clearly associated with sustained viability of intracellular *M.leprae* cultured at 35°C (Fig.3). In the presence of 3 U/ml of IL-10, *M.leprae* maintained their viability, whereas viability was steadily lost without IL-10. We also examined the effect of TGF-β, another suppressive cytokine for macrophage activation, on the viablility of the bacilli. To the contrary, supplementation of TGF-β

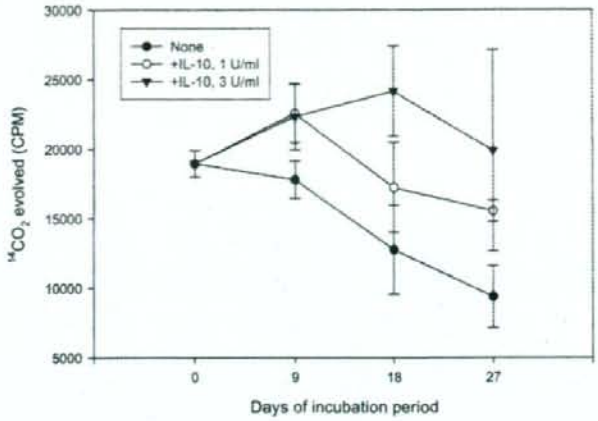

Fig.3. Effect of IL-10 on *M.leprae* survival in mouse macrophages. Mouse peritoneal resident macrophages were incubated with 1×10^7 per well of *M.leprae* (MOI, 10:1) at 35°C for 4 hr to allow phagocytosis, and the culture continued at 35°C for 9, 18 and 27 days. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.

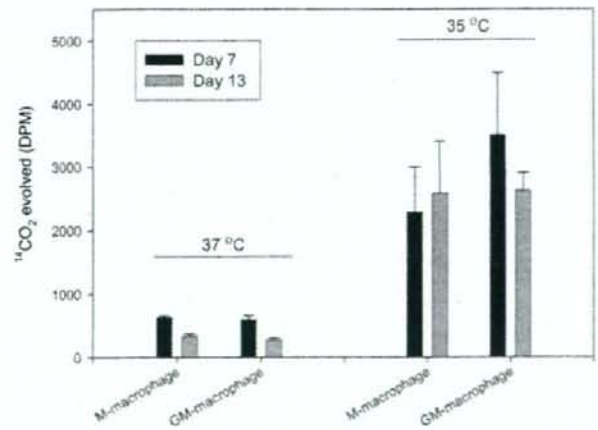

Fig.4. Viability of *M.leprae* in human macrophages cultured *in vitro*. Human M- or GM-macrophages were incubated with *M.leprae* (MOI, 50:1) for 20 hr either at 35°C or 37°C for infection and incubated at the same temperatures for indicated periods. By observation of the acid fast-stained cells under light microscopy, no difference was recognized in the number of *M.leprae* phagocytosed by macrophage cultured between at 35°C and at 37°C. So the viability at day 0 is considered equal. After 7 days and 13 days incubation period, the cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry (dpm: disintegrations per minute).

significantly decreased the viability of *M.leprae*, when incubated for longer than 28 days post infection (Table 1).

Viability of *M.leprae* in human macrophages cultured *in vitro*: Human macrophages were obtained by culturing monocytes in the presence of either M-CSF or GM-CSF for 7 days. These macrophages (1×10^5 cells per well) were incubated with *M.leprae* (MOI, 50:1) for 20 hr either at 35°C or 37°C for infection and incubated again at the same temperatures. By observation of the acid fast-stained cells under light microscopy,

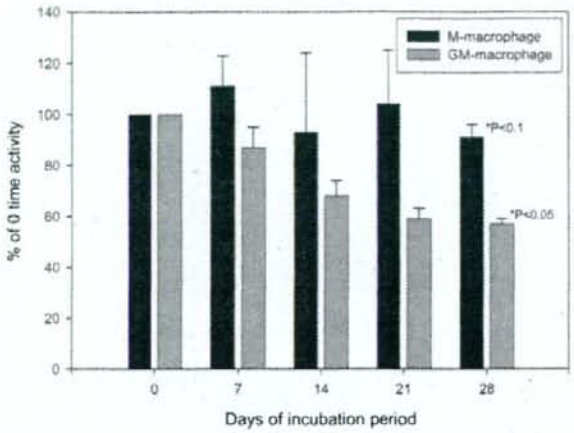

Fig.5. Viability of *M.leprae* in human macrophages cultured *in vitro*. Human M- or GM-macrophages were infected with *M.leprae* (MOI, 50:1) for 20 hr at 35°C and incubated again at 35°C for indicated periods. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry. The results at day 7, 14, 21 and 28 are expressed as percentages of *M.leprae* metabolic activity at time 0. Radiorespirometry data obtained from *M.leprae* in M-macrophages at time 0 was 5,932±399 and those in GM-macrophages was 3,084±78. *P values calculated in comparison to day 0 viability.

Table 1. Effect of TGF-β on survival of *M.leprae* in mouse macrophages cultured at 35°C[a]

Experiment 1

Days of incubation period	At time 0	7	14	28
Medium only	5,222±936[b]	2,774±295	3,086±425	2,828±1,815
+TGF-β		2,919±535	3,119±1,339	1,973±126

Experiment 2

Days of incubation period	At time 0	14	28	49
Medium only	26,791±1,428	19,103±621	7,420±2,986	5,713±1,144
+TGF-β		14,306±2,240	3,728±410	1,594±317

[a]Mouse peritoneal resident macrophages were incubated with *M.leprae* (MOI, 1:10) for 4 hr to allow phagocytosis, and the culture continued for indicated periods. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.
[b]Radiorespirometry data, cpm.
Dose of TGF-β, 500pg/ml.
N.D., not determined.

no difference was recognized in the number of *M.leprae* phagocytosed by macrophage cultured at 35°C and at 37°C (data not shown). Viability of *M.leprae* was assessed after 7 and 13 days. The results clearly showed that the viability of *M.leprae* incubated at 35°C was maintained, whereas the viability was lost if cultured at 37°C (Fig. 4). Next, *M.leprae*-infected human M- and GM-macrophages were cultured for prolonged periods at 35°C. Viability was sustained well for 4 weeks in human macrophages, especially in M-macrophages (Fig. 5).

