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Polymorphism in the *rpoT* gene in *Mycobacterium leprae* isolates obtained from Latin American countries and its possible correlation with the spread of leprosy

Masanori Matsuoka [a,*], Liangfen Zhang [a], Mary Fafutis Morris [b], Pedro Legua [c], Carlos Wiens [d]

[a] *Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1, Aobacho, Higashimurayama-shi, 189-0002 Tokyo, Japan*
[b] *CIINDE, Department of Physiology, CUCS, University of Guadalajara, Federalismo, Norte, 3102 Guadalajara, Jalisco 44220, Mexico*
[c] *Alexander Von Humboldt Tropical Disease Institute, Peruana Cayetano Heredia University, A.P. 4314 Lima, 100, Peru*
[d] *Mennonita K81 Hospital D.d.C. 166, Asuncion, Paraguay*

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Abstract

The genotypes of *Mycobacterium leprae* isolates originating from Mexico, Peru and Paraguay were analysed for the polymorphism of short tandem repeats in the *rpoT* gene. The genotype with four copies of the six-base tandem repeats in the *rpoT* gene was prominently predominant in Mexico, but the genotype of all isolates from Peru and Paraguay contained three copies of the six-base tandem repeats. These obvious different distributions might reflect the spread of leprosy by the different strains of *M. leprae* harboured by the various human races that moved to the American continent, as has been demonstrated in other infectious diseases.

Keywords: Leprosy; *Mycobacterium leprae*; *rpoT* gene; Genotyping; Latin American countries

1. Introduction

Genotyping of causative agents of infectious diseases is essential for epidemiological analysis of transmission. Until recently, it was believed that there is no genomic diversity among *Mycobacterium leprae* isolates, a useful feature for the analysis of leprosy transmission. However, in 2000, two independent studies reported some genomic polymorphisms suitable for genotyping of *M. leprae* [1,2]. Since then other polymorphic genomic regions which might be applicable for genotyping have been also revealed [3]. One of them is the different copy number of six-base tandem repeats in the *rpoT* gene of *M. leprae* [1]. Specifically, *M. leprae* isolates can be divided into two sub-genotypes based on the polymorphism in the *rpoT* gene, which contains either four or three copies of a six-base tandem repeat. Our previous study showed the prominent distribution of the genotype with four copies of the six-base tandem repeats in the *rpoT* gene in the East Asia, Korea and the main island of Japan [1]. For some infectious diseases, the distribution of microorganisms with specific genotype in specific geographical areas has been noticed to correspond with the worldwide movement of human races

* Corresponding author. Tel.: +81 42 391 8211; fax: +81 42 394 9092.
E-mail address: matsuoka@nih.go.jp (M. Matsuoka).

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[4–8]. Studies of the genomic similarity of microorganisms, other than *M. leprae*, suggest that disease strains were distributed in Korea and the main island of Japan by one of the Mongoloid groups that migrated into these areas [6,9–11]. Our data with *M. leprae* [1] substantiated this observation.

Intrigued with this, it was of interest to determine the genotypes of *M. leprae rpoT* gene that exist in the American continent. Because of the complicated history of invasion, conquest and migration, the American continent consists of multiple races. The genotypes of *M. leprae rpoT* gene distributed in Latin American countries are investigated in this study.

2. Materials and methods

2.1. Source of M. leprae isolates and preparation of genomic DNA

A total of 72 *M. leprae* isolates were used in the present study. Twenty-seven samples from Mexico, 25 samples from Peru and 20 samples from Paraguay were examined. In Mexico, 2 samples from Sinaloa State, 15 samples from Jalisco State and 10 samples from Guanajuato State were collected. Samples from Peru and Paraguay were collected randomly from patients throughout the countries. Almost all of the patients were Mestizo. Twenty-four samples originated from Philippines were also included in the analysis (kindly supplied by Dr. A. Abraham, Dr. Jose N. Rodriguez Memorial Hospital, Philippines). Samples were collected from multibacillary cases [12], since paucibacillary specimens gave poor PCR products in the preliminary study, as reported by others [13]. Furthermore, leprosy disease types are defined by host immune response rather than causative strains. Slit-skin smear specimens were collected from the skin lesion of patient in the same manner as the routine slit-skin smear test for Bacterial Index examination. The sample on the disposable surgical blade was soaked in 70% ethanol and kept at a room temperature until use. The bacilli were removed from the blade and collected as a pellet by centrifugation at 10,000*g* for 20 min and then washed with phosphate-buffered saline. Template was prepared by treatment with lysis buffer as mentioned elsewhere [13].

2.2. rpoT genotyping by PCR and electrophoresis

PCR was carried out using a G mixture of FailSafe PCR System (EPICENTRE, Madison, WI, USA) in a 50 μl volume of reaction mixture. Primers A (5′-ATGCCGAACCGGACCTCGACGTTGA-3′) and B (5′-TCGTCTTCGAGGTCGTCGAGA-3′) (GenBank Accession No. AB019194) were used for amplification to span the 91 or 97-bp fragment containing the target region with three copies of the six-base tandem repeats or four copies of the six-base tandem repeats in the *rpoT* gene [1]. For comparing the differences of the repeats in the *rpoT* gene, 91- or 97-bp products were separated by electrophoresis in a 4% Meta Phore™ agarose gel (FMC Bioproducts, Rockland, ME, USA) using TBE (Tris/Borate/EDTA, pH 8.0) buffer at 50 V.

2.3. Sequencing

The numbers of six-base repeats were confirmed by direct sequencing. DNA samples for sequencing were recovered by MinElute Gel Extraction Kit (QIAGEN, GmbH, Germany) after electrophoresis of PCR products in 1.2% Seakem GTG agarose gel (Cambrex Bio Science Rockland Inc., Rockland, ME, USA). Samples were sequenced as described previously [14].

2.4. Genotyping of TTC repeats

Templates prepared for the *rpoT* genotyping were also analysed TTC repeats [2]. The target region was amplified and sequenced as described previously [14].

2.5. Confirmation of M. leprae

To identify of *M. leprae*, the *groEL* gene was amplified by PCR from the template samples using the method of Plikaytis et al. [15].

2.6. Ethical approval

Informed consent was obtained from all subjects, and the study was approved by the institutional ethics committee of National Institute of Infectious Diseases, Japan. Bacillary samples of slit-skin smears were collected when informed consent was obtained.

3. Results

3.1. Geographic distribution of M. leprae with different rpoT genotypes

PCR products of different sizes, 91- or 97-bp, were obtained according to the number of six-base tandem repeats in the *rpoT* gene (Fig. 1). Sequencing revealed that there were three copies of the six-base tandem repeats in the 91-bp PCR products and four copies in the 97-bp PCR products, respectively. No other *rpoT* genotype was detected. Of 27 samples from Mexico, only two samples from Jalisco state harboured three copies of the six-base tandem repeats. Both samples from Sinaloa state, 13 samples from Jalisco state and 10 samples from Guanajuato state revealed a genotype with four copies of the six-base tandem repeats. In contrast, all samples

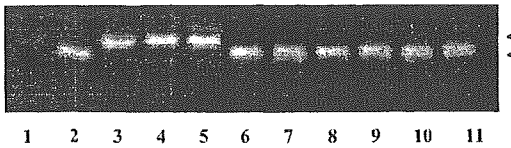

Fig. 1. The *rpoT* gene polymorphism in *M. leprae* obtained from Mexico, Peru and Paraguay. PCR products were resolved on a 4% Meta Phore agarose gel. Samples were: lane 1, the DNA size marker of 100 bp; lane 2, *M. leprae* with 3-copy of 6-bp tandem repeats; lane 3, *M. leprae* with four copies of the 6-bp tandem repeats; lane 4, isolate from Sinaloa state in Mexico; lane 5, isolate from Guanajuato state in Mexico; lane 6, isolate from Jalisco state in Mexico; lane 7, isolate from Peru; lane 8, isolate from Peru; lane 9, isolate from Peru; lane 10, isolate from Paraguay; lane 11, isolate from Paraguay.

Table 2
Frequency (%) of TTC genotypes in each country

No. of repeats	Country		
	Mexico	Peru	Paraguay
9	5		10
10	57	35	36
11	24	22	24
12	3	35	20
13	3	8	5
14	3		5
18	5		
Total	100% (27 cases)	100% (25 cases)	100% (20 cases)

from Peru, Paraguay and the Phillipines showed three copies of the six-base tandem repeats (Table 1).

3.2. Frequency of each TTC genotype

The copy number of TTC repeats varied from 9 to 18 copies (Table 2). No biased distribution of some specific TTC repeat genotypes was recognized in these three countries. The 10-copy TTC genotype was the most frequent in all three countries.

3.3. Verification of bacterial materials

All samples examined showed the 364-bp PCR products of the *groEL* gene and indicated that samples contained *M. leprae.*

4. Discussion

The first stage of our study showed a prominent biased distribution of *M. leprae* with four copies of the six-base tandem repeats in the *rpoT* gene in the main island of Japan and Korea. The predominance of three copies of the same repeats in Okinawa, the island off the southern end of Japan, was associated with human migration [1]. It has been a very influential hypothesis that modern Japanese are derived from a base of Jomon ancestry compounded with later Yayoi immigrants who had migrated to Japan through the Korean Peninsula [16]. Additional data to support the hypothesis that *M. leprae* was spread by one of the Mongoloid lineage which migrated to Japan through the Korean Peninsula include the similarity of genetic marker of Korean and Japanese [17]; low seroprevalent frequency of HTLV-1 carriers in Koreans and Japanese [18]; and the resemblance between isolates from Korea and Japan on genotypes of JC virus, *Mycobacterium tuberculosis*, *Helicobacter pylori* and Hepatitis B virus [6,7,9–11].

In this study, the geographical distribution of *M. leprae* discriminated by *rpoT* gene polymorphism was compared in some Latin American countries. The most noticeable finding of this study was that even although all of the isolates from Peru and Paraguay harboured three copies of the six-base tandem repeats of *M. leprae rpoT* gene, in Mexico, was a predominance of bacilli with four copies of the repeats.

Genotyping of microorganisms distributed throughout the American continent and Asia has proved the concordant spread of some infectious diseases with the migration of human races [4–8]. It must be of interest to pursue whether there were any correlations between the genotypes of *M. leprae rpoT* gene distribution in the American continent and the spread of leprosy with the intercontinental movement of human races in the past because there have been reports that the movement of leprosy patients initiated the transmission of *M. leprae* [19]. Notably, the genotype of four copies of the six-base tandem repeats of the *M. leprae rpoT* gene was typically dominant in Mexico whilst in all isolates from Peru and Paraguay there were only three copies. According to a random analysis of *M. leprae rpoT* gene on an isolate from armadillo in the USA, three isolates from Brazil and an isolate from Haiti, it was supposed that *M. leprae* with three copies of the six-base tandem repeats existed on the American

Table 1
Number of each *rpoT* genotype isolated in the three Latin American countries

rpoT Genotype	Mexico			Peru	Paraguay
	Sinaloa state	Guanajuato state	Jalisco state		
4-copy	2	10	13		
3-copy			2	25	20

continent [1]. For this reason, the predominance of four copies of the six-base tandem repeats in Mexico was unexpected. These limited data might indicate that leprosy was introduced into Mexico and other countries in different ways, for example, from different human races such as from Africa by slaves, from Asia by different groups of Mongoloid people and from Europe countries by Caucasians. Such ideas have been explored in the context of other infectious diseases [4,5,8,20]. However, more samples from more other related countries would help to develop a more comprehensive correlation between the genotype of *M. leprae* and the history of introduction of leprosy to Latin American countries. This would with no doubt contribute to the study of anthropology and archaeology.

