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.

References

1) WHO: Global leprosy situation, beginning 2008. Weekly epidemiological record. 83: 293-300, 2008 (http://www.who.int/wer/en/).

2) Van Duijkeren E, Wannet WJ, Housewers DJ, van Pelt W: Serotype and phage type distribution of *salmonella* strains isolated from humans, cattle, pigs, and chicken in Netherland from 1984 to 2001. J Clin Micrbiol 40: 3980-3985, 2002.

3) Van Soolingen D: Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. J Int Med 249: 1-26, 2001.

4) Matsuoka M, Maeda S, Kai M, Nakata N, Chae GT, Gillis TP, Kobayashi K, Izumi S, Kashiwabara Y: *Mycobacterium leprae* typing by genomic diversity and global distribution of genotypes. Int J Lepr 68: 121-128, 2000.

5) ShinYC, Lee H, Lee H, Walsh GP, Kim JD, Cho SN: Variable numbers of TTC repeats in *Mycobacterium leprae* DNA from leprosy patients and use in strain differentiation. J Clin Microbiol 38: 4535-4538, 2000.

6) Monat M, Honore N, Garmier T, Araoz R, Copee JY, Lacroiz C, Sow S, Spencer JS, Truman RW, Williams DL, Gelber R, Virmond M, Flageul B, Cho SN, Ji B, Paniz-Mondolf A, Convit J, Young S, Fine PE, Rasolofo V, Brennan PJ, Cole ST: On the origin of leprosy. Science 308: 1040-1042, 2005.

7) Aseffa A, Brennan P, Dockrell H, Gills T, Hussain R, Oskam L, Richardson JH: Report on the first meeting of the IDEAL (Initiative for Diagnostic and Epidemiological assays for Leprosy) Consortium held at Armauer Hansen Research Institute, ALERT, Addis Ababa, Ethiopia on 24-27 October 2004. Lepr Rev 76: 147-159, 2005.

8) Matsuoka M, Zhang L, Budiawan T, Saeki K, Izumi S: Genotyping of *Mycobacterium leprae* on the basis of polymorphism of TTC repeats for analysis of leprosy transmission. J Clin Microbiol 42: 741-745, 2004.

9) Young SK, Taylor GM, Jain S, Suneetha

LM, Suneetha S, Lockwood DN, Young DB: Microsatellite mapping of *Mycobacterium leprae* populations in infected humans. J Clin Microbiol 42: 4931-4936, 2004

10) Zhang L, Budiawan T, Matsuoka M: Diversity of potential short tandem repeats in *Mycobacterium leprae* and application for Molecular typing. J Clin Microbio 43: 5221-5229, 2005.

11) Weng X Wang Z, Liu J, kimura M, Black IV WC, Brennan PJ, Li H, Vissa VD: Identification and distribution of *Mycobacterium leprae* genotypes in a region of high leprosy prevalence in China: a 3-year molecular epidemiological study. J Clin Microbio 45: 1728-1734, 2007.

12) Chae GT. Lee SB, Kang TJ, Shin HK, Kim JP, Ko YH, Kim SH, Kim NH: Typing of clinical isolates of *Mycobacterium leprae* and their distribution in Korea. Lep Rev 73: 41-46, 2002.

13) Matsuoka M, Zhang L, Fafutis M, Legua P, Wiens C: Polymorphism in the rpoT gene in *Mycobacterium leprae* isolates obtained from Latin American countries and its possible correlation with the spread of leprosy. FEMS Microbiol Lett 243: 311-315, 2005.

14) Matsuoka M, Lopez Roa RI, Budiawan T, Kyaw K, Chae GT: Genotyping analysis of *Mycobacterium leprae* isolates from Japan and other Asian countries reveals a global transmission pattern of leprosy. FEMS Microbiol lett 261: 150-154, 2006.

15) Lavania M, Katoch K, Sigh H, Das R, Gupta AK, Sharma R, Chauhan DS, Sharma VD, Sachan P, Sachan S, Katoch VM: Predominance of three copies of tandem repeats in proT gene of *Mycobacterium leprae* from Northern India. Infect Genet Evol 7: 627-631, 2007.

16) Srisungam S, Rudeeaneksin J, Wattanpokayakit S, Pasadorn S, Tragoolpua R, Suwanrit S, Sawanpanyalert P, Phetsuksiri B: Typing of Thai clinical isolates of and analysis of leprosy transmission by polymorphism of tandem repeat. Southeast Asian J Trop Med Public Health 38: 714-720, 2007.

17) Abe M, Ozawa T, Minagawa F, YoshinoY: Immunoepidemiological studies on studies on substantial infection in leprosy II. Geographical distribution of seropositive responders with special reference to their possible source of infection. Int J Lepr 59:162-168, 1990.

18) Moet FJ, Pahan D, Oskam L, Richardus JH: Effectiveness of single dose rifampicin in preventing leprosy in close contacts of patients with newly diagnosed leprosy: cluster randomised controlled trial. BMJ doi:10.1136/bmj. 395000.885752. BE, 2008.

19) Smith CM, and Smith WCS: Chemoprophylaxis is effective in the prevention of leprosy in endemic countries: a systematic review and meta-analysis. J Infect 41:137-142, 2000.

20) Fine PE, Sterne JM, Ponnighaus JM, Bliss L, Saui J, Chihana A, Munthali M, Warandorff DK: Household and Dwelling contacts as risk factors for leprosy in northern Malawi. Am J Epidemiol 146:91-102, 1997.

21) Nordeen SK The epidemiology of leprosy. In: Hastings RC (eds) Leprosy, 2nd edn. Churchill Livingstone, cp 3, pp 29-45

22) Saeki K, Budiawan T, Matsuoka M, Izumi S: Epidemiological significance of *Mycobacterium leprae* in the residential environment: Detection of *M. leprae* on the surface of nasal cavity of inhabitants in a leprosy endemic area using the polymerase chain reaction (in Japanese). Jap J Dermatol 110:153-160, 2000.

23) van Beers SM, Izumi S, Madjid B, Maeda Y, Day R, Klatser PR: An epidemiological study of leprosy infection by serology and polymerase chain reaction. Int J Lepr 62:1-91, 1994.

24) Monot M, Honore N, Baliere, Ji B, Sow S, Brennan, PJ, Cole ST: Are variable-number tandem repeats appropriate for genotyping *Mycobacterium leprae*. J Clin Microbiol 46: 2291-2297, 2008.

25) Young SK, Ponnighaus LM, Jain S, Lucas S, Suneetha S, Lockwood DNJ, Young DB, Fine PEM: Use of short tandem repeat sequences to study *Mycobacterium leprae* in leprosy patients in Malawi and India. PLoS Ngl Trop Dis 2:e214, 2008.

26) Goslee S, Wolinsky E: Water as a source of potentially pathogenic mycobacteria. Am rev Resp Dis 113:287-292, 1976.

27) Gruft H, Loder A, Osterhout M, Parker B, Falkinham III JO: Postulated source of *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum* infection: Isolation of Mycobacteria from estuaries and ocean waters. Am Rev Resp Dis 120:1385-1388, 1979.

28) Stinear T, Johson PDR: First isolation of *Mycobacterium ulcerans* from an aquatic environment: The end of a 60-years search. PLoS Ngl Trop Dis 2:e216, 2008.

29) Matsuoka M, Izumi S, Budiawan T, Nakata N, Saeki K: *Mycobacterium leprae* DNA in daily using water as a possible source of leprosy infection. Ind J Lepr 71:61-67, 1999.

30) Kurabachew M, Wondimu A, Ryon JJ: Reverse transcription-PCR detection of *Mycobacterium leprae* in clinical specimens. J Clin Micobiol 36: 1352-1356, 1998.

31) Matsuoka M, Budiawan T, Mukai T, Gidoh M, Izumi S: Quantification and evaluation of *Mycobacterium leprae* viability found in water in a leprosy endemic area. Abstract of U.S.-Japan cooperative medical sciences program, Forty-third Tuberculosis and leprosy research conference. 2008.

32) Lavania M, Katoch K, Sachan P, Dubey A, Kapoor S, Kashyap M, Chauhan DS, Sigh HB, Sharma VD, Jadhav RS, Katoch VM: Detection of DNA from soil samples by PCR targeting RLEP sequences. J Comm Dis 38: 269-373, 2006.