Discussion

In vivo M.leprae is able to enter and survive in a wide variety of tissues and cell types[12]. The preferred host cell for the leprosy bacillus appears to be the macrophages and a number of unsuccessful attempts have been made to grow *M.leprae* in macrophages *in vitro*. For example, Sharp and Banerjee[13] employed macrophages from conventional mice and rats, *nu/nu* mice or *nu/nu* rats and armadillos. The *M.leprae* inocula were derived from 3 sources (human leproma, *nu/nu* mouse footpad and frozen infected armadillo tissue). Incubation temperature was varied from 31°C to 35°C and *M.leprae*-infected cells were maintained for up to 200 days. Fieldsteel and McIntosh [14] employed a range of rat, mouse and human tissue. The conclusion of these reports is that no significant multiplication of *M.leprae* occurred in any of the cells or tissues.

Previously, we reported that metabolically active *M.leprae* could be maintained in monolayer cultures of mouse peritoneal macrophages and that supplemental IL-10 bolstered *M.leprae* metabolism in the macrophages for as long as 8 weeks. In the cell culture system, temperature is an extremely important factor for growth and 31-33°C incubation temperature is more permissive than 37°C [5]. In the present study, we further observed that incubation of mouse macrophages infected with *M.leprae* at 35°C was also more growth permissive than at 37°C. We chose 35°C as the incubation temperature, and not 31°C, because the maintenance of the integrity of the macrophage monolayer was better at 35°C than at 31-33°C. Moreover, the monolayer of *M.leprae*-infected human macrophages at 31-33°C could not be maintained for longer than one week. We observed that maintenance of the monolayer was good at 35°C, and *M.leprae* at 35°C was also more growth permissive than those at 37°C in human macrophages (Fig. 4 and 5). Our starting inoculum of *M.leprae* was freshly obtained for each experiment from infected *nu/nu* mice. We also were able to rapidly quantify the metabolic activity of *M.leprae* using the radiorespirometry technique adapted by Franzblau [11]. This assay is accurate and highly sensitive with the results available in a short duration of 1 wk (compared to 6-12 months when titrated in mouse footpads). Radiorespirometry data correlates well with other *in vitro* systems [11] but, more importantly, the data correlated well with "viability" as observed in the mouse footpad system [12].

Various clinical evidence suggests that *M.leprae* prefer a growth temperature of less than 37°C [1]. In animal models, *M.leprae* multiplies in the mouse foot pad where the temperature is lower than the body temperature [2]. In addition, *Dasypus novemcinctus*, the nine-banded armadillo has a core temperature of ~33°C, which renders it permissive as a host for the leprosy bacillus [13]. Mononuclear phagocytes in virtually every organ of the natural or experimentally infected armadillo become heavily parasitized with propagating *M. leprae* [14]. Whether intracellular or extracellular, *M.leprae* clearly prefers temperatures cooler than

normal human body temperature [12], and 37°C appeared to be highly detrimental to *M.leprae* viability. The *in vitro* results obtained in the present study confirmed the preference of lower temperature (35°C) by *M.leprae* residing in human macrophages.

In this study, supplemental IL-10, but not TGF-β supported the metabolic activity of *M.leprae* in mouse macrophages for several weeks, similar to the results obtained previously [5]. In choosing TGF-β and IL-10 as the cytokines that might bolster the intracellular survival of *M.leprae*, we were attempting to down-regulate any innate ability of the normal macrophages to cope with the organism. TGF-β is produced by activated macrophages and other inflammatory cells and has a broad array of modulatory functions on the immune response. TGF-β has been shown to interfere with macrophage antimicrobial mechanisms including the generation of reactive oxygen intermediates [15] and reactive nitrogen intermediates [16], and has been shown to enhance the intracellular growth of *M.tuberculosis* in human monocytes [17]. However, in the present studies with mouse macrophages, exogenous TGF-β had no detectable effect on sustaining intracellular *M.leprae* viability, and in fact decreased the viability (Table 1). In contrast, supplementing media with IL-10 clearly affected the long term viability of *M.leprae* in mouse macrophages (Fig. 3). IL-10 is produced by T cells, B cells and macrophages [18, 19]. IL-10 has been shown to be a potent down-regulator of cell-mediated immunity to intracellular pathogens [20]. *In vivo*, endogenous IL-10 dampened the cell-mediated immune response to avirulent mycobacterial infection [4] and appeared to lead to loss of control of *M.tuberculosis* infection with widespread dissemination [21]. IL-10 functions in part at the level of the macrophage by attenuating iNOS mRNA expression, iNOS activity and, by inference, NO production [22]. In human macrophages, however, the viability of *M.leprae* was maintained for 4 weeks in the absence of IL-10 (Fig. 5), suggesting that human cells seem to be better hosts than mouse cells for *M.leprae* survival. Viability of *M.leprae* in M-macrophages seems to be maintained for a longer period (up to one month) than that in GM-macrophages (Fig. 5). One of the reasons for this may be due to the production of IL-10 by M-macrophages [23], although the mechanism by which IL-10 contributes to the maintenance and growth of *M.leprae* is unclear.

In conclusion, the present study showed that the metabolism, and presumably the viability, of *M.leprae* could be sustained under culture conditions at 35°C, which is also a moderate temperature necessary to maintain the integrity of macrophages.

Acknowledgments

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マクロファージ内におけるらい菌生存の温度依存性

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キーワード：生存、ヒト、マウス、マクロファージ、らい菌

ハンセン病は細胞内寄生菌であるらい菌によって引き起こされる感染症である。らい菌は主にマクロファージとシュワン細胞に感染する。しかしながら、マウスやヒトマクロファージ内における生存・発育機構について詳細は明らかになっていない。本研究では放射性同位元素を用いた方法によりらい菌の生存率を評価した。そして、らい菌感染マクロファージを35度で培養する方が37度で培養するよりもらい菌の生存率を高い状態に保つことができることが判明した。また、免疫抑制性サイトカインであるIL-10を添加することにより3週間程度生存が維持されることが分かった。一方、IL-10未添加の場合、生存率は徐々に低下した。ヒトマクロファージの場合は、IL-10未添加の場合でも少なくとも4週間生存は維持された。しかしながら、37度で培養すると2週間以内に生存率は著明に低下した。これらの結果から、らい菌の細胞内における生存には温度が決定的な要因のひとつであることが判明した。

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Recent advances in the molecular epidemiology of leprosy

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Recent advances in the molecular epidemiology of leprosy through genotyping of variable number tandem repeats (VNTRs) and single nucleotide polymorphisms (SNPs) are described. VNTRs with a broad range of diversity are useful genotyping tools for analyzing transmission in community areas, and SNPs and VNTRs with a small degree of variation are favorable for investigating the global transmission of leprosy. We expect that the transmission of leprosy can be fully analyzed by the application of these new methodologies.