It is generally said that leprosy was introduced to Mexico from the Philippines during the Spanish colonial era. Until now, the preliminary study of all 24 isolates from the Philippines has shown three copies of the six-base tandem repeats, which does not support this view. More isolates of *M. leprae* from Mexico and other countries around Mexico for genotyping are required to resolve this point.

Polymorphism of TTC repeats exhibited a wide range of variation; however, the variation of samples from Peru was relatively limited, and there was no biased distribution of some specific genotypes in any countries. The bacilli with 10 copies of TTC repeats were detected most frequently and this was same with our previous results obtained in Indonesia [14].

Genotypes of *M. leprae* that are particularly distributed in some specific areas, other than three or four copies of six base tandem repeats in the *rpoT* gene, might facilitate the epidemiological and anthropological analysis of the origin and spread of leprosy. Unfortunately, even though microsatellite and minisatellite nucleotide sequences with polymorphism have been used for genotyping [3], none of them showed such biased geographical distribution as *rpoT* genotyping in our preliminary study (data ware not shown). Other short tandem repeats with polymorphism that are valuable for genotyping are under investigation for epidemiological analysis of leprosy transmission and the worldwide spread in the past.

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Diversity of Potential Short Tandem Repeats in *Mycobacterium leprae* and Application for Molecular Typing

Liangfen Zhang,[1] Teky Budiawan,[2] and Masanori Matsuoka[3]*

Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1, Aobacho, Higashimurayama-shi, 189-0002, Tokyo, Japan[1]; Leprosy-TB Program, Provincial Health Service, Jl 17, Agustus, Manado, North Sulawesi, 95117, Indonesia[2]; and Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1, Aobacho, Higashimurayama-shi, 189-0002, Tokyo, Japan[3]

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A recent advance in molecular typing for tracing the transmission of leprosy is the discovery of short tandem repeats (STRs) in *Mycobacterium leprae*. To substantiate polymorphic loci from STR as promising candidates for molecular typing tools in leprosy epidemiology, 44 STR loci including 33 microsatellites and 11 minisatellites were investigated among 27 laboratory strains by sequencing PCR products. Not all STRs were necessarily polymorphic. Thirty-two out of the 44 loci were polymorphic. Nine polymorphic loci were suitable for identifying genotypes according to the discriminatory capacity, stability, and reproducibility. All the strains were classified into independent genotypes by the selected nine loci. Three multicase households were subjected to molecular typing. *M. leprae* obtained from household cases showed identical copy numbers by TTC triplet alone, but the isolates from one family contact case were divided into different genotypes by adding eight other polymorphic loci. The combination of information from multiple loci allows increasing levels of discrimination and it is likely that the generation and documentation of data will result in the choice of a potential molecular typing tool for leprosy epidemiology.

Mycobacterium leprae is an obligate intracellular parasite with tropism for macrophages and Schwann cells and the only species of mycobacteria to infect peripheral nerves (19). It causes leprosy, a chronic granulomatous infection of the skin and peripheral nerves with characteristic deformities and disability (1). It is generally accepted today that the worldwide implementation of standardized multidrug therapy for leprosy has decreased the number of registered leprosy cases from a peak of 10 to 15 million to a current total of less than 1 million. However, the annual confirmed new cases remain at 500,000 to 700,000. This continuing number suggests that effective multidrug therapy fails to disrupt the chain of leprosy transmission (26).

Even though leprosy is one of the oldest recorded diseases, the source for *M. leprae*, the portal of its exit/entry, and the mode of transmission are still under investigation. Some regard human beings as a host for the bacteria, while others are still considering more possibilities. It was proposed that the nasal mucosa are the exit/entry pathway of *M. leprae* (9, 17, 18). In light of the transmission mode, human-to-human direct contact was first generally accepted (6, 16), with time as airborne (23), as vector-borne (14, 25) and as vehicle-borne (3, 12) routes from evidence that has been obtained. All the progress in leprosy epidemiology was helpful in understanding the chain of transmission, yet at the same time was overwhelming.

In the past few years, studies focusing on leprosy transmission by molecular genotyping have shed new light on it (8, 10, 11, 22, 24, 27, 28). The most recent one was the report from Groathouse et al. (8). By in silico analysis, 44 promising polymorphic short tandem repeat (STR) sites, including both 33 microsatellite loci (repeat units of 1 to 5 bp) and 11 minisatellite (repeat units of >5 bp) were selected from the *M. leprae* TN genome sequence. Variable-number tandem repeats (VNTRs) at 9 of 11 STR loci from four clinical isolates of *M. leprae* were found. Clearly, it is urgent to accumulate extensive data for developing powerful typing tools to tracking the transmission of leprosy to finally reach a world free of leprosy.

In this study, we identified 32 polymorphic loci throughout the 44 STRs in a batch of 27 laboratory strains by sequencing and demonstrating the applicability and feasibility of nine potential loci acting as genetic markers to discriminate different *M. leprae* strains.

MATERIALS AND METHODS

***M. leprae* strains.** A panel of 27 laboratory strains of *M. leprae* was subjected for genetic analysis. Strains were maintained by inoculation into nude mice footpads annually in our laboratory. All the strains used in this study were recovered from multibacillary cases. Excluding Thai-53, Thai-311 and Thai-237 were from Thailand, Indonesia-1 was from Indonesia, and Korea 3-2 was from Korea, and the others were from Japan.

All patients were from geographically distinct regions. Four strains, Thai-53, Kyoto-1, Zensho-4, and Korea3-2, and 17 samples, namely, the fourth generation of Thai-53 (Thai-53 4th), Thai-53 7th, Thai-53 11th, Kyoto-1 3rd, Kyoto-1 5th, Kyoto-1 7th, Kyoto-1 8th, Zensho-4 (biopsy specimen), Zensho-4 1st, Zensho-4 2nd, Zensho-4 3rd, Zensho-4 4th, Korea3-2 (biopsy specimen), Korea3-2 1st, Korea3-2 2nd, Korea3-2 3rd, and Korea3-2 4th (Table 6) were employed for the stability testing of loci. Partially purified bacterial materials were prepared from the inoculated footpads by deferential centrifuging and suspension in a phosphate buffered saline at concentration of 10^{5-6}/ml.

Primer selection. Primer sets for the amplification regions of DNA containing the STR sites were referred to the study of Groathouse et al. (8). The sequences of primer pairs were listed (Table 1).

* Corresponding author. Mailing address: Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1, Aobacho, Higashimurayama-shi, Tokyo 189-0002, Japan. Phone: 81-42-391-8211. Fax: 81-42-394-9092. E-mail: matsuoka@nih.go.jp.

TABLE 1. Primers used in this study[a]

Primer	Sequence (5′ to 3′)	Repeat unit (bp)	Locus	Amplicon size (bp)	Accession no.[b]
RP1-1	ATA ACC AAC CGT TTC GCT GC GCA GAT TGC ACC CTG CAG TA	1	(T)8(A)6	108	*ML0258-ML0259*
RP1-2	ATG ATC CCG GTG TGA GCT CG TCT CTG CAG CCC ACA CCC TG	1	(T)6(N)7(T)8	149	ML0415/−
RP1-3	AAG AAC GTA CAA TCA CC AGC GAC CAC CCA CTA GCA TC	1	(A)9	145	*ML1196-ML1197*
RP1-4	TTG TGC TGG TCT CGT AGG CG TGC CGA CCT CGG TCT GCT TC	1	(G)9	150	ML0823/+
RP1-5	CGG ATA TAA TTA ACC TTC GG GTT GCG GGT CTA CTT GGT TG	1	(C)9	131	ML2238/−
RP1-6	GGG CGA TGC CGC CTT CGA AC CAG ACC CGG AAA CAG GAT AC	1	(G)10a	128	ML0266/+
RP1-7	AAT CCA AGC TGA TCG GCC AG TGC CCT GCA ATG TGC CGA TG	1	(G)10b	137	ML0350/−
RP1-8	GCG CAC TCT GTT AAT GAT GC ACA CCG ACA ACC GGT TAC CG	1	(G)11	136	ML1126/−
RP1-9	TGG CCA TCG TGG TGC TGT TC CAA CCT CGG CGG ACG CAT AC	1	(G)12	119	**ML0946/+**
RP1-10	TCG AGT TTT GGA CGG CAC CG AGT GCT TGT GCG GTC CAC AC	1	(C)16(G)8	152	*ML1647-ML1648*
RP1-11	ACT TCG GCG ACT GCG GTA AC GGT CAC TGG GCG TTG TGG TC	1	(C)20	159	*ML0237-ML0238*
RP1-12	GAA CTG GCC GGG TTA AAA GG TGC GCC TTC ACT GTG AAA TG	1	(G)22	176	ML0164/+
RP2-1	CAG GTC TTC GCG CCA ATT CT CCC GCG ACT CCC TGG CTT GC	2	(CG)6	140	**ML2472/−**
RP2-2	GTG TTA CGC GGA ACC AGG CA CCA TCT GTT GGT ACT ACT GA	2	(AC)8a	124	ML1285/−
RP2-3	GAT GCG ACT ATC ACT CGC AC GCT GGT TTC CTT CTA GTC CC	2	(AC)8b	140	*ML1824-ML1825*
RP2-4	GCC TGG TGC CCG GAC AAT GC ACT GAT CTC GCC GGC GCT GT	2	(AC)9	140	*ML1227-ML1228*
RP2-5	CAA CAG ATC GGT CGG AGA GG CTG GGT AGC CAT CTG GCT GC	2	(CA)6	160	*ML2106-ML2107*
RP2-6	GAC CCG GTT GCT TCA TGT AT GTA AGT TAC AAA CAA GCT GT	2	(TA)8	143	*ML2676-ML2677*
RP2-7	CGG AAA GTG TTG TGG TCG AC CCA AAC ACT TAG TCC ACT AA	2	(TA)9	189	*ML2375-ML2376*
RP2-8	TAA TTT TCT TAA AGA TAG AG TGT GAT CTT TGC ACT GTC GT	2	(TA)10	139	*ML1450-ML1451*
RP2-9	ATG AGC CGT AAG GAA TTA AC ATG CTG CCA CTC ATT AGA GG	2	(TA)13	158	*ML0235-ML0236*
RP2-10	CTG AGT AGA CCA GTC GAC AG ATC GCG GAG CAA CAA TTT CT	2	(AT)10	150	*ML2476-ML2477*
RP2-11	TTG GCG CTT CTG ATA TGG CT GGC GAG CCC GTC TGG CTC GA	2	(AT15)	164	*ML0798-ML0799*
RP2-12	TTA GCA GGA CGA TTG TAC AG ACC CGG AAT TCC TCC AAG	2	(AT)17	160	ML2183/−
RP2-13	CCG ACT CGC CGA AGC GAA AC CCG CGT TAG CAT AGG CAA T	2	(TA)18	119	ML0830/−
RP3-1	GTC ACG GCG CCG GGA ACG CA CCG ATT CTG CTG GGC TGC TG	3	(ACC)5	150	ML1645/−
RP3-2	TCA CCA TCG ACG CTC CGG GT TCG GCC TGG TTG TCT GCC TT	3	(GGT)5	161	*ML2159-ML2160*
RP3-3	CGT GAC AAT TCC TGG CAC AT CGA GAA GTG TAC CAC CAT CC	3	(AGT)5a	143	ML1073/−
RP3-4	GGC CAA ACG GTC AGG TTG TT ACA TCG AAT CGC TGT TGT AC	3	(AGT)5b	149	ML1118/−
RP3-5	GTA CGC CAA GGA CCG TGA CT GGT TGT TGA TGT TGG TGG TG	3	(ACT)5	120	ML2236/+
RP3-6	ACG CTG CGG TTT CGC AGC CT AAT ATG CAT GCC GGT GGT	3	(GTA)9	148	*ML2172-ML2173*
RP3-7	GGA CCT AAA CCA TCC CGT TT CTA CAG GGG GCA CTT AGC	3	(AGA)20	168	*ML2344-ML2345*
RP5-1	GAA GTT GAA TTC TTA TTG CC GCG GAG GGC ATC ACA CAG AA	5	(CACCG)3	130	*ML2158-ML2159*