33) Falush D, Wirth T, Linz B, Pritchard JK, Stephens M, Kidd M, Blaser MJ, Graham DY, Vacher S, Perez-Perez GI, Yamaoka Y, Megraud F, Otto K, Reichard U, Katzowitsch E, Wang X, Achtman M, Suerbaum S: Traces of human migrations in *Helicobacter pylori* populations. Science 299: 1582-1585, 2003

34) Sugimoto C, Kitamura T, Guo J, Al-Ahdal MN, Shchelkunov SN, Otova B, Ondrejka P, Chollet J-Y, El-Safi S, Ettayebi M, Grésenguet G, Kocagöz T, Chaiyarasamee S, Thant KZ, Thein S, Moe K, Kobayashi N, Taguchi F, Yogo Y: Typing of urinary JC virus DNA offers a novel means of tracing human migrations. Proc Nat Aca Sci USA 94: 9191-9196, 1997

35) Hanihara K: Dual structure model for the population history of the Japanese. Japan Review 2: 1-33, 1991.

36) Trautman JR: The history of leprosy. In: Hastings RC (eds) Leprosy, 2nd edn. Churchill Livingstone, cp 2, pp 11-25

Jpn.J.Leprosy 78, 67-73(2009)

ハンセン病の分子疫学解析における最近の知見

松岡　正典*
国立感染症研究所ハンセン病研究センター生体防御部

〔受付・掲載決定：2008 年 9 月 24 日〕

キーワード：ハンセン病、分子疫学、らい菌、SNPs、VNTRs

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。

* Corresponding author:
国立感染症研究所ハンセン病研究センター生体防御部
〒 189-0002　東京都東村山市青葉町 4-2-1
TEL：042-391-8211　FAX：042-394-9092
E-mail：matsuoka@nih.go.jp

Journal of Medical Microbiology (2008), **57**, 1213–1219 DOI 10.1099/jmm.0.2008/002600-0

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

Masanori Matsuoka,[1]† Khin Saw Aye,[2] Kyaw Kyaw,[3] Esterlina Virtudes Tan,[4] Ma Victoria Balagon,[4] Paul Saunderson,[4] Robert Gelber,[4] Masanao Makino,[5] Chie Nakajima[6] and Yasuhiko Suzuki[6]†

[1]Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan

[2]Department of Medical Research, Yangon, Myanmar

[3]Central Special Skin Clinic, Yangon General Hospital, Yangon, Myanmar

[4]Leonard Wood Memorial, Cebu, The Philippines

[5]National Leprosy Hospital Okukomyoen, Okayama, Japan

[6]Department of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan

Correspondence
Masanori Matsuoka
matsuoka@nih.go.jp

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.

Received 14 April 2008
Accepted 18 June 2008

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

†These authors contributed equally to this work.

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
				FM : 8GACCGGCTCGGTGCC
Rifampicin	*rpoB*	407	RW1 : AGCTGTCGCAGTTCATG	RM1 : AGCTGTCGGTGTTCAT
		410	RW2 : TTCATGGATCAGAA	RM2 : TTCATGAATCAGAACAA
				RM3 : TTCATGTATCAGAACAA
		420	RW3 : CCTGACCCACAAGCGC	RM4 : GCCTGACCTACAAGCGC
				RM5 : GCCTGACCGACAAGCGC
		425	RW4 : CGCCGACTGTCGGCGCTG	RM : 6CCGACTGATGGCGC
				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)

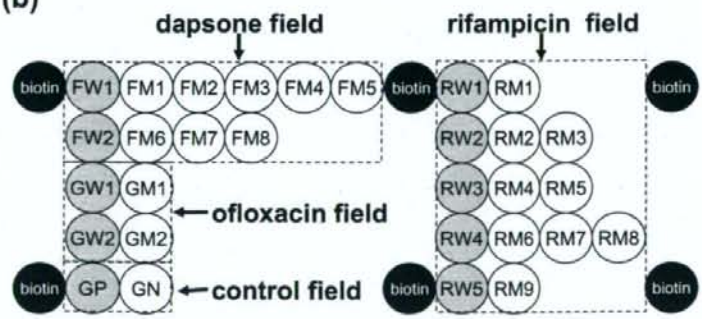

Fig. 1. Development of the LDS-DA. (a) The oligonucleotide sequences used in the test. Codons relating to drug resistance are underlined. Oligonucleotide GP, positive control for PCR amplification and hybridization; GN, negative control for hybridization. (b) Schematic representation of the array of 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-

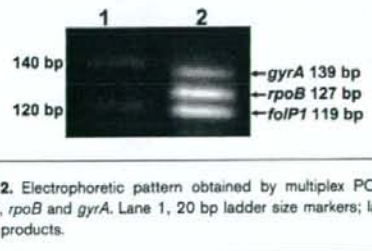

Fig. 2. Electrophoretic pattern obtained by multiplex PCR for *folP1*, *rpoB* and *gyrA*. Lane 1, 20 bp ladder size markers; lane 2, PCR products.

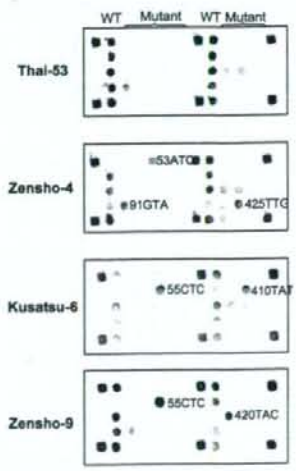

Fig. 3. Signals obtained by the LDS-DA with: a susceptible strain, Thai-53; strain Zensho-4, with mutation from ACC to ATC at codon 53 in the *folP1* gene, from TCG to TTG at codon 425 in the *rpoB* gene and from GCA to GTA at codon 91 in the *gyrA* gene; strain Kusatsu-6, with mutation from CCC to CTC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

ACKNOWLEDGEMENTS

This study was supported by the following grants: a Health Research Grant for Emerging and Re-emerging Infectious Diseases, Ministry of Health, Labour and Welfare, Government of Japan; a grant from the International Medical Center, Ministry of Health, Labour and Welfare; a grant from the US–Japan Cooperative Medical Science Programs; and also by the Grants-in-Aid Program of the Founding Research Center for Emerging and Reemerging Infectious Diseases from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, to Y. S.

REFERENCES

Cambau, E., Perani, E., Guillemin, I., Jamet, P. & Ji, B. (1997). Multidrug rtesistance to dapsone, rifampicin, and ofloxacin in *Mycobacterium leprae*. *Lancet* **349**, 103–104.

Cambau, E., Bonnafous, P., Perani, E., Sougakoff, W., Ji, B. & Jarlier, V. (2001). Molecular detection of rifampicin and ofloxacin resistance for patients who experience relapse of multibacillary leprosy. *Clin Infect Dis* **34**, 39–45.

Cambau, E., Carthagena, L., Chauffour, A., Ji, B. & Jarlier, V. (2006). Dihydropteroate synthase mutations in the *folP1* gene predict dapsone resistance in relapsed cases of leprosy. *Clin Infect Dis* **42**, 238–241.

de Wit, M. Y. L., Faber, W. R. S., Krieg, R., Douglas, J. T., Lucas, S. B., Montreewasuwat, N., Pattyn, S. R. N., Hussain, R., Ponnighaus, J. M. & other authors (1991). Application of polymerase chain reaction for the detection of *Mycobacterium leprae* in skin tissue. *J Clin Microbiol* **29**, 906–910.

Honoré, N. & Cole, S. T. (1993). Molecular basis of rifampicin resistance in *Mycobacterium leprae*. *Antimicrob Agents Chemother* **37**, 414–418.

Honoré, N., Perani, E., Telenti, A., Grosset, J. & Cole, S. T. (1993). A simple and rapid technique for the detection of rifampicin resistance in *Mycobacterium leprae*. *Int J Lepr Other Mycobact Dis* **61**, 600–604.

Ji, B. (2002). Rifampicin resistant leprosy: a review and research proposal of a pilot study. *Lepr Rev* **73**, 2–8.

Kai, M., Matsuoka, M., Nakata, N., Maeda, S., Gidoh, M., Maeda, Y., Hashimoto, K., Kobayashi, K. & Kashiwabara, Y. (1999). Diaminodiphenilsulfone resistance of *Mycobacterium leprae* due to mutations in the dihydropteroate synthase gene. *FEMS Microbiol Lett* **177**, 231–235.