Introduction

The number of newly detected cases of leprosy has been gradually decreasing around the world in recent years [1]. Even so, some details of disease transmission remain to be elucidated. One of the important subjects still under dispute, and essential for establishing a better strategy for preventing new cases, is the transmission mode of leprosy including the infectious source. Phenotype, serotype, phage type and genotype are robust metrics for analyzing and tracing transmission in many infectious diseases [2, 3]. These useful tools were not available for understanding the transmission of leprosy until two kinds of variable number tandem repeat (VNTR) were identified [4, 5], followed by the discovery of single nucleotide polymorphisms (SNPs) in the *Mycobacterium leprae* genome [6]. Epidemiological studies of leprosy using these two assays have been gradually accumulating since 2004, and findings generated by these breakthroughs in molecular epidemiology are now taking the place of classical epidemiological methods. An international study group for investigating the transmission of leprosy through the application of advances in molecular biology, including genotyping, has been organized and collaborative studies have begun [7]. VNTRs with broad allelic diversity are useful for analyzing transmission patterns in the community [8-11]. SNPs and VNTRs with a narrow range of diversity are practical for characterizing the global geographical distribution of different genotypes of *M. leprae*,

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and are beneficial in tracing the dissemination of leprosy in prehistoric times and following current global leprosy migration patterns [12-16]. This article describes recent advances in the molecular epidemiology of leprosy.

Application of VNTRs to Analyze the Transmission of Leprosy

It has long been believed that leprosy is transmitted by heavy contact with leprosy patients; however, there are some findings which contradict this premise. For instance, a study on the geographical distribution of responders to *M. leprae* antibodies showed a distribution of positive responders in houses remote from those with known patients [17]. The limited effect of chemoprophylaxis [18] means that drugs prevent the progression of an active infection to overt disease [19] but do not interrupt transmission. Attempts to curtail transmission and reduce new infections by chemoprophylaxis in the Federated States of Micronesia (FMS) failed. The number of new cases detected in FMS has increased again after 1999 and over the last several years has risen to nearly the same level as before execution of the chemoprophylaxis program [1]. Many new cases without known contact with leprosy patients [20, 21] have also been reported. These findings suggest gaps in the traditional concept of leprosy transmission and imply the existence of an infectious source of *M. leprae* other than leprosy patients.

PCR studies have shown that in areas with a high prevalence of leprosy, many individuals harbor *M. leprae* in their nasal mucus [22, 23]. These findings indicate that people in such areas are frequently exposed to *M. leprae* infection. The hypothesis that the bacterial genotypes from household members or other contacts of patients should be identical if the bacilli are transmitted by contact with the patient, was tested by a comparison of genotypes from the nasal mucus of patients and family members, and also from new patients and the supposed index case of a multifamily case [8]. Genotyping based on the copy number of a TTC repeat [5, 8] and another genotyping method with higher discriminatory power were applied [10]. Results revealed that the genotype of *M. leprae* in the nasal mucus differed between the patient and contacts in a given household. The existence of *M. leprae* strains with allelic diversity in a multifamily case in China was also shown [11]. On the other hand, a study conducted in India revealed no divergence among the three VNTRs from two patients in a multifamily case [9]. The lack of divergence among the patients of this case could be explained by the low discriminatory power of genotyping using only three polymorphic VNTRs. In another study, isolates determined as having identical genotypes using a few VNTRs were separated into different genotypes by a method with high resolution power using a combination of other appropriate VNTRs [10]. Thus, we theorize that the Indian clinical isolates with identical genotypes might be classified into different genotypes with the improved methodology. Although VNTRs seem to fluctuate in biopsy samples from the same patient [24, 25], co-infection of *M. leprae* strains with different VNTRs may occur. While the variation of VNTRs in a given patient is often by only one or two copies, the TTC copy number of the isolate from a supposed index case was 18 and the TTC copy number of the bacilli from his son was 10 in one multifamily case report [8]. It is clear that the genotypes of the bacilli from father and son were distinct and hence did not come from the same infectious source [8]. This suggests the presence of an alternate source of infection.

Water sources used daily by people in an endemic area are thought to be a potential source

of *M. leprae* infection, since many water-borne mycobacterial infectious diseases exist [26, 27, 28]. Water samples collected at 6 villages with a high prevalence of leprosy in Indonesia were assayed by PCR. Of 44 water samples, 21 were positive by PCR assay for a 65 kDa protein coding sequence [29]. The relative risk of leprosy infection was 3.24 for those using the PCR-positive water source for washing and bathing, but no difference was shown in risk for water used for drinking and cooking. Water is heated for drinking and cooking, and consequently bacilli in the water are inactivated [29]. PCR positivity does not prove the existence of live *M. leprae* in the water. However, reverse transcription-PCR (RT-PCR) targeting 16s rRNA did detect the presence of *M. leprae* organisms [30]. This method was used to analyze the water from wells in a village on the Indonesian island of North Sulawesi that has many newly detected patients every year. Of 51 well water samples, 17 were positive by RT-PCR [31]. These results strongly suggest that water is an environmental reservoir of *M. leprae* and an infectious source of leprosy. Possible infection from the soil has also not been ruled out [32].