Continued on facing page

TABLE 1—*Continued*

Primer	Sequence (5′ to 3′)	Repeat unit (bp)	Locus	Amplicon size (bp)	Accession no.[b]
RP6-1	AAG CGT CGA TAC AAA GGC ACC GT AGT AGC TTC GCCATC CTC GGT TT	6	6-3a	91	**ML1022/−**
RP6-2	GTT TGT CAA CAT TGG CAG GT CTG GAA CCG CGT GGC CCA CT	6	6-3b	148	**ML1918/−**
RP6-3	CTA CTT GCG CGC CAC CGC CA CCG TCG CCA GGT TTT GCA GA	6	6-7	191	**ML1505/+**
RP7-1	CTG GGG CGC GCT CAA TCG CT CGG GTT CGG GTG TAA CGA CA	7	7-3	160	**ML0213/+**
RP10-1	GTA CGC CAC CAG GAC AAC TC TGG CGG GCG TGC AGC CAA AC	10	10-4	203	ML0970/+
RP12-1	AGT AGC TTC CAT CCC CTC AT GCG ACG AAA GCA TTT ACG GC	12	12-5	180	**ML1182**
RP15-1	GCT GAG GTT AGG CGC CGA TC TCG GCA GAG TCC TGG CCA TC	15	15-3	195	*ML2454-ML2455*
RP18-1	GCT ATG GGC AGC CTG GGT AT AGC CGG TTA CCA AGA TGG CA	18	18-8	330	**ML1334/+**
RP21-1	TGT TGA AAT TTG GCG GCC AT TGC AAG GAG TGC TCA GCT AT	21	21-3	179	ML0058/−
RP23-1	CAG TCG CCC GGA TAC TGT TA TAA ATC CGC TCC CAA ATC TT	23	23-3	190	*ML2469-ML2470*
RP27-1	GTG CTG TGC CTG CCG TT TCC CCA AAG CCG CCG AAT CC	27	27-5	270	**ML0568/+**

[a] Primers were designed to amplify STRs reported by Groathouse et al. (8).
[b] Protein coding genes are in bold type. Intergenic regions are in italic type. Pseudogenes are underlined.

Slit-skin smears from multicase household. Eleven slit skin smears were collected as the same manner as that for Bacterial Index examination from leprosy patients in a total of three households. Among them, five smears from five patients in household I; four smears from two patients in household II, sample 6 from the left earlobe and sample 7 from the right earlobe within a single individual; sample 8 from the earlobe and sample 9 from the back in another individual; and two smears from two patients in household III (Table 7).

Preparation of *M. leprae* DNA from strains and slit skin smears and sequencing analysis. *M. leprae* templates from both strains and slit-skin smears were prepared by treatment with lysis buffer at 60°C overnight as described previously (13). PCR amplification of STR sites as well as sequencing analysis was performed under the same condition as described elsewhere using the listed primer pairs (10, 11, 12, 13). Briefly, target loci were amplified using a G mixture and a FailSafe PCR system (EPICENTRE, Madison, Wis.). DNA samples for sequencing were recovered with a MinElute gel extraction kit (QIAGEN GmbH, Hilden, Germany) after electrophoresis of PCR products. Samples were sequenced with a BigDye terminator cycle sequencing FS Ready Reaction kit (Perkin-Elmer Applied Biosystems, Norwalk, Conn.) and an ABI Prism 310 genetic analyzer (Perkin-Elmer). The nucleotide sequences obtained were analyzed using DNASIS software (Hitachi Software Engineering, Yokohama, Japan).

Multiplication of *M. leprae* strains in nude mice footpad. Inoculums of each strain were prepared from BALB/c-*nu/nu* nude mice which were inoculated with isolates of the third to fifth generations about 8 to 10 months before by Nakamura's method as described previously (15). Five-week-old male nude mice were injected with the inoculums containing 10^4 bacilli/0.05 ml of Hanks' balanced salt solution. Bacillary number in the footpads of six nude mice at 10, 20, 30, 40, and 50 weeks growth were examined by Shepard's method (21).

Ethical approval. Informed consent was obtained from all subjects, and the study was approved by the institutional ethics committee of National Institute of Infectious Diseases, Tokyo, Japan. Bacillary samples of nasal mucus and slit skin smears were collected when informed consent was obtained. Animal experiments were conducted under the approval of the institutional animal experiment committee.

RESULTS

Allelic diversity of 33 microsatellite loci in *M. leprae* strains. In the 27 *M. leprae* strains, 8 out of 33 selected microsatellite loci showed the same copy number of allele whereas the other 25 loci, (A)9, (G)9, (C)9, (G)10a, (G)10b, (G)11, (G)12, (C)16(G)8, (C)20, (G)22, (AC)8a, (AC)8b, (AC)9, (CA)6, (TA)8, (TA)9, (TA)10, (TA)13, (AT)10, (AT)15, (AT)17, (TA)18, (GGT)5, (GTA)9, and (AGA)20, presented at least two types of allele. All locus identifications are from Groathouse et al. (8) to maintain integrity. This result validated the comments from Groathouse group on the polymorphic (C)20, (AT)17, (TA)18, (GTA)9, and (AGA)20 and no diversity of (CG)6, albeit (AC)9 was also examined for considerable polymorphism here which was predicted as nonpolymorphic loci by them. However, the last repeat unit of some results was not easily defined for unknown reasons (Table 2).

Allelic diversity of eleven minisatellite loci in *M. leprae* strains. Throughout the 27 *M. leprae* strains, four out of 11 selected minisatellite loci showed no diversity while the other seven loci, 6-3a, 6-7, 12-5, 18-8, 21-3, 23-3, and 27-5, exhibited variable characteristics, which also verified Groathouse et al. (8) reports on the polymorphism of loci 6-7, 12-5, 18-8, 21-3, and 27-5 (Table 3).

Variations of allelic diversity at STR loci in *M. leprae* strains. Forty-four selected STR loci consisted of nine protein coding genes, 19 intergenic regions, and 16 pseudogenes. Polymorphism was revealed in six among nine (67%) protein coding genes, 16 out of 19 (84%) intergenic genes, and 10 of 16 (63%) pseudogenes. Almost all of the STR loci located in intergenic regions or in pseudogenes and were unlikely involved in biological functions. Overall, thirty-two out of 44 STR loci were polymorphic and 12 loci were invariable that might be of limited value as epidemiological markers. The variations were between 2 and 11 alleles (Table 4). Notably, at locus (CA)6, isolate Thai-237 differed from the other 26 strains by one repeat unit (Table 2). Similarly, at locus 23-3, isolate Hoshizuka-1 alone, and at locus 27-5, isolate Airaku-2 alone

TABLE 2. Allelic diversity of microsatellite loci in *M. leprae* strains

Strain	Copy no. of repeat element(s) at locus[a]:																
	(T)8(A)6	(T)6(N)7(T)8	(A)9	(G)9	(C)9	(G)10a	(G)10b	(G)11	(G)12	(C)16(G)8	(C)20	(G)22	(CG)6	(AC)8a	(AC)8b	(AC)9	(CA)6
Zensho-2	8 & 6	6 & 7 & 8	8	9	10	10	10	13†	9	16† & 7	16†	17†	6	7	8	7	6
Airaku-2	8 & 6	6 & 7 & 8	8	9	10	9	9	13	14†	12† & 8	9	14†	6	9	6	8	6
Airaku-3	8 & 6	6 & 7 & 8	8	9	10	9	11	11	10	16† & 8	13†	18†	6	10	6	8	6
Ryukyu-2	8 & 6	6 & 7 & 8	8	9	12	7	9	14	11	10 & 8	8	16†	6	12	7	8	6
Zensho-9	8 & 6	6 & 7 & 8	8	9	9	9	11	13	10	16† & 8	8	19†	6	9	7	8	6
Gushiken	8 & 6	6 & 7 & 8	9	9	10	12	11	10	9	14† & 8	9	16†	6	11	7	7	6
Thai-53	8 & 6	6 & 7 & 8	9	9	10	12	11	10	9	14† & 8	9	17†	6	11	7	7	6
Thai-311	8 & 6	6 & 7 & 8	8	10	10	9	11	10	9	16† & 8	13†	16†	6	9	6	8	6
Amami-1	8 & 6	6 & 7 & 8	8	9	9	8	9	11	9	17† & 8	8	12†	6	8	7	8	6
Indonesia-1	8 & 6	6 & 7 & 8	9	9	13†	11	9	14	9	18† & 8	12†	9	6	11	7	9	6
Thai-237	8 & 6	6 & 7 & 8	9	9	9	9	11	9	9	14† & 8	9	13†	6	9	7	7	7
Zensho-12	8 & 6	6 & 7 & 8	9	9	9	10	9	11	9	12 & 7	10	14†	6	10	7	10	6
Hoshizuka-1	8 & 6	6 & 7 & 8	8	9	12†	9	10	13	9	10 & 7	10	14†	6	8	8	7	6
Hoshizuka-5	8 & 6	6 & 7 & 8	8	10	12†	9	10	13	11	14† & 7	17†	13†	6	10	8	7	6
Izumi	8 & 6	6 & 7 & 8	8	9	10	13†	11	11	9	17† & 7	9	17†	6	11	8	7	6
Kanazawa	8 & 6	6 & 7 & 8	8	9	10	9	13†	14†	9	16† & 7	11	13†	6	6	8	7	6
Keifu-4	8 & 6	6 & 7 & 8	8	9	11	9	10	11	9	14† & 7	16†	12†	6	8	10	7	6
Kitazato	8 & 6	6 & 7 & 8	8	9	9	13†	12	13†	10	15† & 7	15†	14†	6	9	7	7	6
Zensho-4	8 & 6	6 & 7 & 8	8	9	10	11	12	11	9	17† & 7	14†	14†	6	8	8	7	6
Kusatsu-3	8 & 6	6 & 7 & 8	8	9	10	12	10	10	12†	18† & 7	13†	12†	6	9	8	8	6
Kyoto-2	8 & 6	6 & 7 & 8	8	9	11	11	9	13	10	19† & 7	10	15†	6	6	8	7	6
Oku-4	8 & 6	6 & 7 & 8	8	9	9	10	13†	14	9	10 & 8	15†	14†	6	9	8	7	6
Zensho-5	8 & 6	6 & 7 & 8	8	9	12	11	11	13	11	16† & 7	13†	14†	6	10	8	7	6
Kusatsu-6	8 & 6	6 & 7 & 8	8	9	10	10	12	10	10	17† & 7	14†	16†	6	9	8	7	6
Korea3-2	8 & 6	6 & 7 & 8	8	9	11	11	13†	12	9	12† & 7	9	18†	6	7	8	7	6
Hoshizuka-4	8 & 6	6 & 7 & 8	8	9	10	10	11	13	9	19† & 7	10	10	6	8	8	7	6
Kyoto-1	8 & 6	6 & 7 & 8	8	9	11	10	12	13	9	13† & 8	11	18†	6	7	8	7	6

[a] †, difficult to read the sequence across the junction of stretch of repeat.

had one copy difference allele (Table 3). Additionally, at locus 12-5, a five-copy-repeat unit was obtained from *M. leprae* TN, but all of the strains in this study showed three- and four-copy-repeat units instead of the five-copy-repeat unit (Table 3).