Lee, S. B., Kim, S. K., Kang, T. J., Chae, G. T., Chun, J. H., Shin, H. K., Kim, J. P., Ko, Y. H. & Kim, N. H. (2001). The prevalence of *folP1* mutations associated with clinical resistance to dapsone, in *Mycoabcterium leprae* isolates from South Korea. *Ann Trop Med Parasitol* **95**, 429–432.

Maeda, S., Matsuoka, M., Nakata, N., Kai, M., Maeda, Y., Hashimoto, K., Kimura, H., Kobayashi, K. & Kashiwabara, Y. (2001). Multidrug resistant *Mycobacterium leprae* from patients with leprosy. *Antimicrob Agents Chemother* **45**, 3635–3639.

Matsuoka, M., Kashiwabara, Y. & Namisato, Y. (2000). A *Mycobacterium leprae* isolate resistant to dapsone, rifampicin, ofloxacine and sparfloxacin. *Int J Lepr Other Mycobact Dis* **68**, 452–455.

Matsuoka, M., Kashiwabara, Y., Zhang, L., Gotoh, M. & Kitajima, S. (2003). A second case of multidrug resistant *Mycobacterium leprae* isolated from a Japanese patient with relapsed lepromatous leprosy. *Int J Lepr Other Mycobact Dis* **71**, 240–243.

Matsuoka, M., Zhang, L., Morris, M. F., Legua, P. & Wiens, C. (2005). Polymorphism in the *rpoT* gene in *Mycobacterium leprae* isolates obtained from Latin American countries and its possible correlation with the spread of leprosy. *FEMS Microbiol Lett* **243**, 311–315.

Matsuoka, M., Teky, B., Khin, S. A., Kyaw, K., Tan, E. V., dela Cruz, E., Gelber, R., Saunderson, P., Balagon, M. A. & Pannikar, V. (2007). The frequency of drug resistance mutations in *Mycobacterium leprae* isolates in untreated and relapsed leprosy patients from Myanmar, Indonesia and the Philippines. *Lepr Rev* **78**, 343–352.

Rossau, R., Traore, H., de Beenhouwer, H., Mijs, W., Jannes, G., De Rijk, P. & Portaels, F. (1997). Evaluation of the INNO-LiPA Rif. TB assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampicin. *Antimicrob Agents Chemother* **41**, 2093–2098.

Sapkota, B. R., Ranjit, C., Neupane, K. D. & Macdonald, M. (2008). Developing and evaluation of a novel multiple-primer PCR amplification refractory mutation system for the rapid detection of mutations conferring rifampicin resistance in codon 425 of the *rpoB* gene of *Mycobacterium leprae*. *J Med Microbiol* **57**, 179–184.

Shepard, C. (1960). The experimental disease that follows the injection of human leprosy bacillus into footpads of mice. *J Exp Med* **112**, 445–454.

WHO (1998). WHO Expert Committee on Leprosy, seventh report. Technical report series 87. Geneva: World Health Organization.

Williams, D. L. & Gillis, T. P. (2004). Molecular detection of resistance in *Mycobacterium leprae*. *Lepr Rev* **75**, 118–130.

Williams, D. L., Waguespack, C., Eisenack, K., Crawford, J. T., Portaels, F., Salfinger, M., Nolan, M. C., Abe, C., Stich-Groh, V. & Gillis, T. P. (1994). Characterization of rifampicin resistance in pathogenic mycobacteria. *Antimicrob Agents Chemother* **38**, 2380–2386.

Williams, D. L., Spring, L., Harris, E., Roche, P. & Gillis, T. P. (2000). Dihydropteroate synthase of *Mycobacterium leprae* and dapsone resistance. *Antimicrob Agents Chemother* **44**, 1530–1537.

Williams, D. L., Pittman, T. L., Gillis, T. P., Matsuoka, M. & Kashiwabara, Y. (2001). Simultaneous detection of *Mycobacterium leprae* and its susceptibility to dapsone using DNA heteroduplex analysis. *J Clin Microbiol* **39**, 2083–2088.

Zhang, L., Namisato, M. & Matsuoka, M. (2004). A mutation at codon 516 in the *rpoB* gene of *Mycobacterium leprae* confers resistance to rifampicin. *Int J Lepr Other Mycobact Dis* **72**, 468–472.

CLINICAL AND VACCINE IMMUNOLOGY, Dec. 2008, p. 1755–1759 Vol. 15, No. 12
1556-6811/08/$08.00+0 doi:10.1128/CVI.00148-08

Serological Diagnosis of Leprosy in Patients in Vietnam by Enzyme-Linked Immunosorbent Assay with *Mycobacterium leprae*-Derived Major Membrane Protein II[▽]

Masanori Kai,[1]* Nhu Ha Nguyen Phuc,[2] Thuy Huong Hoang Thi,[2] An Hoang Nguyen,[2] Yasuo Fukutomi,[1] Yumi Maeda,[1] Yuji Miyamoto,[1] Tetsu Mukai,[1] Tsuyoshi Fujiwara,[3] Tan Thanh Nguyen,[2] and Masahiko Makino[1]

Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aoba-cho, Higashimurayama, Tokyo 189-0002, Japan[1]; Quyhoa National Leprosy & Dermato-Venereology Hospital, Ghenhrang District, Quynhon City, Binhdinh, Vietnam[2]; and Institute for Natural Science, Nara University, Nara 631-8502, Japan[3]

Received 21 April 2008/Returned for modification 13 June 2008/Accepted 12 October 2008

A serological diagnostic test using phenolic glycolipid-I (PGL-I) developed in the 1980s is commercially available, but the method is still inefficient in detecting all forms of leprosy. Therefore, more-specific and -reliable serological methods have been sought. We have characterized major membrane protein II (MMP-II) as a candidate protein for a new serological antigen. In this study, we evaluated the effectiveness of the enzyme-linked immunosorbent assay (ELISA) using the MMP-II antigen (MMP-II ELISA) for detecting antibodies in leprosy patients and patients' contacts in the mid-region of Vietnam and compared to the results to those for the PGL-I method (PGL-I ELISA). The results showed that 85% of multibacillary patients and 48% of paucibacillary patients were positive by MMP-II ELISA. Comparison between the serological tests showed that positivity rates for leprosy patients were higher with MMP-II ELISA than with PGL-I ELISA. Household contacts (HHCs) showed low positivity rates, but medical staff members showed comparatively high positivity rates, with MMP-II ELISA. Furthermore, monitoring of results for leprosy patients and HHCs showed that MMP-II is a better index marker than PGL-I. Overall, the epidemiological study conducted in Vietnam suggests that serological testing with MMP-II would be beneficial in detecting leprosy.

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* infection, which sometimes leads to progressive peripheral nerve injury and systematic deformity (16, 30). Early detection of *M. leprae* infection and early start of treatment are key in avoiding deformities. Also, in order to decrease the incidence of new cases, it is important to find and treat the sources of the infection as soon as possible. Thus, early detection of these infected individuals who cannot be clinically diagnosed is critical (34). The diagnosis of leprosy is based on microscopic detection of acid-fast bacilli in skin smears or biopsies, along with clinical and histopathological evaluation of suspected patients. Recently, diagnostic methods for leprosy based on *M. leprae* DNA sequences have been developed (10, 20, 25). However, it is difficult to use these methods in developing countries which still have leprosy hot spot areas, because such methods require expensive machines and materials as well as skilled technicians. Although many developing countries have recently established laboratories for DNA-based diagnosis, it is harder to perform DNA tests than serodiagnostic tests. Thus, in countries where leprosy is endemic, diagnosis still relies on clinical observations and easy, inexpensive tests.

