesion: identification based on 16S rRNA gene sequencing. *J Clin Microbiol* 2007; **45** (11): 3840–3843.
25 World Health Organization. *Provisional Guidance on the Role of Specific Antibiotics in the Management of Mycobacterium ulcerans Disease (Buruli ulcer) (WHO/CDS/CPE/GBUI/2004).* Geneva, Switzerland: World Health Organization, 2004.
26 Nienhuis WA, Stienstra Y, Thompson WA *et al.* Antimicrobial treatment for early, limited *Mycobacterium ulcerans* infection: a randomized controlled trial. *Lancet* 2010; **37**: 664–672.
27 Nakanaga K, Hoshino Y, Yotsu RR *et al.* Nineteen cases of Buruli ulcer diagnosed in Japan from 1980 to 2010. *J Clin Microbiol* 2011; **49** (11): 3829–3836.
28 O'Brien DP, Robson ME, Callan PP, McDonald AH. "Paradoxical" immune-mediated reactions to *Mycobacterium ulcerans* during antibiotic treatment: a result of treatment success, not failure. *Med J Aust* 2009; **191**: 564–566.
29 World Health Organization. Buruli ulcer: progress report, 2004–2008. *Wkly Epidemiol Rec* 2008; **83**: 145–156.
30 The Uganda Buruli study group. BCG vaccination against *Mycobacterium ulcerans* infection (Buruli ulcer): first results of a trial in Uganda. *Lancet* 1969; **293**: 111–115.
31 Smith PG, Revill WD, Lukwago E, Rykushin YP. The protective effect of BCG against *Mycobactenum ulcerans* disease: a controlled trial in an endemic area of Uganda. *Trans R Soc Trop Med Hyg* 1977; **70**: 449–457.
32 Portaels F, Aguiar J, Debacker M *et al.* Prophylactic effect of *Mycobacterium bovis* BCG vaccination against osteomyelitis in children with *Mycobacterium ulcerans* disease (Buruli ulcer). *Clin Diagn Lab Immunol* 2002; **9**: 1389–1391
33 Portaels F, Aguiar J, Debacker M *et al. Mycobacterium bovis* BCG vaccination as prophylaxis against *Mycobacterium ulcerans* osteomyelitis in Buruli ulcer disease. *Infect Immun* 2004; **72**: 62–65.
34 Nackers F, Dramaix M, Johnson RC *et al.* BCG vaccine effectiveness against Buruli ulcer: a case-control study in Benin. *Am J Trop Med Hyg* 2006; **75**: 768–774.
35 van der Werf TS, van der Graaf WT, Tappero JW, Asiedu K. *Mycobactenum ulcerans* infection. *Lancet* 1999; **354**: 1013–1018.
36 Faber WR, Arias-Bouda LM, Zeegelaar JE *et al.* First reported case of *Mycobacterium ulcerans* infection in a patient from China. *Trans R Soc Trop Med Hyg* 2000; **94**: 277–279.

motifs V–IX at the C-terminal region (Table 1), indicating that this segment is specially important for Gasdermin A3 functions in the skin and hair follicles. Another common feature of these lines is a pronounced hair loss between the first and the third weeks of age. However, considerable differences have been reported regarding the severity of the phenotype, whether the anagen or the catagen stages of the first hair cycle are affected by the mutation, and whether the length of the hair shafts are affected or not. These differences might be due to dissimilarities in the genetic background of the various *Gsdma3* mutant lines, and also to the fact that they have been studied in a variety of laboratories employing different protocols.

While the exact function of Gasdermin A3 in skin and hair follicle physiology remains to be determined, mouse lines carrying mutations in the *Gsdma3* gene have been already useful for studying the mechanisms underlying hair follicle destruction in cicatricial alopecia [3]. Also, since there are some parallels with psoriasis (including an acanthothic and hyperkeratotic epidermis and the presence of numerous cells of the immune system in the dermis), a *Gsdma3* mutant line was employed as a model for evaluating therapies for this disease [4]. Thus, we believe that the newly described mouse line Gsdma3^{I359N} will be useful as an additional member of the allelic series for unraveling the functions of Gasdermin A3 in the skin and its appendages and to study a range of processes associated with different dermatological diseases.

References

[1] Hrabe de Angelis MH, Flaswinkel H, Fuchs H, Rathkolb B, Soewarto D, Marschall S, et al. Genome-wide, large-scale production of mutant mice by ENU mutagenesis. Nat Genet 2000;25:444–7.

[2] Tamura M, Tanaka S, Fujii T, Aoki A, Komiyama H, Ezawa K, et al. Members of a novel gene family, Gsdm, are expressed exclusively in the epithelium of the skin and gastrointestinal tract in a highly tissue-specific manner. Genomics 2007;89:618–29.

[3] Ruge F, Glavini A, Gallimore AM, Richards HE, Thomas CP, O'Donnell VB, et al. Delineating immune-mediated mechanisms underlying hair follicle destruction in the mouse mutant defolliculated. J Invest Dermatol 2011;131:572–9.