Comparison among the loci fall within protein coding genes with two variations. Locus 6-3a is in the *rpoT* gene coding RNA polymerase sigma factor in *M. leprae* contained two variations, three- and four-copy alleles (2, 13). Twelve strains, Zensho-2 to Zensho-12, harbored the three-copy allele whereas 15 strains, Hoshizuka-1 to Kyoto-1, had a four-copy allele in the *rpoT* gene (Table 3). Geographic distribution of each genotype of *M. leprae rpoT* revealed the distinguished distribution in several countries in the world (13, 10). Intriguingly, we compared the copy numbers of the other two protein coding genes 12-5 and 18-8 having two alleles with the strains carrying three-copy and four-copy *rpoT* that were closely associated with geographic distributions and no correlation was exploited.

Discriminatory capacity, stability, and reproducibility of nine potential polymorphic loci detected among serial passage strains by nude mice. Nine polymorphic microsatellite loci, (AC)9, (AC)8b, (AC)8a, (TA)10, (AT)17, (AT)15 (GTA)9, (TA)18, and (AGA)20 (also named TTC) were selected for discriminatory capacity analysis. All 27 strains were divided into two groups, three-copy *rpoT* and four-copy *rpoT*, based on the *rpoT* polymorphism. By adding the conjunction of 9 loci, they were distinguished from each other (Table 5). Then stability and reproducibility testing was carried out among them through serial passage strains by nude mice owing to the nature of susceptibility of replication slippage of microsatellite (28). One generation designated approximately 1 year, these strains have been in passage for 4 to 11 years. Not only the identical profile of allele was shared among the different generations of the strains at each locus, but also the copy number of repeats was in agreement with that in the repeated experiment of the same strains, which ensured these polymorphic microsatellite loci were highly stable and reproducible (Tables 1 and 6). The stability of TTCwas done previously (11)]. It was the rationale for these nine polymorphic microsatellite loci to be a significant source of informative markers for the identification and discrimination of *M. leprae* strains.

Application of polymorphic microsatellite loci for multicase households. Based on the condition of leprosy patients living in same household and possessing the identical TTC pattern, eleven smears from three household were chosen (Table 7). Five bacterial materials from household I shared identical 13-copy of TTC repeat were also subjected to loci (AC)8a, (AC)8b, (AC)9, (TA)10, (AT)15, (AT)17, (TA)18, and (GTA)9 and presented the identical copy number of 12, 7, 10, 10, 13, 15, 14, and 9, respectively. In household II which had 12 copies of the TTC repeat, four smears at the above loci showed equal repeats of 10, 7, 9, 10, 13, 13, 11, and 11. There was an exact match by copy number at each locus between samples 6 and 7 as well as samples 8 and 9 within one individual. In household III, two smears harboring eight copies of the TTC repeat, the copy number of loci (AC)8a, (AC)8b, (AC)9, (TA)10, (AT)15 and (TA)18 was 10, 7, 9, 8, 16, and 15. respectively. Strikingly, at locus (AT)17, a difference of 9 and 10 copies and a mismatch of 11 and 12 copies at locus (GTA)9 were found in these two smears, respectively.

TABLE 2—*Continued*

Copy no. of repeat element(s) at locus[a]:														
(TA)9	(TA)10	(TA)13†	(AT)10	(AT15)	(AT)17	(TA)18	(ACC)5	(GGT)5	(AGT)5a	(AGT)5b	(ACT)5	(GTA)9	(AGA)20	(CACCG)3
8	14	23	9	14	15	15	5	4	5	5	5	14	9	3
12	7	19	7	15	14	17	5	4	5	5	5	10	13	3
10	7	21	7	18	13	17	5	4	5	5	5	11	13	3
6	10	22	7	19†	13	13	5	4	5	5	5	8	14	3
7	13	15	7	21	16	12	5	4	5	5	5	11	14	3
8	11	23	8	13	13	20	5	5	5	5	5	11	14	3
9	11	23	8	13	13	21	5	5	5	5	5	11	14	3
12	7	19	7	14	11	19	5	4	5	5	5	9	25	3
6	10	24	8	17	10	15	5	4	5	5	5	9	15	3
8	10	19	9	14	13	10	5	5	5	5	5	9	15	3
9	11	18	9	11	15	10	5	5	5	5	5	9	16	3
10†	12	22	9	16	18	16	5	6	5	5	5	9	11	3
9	15	15	8	21†	14	13	5	4	5	5	5	15	9	9
14	13	19	8	15	14	12	5	4	5	5	5	15	9	3
8	12†	16	8	13	13	16†	5	4	5	5	5	11	9	3
9	14	19	8	17	13	11	5	4	5	5	5	12	10	3
7	11	19	9	21†	15	19	5	4	5	5	5	13	10	3
14†	13	18	8	12	12	14	5	4	5	5	5	16	10	3
8	10	17	9	20	13	15	5	4	5	5	5	13	10	3
10	9	24	8	13	12	17	5	4	5	5	5	16	11	3
14	16	18	9	18	15	14	5	4	5	5	5	16	11	3
10	13	19	9	14	15	12	5	4	5	5	5	13	11	3
8	12†	18	8	14	14	14	5	4	5	5	5	18	11	3
12	16	19	8	18	12	17	5	4	5	5	5	16	16	3
11	15	20	8	17	14	15	5	4	5	5	5	14	13	3
10	12†	20	8	20†	14	17	5	4	5	5	5	13	12	3
12	12	23	9	15	12	16	5	4	5	5	5	15	9	3

TABLE 3. Allelic diversity of minisatellite loci in *M. leprae* strains

Strain	No. of copies at locus:										
	6-3a	6-3b	6-7	7-3	10-4	12-5	15-3	18-8	21-3	23-3	27-5
Zensho-2	3	3	7	3	4	3	3	7	2	2	5
Airaku-2	3	3	6	3	4	4	3	7	2	2	3
Airaku-3	3	3	6	3	4	4	3	8	1	2	5
Ryukyu-2	3	3	6	3	4	4	3	8	2	2	5
Zensho-9	3	3	6	3	4	4	3	8	2	2	5
Gushiken	3	3	6	3	4	4	3	7	3	2	5
Thai-53	3	3	6	3	4	4	3	8	3	2	5
Thai-311	3	3	6	3	4	4	3	8	2	2	5
Amami-1	3	3	6	3	4	3	3	8	2	2	5
Indonesia-1	3	3	6	3	4	4	3	8	3	2	5
Thai-237	3	3	6	3	4	4	3	8	3	2	5
Zensho-12	3	3	7	3	4	4	3	8	3	2	5
Hoshizuka-1	4	3	6	3	4	3	3	7	2	3	5
Hoshizuka-5	4	3	6	3	4	3	3	7	1	2	5
Izumi	4	3	6	3	4	3	3	7	1	2	5
Kanazawa	4	3	10	3	4	3	3	7	2	2	5
Keifu-4	4	3	6	3	4	3	3	7	2	2	5
Kitazato	4	3	6	3	4	3	3	7	1	2	5
Zensho-4	4	3	9	3	4	3	3	7	3	2	5
Kusatsu-3	4	3	6	3	4	3	3	7	2	2	5
Kyoto-2	4	3	8	3	4	3	3	7	3	2	5
Oku-4	4	3	5	3	4	3	3	7	2	2	5
Zensho-5	4	3	6	3	4	3	3	7	1	2	5
Kusatsu-6	4	3	6	3	4	3	3	7	2	2	5
Korea3-2	4	3	7	3	4	3	3	7	1	2	5
Hoshizuka-4	4	3	9	3	4	3	3	7	2	2	5
Kyoto-1	4	3	6	3	4	3	3	7	3	2	5

Linkage between the growth rate of *M. leprae* in nude mice footpad and allelic diversity. Shepard et al. (20) reported the hereditary fast-slow growth difference among *M. leprae* strains in conventional mouse footpads, which was also observed in nude mice footpads in our laboratory. However, in this study, the growth curve of *M. leprae* in nude mice footpads gave no difference between strains with three-copy *rpoT* (Zensho-2, Airaku-3, and Thai-53) and those with four-copy *rpoT* (Izumi and Zensho-4) except Kyoto-2, which revealed the copy number of *rpoT* was irrelevant to the fastidious growth. In addition, no correlation was found between the VNTR of the other protein coding genes and *M. leprae* growth.

DISCUSSION

Poor understanding of leprosy transmission has hindered us from eradicating the disease even though *M. leprae* was identified as the pathogen as early as 1873 by Hansen (19). The unusual biological traits of being especially slow growing as well as the inability to culture *M. leprae* in vitro have partially accounted for the setbacks of detailed studies on leprosy. However, the determination of the complete *M. leprae* TN genome sequence (4) is a breakthrough for leprosy research, which has served as a public domain used by the Groathouse study group for the screening of potential VNTR for molecular typing (8). Tandem repeats are usually classified among satellites (spanning megabases of DNA, associated with heterochromatin), minisatellites (repeat units in the range from 6 to 100 bp, spanning hundreds of base pairs) and microsatellites (repeat units in the range from 1 to 5 bp, spanning a few tens of nucleotides) (5, 7). Both microsatellite and minisatellite loci

TABLE 4. Variation of allelic diversity at STR loci in *M. leprae* strains[a]