Serodiagnosis is generally accepted as the easiest way of diagnosing a disease. For leprosy serodiagnosis, the only antigen currently used is phenolic glycolipid I (PGL-I), which is supposedly specific to *M. leprae* (21, 26, 27). Since the identification of PGL-I in 1981 by Hunter and Brennan (14), a number of serological tools have been developed. Simple assays, such as the Serodia-Leprae method, a dipstick assay, and lateral flow tests based on the PGL-I antigen, have been used to detect leprosy patients in areas where leprosy is endemic (3, 15, 17, 32). However, these tests seem to be insufficient for detection of both multibacillary (MB) and paucibacillary (PB) patients, as well as for early diagnosis, and have not been used as widely as would be expected in field situations (6, 29). Therefore, we have begun the search for a more sensitive antigen. Major membrane protein II (MMP-II; encoded by the ML2038c gene, named *bfrA*, also known as bacterioferritin) was previously identified from the cell membrane fraction of *M. leprae* as an antigenic molecule capable of activating both antigen-presenting cells and T cells (19, 24). A homology search of the mycobacteria nucleotide database revealed that MMP-II is conserved between *M. leprae*, *M. tuberculosis*, and *M. avium*. The amino acid identity is about 86% among the three species. However, we have previously examined the role of MMP-II in the humoral responses of Japanese patients and showed that MMP-II could contribute to the specific serodetection of leprosy patients (18).

In the present study, we performed a serological test using serum samples collected in regions of leprosy endemicity in Vietnam and evaluated the use of MMP-II as an antigen for serodiagnosis of leprosy. We believe that identifying the appropriate antigens for serodiagnosis could facilitate the devel-

* Corresponding author. Mailing address: Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aoba-cho, Higashimurayama, Tokyo 189-0002, Japan. Phone: 81-42-391-8211. Fax: 81-42-391-8807. E-mail: mkai@nih.go.jp.
▽ Published ahead of print on 22 October 2008.

opment of simple diagnostic tests, like dip-stick assays, for use in developing countries.

MATERIALS AND METHODS

Serum samples. A total of 974 serum samples from various individuals, including in- and out-patients of Quyhoa National Leprosy & Dermato-Venereology Hospital (NDH), were obtained under informed consent. The sera were donated by 205 leprosy patients (163 patients undergoing treatment and 42 new patients), 428 household contacts (HHCs), 130 medical staff members, and 211 noncontact healthy individuals. Sera of leprosy patients and their contacts were taken at regional medical centers in the midregion of Vietnam, including those in the Danang, Quangnam, Quangngai, Binhdinh, Phuyen, Khanhhoa, Ninhthuan, Gialai, Kontum, Daklak, and Daknong provinces, where the average prevalence rate is 0.17 (number of cases/10,000 persons) and the average detection rate is 2.13 (number of cases/10,000 persons). Among these provinces, Binhdinh, Ninhthuan, Gialai, and Kontum had hot spot areas. The medical staff members consisted of workers in Quyhoa NDH, including medical doctors, nurses, pharmacists, technicians, and helpers. Only the sera from medical staff members who were not HHCs of leprosy patients were used in this study. Sera were also obtained from healthy persons living in the Binhdinh province (n = 126) and the Longan province (n = 85), which are distantly located from each other. Out of 205 leprosy patients, 121 had MB leprosy and 84 had PB leprosy. We made the initial diagnosis according to the Ridley-Jopling classification system and classified patients as MB and PB types based on the WHO recommendation. In Vietnam, the *M. bovis* bacille Calmette-Guérin (BCG) vaccination against tuberculosis has been undertaken in earnest since 1976. Almost all medical staff personnel who donated their blood for this study were vaccinated with BCG.

MMP-II and PGL-I antigens. The MMP-II gene (ML2038c, or *bfrA*) was expressed in *Escherichia coli* as a fusion construct by using a pMAL-c2X expression vector (New England BioLabs) (18). Synthetic bovine serum albumin-conjugated trisaccharide-phenyl propionate for the detection of PGL-I antibodies was produced by our laboratory. The procedure for synthesis of the antigen is described elsewhere (12).

ELISAs for detection of antibodies. MaxiSorp (Nalge Nunc) microtiter plates were coated with 50 μl antigen solution (MMP-II [0.4 μg/ml] and PGL-I [0.2 μg/ml]) in carbonate-bicarbonate buffer (pH 9.4) and kept at 4°C overnight. The optimal concentrations of these antigens were determined in advance. The enzyme-linked immunosorbent assay (ELISA) protocol was performed as described previously (18). We measured anti-MMP-II immunoglobulin G (IgG) antibodies and anti-PGL-I IgM antibodies. Plate-to-plate variations in optical density (OD) readings were controlled for by using a common standard serum.

Monitoring. One hundred forty-eight leprosy patients have been monitored using MMP-II ELISA and PGL-I ELISA during their multidrug therapy (MDT) treatment since 2001. Twelve-month MDT for MB was carried out, and sampling was performed three to five times. Also, HHCs were monitored once every 3 or 6 months by both the MMP-II and the PGL-I ELISA methods from 2001 to 2004.

Statistics. The data were analyzed using a statistical software package (version 9.3.2.0; MedCalc software). A receiver operator characteristic (ROC) curve was drawn to calculate the cutoff levels (2). Additionally, the statistically significant differences between assays were confirmed by the chi-square test (28).

RESULTS

Comparison of the distribution of ELISA values between MMP-II and PGL-I. We focused on the distribution of ELISA values derived from MB leprosy patients and compared them to those from healthy individuals (Fig. 1). The cutoff OD_{405} value for anti-MMP-II antibody was defined as 0.103 (95% confidence interval, 85.2 to 93.7), and that for anti-PGL-I antibody was defined as 0.452 (95% CI, 85.2 to 93.7), by ROC curve analysis (MedCalc software) using OD titers from 211 healthy individuals and 205 leprosy patients. The distribution pattern of MMP-II ELISA values was quite different from that of PGL-I ELISA for healthy individuals. While the OD values of most healthy individuals were in the low range for MMP-II ELISA (Fig. 1A), the titers obtained by PGL-I ELISA showed a bell-shaped curve which was similar to that of MB leprosy patients (Fig. 1B). The PGL-I ELISA values for PB leprosy patients also showed a similar bell-shaped curve (data not shown).

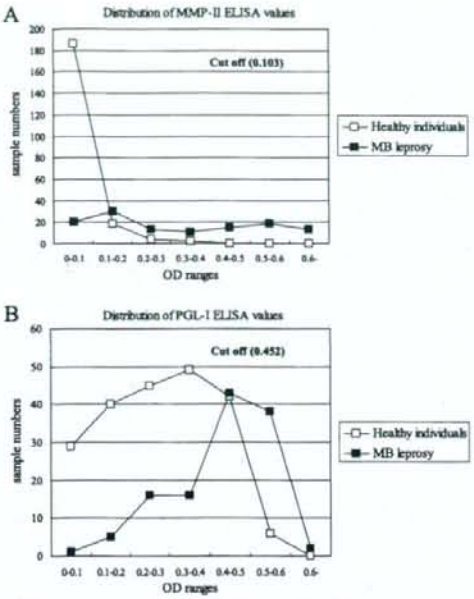

FIG. 1. Comparison of distributions of OD values in MB leprosy patients and normal individuals. (A) Distribution pattern of MMP-II ELISA values in patients and healthy individuals. (B) Distribution pattern of PGL-I ELISA values in patients and healthy individuals. The solid squares show the number of MB leprosy patients in each OD value range, and the open squares show the number of healthy individuals.

Detection rate of antibodies in sera of leprosy patients. Among the MB patients, 85.1% were positive by MMP-II ELISA and 57.0% were positive by PGL-I ELISA; 47.6% of PB patients were positive by MMP-II ELISA, and 20.2% were positive by PGL-I ELISA (Fig. 2). The MMP-II ELISA values for both MB and PB patients were significantly higher than the PGL-I ELISA values ($P < 0.001$) (Fig. 2). Patients undergoing treatment and new cases showed a similar difference (data not shown).

Seropositivity rates of contacts, medical staff members, and healthy volunteers. There was no significant difference in positivity rate between MMP-II ELISA and PGL-I ELISA for healthy individuals and HHCs (Fig. 3). Also, there was no significant difference in positivity rate between MMP-II ELISA and PGL-I ELISA for healthy individuals from different provinces, namely, Binhdinh and Longan (data not shown). In contrast, the medical staff showed a significantly higher rate of positivity by MMP-II ELISA (26.2%) than by PGL-I ELISA. The anti-MMP-II antibody positivity rate for the medical staff

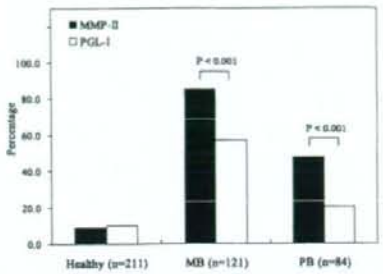

FIG. 2. Comparison of positivity rates of patients as determined by MMP-II and PGL-I ELISA. Black bars show percentages of healthy individuals and patients positive by MMP-II ELISA, and white bars show those for PGL-I ELISA. Statistically significant differences were confirmed by the chi-square test and are indicated as *P* values.

was significantly higher than those for healthy individuals and HHCs.