Application of SNPs and VNTRs with a Small Range of Diversity to Analyze the Global Dissemination of Leprosy

Using a VNTR with either 3 or 4 copies of a 6-bp sequence, a pattern in the global geographical distribution of *M. leprae* has been found [4, 12, 13, 15, 16]. The 3-copy strain is overwhelmingly predominant in most countries around the world, while the 4-copy strain is predominantly found in East Asia, including the mainland of Japan, Korea and eastern parts of China, as well as western parts of Mexico [4, 12-14]. No 4-copy isolates have been obtained from the Okinawa and Amami Islands of Japan. These islands are located at the southern end of Japan and have their own unique culture. The presence of different genotypes between these two regions of Japan is also found in other species of microorganisms [33, 34]. It is thought that the predominance of the 4-copy strain in East Asian countries was established in prehistoric times by the movement of Mongoloid people in the region. In Japan, it is likely that the characteristic distribution of each genotype was established by the movement of two ethnic groups which migrated to Japan about 30,000 years ago through the Okinawa Islands, and 2,800 to 1,700 years ago through the Korean peninsula [35]. Specifically, *M. leprae* of the 4-copy strain might have been carried by the people who migrated to Japan through the Korean Peninsula, while the 3-copy strain was spread to the Okinawa Islands by the people who migrated to Japan from Southeast Asia 30,000 years ago. This example demonstrates how VNTRs with small range of polymorphic variation, such as that of the *rpoT* gene, are useful in tracing the global transmission of leprosy.

The geographical distribution of *M. leprae* SNP alleles 1-4 is also suitable for analyzing the global spread of leprosy [6]. An assay using a combination of SNPs and the *rpoT* gene VNTR was applied to determine whether Japanese Brazilian patients newly diagnosed in the mainland of Japan had been infected in Japan or Brazil [14]. SNP type 4, detected in two Japanese Brazilian patients, was not found among 46 Japanese patients from the mainland. The isolate with SNP type 3 and the 3-copy polymorphism of the *rpoT* gene, found in 7 isolates from Japanese Brazilian cases, is also uncommon in mainland Japan. Thus it is most likely that the patients were infected in Brazil and developed symptoms of leprosy after they came to Japan. It had been generally assumed that this was the case since the prevalence of leprosy is high in

Brazil, but there was no substantial microbiological evidence to support the idea. The assay combining SNPs and the *rpoT* gene VNTR clearly contributed to the analysis of the global transfer of leprosy incident to the current movement of people.

The distribution of each SNP genotype in Asian countries was also examined in another study [14]. Most of the isolates (26 of 29) from Myanmar had SNP type 1. The frequency of this SNP declines in proportion to the distance of the countries from India, as is seen by low proportions in Indonesia, Korea and Japan. Logically, the SNP type 2 isolate could be derived from a type 1 progenitor, followed by evolution to type 3 and type 4 derivatives. All isolates from the southern part of India had SNP type 1 [6]. Taken together with data from other studies, it seems likely that leprosy originated in the Indian subcontinent and spread outward to other areas of the world. This idea is compatible with the argument that leprosy originated in the Indian subcontinent and was introduced to Egypt as a result of Persian invasion during the sixth century BC or by the troops of Alexander the Great in the fourth century BC [36].

The use of molecular epidemiology through the application of genotyping as a means of tracking the transmission of leprosy began just 4 years ago in 2004, on the basis of a milestone discovery of two VNTRs in the genomic DNA of *M. leprae*. This new field of leprosy research is expected to illuminate obscure mechanisms of leprosy transmission with respect to sources, reservoirs, modes of transmission and the history of leprosy.

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ハンセン病の分子疫学解析における最近の知見

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らい菌遺伝子の直列繰返配列多型（variable number tandem repeat：VNTRs）及び 1 塩基多型（single nucleotide polymorphism: SNPs）を用いたハンセン病の分子疫学の最近の知見が述べられた。VNTRs は地域社会を対象としたハンセン病の伝搬の解析にまた、SNPs 並びに多様性の小さな VNTRs はハンセン病の地球規模での伝搬の解析に有用であることが述べられた。これらの手技をもちいることにより、ハンセン病の感染様式がより明確にされることが期待される。

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A novel method for simple detection of mutations conferring drug resistance in *Mycobacterium leprae*, based on a DNA microarray, and its applicability in developing countries

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A simple method to detect mutations in the genome of *Mycobacterium leprae* that confer resistance to key drugs for leprosy was exploited on the basis of a reverse hybridization system. A series of oligonucleotide probes corresponding to each mutation in the *folP1*, *rpoB* and *gyrA* genes for dapsone, rifampicin and ofloxacin resistance, respectively, were selected and fixed on a glass slide as capture probes, to develop a DNA microarray termed the leprosy drug susceptibility-DNA microarray (LDS-DA). Mutations in clinical isolates of *M. leprae* were successfully identified by the LDS-DA. Feasibility studies were conducted to evaluate the performance of the LDS-DA in two developing countries, Myanmar and the Philippines. The high concordance of the results obtained by this method with the results of nucleotide sequencing strongly supports the applicability of the LDS-DA as a drug susceptibility test in place of sequencing, a time-consuming and costly procedure. This is a rapid and simple method for the simultaneous susceptibility testing of three front-line drugs for leprosy, and solves the problems of previously reported methods.

INTRODUCTION

The current strategy for leprosy control relies mainly on multidrug therapy (MDT) (WHO, 1998). However, cases of leprosy caused by drug-resistant *Mycobacterium leprae* have been documented as the result of therapeutic failure (Cambau *et al.*, 2001; Maeda *et al.*, 2001; Matsuoka *et al.*, 2000, 2003). Although information on the drug susceptibility of clinical isolates contributes to the better outcome of treatment, susceptibility testing has rarely been done because of its difficulty. Antibiotic susceptibility testing of *M. leprae* still relies on a time-consuming method based on the growth of bacteria in mouse footpads (Shepard, 1960), which takes up to 12 months to give a result. This has hindered the comprehensive surveillance that would offer useful information to evaluate the efficacy of MDT and to prevent the spread of drug-resistant strains. Recent advances in the molecular biology of drug-resistant *M. leprae* have enabled the development of drug susceptibility tests for key component drugs of MDT, by the detection of relevant gene mutations that confer resistance (Williams & Gillis, 2004). The molecular mechanism of rifampicin resistance was first demonstrated in *Escherichia coli* and thereafter in *M. leprae* (Honoré & Cole, 1993). Rifampicin resistance is strongly correlated with mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase (Honoré & Cole, 1993; Honoré *et al.*, 1993; Williams *et al.*, 1994, 2001; Matsuoka *et al.*, 2000, 2003; Cambau *et al.*, 2001; Maeda *et al.*, 2001; Zhang *et al.*, 2004). Resistance to fluoroquinolones has been proved to correlate with