No.	Locus	No. of repeats and range	No.	Locus	No. of repeats and range	No.	Locus	No. of repeats and range
1	(T)8(A)6	No variation	16	(AC)**9***	7, 8, 9, 10	31	(GTA)**9***	8, 9, 10, 11, 12, 13, 14, 15, 16, 18
2	(T)6(N)7(T)8	No variation	17	(CA)6	6, 7	32	(AGA)20*	9, 10, 11, 12, 13, 14, 15, 16, 25
3	(A)9	8, 9	18	(TA)8	6, 7, 8, 9, 10	33	(CACCG)3	No variation
4	(G)9	9, 10	19	(TA)9	6, 7, 8, 9, 10, 11, 12, 14	**34**	**6-3a**	3, 4
5	(C)9	9, 10, 11, 12	20	(TA)10*	7, 9, 10, 11, 12, 13, 14, 15, 16	**35**	**6-3b**	No variation
6	(G)10a	7, 8, 9, 10, 12, 13	21	(TA)13	15, 16, 17, 18, 19, 20, 21, 22, 23, 24	**36**	**6-7**	5, 6, 7, 8, 9, 10
7	(G)10b	9, 10, 11, 12, 13,	22	(AT)10	7, 8, 9, 10, 12, 14	**37**	**7-3**	No variation
8	(G)11	10, 11, 12, 13, 14	23	(AT15)*	13, 14, 15, 16, 17, 18, 19, 20, 21	38	10-4	No variation
9	**(G)12**	9, 10, 11, 12, 14	24	(AT)17*	10, 11, 12, 13, 14, 15, 16, 18	**39**	**12-5**	3, 4
10	(C)16(G)8	7, 8 & 10, 12, 13, 14, 15, 16, 17, 18, 19	25	(TA)18*	11, 12, 13, 14, 15, 16, 17, 19	40	15-3	No variation
11	(C)20	8, 9, 10, 11, 12, 13, 14, 15, 14, 15, 16	26	(ACC)5	No variation	**41**	**18-8**	7, 8
12	(G)22	9, 10, 12, 13, 14, 15, 16, 17, 18, 19	27	(GGT)5	4, 5, 6	42	21-3	1, 2, 3
13	**(CG)6**	No variation	28	(AGT)5a	No variation	43	23-3	2, 3
14	(AC)8a*	6, 7, 8, 9, 10, 11, 12	29	(AGT)5b	No variation	**44**	**27-5**	3, 5
15	(AC)8b*	6, 7, 8, 10,	30	(ACT)5	No variation			

[a] Protein coding genes are in bold type. Pseudogenes are underlined. Intragenic genes are in standard type. *, selected loci as the combined one for genotyping.

have been selected for this study to substantiate the polymorphic loci as promising candidates used as the molecular typing tools for leprosy epidemiology.

In order to generate comprehensive and reliable data, a battery of 27 laboratory strains was used to develop PCR systems to amplify 44 STR target loci and the PCR products were sequenced. Unexpectedly, the results disclosed that not all the STR loci were polymorphic. Some of the loci were with

TABLE 5. Discriminatory capacity of polymorphic microsatellite loci[a]

Strain	No. of repeats									
	6-3a	(AC)9	(AC)8b	(AC)8a	(TA)10	(AT)17	(AT)15	(GTA)9	(TA)18	(AGA)20 or TTC
Gushiken	3	7	7	11	11	13	13	11	20	14
Thai-53	3	7	7	11	11	13	13	11	21	14
Thai-237	3	7	7	9	11	15	11	9	10	16
Zensho-2	3	7	8	7	14	15	14	14	15	9
Thai-311	3	8	6	9	7	11	14	9	19	25
Airaku-2	3	8	6	9	7	14	15	10	17	13
Airaku-3	3	8	6	10	7	13	18	11	17	13
Amami-1	3	8	7	8	10	10	17	9	15	15
Zensho-9	3	8	7	9	13	16	21	11	12	14
Ryukyu-2	3	8	7	12	10	13	19	8	13	14
Indonesia-1	3	9	7	11	10	13	14	9	10	15
Zensho-12	3	10	7	10	12	18	16	9	16	11
Kitazato	4	7	7	9	13	12	12	16	14	10
Kanazawa	4	7	8	6	14	13	17	12	11	10
Kyoto-2	4	7	8	6	16	15	18	16	14	11
Kyoto-1	4	7	8	7	12	12	15	15	16	9
Korea3-2	4	7	8	7	15	14	17	14	15	13
Zensho-4	4	7	8	8	10	13	20	13	15	10
Hoshizuka-4	4	7	8	8	12	14	20	13	17	12
Hoshizuka-1	4	7	8	8	15	14	21	15	13	9
Oku-4	4	7	8	9	13	15	14	13	12	11
Kusatsu-6	4	7	8	9	16	12	18	16	17	16
Zensho-5	4	7	8	10	12	14	14	18	14	11
Hoshizuka-5	4	7	8	10	13	14	15	15	12	9
Izumi	4	7	8	11	12	13	13	11	16	9
Keifu-4	4	7	10	8	11	15	21	13	19	10
Kusatsu-3	4	8	8	9	9	12	13	16	17	11

[a] Strains with identical *rpoT* genotypes were discriminated by the combination of STRs with polymorphism. All isolates were divided into independent genotypes.

TABLE 6. Stability of polymorphic microsatellite loci detected among strains subjected to serial passage in nude mice

Generation	No. of copies at locus:							
	(AC)8a	(AC)8b	(AC)9	(TA)10	(AT)15	(AT)17	(TA)18	(GTA)9
Thai53 4th	11	7	7	11	13	13	20	11
Thai53 7th	11	7	7	11	13	13	20	11
Thai53 11th	11	7	7	11	13	13	20	11
Kyoto-1 3rd	7	8	7	12	15	12	16	15
Kyoto-1 5th	7	8	7	12	15	12	16	15
Kyoto-1 7th	7	8	7	12	15	12	16	15
Kyoto-1 8th	7	8	7	12	15	12	16	15
Zensho-4[a]	8	8	7	10	20	13	15	13
Zensho-4 1st	8	8	7	10	20	13	15	13
Zensho-4 2nd	8	8	7	10	20	13	15	13
Zensho-4 3rd	8	8	7	10	20	13	15	13
Zensho-4 4th	8	8	7	10	20	13	15	13
Korea3-2[a]	7	8	7	15	17	14	15	14
Korea3-2 1st	7	8	7	15	17	14	15	14
Korea3-2 2nd	7	8	7	15	17	14	15	14
Korea3-2 3rd	7	8	7	15	17	14	15	14
Korea3-2 4th	7	8	7	15	17	14	15	14

[a] Biopsy specimen.

no polymorphism which may have little or no long-term epidemiological value. In bacteria, loci containing a tandem repeat from the microsatellite class have been called simple sequence contingency loci. Altered number of repeats allows reversible on and off states of expression for the corresponding gene. In such an extreme situation, the microsatellite is not suitable for strain identification, epidemiological, or phylogenetic studies (7). The slight allele shift found in a few loci, (CA)6, 23-3, 27-5, and 12-5, was on one hand supposed to be a result of replication slippage, which can lead to an increase or a decrease in the copy number of the repeat element during cell division and inclined to be attributed to the locus with no polymorphism, and on the other hand, there might be different subpopulations of *M. leprae*.

As far as we are concerned, the nine combined microsatellite loci with strong discriminatory capacity which have been also tested for its stability and reproducibility in this study were the most likely and reliable sites for genetic diversity for *M. leprae*. Our previous study showed the 10-copy TTC repeat was the predominant genotype in a leprosy-endemic village (11). Nonetheless, by TTC alone, no conclusion could be drawn that bacteria were from the same population. Meanwhile, the multicase household which was examined the identical genotype for TTC alone does not mean an identical *M. leprae* isolate at all. As a matter of fact, when eight polymorphic loci rather than TTC, (AC)8a, (AC)8b, (AC)9, (TA)10, (AT)15, (AT)17, (TA)18, and (GTA)9, were added to the genotype, *M. leprae* could be further subdivided into two populations. Moreover, all 27 strains could also be identified individually by these combined nine polymorphic loci. Hence, this group of locus is

TABLE 7. Application of potential polymorphic loci for household leprosy patients

Patients[a]	Sample no.	No. of copies at polymorphic locus[b]:								
		TTC or (AGA)20	(AC)8a	(AC)8b	(AC)9	(TA)10	(AT)15	(AT)17	(TA)18	(GTA)9
Son*	1	13	12	7	10	10	13	15	14	9
Father	2	13	12	7	10	10	13	15	14	9
Sister	3	13	12	7	10	10	13	15	14	9
Brother	4	13	12	7	10	10	13	15	14	9
Brother	5	13	12	7	10	10	13	15	14	9
Son*	6 (left earlobe)	12	10	7	9	10	13	13	11	11
	7 (right earlobe)	12	10	7	9	10	13	13	11	11
Brother	8 (earlobe)	12	10	7	9	10	13	13	11	11
	9 (back)	12	10	7	9	10	13	13	11	11
Son*	10	8	10	7	9	8	16	**9**	15	**11**
Brother	11	8	10	7	9	8	16	**10**	15	**12**

[a] *, supposed index case.
[b] STRs with variation in the sane TTC repeat strains were indicated by boldface type.

anticipated to a wide application for clinical samples as a preliminary molecular typing system in leprosy epidemiology.

It is thought that tandem repeats located within the regulatory region of a gene can constitute an on/off switch of gene expression at the transcriptional level (7). In spite of this, the intergenic or interpseudogenic location of the microsatellite loci makes it unlikely that a difference in repeat copy number would itself confer any biological advantage, but it is possible that it acts as a marker for some biologically distinct subpopulation of bacteria and that it is carried along during selective expansion of this subpopulation (28). On the basis of this, the repeat unit which was differed by one copy number at (AT)17 and (GTA)9 loci when multicase household were subjected to the nine combined polymorphic loci indicated the presence of two subpopulations of *M. leprae* which have different dominant genotypes and presumably have propagated independently of each other. With regard to the fully matching polymorphism profile in each sample from the same dwelling at each polymorphic microsatellite loci, it was possible that the patients were infected by the same strain of *M. leprae* that survived in some common infectious sources. Taking these into consideration, there might be certain publicly shared infectious sources other than multibacillary cases played a role in the transmission of leprosy.

The unique function of *rpoT* was precisely evaluated in tracking the possible worldwide spread of leprosy and its contribution to the study of anthropology and archaeology (10, 13). We analyzed the other two loci with characteristics common to locus 6-3a in *rpoT* and found no regularity and significance such as *rpoT* had.

Young et al. (28) compared microsatellite profiles for samples taken from different anatomic sites from the same individual and observed that while skin, blood, and nasal cavity samples consistently generated matching profiles, frequent mismatches were found when bacteria in skin and nerves were compared. Mostly, we obtained identical repeats from different skin lesions on the same individual such as samples 6 (left earlobe), 7 (right earlobe) and 8 (earlobe) and 9 (back). Rarely, different skin lesions from the same patient exhibited mismatch profiles at the TTC locus (data was not shown). This probably suggested different bacterial populations coexisted instead of resulting from replication slippage. Nevertheless, the underlying mechanisms are awaiting to be elucidated.

Molecular typing systems are undergoing rapid technical improvements. Advance in the understanding of the biological basis of microbial biodiversity at subspecies levels will improve the conceptual framework required for proper epidemiologic interpretation of disease transmission. Wider application of these systems will surely contribute to the epidemiology of leprosy transmission and, therefore, allow for more effective control and prevention strategies to reach a world free of leprosy.