Monitoring of HHCs. Previous studies suggested the usefulness of PGL-I ELISA in monitoring the effects of leprosy treatment (5, 8, 9, 22). Therefore, we monitored anti-MMP-II antibody titers in patients after treatment and compared them to anti-PGL-I antibody titers. Ninety-two MB and 56 PB patients were monitored. The anti-MMP-II antibody value of approximately 30% of monitored MB patients declined within 1 to 2 years after the start of treatment, in accordance with changes in bacterial index values (data not shown), although approximately 50% of MB patients showed no reduction in ELISA values and 20% of patients showed mild increases in value. Three representative samples of MB patients are shown in Fig. 4. Among PB patients, 18% of the monitored patients had reduced anti-MMP-II antibody titers. On the other hand, anti-PGL-I antibody titers were reduced approximately only 20% in both MB and PB patients during the monitoring period. Therefore, anti-MMP II antibody may reflect the efficacy of treatment similarly to or slightly better than anti-PGL-I antibody in some cases. Furthermore, 9 individuals out of 428

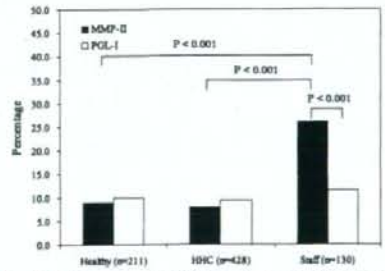

FIG. 3. Positivity rates of HHCs and medical staff members as determined by MMP-II and PGL-I ELISA. Black bars show percentages of HHCs and medical staff members positive by MMP-II ELISA, and white bars show those by PGL-I ELISA. Statistically significant differences were confirmed by the chi-square test and are indicated as *P* values.

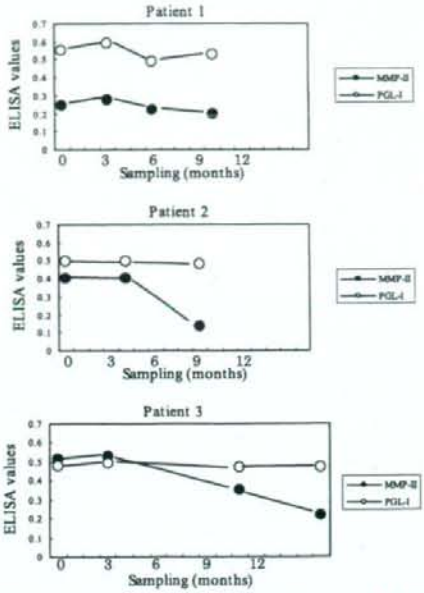

FIG. 4. Monitoring of three MB leprosy patients by MMP-II and PGL-I ELISAs. Three cases of monitored leprosy patients are shown. The closed circles show MMP-II ELISA values, and the open circles show PGL-I ELISA values. Note that the cutoff value for MMP-II is 0.103 and that of PGL-I is 0.452.

HHCs developed leprosy after several years of monitoring. Among the nine cases, two individuals had increasing antibody titers by MMP-II and/or PGL-I ELISA 1 year before manifesting clinical symptoms (data not shown). Patient HHC192 showed a prominent rise in anti-MMP-II antibody values during the asymptomatic period. Both patients developed MB leprosy. The other seven, whose antibody levels did not show an apparent increase during the observation period, developed PB leprosy.

DISCUSSION

Serodiagnosis is the easiest, cheapest, and least invasive diagnostic tool for infectious diseases. Currently, PGL-I is used as a specific antigen for *M. leprae*, but in practice, its sensitivity and specificity are not as high as expected, even though previous studies using stock sera reported that the detection rate for MB patients was more than 80% (1, 3, 4, 7). The present study involving Vietnamese leprosy patients indicated that there is a significant difference between MMP-II ELISA and PGL-I ELISA in detecting both MB and PB leprosy. The positivity rate of anti-MMP-II antibody for MB leprosy was approximately 85%, and that for PB leprosy was 48%; these titers were significantly higher than the titers obtained by PGL-I ELISA (57% and 20%, respectively). The detection rates obtained by

MMP-II ELISA were similar to those for a previous study using stock sera from Japanese leprosy patients (18). However, the positivity rates of anti-PGL-I antibody in the present study were significantly lower than those for the Japanese patients, although the same antigens for both MMP-II and PGL-I were used in the two studies.

There are several possible reasons why the sensitivity of PGL-I ELISA was low in the present study. One reason may be that some healthy Vietnamese individuals have high anti-PGL-I antibody titers. Although we could not conduct further detailed analysis on the subjects, these individuals might be highly exposed to *M. leprae*, and so their B lymphocytes might be repeatedly stimulated with *M. leprae*-derived antigens, including PGL-I. It seems quite difficult to discriminate the healthy individuals from MB or PB leprosy patients by PGL-I ELISA, as shown in Fig. 1. Furthermore, we concluded that a reasonable cutoff point for PGL-I ELISA was an OD_{405} of 0.452, as deduced from Fig. 1 and the ROC values, but this resulted in lower sensitivity. The difference in sensitivity between PGL-I ELISA and MMP-II ELISA may also be due to differences in the biochemical features of the antigens. PGL-I is a glycolipid component, and as such, it might be retained in some infected cells for a long time after the initial exposure (13, 33). This speculation is supported by previous reports showing that healthy individuals residing in areas where leprosy is endemic had high anti-PGL-I antibody titers, and *M. leprae* DNA was recovered by PCR from the nasal swabs of these individuals (31, 32). Also, it has been reported that the usefulness of PGL-I-based tests for early diagnosis is limited, since 7 to 10% of individuals testing positive do not develop the disease (14).

In contrast, MMP-II is a protein antigen and is considered to be one of the immunodominant antigens of *M. leprae* (19). Therefore, in individuals who have been exposed to *M. leprae* but have not developed leprosy, antigen-presenting cells expressing MMP-II might feasibly be eliminated from the body by immune cells such as cytotoxic T lymphocytes and thus lack the ability to produce anti-MMP-II antibodies through antigen-presenting-cell-dependent mechanisms. These speculations seem to be supported by our present observations with sera from patients monitored over time. Anti-MMP-II antibody titers of MB patients declined earlier than PGL-I titers with MDT treatment, indicating the disappearance of MMP-II antigens, while no apparent reduction in PGL-I antigens was observed during the 12 months of observation (Fig. 4). Furthermore, in one case the anti-MMP-II antibody titer increased drastically before manifestation of clinically apparent leprosy (data not shown).

Medical staff members (n = 130) showed a high positivity rate by MMP-II ELISA, compared with healthy individuals or HHCs. These medical staff members were mostly BCG vaccinated, as were the HHCs. Therefore, it seems that BCG vaccination has no effect on anti-MMP-II antibody titers. Although we could not determine a conclusive reason for the high positivity rate, these medical personnel may be repeatedly exposed to *M. leprae* in hospitals. However, we cannot eliminate the possibility that they have produced the antibody in response to exposure to other mycobacteria, since the MMP-II protein is conserved in other pathogenic mycobacterial species, such as *M. tuberculosis* and *M. avium*, though the staff members with high anti-MMP-II antibody titers did not manifest any clinical signs or features indicating infection with other mycobacteria. We tried to perform nested PCR using the *M. leprae*-specific repetitive element for DNA extracted from nasal swabs of some hospital staff members (n = 25). However, because the sampling dates for the serological test and the PCR test were not coordinated, we could not come to a definite conclusion. Nevertheless, we were surprised to find that ≅40% (n = 25) of the nasal swab samples were positive (data not shown). As for tuberculosis, it is said that one-third of the world population is infected with *M. tuberculosis*. The same may be the case with leprosy, although further studies are needed with larger populations, including medical staff members as well as contacts and noncontacts of leprosy.