[4] Zulfakar MH, Alex A, Povazay B, Drexler W, Thomas CP, Porter RM, et al. In vivo response of GsdmA3Dfl/+ mice to topically applied anti-psoriatic agents: effects on epidermal thickness, as determined by optical coherence tomography and H&E staining. Exp Dermatol 2011;20:269–72.

[5] Porter RM, Jahoda CA, Lunny DP, Henderson G, Ross J, McLean WH, et al. Defolliculated (dfl): a dominant mouse mutation leading to poor sebaceous gland differentiation and total elimination of pelage follicles. J Invest Dermatol 2002;119:32–7.

[6] Lunny DP, Weed E, Nolan PM, Marquardt A, Augustin M, Porter RM. Mutations in gasdermin 3 cause aberrant differentiation of the hair follicle and sebaceous gland. J Invest Dermatol 2005;124:615–21.

[7] Runkel F, Marquardt A, Stoeger C, Kochmann E, Simon D, Kohnke B, et al. The dominant alopecia phenotypes Bareskin, Rex-denuded, and Reduced Coat 2 are caused by mutations in gasdermin 3. Genomics 2004;84:824–35.

[8] Tanaka S, Tamura M, Aoki A, Fujii T, Komiyama H, Sagai T, et al. A new Gsdma3 mutation affecting anagen phase of first hair cycle. Biochem Biophys Res Commun 2007;359:902–7.

[9] Li J, Zhou Y, Yang T, Wang N, Lian X, Yang L. Gsdma3 is required for hair follicle differentiation in mice. Biochem Biophys Res Commun 2010;403:18–23.

[10] Sun L, Li XH, Du X, Benson K, Smart NG, Beutler B. Record for "Fuzzy", updated March 30, 2009. MGI direct data submission; 2009. www.informatics.jax.org.

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Letter to the Editor

Present situation of leprosy in Japan, 2006–2010: Analysis of drug resistance in new registered and relapsed cases by molecular biological methods

Keywords:
Leprosy;
Drug resistance;
New registered cases;
Relapsed cases;
Dapsone;
Rifampicin

Leprosy is a chronic infectious disease caused by an obligate intracellular pathogen *Mycobacterium leprae*. The present strategy for leprosy control is based on the multidrug therapy (MDT), recommended by the World Health Organization (WHO), which has successfully reduced the number of leprosy cases in the world.

Newly reported cases in Japan have markedly decreased during the last two decades. There have been fewer than 10 cases per year in the recent three years. Amongst these newly registered cases, the proportion of imported cases. Relapse cases in Japan are limited to only a few in the recent years.

Although MDT is an effective treatment for leprosy, drug-resistance are known to occur for each agents. Rapid detection and control of such drug-resistant strains is essential in control of leprosy. However, the drug-resistance situation of *M. leprae* has not yet been well informed in Japan.

Table 1A
Number of newly registered patients in Japan. The number of newly reported leprosy in Japan between 2006 and 2010 shows decline whilst the proportion of imported cases increased. The percentage of non-Japanese patients in 2006, 2007, 2008, 2009 and 2010 were 85.7% (6/7), 91.7% (11/12), 57.1% (4/7), 100% (2/2) and 100% (4/4), respectively.

Year	Japanese	Non-Japanese	Ratio of Non-Japanese (%)
2006	1	6	85.7
2007	1	11	91.7
2008	3	4	57.1
2009	0	2	100
2010	0	4	100
Total	5	27	84.4

Table 1B
PCR result in newly registered patients. First, *hsp-70* PCR method was applied to detect *M. laprae* on 27 samples obtained from newly registered patients, excluding five cases registered in 2006. The positive rate was 85% (23/27). Then, mutation analyses on the DRDRs of *folP1*, *rpoB* and *gyrA* genes were applied to samples positive for *hsp-70* with PCR. Cases of mutations detected on *folP1*, *rpoB* and *gyrA* were 8.7% (2/23), 0% (0/23) and 4.3% (1/23), respectively.

Country	PCR		Mutation			
	Positive	Negative	No mutation	*fol P (dapsone)*	*rpo B* (RFP)	*gyr A (quinolones)*
Philippines	6	0	5	0	0	1
Brazil	6	1	5	1	0	0
Indonesia	3	1	3	0	0	0
Vietnam	1	0	1	0	0	0
Korea	1	0	0	1	0	0
Nepal	1	0	1	0	0	0
Thailand	1	0	1	0	0	0
Myanmar	0	1	0	0	0	0
Japan	4	1	4	0	0	0
Total	23	4	20	2	0	1
%	100	-	87	8.7	0	4.3

We investigated the present situation of leprosy in the aspect of drug-resistance mutation in new and relapse cases of leprosy by molecular biological methods. In this study, drug-resistant mutation was investigated amongst the patients presenting positive PCR tests in the years from 2006 to 2010. A total 49 patients (27 new and 22 relapse cases) met the criteria and included in this study.