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Identification of an Immunomodulating Agent from *Mycobacterium leprae*

Yumi Maeda,[1] Tetsu Mukai,[1] John Spencer,[2] and Masahiko Makino[1]*

Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan,[1] and Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523-1677[2]

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A search for an immunomodulating agent from mycobacteria was carried out using *Mycobacterium leprae*. The antigenicity of each fraction of the bacterial membrane, which contains the most antigenic components of *M. leprae*, was assessed by using sera from paucibacillary leprosy. N-terminal sequencing of the serum-reactive protein and functional assessment of the membrane fractions using monocyte-derived dendritic cells (DCs) identified major membrane protein II (MMP-II) as one of the efficient T-cell-activating candidates. Purified MMP-II stimulated DCs from healthy individuals to produce interleukin-12 p70 and up-regulated the surface expression of major histocompatibility complex class I and II, CD86, and CD83 molecules. Also, there was an increase in the percentage of $CD83^+$ cells in the DC population. Furthermore, MMP-II-pulsed DCs expressed their derivatives on their surfaces. Using Toll-like receptor 2 (TLR-2)-dependent receptor constructs, we found that TLR-2 signaling was involved in DC maturation induced by MMP-II. Taken together, MMP-II can be recognized as an immunomodulating protein in terms of activation of antigen-presenting cells and innate immunity.

Mycobacterial infection is a major public health risk worldwide, and around one-third of the world's population is estimated to be latently infected. *Mycobacterium tuberculosis*, the causative agent of human tuberculosis, accounts for 8 to 10 million new active cases and 2 million deaths annually (12, 41). Nontuberculoid mycobacterial infections of immunocompromised individuals, such as human immunodeficiency virus type 1 (HIV-1)-infected patients (8, 28), evoke serious concern, and *Mycobacterium leprae* induces a chronic progressive peripheral nerve injury which leads to systemic deformity (16, 37). The sole immunomodulating agent currently available for human use against mycobacterial diseases is *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). However, its protective effect against mycobacterial infection is unconvincing, especially to elderly people, and thus, its use is limited (3). Various efforts are currently being made to develop immunomodulating agents, but convincing protection against mycobacterium-induced diseases has not been achieved to date. The development of other useful molecules as immunomodulating agents is greatly desired. In this study, we attempted to find such antigenic molecules in *M. leprae* subfractions.

M. leprae is the causative agent of human leprosy, for which a broad disease spectrum is clinically observed (34). Most individuals infected with *M. leprae* do not manifest leprosy, but a few manifest the disease, depending on their immunological status. The representative spectra are the tuberculoid, or paucibacillary (PB), leprosy and the lepromatous, or multibacillary (MB), leprosy. In the former disease spectrum, localized skin and nerve lesions are observed, and T cells act chiefly to localize bacterial spread and, thus, disease lesions (20, 31, 36). In contrast, in the latter disease spectrum, such cell-mediated immune responses are not efficiently evoked; rather, T cells show *M. leprae* antigen (Ag)-specific anergic responses. In both types of leprosy, the protective effect of antibody (Ab) is not observed. These observations indicate that the bacterial component Ags capable of modulating immune responses should be identified. Previously it has been demonstrated that monocyte-derived dendritic cells (DCs), which are the most potent antigen-presenting cells (APCs), are capable of stimulating both memory and naïve $CD4^+$ and $CD8^+$ T cells (14, 15, 21). Also, we reported that DCs played a central role in stimulating T cells (10, 18, 22); however, macrophages stimulated T cells less efficiently. Furthermore, we showed that among subcellular components of *M. leprae*, the cell membrane fraction was quite antigenic and contained molecules which stimulated DCs to produce interleukin-12 (IL-12) p70 (22). However, the molecules associated with DC activation have not been elucidated. For identification of an APC-associated immunomodulator, the following issues should be addressed: (i) the ability of the immunogen to activate APCs, including both DCs and macrophages; (ii) the ability of the immunogen to be processed and presented on the surfaces of these APCs, because mycobacteria are intracellular parasitic pathogens. In this context, we fractionated the *M. leprae*-derived cell membrane fraction, screened the fractions for such a protein by using DCs as APCs, and subsequently evaluated the newly identified molecule, major membrane protein II (MMP-II), in terms of innate immunity.

* Corresponding author. Mailing address: Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan. Phone: 81-42-391-8059. Fax: 81-42-391-8212. E-mail: mmaki@nih.go.jp.

MATERIALS AND METHODS

Preparation of cells and bacteria. Peripheral blood was obtained with informed consent from healthy individuals who were positive for purified protein derivative (PPD) due to *M. bovis* BCG vaccination. We are aware that PPD-negative individuals would help to provide full information for these experi-

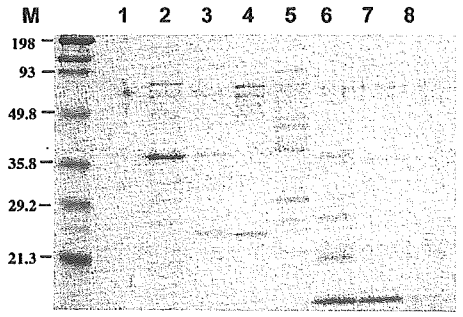

FIG. 1. *M. leprae* membrane fractions were separated into eight fractions by gel filtration as described in Materials and Methods. Then 3 μg of each fraction was run on a 12% SDS-polyacrylamide gel, and silver staining of the gel was performed.

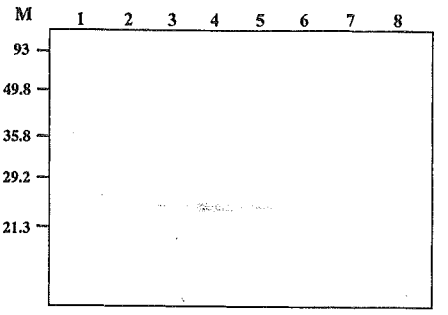

FIG. 3. Western blot of *M. leprae* membrane fractions. Five micrograms of various membrane fractions of *M. leprae* was run on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane, which was further probed with an anti-MMP-II MAb. An alkaline phosphatase-conjugated anti-mouse IgG Ab was used as the secondary Ab, and the protein was detected with NBT/BCIP reagent.

ments, however, in Japan, such individuals are not available for study, because *M. bovis* BCG vaccination is compulsory for children (0 to 4 years old). Moreover, PPD-negative individuals in the Japanese population are those who do not respond to BCG vaccination, and therefore, it is likely that they suffer from an unknown human disease or immune insufficiency. Therefore, these individuals cannot be used as controls for our experiments. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described (23). Macrophages were differentiated by culturing plastic-adherent $CD14^+$ monocytes with RPMI 1640 medium containing 20% of fetal calf serum (FCS) and 1% penicillin G (Katayama Chemical, Osaka, Japan) in the presence of 5 ng/ml of macrophage colony-stimulating factor (R & D Systems, Abingdon, United Kingdom), as previously described (40). For preparation of the monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood or cryopreserved PBMCs using immunomagnetic beads coated with an anti-CD3 monoclonal antibody (MAb) (Dynabeads 450; Dynal, Oslo, Norway). The $CD3^-$ fraction of the PBMCs was plated on collagen-coated plates and cultured for 60 min at 37°C. The non-plastic-adherent cells were then removed by extensive washing, and the remaining adherent cells were used as monocytes and precursors of macrophages and DCs (23). Monocyte-derived DCs were differentiated from the plastic-adherent cells as described previously (23, 24). Briefly, the plastic-adherent cells were cultured in 3 ml of RPMI 1640 medium containing 10% FCS for 5 days in the presence of 50 ng of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) (Pepro Tech EC Ltd., London, England) and 10 ng of rIL-4 (Pepro Tech) per ml. rGM-CSF and rIL-4 were supplied every 2 days, and 400 μl of medium was replaced as described previously (24). In some cases, DCs unpulsed or pulsed with Ags were further treated with a soluble form of CD40 ligand (CD40L) (Pepro Tech) to obtain fully matured DCs capable of efficiently activating T cells. The purity of DCs obtained was 90.5% as judged by the expression of CD1a. Since *M. leprae* cannot be cultivated or grown in vitro, *M. leprae* (Thai-53) was obtained from an armadillo liver that had been previously infected with *M. leprae*. The isolated bacteria were counted by Shepard's method (35) and were frozen at −80°C until use. The viability of *M. leprae* was assessed by using a fluorescent diacetate/ethidium bromide test (17).

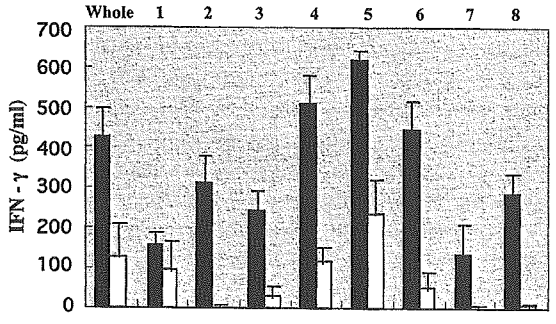

FIG. 2. IFN-γ production by T cells stimulated with membrane fraction-pulsed DCs. The responder $CD4^+$ and $CD8^+$ T cells (10^5/well) were stimulated for 4 days with autologous DCs which had been previously stimulated with 10 μg/ml of various membrane fractions of *M. leprae* obtained by gel filtration. Solid bars, IFN-γ production from $CD4^+$ T cells; open bars, IFN-γ production from $CD8^+$ T cells.

Fractionation of *M. leprae* protein and N-terminal sequencing. The fractionation of the mycobacterial proteins into cell wall, membrane, and cytosolic fractions was carried out according to previous reports (13, 18, 22). Briefly, the mycobacterial suspension was mixed with zirconium beads in the presence of protease inhibitors at a ratio of approximately 1:1 (vol/vol) and homogenized using Beads Homogenizer, model BC-20 (Central Scientific Commerce, Tokyo, Japan), at 1,500 rpm for 90 s three to four times. The beads were separated, and the suspension was centrifuged at 10,000 × *g* for 30 min. The supernatant was then further ultracentrifuged at 100,000 × *g* for 1 h. The resulting pellet was suspended in phosphate-buffered saline, washed twice, and taken as the membrane fraction. For the identification of *M. leprae* antigenic molecules, the membrane fraction was further fractionated using a fast protein liquid chromatography system (Amersham Bioscience, New Jersey). Four hundred micrograms of protein was run on a Superose 12 column (Amersham Bioscience) in 50 mM Tris-HCl, 0.5 M NaCl, and 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) at a flow rate of 0.5 ml/min. Fractions were collected, concentrated, buffer exchanged to 50 mM Tris-HCl using Microcon centrifuge filter units YM-3 (Millipore, Bedford, MA), and run on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The gel was stained with "Daiichi" silver stain (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Western blotting was performed using PB patients' pooled sera, at a dilution of 1:25, which had been preadsorbed with the *M. leprae* cytosolic fraction. Alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) (Biosource, Camarillo, CA) was used as the secondary Ab, and detection was performed by using the nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) reagent (Calbiochem, San Diego, CA). N-terminal peptide sequencing of the protein which reacted to the sera was performed at the Center for Instrumental Analysis, Hokkaido University.

Analysis of cell surface Ags. The expression of cell surface Ags on DCs was analyzed using FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical Co., St. Louis, MO), and 10^4 live cells were analyzed. For analysis of cell surface Ags, fluorescein isothiocyanate-conjugated MAbs against HLA-ABC (G46-2.6; PharMingen, San Diego, CA), HLA-DR (L243; PharMingen), CD86 (FUN-1; PharMingen), and CD83 (HB15a; Immunotech, Marseille, France) were used. A murine MAb against MMP-II was raised by immunizing a mouse with purified MMP-II. The optimal concentrations of MAbs were determined in advance.