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Abbreviations: BI, bacterial index; DRDR, drug-resistance-determining region; LDS-DA, leprosy drug susceptibility-DNA microarray; MDT, multidrug therapy.

mutations in the *gyrA* gene, encoding the A subunit of DNA gyrase in *M. leprae* (Cambau *et al.*, 1997; Matsuoka *et al.*, 2000; Cambau *et al.*, 2001), as in many other bacteria. In addition, mutations in the *folP1* gene, encoding dihydrofolate synthetase, have been shown to be responsible for dapsone resistance (Kai *et al.*, 1999; Matsuoka *et al.*, 2000, 2003, 2007; Williams *et al.*, 2000; Lee *et al.*, 2001; Maeda *et al.*, 2001; Cambau *et al.*, 2006). The prevalence of drug resistance in selected areas was surveyed through the application of molecular analysis to detect mutations conferring drug resistance (Matsuoka *et al.*, 2007). Analysis of mutations is generally performed by sequencing the target genomic region, amplified by PCR, although the implementation of sequencing is not easy in many developing countries. Therefore, a simple and rapid method to detect mutations conferring drug resistance has been long awaited. In the current study, a DNA microarray method was developed and the applicability of this system was evaluated in Myanmar and the Philippines.

METHODS

Design of capture probes. Mutant nucleotide sequences conferring resistance to dapsone, rifampicin and ofloxacin and their corresponding wild-type sequences in *Mycobacterium leprae* (Table 1) were employed in this study. Nearly all the drug-resistant strains of *M. leprae* reported so far are covered by the mutations selected. Capture oligonucleotide probes (14- to 18-mer) for the detection of the mutations were designed according to these data. Optimal sequences of oligonucleotides corresponding to each missense mutation were designed empirically as shown in Fig. 1(a). The array of capture oligonucleotide probes was covalently bound to the surface of a glass slide coated with polycarbodiimide and the resulting DNA microarray was designated the leprosy drug susceptibility-DNA microarray (LDS-DA), as shown in Fig. 1(b).

Amplification of three target gene fragments. Target regions of *folP1* (accession no. AL 583917, gene ML583917), *rpoB* (accession no. AL583923, gene ML1891) and *gyrA* (accession no. AL583917, gene ML0006) were simultaneously amplified with three primer pairs in one PCR. The sequences of the primers are listed in Table 2. PCR was carried out using the G mixture of the FailSafe PCR System (EPICENTRE) in a volume of 25 µl with 1 µM of each primer. Cycling conditions began with an initial incubation at 94 °C for 4 min, followed by 40 cycles of annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and denaturation at 94 °C for 30 s. Finally, incomplete PCR products were extended for 5 min at 72 °C. The amplified DNA fragments were confirmed by gel electrophoresis through 4.0 % Metaphor Agarose (FMC Corp.) in TBE (Tris/borate/EDTA, pH 8.0) buffer.

LDS-DA assay. A 2 µl aliquot of the resulting PCR mixture was mixed with 38 µl UniHyb Hybridization Solution (TeleChem International), heat denatured at 98 °C for 5 min and quickly chilled. The solution was then applied to the LDS-DA and incubated at 42 °C for 60 min followed by stringent washing with 50 µl washing solution (3 M tetramethylammonium chloride; Sigma-Aldrich) at 47 °C for 60 min. The biotin-labelled DNA fragments hybridizing to the capture probes on the LDS-DA were detected by avidin-biotin-horseradish peroxidase complex (VECTASTAIN Elite ABC kit, Vector

Table 1. Missense mutations associated with drug resistance in *M. leprae*

Drug	Gene	Codon no.	Susceptible		Resistant		References†
			Codon	AA*	Codon	AA*	
Dapsone	*folP1*	53	ACC	Thr	GCC	Ala	1, 2, 3
					GTC	Val	4
					ATC	Ile	2, 3, 5, 6
					AGG	Arg	3
					AGA	Arg	4
		55	CCC	Pro	TCC	Ser	1, 7
					CGC	Arg	1, 3
					CTC	Leu	2, 3, 8
Rifampicin	*rpoB*	407	CAG	Gln	GTG	Val	9
		410	GAT	Asp	AAT	Asn	2
					TAT	Tyr	8
		420	CAC	His	TAC	Tyr	2
					GAC	Asp	9, 10
		425	TCG	Ser	ATG	Met	9, 10
					TTG	Leu	2, 9
					TTC	Phe	11, 2, 6, 7, 3
		427	CTG	Leu	CCG	Pro	2
Ofloxacin	*gyrA*	89	GGC	Gly	TGC	Cys	2
		91	GCA	Ala	GTA	Val	12, 2, 6

*AA, amino acid.

†1, Lee *et al.* (2001); 2, Maeda *et al.* (2001); 3, Williams *et al.* (1994); 4, Matsuoka *et al.* (2007); 5, Kai *et al.* (1999); 6, Matsuoka *et al.* (2000); 7, Matsuoka *et al.* (2003); 8, Zhang *et al.* (2004); 9, Cambau *et al.* (2001); 10, Honoré & Cole (1993); 11, Honoré *et al.* (1993); 12, Cambau *et al.* (1997).