For the detection of DNA of *M. leprae*, we performed PCR amplification of the *hsp*-70 gene of *M. leprae* [1], and further tested the *hsp*-70 PCR positive sample for drug-resistance determining regions (DRDRs) [2]. Mutations were measured on the *folP1* gene for dapsone [3], the *rpoB* gene for rifampicin (RFP), and the *gyrA* gene for quinolones [4,5]. Nested PCR conditions for drug resistance were different from that of RLEP-nested PCR [6,7].

The number of newly reported leprosy in Japan between 2006 and 2010 shows decline whilst the proportion of imported cases increased (Table 1A). Mutation analyses on the DRDRs of *folP1*, *rpoB* and *gyrA* genes were applied to samples positive for *hsp-70* with PCR (Table 1B). All of the drug resistant samples originated from imported cases (Table 1C).

All (22) of the relapse cases were Japanese nationals, and mutation analyses on the DRDRs of *folP1*, *rpoB*, and *gyrA* genes were performed (Table 2A). All of the drug resistant cases we confirmed were lepromatous leprosy, multibacillary (MB) leprosy case (Table 2B).

The mutation rate in relapse cases in Japanese was higher than that of newly detected cases. This phenomenon is most likely to be the result of prolonged administration of dapsone alone until the 1990s in Japan. The result indicated a strong correlation between mutation rate and relapse. Two possible reasons were conceived regarding the high positive rate of dapsone resistance in patients with relapse: reinfection by the primary drug resistant strain and reactivation of dapsone-resistant strains capable of persisting after chemotherapy, as discussed below. Although it is still unclear whether recurrences are caused by reinfection of *M. leprae* or by reactivation of persistent *M. leprae*, close correlation between drug resistance and relapse have been recognized likewise in several studies [8,9].

The sum of the mutation rates with relapsed case for *folP1*, both *fol P1* and *gyr A*, and *folP1* and *rpoB*, thus dapsone-resistant cases were 23% (Table 2A). This rate falls approximately in the mid portion of the ranges from other reports. Regarding other areas in Southeast Asia, mutation rates for *folP1* amongst the relapse cases were 26% (5/19) in the Philippines (Cebu), 8.3% (2/24) in Myanmar (Yangon), 10% (1/10) in Indonesia (North Maluku and North Sulawesi) [10], and 57% (8/14) in Vietnam (the central and highland regions) [7].

Table 1C
Drug resistant cases in newly registered patients. All of the drug resistant samples originated from imported cases. Case 1: a 32 year-old male from Brazil having borderline lepromatous leprosy presented *folP1* mutation. Case 2: a 69 year-old female from Korea having borderline lepromatous leprosy demonstrated *folP1* mutation. Case 3: a 24 year-old male from Philippines with lepromatous leprosy showed *gyrA* mutation. All of these cases drug resistant mutations were cases of multibacillary (MB) leprosy.

Case	Country	Age	Gender	Classification	Mutation
1	Brazil	32	M	BL[a]	*folP1* (dapsone)
2	Korea	69	F	BL	*folP1* (dapsone)
3	Philippines	24	M	LL[b]	*gyrA* (quinolones)

[a] BL, borderline lepromatous leprosy.
[b] LL, lepromatous leprosy.

Table 2A
PCR results of relapsed leprosy patients. The mutations detected on *fol P1*, *ropB*, *fol P1/gyr A*, and *fol P1/rpo B* were 9.1% (2/22), 9.1% (2/22), 9.1% (2/22), 4.5% (1/22), respectively. These data are summed up that the percentage of dapsone-resistant cases was 23% (5/22), 14% (3/22) for RFP, and 9.1% (2/22) for quinolone.

Mutation	Cases	%
No mutation	15	68.2
Dapsone (*folP1*)	2	9.1
RFP (*rpoB*)	2	9.1
Dapsone and quinolones (*folP1* and *gyrA*)	2	9.1
Dapsone and RFP (*folP1* and *rpoB*)	1	4.5
Total	22	100

Table 2B
Drug resistant cases in relapsed leprosy patients. Cases detected with *folP1* mutation included a 73 year-old male with history of dapsone use and 69 year-old female with history of dapsone and RFP use. Cases detected with *rpoB* mutation were a 77 year-old male with history of dapsone use and a 72 year-old male with history of dapsone and RFP use. Cases that showed both *folP1* and *gyaA* mutations were a 71 year-old male with a history of dapsone and RFP use and a 77 year-old female with history of dapsone use. The case that presented both *folP1* and *rpoB* mutations was a 72 year-old male with history of dapsone and RFP use.

Case	Age	Gender	Classification	Mutation	Past drug history		
					Dapsone	RFP	Quinolones
1	73	M	LL	*folP1*	+	–	–
2	69	F	LL	*folP1*	+	+	–
3	77	M	LL	*rpoB*	+	–	+
4	72	M	LL	*rpoB*	+	+	–
5	71	M	LL	*folP1* and *gyrA*	+	+	–
6	77	F	LL	*folP1* and *gyrA*	+	–	–
7	72	M	LL	*folP1* and *rpoB*	+	+	–