Identification and purification of MMP-II. The MMP-II gene was PCR amplified from *M. leprae* chromosomal DNA and cloned into an *Escherichia coli* expression vector. Briefly, the MMP-II gene was inserted into the expression plasmid pET28 (Novagen Inc., Madison, WI) and transformed into *E. coli* strain ER2566 (New England Biolabs Inc., Beverly, MA). The expressed protein was eluted using Whole Gel Eluter (Bio-Rad Laboratories, Hercules, CA) and used for all experiments. The amount of lipopolysaccharide (LPS) in the purified MMP-II protein was determined by using a *Limulus* amebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD) and found to be less than 70 pg per mg of MMP-II, a level that did not affect the maturation of DCs.

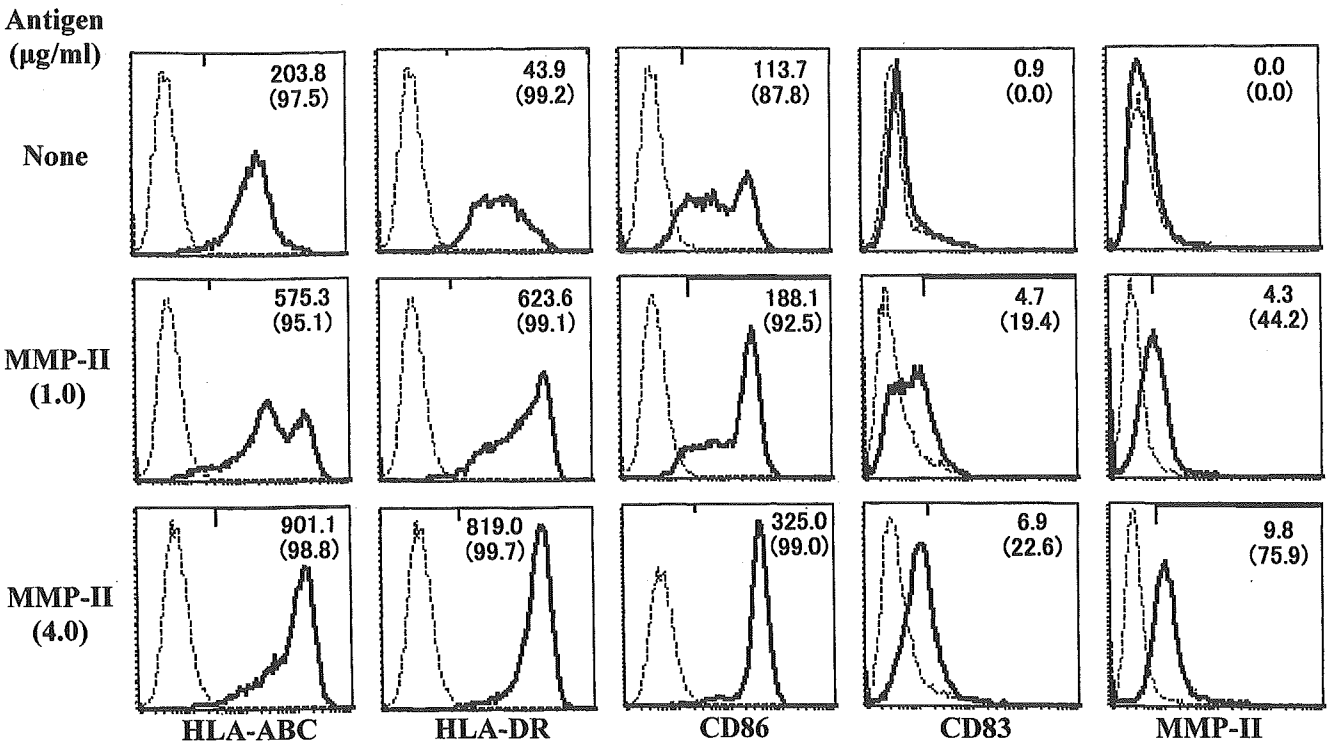

FIG. 4. Expression of various molecules on DCs pulsed with MMP-II. Monocyte-derived DCs from healthy individuals (PPD positive) were pulsed with the indicated dose of MMP-II. DCs were gated and analyzed. Solid curves, isotype-matched control IgG; broken curves, the indicated MAb. The number in the top right corner of each panel represents the difference in mean fluorescence intensity between the control IgG and the test MAb. The number in parentheses is the percentage of positive cells. Results of one experiment representative of three separate experiments are shown.

Assessment of APC function of DCs pulsed with cell membrane fractions. The ability of DCs pulsed with various fractions of the *M. leprae* cell membrane to stimulate autologous T cells was assessed using an autologous stimulator–T-cell mixed reaction as previously described (10, 24). The Ag-pulsed DCs were treated with 50 μg/ml of mitomycin C, washed extensively to remove extracellular Ags, and used as a stimulator. T cells were prepared as follows: freshly thawed PBMCs were depleted of major histocompatibility complex (MHC) class II^+ cells by using magnetic beads coated with a MAb to MHC class II Ag (Dynabeads 450; Dynal) and were further treated with beads coated with either a CD4 or a CD8 MAb to select T cells negatively as previously reported (10). The purity of $CD4^+$ T cells or $CD8^+$ T cells was more than 98%. The supernatant of the stimulator–T-cell mixture was collected on day 4 of coculture, and the level of gamma interferon (IFN-γ) produced was measured by an Opt EIA Human ELISA Set (BD PharMingen International).

Assessment of cytokine production. Levels of the following cytokines were measured: tumor necrosis factor alpha (TNF-α), IL-10, and IL-12 p70 produced from either macrophages or DCs by stimulation with MMP-II for 24 h in the presence or absence of a soluble form of CD40L (Pepro Tech). The murine MAb against TLR-2 (clone 2392; IgG1) with neutralizing activity was obtained from Genentech (San Francisco, CA). The optimal concentration of the anti–Toll-like receptor 2 (anti–TLR-2) Ab was determined in advance. The concentrations of IL-12 p70, IL-10, and TNF-α were quantified using the Opt EIA Human ELISA Set enzyme assay kits, available from BD PharMingen International.

Cell transfection and luciferase assay. Human embryonic kidney cells (HEK293) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% FCS, 50 mg/ml penicillin/streptomycin, and nonessential amino acids (Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator with 5% CO_2. The cDNA of human TLR-2 was PCR amplified using a human spleen cDNA library (BD Biosciences, San Jose, CA) and inserted into pCIneo (Promega, Madison, WI). HEK293 cells (2×10^4) were transiently transfected with a mixture of plasmids—200 ng of pCIneo hTLR2, 25 ng of p5×NF-κB-luc

TABLE 1. Cytokine production from DCs stimulated with MMP-II[a]

DC stimulation (dose)	Concn (pg/ml) of the following cytokine:					
	IL-12 p70		TNF-α		IL-10	
	CD40L (−)	CD40L (+)	CD40L (−)	CD40L (+)	CD40L (−)	CD40L (+)
None	2.6 ± 0.2*,†	17.7 ± 0.4‡,§	2.6 ± 0.4¶,‖	15.4 ± 3.8**,††	2.0 ± 0.1	3.4 ± 0.1
MMP-II (1 μg/ml)	51.2 ± 0.5*	782.0 ± 8.7‡	345.4 ± 9.9¶	345.7 ± 19.3**	2.5 ± 0.3	1.7 ± 0.1
MMP-II (4 μg/ml)	404.0 ± 9.8†	1624.0 ± 11.0§	773.8 ± 11.1‖	747.3 ± 18.7††	2.2 ± 0.1	2.8 ± 0.3
LPS (0.3 pg/ml)	2.8 ± 0.3	18.7 ± 0.6	5.0 ± 1.3	36.0 ± 9.2	2.0 ± 0.3	3.0 ± 0.2

[a] Monocyte-derived DCs (10^5/well) were stimulated for 24 h with the indicated dose of MMP-II in the absence [CD40L (−)] or presence [CD40L (+)] of a soluble form of CD40L (1.0 μg/ml). The DCs were also stimulated for 24 h with 0.3 pg/ml of LPS, which is estimated to be present in 4 μg/ml of MMP-II. Results representative of more than three separate experiments are shown. Assays were done in triplicate, and results are expressed as means ± standard deviations. Titers with the same symbols are statistically compared by Student's *t* test, as follows: *, $P < 0.001$; ‡, $P < 0.001$; ¶, $P < 0.0005$; **, $P < 0.001$; †, $P < 0.001$; §, $P < 0.001$; ‖, $P < 0.0001$; ††, $P < 0.0005$.

TABLE 2. Cytokine production from macrophages stimulated with MMP-II[a]

Macrophage stimulation (dose)	Concn (pg/ml) of the following cytokine:		
	IL-12 p70	TNF-α	IL-10
None	2.0 ± 0.2	19.3 ± 1.8*,†	45.3 ± 8.8‡,§
MMP-II (1.0 μg/ml)	2.1 ± 0.3	122.2 ± 6.8*	149.9 ± 20.3‡
MMP-II (4.0 μg/ml)	1.9 ± 0.2	568.6 ± 12.4†	561.2 ± 31.9§
LPS (0.3 pg/ml)	1.8 ± 0.1	10.0 ± 2.0	15.6 ± 3.2

[a] Monocyte-derived macrophages (10^5/well) were stimulated for 24 h in the presence of a soluble form of CD40L (1.0 μg/ml) with the indicated dose of MMP-II. Results representative of more than three separate experiments are shown. Assays were done in triplicate, and results are expressed as means ± standard deviations. Titers with the same symbols are statistically compared by Student's *t* test, as follows: *, $P < 0.001$; ‡, $P < 0.005$; †, $P < 0.0005$; §, $P < 0.001$.

(Stratagene, La Jolla, CA), and 10 ng of pRL-TK-*Renilla* luciferase plasmid (Promega)—using the FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN), as previously described (38). Thirty-six hours after transfection, cells were treated with various amounts of glutathione *S*-transferase (GST), MMP-II, or peptidoglycan (PGN) as a positive control (for TLR-2-dependent luciferase activity) for a further 6 h. The cells were lysed in 70 μl of 1× passive lysis buffer (Promega), and luciferase activity in 10 μl of the cell lysate was measured using the Promega Dual-Luciferase Reporter Assay System according to the protocol provided by the manufacturer. Data were expressed as fold induction relative to the activity of *Renilla* luciferase, which is an internal control for transfection efficiency.

Statistical analysis. Student's *t* test was applied to demonstrate statistically significant differences.