Taken together, our data indicate that MMP-II ELISA could be useful as a supporting serodiagnostic tool in combination with other clinical diagnostic methods and may also be useful in monitoring disease activity. Furthermore, in this study the correlation between MMP-II and PGL-I was low, with a correlation coefficient among the 205 leprosy patients of only 0.63. If both PGL-I and MMP-II antibodies could be measured simultaneously, the sensitivity of the assay system could be increased. Considering that PGL-I is a sugar antigen (eliciting IgM antibodies) and MMP-II is a protein antigen (eliciting IgG antibodies), assaying for a combination of these antibodies could lead to more-accurate detection of leprosy in the field.

ACKNOWLEDGMENTS

This research was supported in part by Health Sciences research grants for research on emerging and reemerging infectious diseases; by an international cooperation research grant (topic code 18C4) from the Ministry of Health, Labor and Welfare of Japan; and by Quyhoa NDH.

REFERENCES

1. **Agis, F., P. Schlich, J. L. Cartel, C. Guidi, and M. A. Bach.** 1988. Use of anti-*M. leprae* phenolic glycolipid-I antibody detection for early diagnosis and prognosis of leprosy. Int. J. Lepr. Other Mycobact. Dis. **56**:527–535.
2. **Beck, J. R., and E. K. Schultz.** 1986. The use of relative operating characteristic (ROC) curves in test performance evaluation. Arch. Pathol. Lab. Med. **110**:13–20.
3. **Bührer-Sékula, S., H. L. Smits, G. C. Gussenhoven, J. van Leeuwen, S. Amador, T. Fujiwara, P. R. Klatser, and L. Oskam.** 2003. Simple and fast lateral flow test for classification of leprosy patients and identification of contacts with high risk of developing leprosy. J. Clin. Microbiol. **41**:1991–1995.
4. **Cartel, J. L., S. Chanteau, J. P. Boutin, R. Plichart, P. Richez, J. F. Roux, and J. H. Grosset.** 1990. Assessment of anti-phenolic glycolipid-I IgM levels using an ELISA for detection of *M. leprae* infection in populations of the South Pacific Islands. Int. J. Lepr. Other Mycobact. Dis. **58**:512–517.
5. **Chanteau, S., J. L. Cartel, P. Celerier, R. Plichart, S. Desforges, and J. Roux.** 1989. PGL-I antigen and antibody detection in leprosy patients: evolution under chemotherapy. Int. J. Lepr. Other Mycobact. Dis. **57**:735–743.
6. **Chanteau, S., P. Glaziou, C. Plichart, P. Luquiaud, R. Plichart, J. F. Faucher, and J. L. Cartel.** 1993. Low predictive value of PGL-I serology for the early diagnosis of leprosy in family contacts: results of a 10-year prospective field study in French Polynesia. Int. J. Lepr. Other Mycobact. Dis. **61**:533–541.
7. **Chaturvedi, V., S. Sinha, B. K. Girdhar, and U. Sengupta.** 1991. On the value of sequential serology with a *Mycobacterium leprae*-specific antibody competition ELISA in monitoring leprosy chemotherapy. Int. J. Lepr. Other Mycobact. Dis. **59**:32–40.
8. **Cho, S. N., R. V. Cellona, T. T. Fajardo, Jr., R. M. Abalos, E. C. dela Cruz, G. P. Walsh, J. D. Kim, and P. J. Brennan.** 1991. Detection of phenolic glycolipid-I antigen and antibody in sera from new and relapsed lepromatous patients treated with various drug regimens. Int. J. Lepr. Other Mycobact. Dis. **59**:25–31.
9. **Cho, S. N., R. V. Cellona, L. G. Villahermosa, T. T. Fajardo, Jr., M. V. Balagon, R. M. Abalos, E. V. Tan, G. P. Walsh, J. D. Kim, and P. J. Brennan.** 2001. Detection of phenolic glycolipid I of *Mycobacterium leprae* in sera from

leprosy patients before and after start of multidrug therapy. Clin. Diagn. Lab. Immunol. **8**:138–142.
10. **Donoghue, H. D., J. Holton, and M. Spigelman.** 2001. PCR primers that can detect low levels of *Mycobacterium leprae* DNA. J. Med. Microbiol. **50**:177–182.
11. Reference deleted.
12. **Fujiwara, T., S. W. Hunter, S. N. Cho, G. O. Aspinall, and P. J. Brennan.** 1984. Chemical synthesis and serology of disaccharides and trisaccharides of phenolic glycolipid antigens from the leprosy bacillus and preparation of a disaccharide protein conjugate for serodiagnosis of leprosy. Infect. Immun. **43**:245–252.
13. **Gelber, R. H., F. Li, S. N. Cho, S. Byrd, K. Rajagopalan, and P. J. Brennan.** 1989. Serum antibodies to defined carbohydrate antigens during the course of treated leprosy. Int. J. Lepr. Other Mycobact. Dis. **57**:744–751.
14. **Hunter, S. W., and P. J. Brennan.** 1981. A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. J. Bacteriol. **147**:728–735.
15. **Izumi, S., T. Fujiwara, M. Ikeda, Y. Nishimura, K. Sugiyama, and K. Kawatsu.** 1990. Novel gelatin particle agglutination test for serodiagnosis of leprosy in the field. J. Clin. Microbiol. **28**:525–529.
16. **Job, C. K.** 1989. Nerve damage in leprosy. Int. J. Lepr. Other Mycobact. Dis. **57**:532–539.
17. **Kai, M., Y. Maeda, S. Maeda, Y. Fukutomi, K. Kobayashi, Y. Kashiwabara, M. Makino, M. A. Abbasi, M. Z. Khan, and P. A. Shah.** 2004. Active surveillance of leprosy contacts in country with low prevalence rate. Int. J. Lepr. Other Mycobact. Dis. **72**:50–53.
18. **Maeda, Y., T. Mukai, M. Kai, Y. Fukutomi, H. Nomaguchi, C. Abe, K. Kobayashi, S. Kitada, R. Maekura, I. Yano, N. Ishii, T. Mori, and M. Makino.** 2007. Evaluation of major membrane protein-II as a tool for serodiagnosis of leprosy. FEMS Microbiol. Lett. **272**:202–205.
19. **Maeda, Y., T. Mukai, J. Spencer, and M. Makino.** 2005. Identification of an immunomodulating agent from *Mycobacterium leprae*. Infect. Immun. **73**: 2744–2750.
20. **Martinez, A. N., C. F. P. C. Britto, J. A. C. Nery, E. P. Sampaio, M. R. Jardim, E. N. Sarno, and M. O. Moraes.** 2006. Evaluation of real-time and conventional PCR targeting complex 85 genes for detection of *Mycobacterium leprae* DNA in skin biopsy samples from patients diagnosed with leprosy. J. Clin. Microbiol. **44**:3154–3159.
21. **Meeker, H. C., W. R. Levis, E. Sersen, G. Schuller-Levis, P. J. Brennan, and T. M. Buchanan.** 1986. ELISA detection of IgM antibodies against phenolic glycolipid-I in the management of leprosy: a comparison between laboratories. Int. J. Lepr. Other Mycobact. Dis. **54**:530–539.
22. **Meeker, H. C., G. Schuller-Levis, F. Fusco, M. A. Giardina-Becket, E. Sersen, and W. R. Levis.** 1990. Sequential monitoring of leprosy patients with serum antibody levels to phenolic glycolipid-I, a synthetic analog of phenolic glycolipid-I, and mycobacterial lipoarabinomannan. Int. J. Lepr. Other Mycobact. Dis. **58**:503–511.
23. Reference deleted.
24. **Pessolani, M. C., D. R. Smith, B. Rivoire, J. McCormick, S. A. Hefta, S. T. Cole, and P. J. Brennan.** 1994. Purification, characterization, gene sequence, and significance of a bacterioferritin from *Mycobacterium leprae*. J. Exp. Med. **180**:319–327.
25. **Phetsuksiri, B., J. Rudeeaneksin, P. Supapkul, S. Wachapong, K. Mahotarn, and P. J. Brennan.** 2006. A simplified reverse transcriptase PCR for rapid detection of *Mycobacterium leprae* in skin specimens. FEMS Immunol. Med. Microbiol. **48**:319–328.
26. **Schuring, R. P., F. J. Moet, D. Pahan, J. H. Richardus, and L. Oskam.** 2006. Association between anti-PGL-I IgM and clinical and demographic parameters in leprosy. Lepr. Rev. **77**:343–355.
27. **Sekar, B., R. N. Sharma, G. Leelabai, D. Anandan, B. Vasanthi, G. Yusuff, M. Subramanian, and M. Jayasheela.** 1993. Serological response of leprosy patients to *Mycobacterium leprae* specific and mycobacteria specific antigens: possibility of using these assays in combinations. Lepr. Rev. **64**:15–24.
28. **Siegel, S., and N. J. Castellan, Jr.** 1988. Non-parametric statistics for the behavioral sciences, 2nd edition. McGraw-Hill, New York, NY.
29. **Soebono, H., and P. R. Klatser.** 1991. A seroepidemiological study of leprosy in high- and low-endemic Indonesian villages. Int. J. Lepr. Other Mycobact. Dis. **59**:416–425.
30. **Stoner, G. L.** 1979. Importance of the neural predilection of *Mycobacterium leprae* in leprosy. Lancet **ii**(8150):994–996.
31. **van Beers, S., M. Hatta, and P. R. Klatser.** 1999. Seroprevalence rates of antibodies to phenolic glycolipid-I among school children as an indicator of leprosy endemicity. Int. J. Lepr. Other Mycobact. Dis. **67**:243–249.
32. **van Beers, S., S. Izumi, B. Madjid, Y. Maeda, R. Day, and P. R. Klatser.** 1994. An epidemiological study of leprosy infection by serology and polymerase chain reaction. Int. J. Lepr. Other Mycobact. Dis. **62**:1–9.
33. **Verhagen, C., W. Faber, P. Klatser, A. Buffing, B. Naafs, and P. Das.** 1999. Immunohistological analysis of in situ expression of mycobacterial antigens in skin lesions of leprosy patients across the histopathological spectrum. Association of Mycobacterial lipoarabinomannan (LAM) and *Mycobacterium leprae* phenolic glycolipid-I (PGL-I) with leprosy reactions. Am. J. Pathol. **154**:1793–1804.
34. **World Health Organization.** 2007. Global leprosy situation. Wkly. Epidemiol. Rec. **82**:225–232.