(a)

Drug	Gene	Codon no.	Capture probes for wild-type	Capture probes for mutants
Dapsone	*folP1*	53	FW1 : GTGGCGAATCGACCCGG	FM1 : TGGCGAATCGGCCCGG
				FM2 : TGGCGAATCGGTCCGG
				FM3 : TGGCGAATCGATCCGG
				FM4 : GGCGAATCGAGGCGG
				FM5 : TGGCGAATCGAGACGG
		55	FW2 : CGGCCCGGTGCCATTA	FM6 : GACCGGTCCGGTGCC
				FM7 : GACCGGCGCGGTGCC
				FM8 : GACCGGCTCGGTGCC
Rifampicin	*rpoB*	407	RW1 : AGCTGTCGCAGTTCATG	RM1 : AGCTGTCGGTGTTCAT
		410	RW2 : TTCATGGATCAGAA	RM2 : TTCATGAATCAGAACAA
				RM3 : TTCATGTATCAGAACAA
		420	RW3 : CCTGACCCACAAGCGC	RM4 : GCCTGACCTACAAGCGC
				RM5 : GCCTGACCGACAAGCGC
		425	RW4 : CGCCGACTGTCCGCGCTG	RM6 : CCGACTGATGGCGC
				RM7 : GCCGACTGTTGGCG
				RM8 : GCCGACTGTTCGCG
		427	RW5 : GCGCTGGGGCCCGGGTG	RM9 : GGCGCCGGGGCCCGGGTG
Ofloxacin	*gyrA*	89	GW1 : ATCCGCACGGCGACGCA	GM1 : ATCCGCACTGCGACGCA
		91	GW2 : CGGCGACGCATCGATTT	GM2 : CGGCGACGTATCGATTT
Positive hybridization control in *gyrA*			GP : GGACCGTAGCCACGCTAA	
Negative hybridization control in *gyrA*			GN : GGACCGTCATCACGCTAA	

(b)

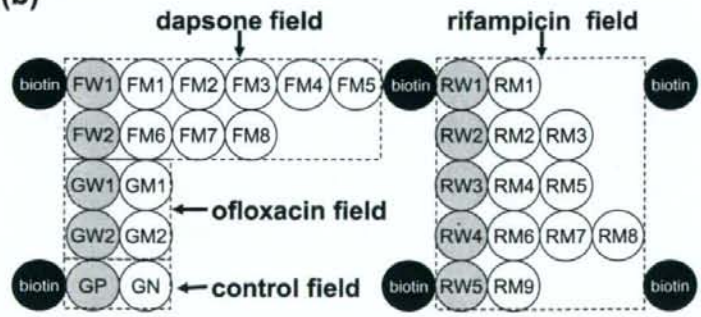

Fig. 1. Development of the LDS-DA. (a) The oligonucleotide sequences used in the test. Codons relating to drug resistance are underlined. Oligonucleotide GP, positive control for PCR amplification and hybridization; GN, negative control for hybridization. (b) Schematic representation of the array of oligonucleotides on the LDS-DA. Black circles represent spots with biotin as landmarks for conjugate reaction control; grey circles are the wild-type spots; white circles are the mutant spots. The region with oligonucleotides designated FW- and FM- is for dapsone resistance detection (the dapsone field); the region designated GW- and GM- is the ofloxacin field; the region designated GP- and GN- is the control field; and the region designated RW- and RM- is the rifampicin field.

Laboratories) and then visualized by TMB peroxidase substrate (Vector Laboratories). The resulting spot patterns were recorded by a conventional scanner and a computer. Only the results of the LDS-DA with proper signals on both positive and negative control spots (GP and GN in Fig. 1) were used for further analysis. The colour intensity of each spot in a row (covering the same region of each gene) was examined. The spot with the highest colour intensity was considered to reflect the sequence of the gene fragment in the sample.

Evaluation of the LDS-DA with clinical specimens. The LDS-DA system was transferred to laboratories in the Department of Medical Research in Yangon, Myanmar, and in the Leonard Wood Memorial in Cebu, the Philippines, and was evaluated on 63 and 73 clinical specimens, respectively, in these laboratories. A majority of the samples in this study had been examined previously (Matsuoka *et al.*, 2007). Of the 63 samples in Myanmar, 44 were from new cases and 19 were from patients with relapse. Of the 19 relapsed patients, one patient had received monotherapy with dapsone for 4 years followed by monotherapy with rifampicin for 4 years, while the other 18 patients had been treated with the standard MDT regimen for multibacillary leprosy. Samples from the Philippines included 64 from new cases and nine from relapsed cases. Of the nine relapsed

Table 2. Sequences of oligonucleotide primers for *M. leprae*

Gene	Primer	Sequence (5′–3′)	PCR products (bp)
folP1	MLfolP1DA-Ft;	GTGAGTTTGGCGCCAGTGCA	119
	MLfolP1DA-RB;	Biotin-GCAAGTTCTTTTACGACAGG	
rpoB	MLrpoBDA-F;	TCGCCGCTATCAAGGAATTC	127
	MLrpoBDA-RB;	Biotin-TCACGCGACAAACCACCCGG	
gyrA	MLgyrADA-F;	TGAGACTCCGGTTTCCGCCC	139
	MLgyrADA-RB;	Biotin-CAGCGACCACGGCTGCGCC	

cases, three patients had been treated with the WHO MDT regimen for 2 years and the other six patients had received dapsone monotherapy or combined treatment with clofazimine and rifampicin. All cases had a positive bacterial index (BI) and were therefore, by definition, multibacillary. Genomic DNA templates were prepared as described previously (Matsuoka *et al.* 2005, 2007). Briefly, slit-skin smear specimens were collected from the skin lesions of patients in the same manner as the routine procedure for BI determination. The bacilli were washed out from the blade into 70 % ethanol and collected as a pellet by centrifugation at 10 000 ***g*** for 20 min. Genomic DNA templates for PCR were prepared by treatment with a lysis buffer as described elsewhere (de Wit *et al.*, 1991). The LDS-DA assays were performed as described above and the results were translated into nucleotide sequences according to the positions of the spots for comparison with the sequence data.

Nucleotide sequencing. To confirm and verify the results obtained by the LDS-DA method, nucleotide sequences of PCR products were determined with the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) using the same primers for PCR amplification with an ABI310 genetic analyser.

Ethical approval and consent. The study was approved by the institutional ethics committee of the National Institute of Infectious Diseases, Japan, and two local institutional review boards. Bacterial samples were collected after informed consent was obtained.