RESULTS

Identification of *M. leprae*-derived antigenic molecules. The cell membrane fraction from *M. leprae* was found to be the most T-cell stimulating (10, 22), although it may also contain some inhibitory molecules (10). The *M. leprae*-derived cell membrane fraction was solubilized and further fractionated using a gel filtration column to search for the antigenic molecules. Figure 1 shows the silver staining of each fraction, which revealed several proteins. Then we pulsed healthy donor-derived DCs with each of these fractions individually and examined the antigenicity of each fraction by monitoring IFN-γ production by DC-stimulated $CD4^+$ and $CD8^+$ T cells derived from PPD-positive healthy individuals (Fig. 2). Among the eight fractions, fractions 4 through 6 seemed to be efficient at stimulating cytokine production by $CD4^+$ T cells, and fractions 4 and 5 appeared to be involved in the activation of both $CD4^+$ and $CD8^+$ T cells. Thus, using these two fractions of cell membrane, we identified one of the antigenic molecules. The pooled PB leprosy sera, preadsorbed with *M. leprae* cytosol fractions, were used for Western blot analysis of the fractions (not shown). N-terminal sequencing of the serum-reactive bands common to fractions 4 and 5 identified MMP-II as one of the candidates. The presence of MMP-II in fractions 4 and 5 was further confirmed by Western blotting using a MAb against MMP-II (Fig. 3). For the purification of the protein, the MMP-II gene was amplified by PCR from the genomic DNA of *M. leprae*, and MMP-II protein was subsequently expressed in *E. coli* by using the T7 expression system (pET-28). The expressed protein was confirmed to be MMP-II by Western blot analysis (not shown), by comparison to purified MMP-II, used as a positive control (donated by P. J. Brennan, Colorado University).

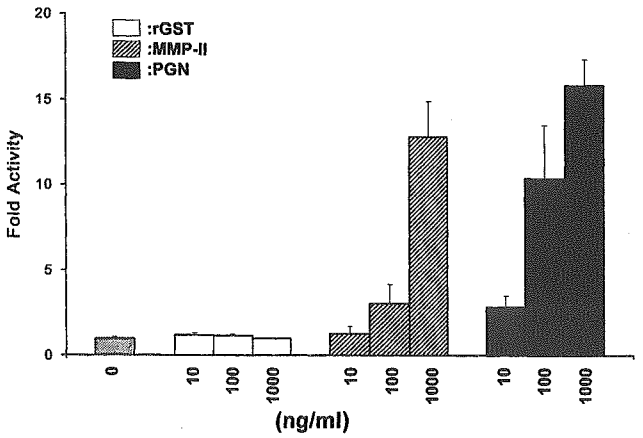

FIG. 5. NF-κB-dependent reporter gene activity of the TLR2 transfectant was measured after stimulation with or without 10, 100, or 1,000 ng/ml of rGST, MMP-II, or PGN, as described in Materials and Methods. Data are expressed as fold induction relative to the activity of *Renilla* luciferase, which is an internal control for transfection efficiency in the dual-luciferase reporter assay. Results of one experiment representative of two separate experiments are shown. Assays were done in triplicate, and the results are expressed as means ± standard deviations.

Antigenicity of *M. leprae*-derived MMP-II. The ability of MMP-II to evoke cellular immunity was assessed using DCs and macrophages as APCs. Previously we demonstrated that the cytosol fraction from *M. leprae* was less efficient at the induction of DC maturation and that the whole cell membrane fraction partially induced DC maturation (22). In contrast, when immature DCs were pulsed with MMP-II, they up-regulated the expression levels of HLA-ABC, HLA-DR, CD86, and CD83 Ags on the surfaces of DCs in an Ag dose-dependent manner, and the percentage of $CD83^+$ cells was found to increase significantly (Fig. 4). The expression of MMP-II on the surfaces of MMP-II-pulsed DCs was revealed using a MAb to MMP-II (Fig. 4). The functional aspects of MMP-II in terms of APC activation were assessed by measuring production of cytokines, such as IL-12 p70, IL-10, and TNF-α, by APCs (Tables 1 and 2). The bioactive form of IL-12 was released from DCs by pulsing MMP-II in the absence of CD40L, and the cytokine production level was enhanced by copulsing DCs with CD40L and MMP-II (Table 1). Obviously IL-12 was not produced from DCs by stimulation with the amount of LPS estimated to be present in 4 μg/ml of MMP-II. Furthermore, DCs produced TNF-α in the presence or absence of CD40L, but they did not produce any significant amount of IL-10 due to MMP-II stimulation. These results suggested that MMP-II could activate DCs and induce their maturation. Macrophages derived from monocytes did not produce IL-12 p70 by stimulation with MMP-II, but they produced TNF-α and IL-10 (Table 2), which are found predominantly in granulomatous mycobacterium-infected lesions. These results indicate that MMP-II also activated macrophages, but macrophages and DCs seem to have distinct functional roles. All cytokines were produced in an Ag dose-dependent fashion.

Involvement of TLR-2 in activation of DCs. In order to elucidate the mechanism by which MMP-II activates DCs, we examined the relationship of MMP-II and TLR-2, because

TABLE 3. Effect of the TLR-2-antagonistic Ab on IL-12 p70 production by DCs[a]

DC stimulation (dose)	IL-12 p70 production (pg/ml) in the presence of:				
	TLR-2-antagonistic Ab at the following concn (μg/ml):			Control IgG at the following concn (μg/ml):	
	0	5.0	10.0	5.0	10.0
MMP-II (1.0 μg/ml)	603.1 ± 11.0*†	491.2 ± 10.2*	178.1 ± 8.8†	658.2 ± 11.3	675.9 ± 10.7
MMP-II (4.0 μg/ml)	1,210.2 ± 20.0‡,§	949.0 ± 9.3‡	805.3 ± 7.9§	1,290.3 ± 12.4	1,403.8 ± 31.5

[a] Monocyte-derived immature DCs (10^6/ml) were treated with the indicated dose of a TLR-2-antagonistic MAb or an isotype-matched control IgG and were subsequently stimulated for 24 h with MMP-II in the presence of CD40L (1.0 μg/ml). Results representative of more than three separate experiments are shown. Assays were done in triplicate, and results are expressed as means ± standard deviations. Titers with the same symbols were statistically compared by Student's *t* test, as follows: *, $P < 0.0001$; †, $P < 0.0005$; ‡, $P < 0.0001$; §, $P < 0.0001$.

TLR-2 is reported to be highly associated with induction of innate immunity against mycobacterial infection (1, 4, 30). When HEK293 cells that had been cotransfected with pCIneo TLR-2, p5×NF-κB-luc, and pRL-Tk-*Renilla* luciferase were pulsed with MMP-II, significant levels of luciferase activity were induced in an Ag dose-dependent manner, levels comparable to those induced by PGN, a well-defined TLR-2-associated bacterial Ag (Fig. 5). Similar results were also obtained using *M. leprae*-derived MMP-II. Such changes were not induced by rGST, a negative-control protein. Furthermore, when the surface TLR-2 Ag on DCs was masked by an antagonistic Ab to TLR-2, IL-12 p70 production by DCs stimulated with MMP-II was significantly, though partially, suppressed (Table 3). The isotype-matched control IgG did not affect IL-12 p70 production by MMP-II-stimulated DCs. As expected, the TLR-2-antagonistic Ab did not suppress IL-12 p70 production by DCs stimulated with LPS (a ligand for TLR-4) (not shown). These results indicate that MMP-II might use TLR-2 as its ligand on APCs, resulting in stimulation of DCs.

DISCUSSION

Leprosy is a broad-spectrum disease (34). One representative manifestation is PB leprosy. Studies on clinical specimens of the skin lesions indicate that the infection is localized and the spread of *M. leprae* is suppressed as a consequence of activation of cellular immune responses (20, 31, 36). On the other hand, MB leprosy usually manifests widespread infection due to the lack of an efficient response to *M. leprae* components. The mechanisms leading to the broad spectrum are not fully understood yet, but these observations suggest the presence of an Ag with immunomodulating activities that modify the immune responses in vivo. So far, however, such Ags have not been identified. Previously we evaluated the APC function of professional APCs and found that DCs were superior to macrophages in activating T cells (10). When we examined the DC-mediated antigenicity of subcellular components of *M. leprae* for identification of immunomodulating molecules, we found that the cell membrane fraction was more suitable than other fractions (22). Therefore, the *M. leprae* membrane fraction was size fractionated, and each fraction was examined for its T-cell-stimulating ability by using DCs as APCs. Two of the fractions with high activity were examined by reaction to PB leprosy sera, and subsequently the N terminus of the reactive protein was sequenced. As a result, MMP-II was identified as one of the antigenic cell membrane proteins, and the result was confirmed by Western blotting of the various fractions using an anti-MMP-II antibody (Fig. 3).

MMP-II was originally identified from *M. leprae* as a major native protein in 1990 (13) and was recognized as being identical to mycobacterial bacterioferritin (32). Purification of MMP-II by reverse-phase chromatography revealed a large molecular mass of 380 kDa and a ferroxidase center residue. MMP-II contains 1,000 to 4,000 atoms of iron per molecule of protein (32). A homology search on the mycobacterial nucleotide database revealed that MMP-II is conserved among *M. leprae*, *M. tuberculosis*, and *M. avium*. The percent homology at the amino acid level is about 86% among these species. The previous studies reported that MMP-II was recognized in vivo by B and T cells. Sera from patients were reported to have higher IgG titers to MMP-II, regardless of the clinical type of leprosy, than sera from healthy individuals (7). Also, T cells from leprosy of both the PB and the MB type were stimulated by MMP-II to proliferate and to produce both IFN-γ and IL-5 (29). However, tuberculosis patients or individuals who have had contact with leprosy patients have not been examined yet. Also, the influence of MMP-II on the innate immune response has not yet been clarified.

MMP-II stimulated DCs to produce TNF-α and a bioactive form of IL-12 (IL-12 p70) (Table 1) and induced their maturation, as observed by their phenotypic changes (Fig. 4). Further, MMP-II also stimulated macrophages to produce TNF-α and IL-10 (Table 1). These cytokines were produced by stimulation with either MMP-II derived from *M. leprae* (not shown) or MMP-II overexpressed in *E. coli* (Table 1). DCs and macrophages play distinct roles in the host defense against mycobacterial infection (9). DCs are central to the initiation of Ag-specific T-cell responses (6, 27, 36), and in our preliminary experiments, DCs pulsed with purified MMP-II stimulated both $CD4^+$ and $CD8^+$ T cells from PPD-positive healthy individuals to produce IFN-γ (not shown). The activated form of macrophage is involved in the formation of tuberculoid granulomatous lesions (5, 9). These results indicate that MMP-II might contribute to the immune regulation of host cells against mycobacteria. Then we investigated what could be the MMP-II ligand that is expressed on APCs. TLR-2 is associated mainly with innate immunity and has been shown to recognize the molecular pattern of pathogens (4, 11, 18, 26, 33). In mycobacterial infection, it has been reported that a 19-kDa lipoprotein isolated from *M. tuberculosis* ligated TLR-2 (4, 19), and the *M. leprae* 33-kDa lipoprotein could be another candidate participating in the TLR-2-associated innate immune system

(19). In our study using the TLR-2 reporter assay with HEK293 cells, we found that TLR-2 is likely to be involved in the recognition of MMP-II in spite of the fact that MMP-II lacks the triacylated region. This finding surprised us, but a similar ligation of protein to TLR-2 has also been reported for neisserial porins HSP60 and HSP70, which have no posttranslational modification of acylation (2, 25, 39). IL-12 production by MMP-II-stimulated DCs was partially inhibited by a TLR-2-antagonistic Ab, which indicates that other receptors are also involved in signals leading to IL-12 production.

The data in this report, taken together, indicate that MMP-II has an immunomodulating activity and contributes to the activation of innate immunity. Further study should be pursued to evaluate its host defense-associated activity against leprosy and other mycobacterial infections that pose a worldwide threat.

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