RESEARCH ARTICLE

$CD4^+$ T-cell activation by antigen-presenting cells infected with urease-deficient recombinant *Mycobacterium bovis* bacillus Calmette-Guérin

Tetsu Mukai, Yumi Maeda, Toshiki Tamura, Yuji Miyamoto & Masahiko Makino

Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan

Correspondence: Masahiko Makino, Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan. Tel.: +81 42 391 8059; fax: +81 42 391 8212; e-mail: mmaki@nih.go.jp

Received 22 November 2007; revised 11 February 2008; accepted 15 February 2008. First published online 9 April 2008.

DOI:10.1111/j.1574-695X.2008.00407.x

Editor: Patrick Brennan

Keywords
BCG; urease; macrophage; dendritic cell.

Abstract

We constructed a recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (BCG-ΔUT) that lacks urease, providing acidic intraphagosomal conditions to drive an effective human immune T-cell response. BCG-ΔUT-infected macrophages stimulated autologous $CD4^+$ T cells more efficiently than parent BCG-infected macrophages. For further T-cell activation, BCG-ΔUT-infected macrophages required pretreatment with exogenous recombinant granulocyte-macrophage colony-stimulating factor or costimulation with either CD40 ligand or interferon-γ. By contrast, BCG-ΔUT-infected dendritic cells induced significant activation of naïve $CD4^+$ T cells without costimulating signals. C57BL/6 mice intradermally inoculated with BCG-ΔUT more efficiently produced memory T cells that responded to recall antigen. Therefore, the depletion of urease from BCG is useful for the activation of T cells.

Introduction

Mycobacteria, such as *Mycobacterium leprae* and *Mycobacterium tuberculosis*, are representative parasitic intracellular pathogens. *Mycobacterium leprae* is a causative agent of human leprosy, in cases of which skin lesions and chronic progressive peripheral nerve injury are usually observed (Stoner, 1979; Job, 1989). At present, around one-third of individuals are infected with *M. tuberculosis* and several millions die as result of tuberculosis each year (Dye *et al.*, 2005; World Health Organization, 2006). *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) has been used as a vaccine against leprosy, although its efficacy is quite limited (Andersen & Doherty, 2005; Setia *et al.*, 2006). The emergence of multidrug-resistant strains of these mycobacteria is of concern (Maeda *et al.*, 2001; Kai *et al.*, 2004; Kaufmann, 2005), and therefore the urgent development of a new vaccine, including a more efficacious recombinant BCG, is desired (Kaufmann, 2005).

Among various immunocompetent cells, $CD4^+$ T cells, especially IFN-γ-producing cells, play an extremely important role in inhibiting the multiplication of mycobacteria, killing them in the early stages of infection, and keeping the bacterial load at a stable level (Orme *et al.*, 1993; Dockrell *et al.*, 1996; Hashimoto *et al.*, 2002). $CD4^+$ T cells that can respond quickly to pathogenic mycobacteria and produce IFN-γ are known as memory T cells. The efficient production of such memory T cells needs pre-exposure to antigenic vaccinating molecules, which share their antigenicity with that of pathogenic mycobacteria (Kaufmann, 2006). BCG has been considered a good candidate for a vaccine against *M. leprae* in this respect, however its efficacy is limited in several aspects, including the ability to activate T cells (Kaufmann & McMichael, 2005). BCG resides in the phagosomes of macrophages and thus attenuates the trafficking of antigenic molecules to the macrophage cell surface (Grode *et al.*, 2005). One possible strategy for improving the ability of BCG to stimulate T cells is to enhance its ability to fuse with the lysosomes. To this end, we knocked out the *urease* gene from BCG. The urease-deficient recombinant BCG (BCG-ΔUT) is expected to allow phagosomal acidification in the host cells, and induce efficient phagosome maturation for cytolytic activity of the antigenic molecules of BCG (Schaible *et al.*, 1998; Honerzu Bentrup & Russell, 2001).

In the present study, we evaluated the ability of BCG-ΔUT to activate IFN-γ-producing type 1 $CD4^+$ T cells through

antigen-presenting cells (APCs), and to produce memory $CD4^+$ T cells. When used as a target of BCG-ΔUT, macrophages fully stimulated $CD4^+$ T cells in the presence of costimulatory agents such as CD40 ligand (L) and IFN-γ. In addition, BCG-ΔUT-infected monocyte-derived dendritic cells (DCs) activated type 1 $CD4^+$ T cells more efficiently than parent BCG-infected cells in the absence of these costimulators. Therefore, BCG-ΔUT was found to be a useful T-cell-stimulating agent.

Materials and methods

Preparation of blood cells

Peripheral blood was obtained from healthy purified protein derivative (PPD)-positive individuals with informed consent. PPD-negative individuals provide more information, however, as healthy individuals are PPD-positive, due to compulsory BCG vaccination for children in Japan (0–4 years old). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described (Makino & Baba, 1997). For the preparation of peripheral monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood, or cryopreserved PBMCs using immunomagnetic beads coated with anti-CD3 monoclonal antibody (mAb) (Dynabeads 450, Dynal, Oslo, Norway). The $CD3^-$ PBMC fraction was plated on collagen-coated plates and nonadherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (Makino & Baba, 1997). Macrophages were generated by culturing monocytes in the presence of 20% fetal calf serum and recombinant (r) macrophage colony-stimulating factor (M-CSF) (R&D Systems, Abingdom, UK) (Makino *et al.*, 2007). Macrophages were pulsed with rBCGs on day 5 of culture, and were used as a stimulator of T cells on day 7 (Makino *et al.*, 2007). Monocyte-derived DCs were differentiated as described previously (Makino *et al.*, 1999). Briefly, monocytes were cultured in the presence of 50 ng recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; Pepro Tech EC Ltd, London, UK) and 10 ng of recombinant interleukin (rIL)-4 (Pepro Tech) per millilitre (Makino *et al.*, 1999). On day 3 of culture, immature DCs were infected with rBCGs at the indicated multiplicity of infection (MOI), and on day 5 of culture, DCs were used for further analyses of surface antigens and for mixed-lymphocyte assays.