RESULTS

Development of a DNA microarray for drug susceptibility testing of *M. leprae*

The target regions of the genes with expected length, 119 bp for *folP1*, 127 bp for *rpoB* and 139 bp for *gyrA*, were amplified simultaneously by mutiplex PCR as shown in Fig. 2. Several oligonucleotides corresponding to each of the wild-type and mutant sequences of *folP1*, *rpoB* and *gyrA* were synthesized, spotted on a glass slide and examined for hybridization with amplicons from the multiplex PCR. The best oligonucleotides, which hybridized with corresponding PCR products without reacting with others, were selected. A DNA microarray with selected oligonucleotides was established as presented in Fig. 1 and designated LDS-DA. The performance of the LDS-DA was examined using PCR products from *M. leprae* isolates and artificially produced DNA fragments with known mutations. PCR products containing the drug-resistance-determining region (DRDR) for each gene were obtained by multiplex PCR (Fig. 2). Fig. 3 shows the hybridization patterns obtained from isolates grown in nude mice footpads, Thai-

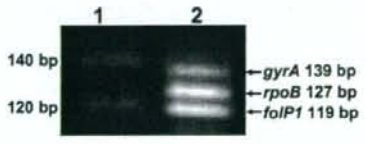

Fig. 2. Electrophoretic pattern obtained by multiplex PCR for *folP1*, *rpoB* and *gyrA*. Lane 1, 20 bp ladder size markers; lane 2, PCR products.

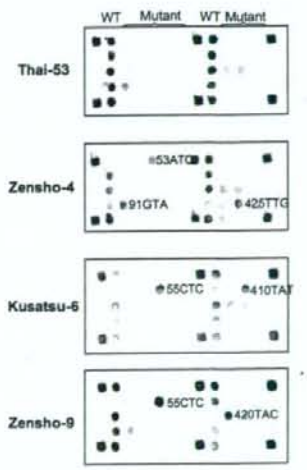

Fig. 3. Signals obtained by the LDS-DA with: a susceptible strain, Thai-53; strain Zensho-4, with mutation from ACC to ATC at codon 53 in the *folP1* gene, from TCG to TTG at codon 425 in the *rpoB* gene and from GCA to GTA at codon 91 in the *gyrA* gene; strain Kusatsu-6, with mutation from CCC to CTC at codon 55 in the *folP1* gene and from GAT to TAT at codon 410 in the *rpoB* gene; and strain Zensho-9, with mutation from CCC to CTC at codon 55 in the *folP1* gene and from CAC to TAC at codon 420 in the *rpoB* gene.

53, Zensho-4 (Matsuoka *et al.*, 2000) and two other strains with known nucleotide mutations (Zhang *et al.*, 2004; Maeda *et al.*, 2001). In Thai-53, which is susceptible to dapsone, rifampicin and ofloxacin, positive signals were observed on all of the wild-type spots. In contrast, the highest colour intensity was seen on the spot with the mutant oligonucleotide in drug-resistant *M. leprae*. In Zensho-4, with a three-drug-resistant phenotype, three positive signals shifted from the wild-type to the mutant spots. In the dapsone field, a mutation at codon 53 was identified by a positive signal on spot FM3 instead of FW1. In the ofloxacin and rifampicin fields, similar events were observed. Spots corresponding to mutant-type and wild-type sequences were also found in the other two isolates. Likewise, all the spots with mutant oligonucleotides were verified as to their proper reactivity with the PCR products carrying corresponding known mutations (data not shown).

Evaluation of the LDS-DA system in two countries with high leprosy prevalence

The LDS-DA system was successfully transferred to a laboratory in Yangon, Myanmar, and a laboratory in Cebu, the Philippines. The BI values of the samples from Myanmar varied from 1 to 6. Most were more than 3. Almost all samples from the Philippines showed a BI of more than 4, with a few samples of BI 2. Positive PCR results were obtained even from samples with a BI of 1, although it was usually hard to obtain good results from PCR and colouring from samples with a BI of less than 3. One of the relapsed cases from Myanmar harboured *M. leprae* with mutations CCC to CGC at position 55 in the *folP1* gene and TCG to ATG at position 425 in the *rpoB* gene. One resistant isolate with the mutation ACC to GCC at position 53 in the *folP1* and another isolate with the mutation GAT to TAT at position 410 in the *rpoB* gene were new cases. In the samples from the Philippines, three *M. leprae* with mutations in the *folP1* gene, CCC to CTC and CCC to TCC at position 55, were from relapsed cases. Two resistant isolates with mutation CCC to CGT at position 55 in the *folP1* were from new cases. The results obtained by the LDS-DA system in these laboratories were compared with the nucleotide sequences of the corresponding genes, as shown in Table 3. All the samples possessing wild-type sequences were judged to be wild-type by both the LDS-DA and sequencing. Concordant results were also observed with seven specimens carrying mutations, five in Myanmar and two in the Philippines. Two unclear results were obtained in *folP1* in Myanmar and one in *rpoB* in the Philippines. In these three samples, the signals were not strong enough to be judged. In the row of codon 55 in *folP1*, no signal was observed with two specimens in the Philippines. Overall, the concordance between the LDS-DA and sequencing results on *folP1* in Myanmar and the Philippines is 96.8 % (61/63) and 97.3 % (71/73), respectively. The LDS-DA results on *rpoB* exhibited good agreement with sequencing results, 100 % (63/63) and 98.6 % (72/73) in Myanmar and the Philippines, respectively. No discordance was found between the LDS-DA and sequencing results on *gyrA* in either country (Table 4).

DISCUSSION

Detection of drug resistance in *M. leprae* is crucial for the efficient treatment of leprosy and the prevention of the spread of drug-resistant strains. The elucidation of the genetic background of resistance by molecular methods has enabled the prediction of drug susceptibility of *M. leprae*. Drug resistance to dapsone, rifampicin and ofloxacin has evolved by mutation in the DRDR in the *folP1*, *rpoB* and *gyrA* genes respectively (Williams & Gillis, 2004). A total of

Table 3. Comparison of results obtained by the LDS-DA and sequencing on clinical specimens in Myanmar and the Philippines

Study site	Status	*folP1*			*rpoB*			*gyrA*		
		No. of specimens	LDS-DA	Sequence	No. of specimens	LDS-DA	Sequence	No. of specimens	LDS-DA	Sequence
Myanmar	Concordant	59	WT*	WT	61	WT	WT	62	WT	WT
		1	53:GCC†	53:GCC	1	410:TAT	410:TAT	1	91:GTA	91:GTA
		1	55:CGC	55:CGC	1	425:ATG	425:ATG			
	Discordant	2	Unclear‡	WT						
Philippines	Concordant	68	WT	WT	72	WT	WT	73	WT	WT
		2	55:CTC	55:CTC						
		1	55:TCC	55:TCC						
	Discordant	2	55:null§	55:CGT	1	Unclear	WT			

*Wild-type sequence.
†Codon number:codon sequence.
‡Data could not be translated because of weak signals.
§No signal was observed on the spots in raw *folP1* 55.

Table 4. Concordance of LDS-DA results with sequencing in clinical specimens in Myanmar and the Philippines

Study site	Target gene		
	folP1	*rpoB*	*gyrA*
Myanmar	61/63 (96.8 %)	63/63 (100 %)	63/63 (100 %)
The Philippines	71/73 (97.3 %)	72/73 (98.6 %)	73/73 (100 %)
Total	132/136 (97.1 %)	135/136 (99.3 %)	136/136 (100 %)

106 isolates without mutation in the *rpoB* gene and 63 isolates without mutation the *gyrA* gene were susceptible to rifampicin and ofloxacin, respectively. All isolates resistant to rifampicin or ofloxacin harboured mutations in the DRDR of *rpoB* or *gyrA*, respectively (Honoré & Cole, 1993; Honoré *et al.*, 1993; Williams *et al.*, 1994, 2001; Cambau *et al.*, 1997, 2001; Matsuoka *et al.*, 2000, 2003; Maeda *et al.*, 2001; Zhang *et al.*, 2004). Resistance of *M. leprae* to dapsone in the mouse footpad is classified into three degrees, namely, low, intermediate and high. A total of 84 isolates without mutation in the *folP1* gene were susceptible to dapsone, but one isolate was resistant with intermediate degree and five isolates were resistant with low degree (Cambau *et al.*, 2006). On the other hand, a total of 24 isolates resistant to dapsone with high or intermediate degree revealed amino acid substitution at the DRDR of the *fop1* gene (Kai *et al.*, 1999; Matsuoka *et al.*, 2000, 2003, 2007; Williams *et al.*, 2000; Lee *et al.*, 2001; Maeda *et al.*, 2001; Cambau *et al.*, 2006). An isolate with mutation ACC to GCC at codon 53 was demonstrated to be resistant with low degree (Cambau *et al.*, 2006), though it is not clear whether dapsone resistance with low degree is true resistance (Matsuoka *et al.*, 2007). Other isolates with this mutation were found to be resistant to dapsone with intermediate degree. Therefore contradiction between mutation in the *folP1* gene and the results obtained by the mouse footpad drug susceptibility test has been encountered for only one case so far.

Although the direct sequencing of PCR products is definitive and allows rapid detection of resistant cases, it has the disadvantage of requiring expensive apparatus and high sequencing costs, so it is not practical in many developing countries. The heteroduplex method (HAD) (Williams *et al.*, 2001) and the PCR-single-strand conformation polymorphism method (SSCP) (Honoré *et al.*, 1993) have been applied to the detection of mutants to overcome these disadvantages. The HAD method can identify mutations in the PCR-amplified fragments by the electrophoretic mobility difference of heteroduplexes of wild-type products and test sample products, while the SSCP method analyses that of single-stranded products. However, neither the HAD nor the SSCP method fully meets the required conditions in developing countries, since these methods demand complicated procedures and both detect silent mutations as resistant mutations. The recently developed LineProbe assay based on reverse hybridization can detect rifampicin-resistant *M. leprae* simply and rapidly, but it cannot provide susceptibility information for other anti-leprosy drugs. The multiple-primer PCR amplification refractory mutation system is relatively simple but detects only nucleotide mutations and cannot distinguish silent mutations from missense mutations (Sapkota *et al.*, 2008)

Our present study aimed to exploit a rapid, simple and simultaneous drug susceptibility test for three key anti-leprosy drugs to solve defects of each method previously reported, based on DNA–DNA hybridization using a DNA microarray. The novel method, designated LDS-DA, allows the simultaneous identification of mutations in three genes, responsible for resistance to dapsone, rifampicin and the quinolones. Easy accessibility and high reproducibility demonstrated by the studies with clinical materials in two developing countries revealed the superior applicability of this method. Only five discordant results were found in 136 specimens examined. Three discordant results, two in Myanmar on *folP1* and one in the Philippines on *rpoB*, showed faint reactions on multiple spots probably caused by some technical errors. In the remaining two discordant results found in the Philippines, no signal was found at any position in row 55 of *folP1*. These samples were shown to carry a mutation at codon 55 in *folP1* from the wild-type CCC to CGT (Table 2), which was recently revealed to be associated with dapsone resistance (Cambau *et al.*, 2006) and was not covered by the oligonucleotide array on the LDS-DA. Although the signal was found neither at the wild-type nor at the mutant position in row 55 of *folP1*, this result can be taken as suggestive of dapsone resistance. The absence of a positive signal in the wild-type position implies the existence of base substitutions in the region covered by the oligonucleotide. Similar translation criteria have been applied to rifampicin-resistant *M. tuberculosis* by the commercially available INNO-LiPA Rif TB assay (Rossau *et al.*, 1997). Other possible mutation(s) related to drug resistance can also be distinguished under the same criteria.

The monitoring of drug-resistant leprosy cases has been recommended in order to maintain the effectiveness of chemotherapy for leprosy (Ji, 2002; Matsuoka *et al.*, 2007). The LDS-DA method developed in this study seems to be a simple and robust tool to assess the drug susceptibility of *M. leprae* in developing countries, where susceptibility testing is rarely applied. Comprehensive data on the prevalence of resistant cases shows that the level of drug resistance is low in some endemic countries (Matsuoka *et al.*, 2007). It is therefore recommended to apply this method to samples from intractable cases and relapsed cases, to examine the susceptibility to anti-leprosy drugs and ensure effective treatment. Additionally, the capacity of the LDS-DA method to identify the positions of mutations can be utilized for molecular epidemiological and geographical studies on the spread of drug-resistant *M. leprae*.

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