BCG culture and DNA manipulation

The mycobacterial strain, BCG substrain Tokyo, for DNA manipulation was grown in Middlebrook 7H9 broth (Difco Laboratories) with 0.05% Tween 80 or Middlebrook 7H10 agar (Difco) with 0.5% glycerol, each supplemented with 10% albumin–dextrose–catalase enrichment (Difco). DNA manipulations including isolation of DNA, transformation and PCR, were carried out as described previously (Miyamoto *et al.*, 2004). *Escherichia coli* strain DH5α was used for routine manipulation and the propagation of plasmid DNA. *Escherichia coli* strain STBL4 was used for the construction of plasmid vectors derived from phAE87. Antibiotics were added as required: hygromycin B, 150 μg mL^{-1} for *E. coli* and 75 μg mL^{-1} for *Mycobacterium smegmatis* (mc^2155) and *M. bovis* BCG. A recombinant BCG that lacks a *urease* gene was constructed. The sequence of the targeted gene, *ureC* (BCG 1886), was obtained from the BCG list (http://genolist.pasteur.fr/BCGList/). The *ureC* gene was inactivated by inserting a hygromycin-resistance cassette (*hyg*) using a specialized transducing phage system for homologous recombination (Bardarov *et al.*, 2002; Miyamoto *et al.*, 2006). To construct the disrupted sequence, fragments of around 0.9 kb both upstream and downstream of *ureC* were amplified from BCG-Tokyo genomic DNA using the following two pairs of primers: F UureC and R UureC for upstream of *ureC*, and F DureC and R DureC for downstream of *ureC*. The PCR products were digested with each restriction enzyme and cloned into the corresponding site flanking *hyg* of pYUB854 to give pYUB854-ureC-UD. This plasmid was used for packaging into the phasmid vector phAE87 to construct a specialized transducing mycobacteriophage for gene disruption as described previously (Bardarov *et al.*, 2002; Miyamoto *et al.*, 2006). BCG-Tokyo infected with the mycobacteriophage at an MOI of 50 was incubated at 37 °C for 3 h in 7H9 broth without Tween 80. Harvested bacterial cells were then plated and cultured on 7H10 agar containing hygromycin B (75 μg mL^{-1}) for 3 weeks. The hygromycin B-resistant colonies were selected and evaluated with a conventional urease assay. A change in the color of the assay medium from yellowish to red was scored as urease-positive. Furthermore, genomic DNA obtained from these colonies was subjected to PCR to confirm the disruption of the gene using primers F ureC and R ureC (Fig. 1). The colony which tested negative in the urease assay was named BCG-ΔUT, while the parental BCG substrain Tokyo is referred to as BCG-Tokyo.

Preparation of *M. leprae*

Mycobacterium leprae (Thai-53) was isolated from the footpads of BALB/c-*nu/nu* mice (McDermott-Lancaster *et al.*, 1987). The isolated bacteria were counted by Shepard's method (Charles & Shepard, 1960). The MOI for infection to host cells was determined based on the assumption that macrophages and DCs were equally susceptible to infection with BCG or *M. leprae* (Hashimoto *et al.*, 2002).

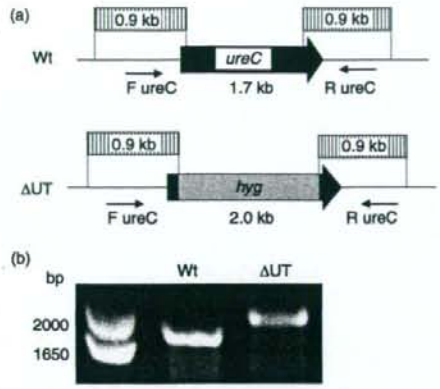

Fig. 1. Disruption of the *ureC* gene. (a) Schematic diagram of the *ureC* region on the chromosome of the wild-type *Mycobacterium bovis* BCG Tokyo strain and its gene disruptant, ΔUT. The shaded boxes indicate the regions included in the recombinant phage for gene disruption. The black arrow represents the coding region of the *ureC* gene. The gray box represents the hygromycin-resistace cassette (*hyg*). The primers used for PCR analysis are indicated by small arrows. (b) PCR analysis of the wild-type and the disruptant using the primers indicated above.

Preparation of mycobacterial antigen

The cytosolic fraction of BCG-Tokyo (BCC) was obtained as described previously (Maeda *et al.*, 2003). Briefly, the mycobacterial suspension containing the protease inhibitors was mixed with zirconium beads at a ratio of *c.* 1 : 1 (v/v) and homogenized using a Beads Homogenizer Model BC-20 (Central Scientific Commerce, Tokyo). The suspension was centrifuged at 10 000 ***g*** to remove the cell-wall fractions. The supernatant was then ultracentrifuged at 100 000 ***g*** and the resulting supernatant was taken as the cytosolic fraction. For preparation of the *M. leprae* membrane (MLM) fraction, *M. leprae* was used instead of BCG and treated similarly. The pellet obtained by ultracentrifugation (100 000 ***g*** for 1 h) was used as a membrane fraction (MLM). The optimal concentration of BCC and MLM for stimulating T cells was determined in advance.

Analysis of cell surface antigens

The expression of cell surface antigens on macrophages and DCs, either untreated or treated with exogenous rIFN-γ (R&D Systems), was analyzed using a FACSCalibur flow cytometer. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical Co., St. Louis, MO), and 1×10^4 live cells were analyzed. For the analysis of cell surface antigens, the following mAbs were used: fluorescein isothiocyanate (FITC)-conjugated mAbs against HLA-ABC (G46-2.6), HLA-DR (L243), CD14 (M5E2), CD40 (5C3) and CD86 (FUN-1). These mAbs were obtained from BD PharMingen (San Diego, CA).

APC function of rBCG-infected macrophages and DCs

The ability of rBCG-infected macrophages to stimulate T cells was assessed using an autologous mixed-lymphocyte assay as previously described (Wakamatsu *et al.*, 1999; Hashimoto *et al.*, 2002). The responder $CD4^+$ T cells were purified from freshly thawed PBMCs by using a CD4-negative isolation kit (Dynabeads 450; Dynal) (Wakamatsu *et al.*, 1999). The purity of $CD4^+$ T cells was more than 95% as assessed by fluorescence-activated cell sorting (FACS) analysis. Naïve $CD4^+$ T cells were produced by further treatment of $CD4^+$ T cells with an mAb to CD45RO antigen, followed by incubation with beads coated with goat antimouse IgG. Memory-type T cells were similarly produced by the treatment of cells with an mAb to CD45RA antigen. The purified responder cells (1×10^5 well^{-1}) were plated in 96-well round-bottom tissue culture plates and macrophages or DCs were added to give the indicated APC/$CD4^+$ T-cell ratio. Supernatants of the cocultures were collected on day 4 and the concentration of cytokines was determined. In some cases, macrophages were treated with the indicated dose of exogenous rGM-CSF (Pepro Tech) in advance of infection with rBCGs. Further, macrophages were infected with rBCGs in the presence of neutralizing mAb to IL-10 (JES3-9D7; Rat IgG, BD PharMingen) or control normal rat IgG. Macrophages infected with BCGs were further costimulated with either rCD40L (Pepro Tech) or rIFN-γ (R&D Systems), and in some cases, the macrophages were stimulated with rIFN-γ in the presence of anti-IFN-γ receptor α chain (CD119) (GIR-208, mouse IgG1, BD PharMingen) or control normal mouse IgG. In other cases, macrophages infected with BCG-ΔUT in the presence of exogenous rIFN-γ were treated with either mAb to HLA-DR (L243, mouse IgG2a), CD86 (IT2.2, mouse IgG2b, BD PharMingen) or control normal mouse IgG, and subsequently cocultured with responder $CD4^+$ T cells. The concentration of IFN-γ produced by $CD4^+$ T cells was quantified using an enzyme assay kit [OptEIA Human enzyme linked immunosorbent assay (ELISA) Set; BD Biosciences].

Production of IL-12p70 and IL-1β by DCs

The ability of DCs to produce IL-12p70 and IL-1β on stimulation with BCG-Tokyo or BCG-ΔUT was assessed. The DCs were stimulated with BCGs at the indicated MOI for 24 h, and the concentration of these cytokines was quantified using the Opt EIA Human ELISA Set.