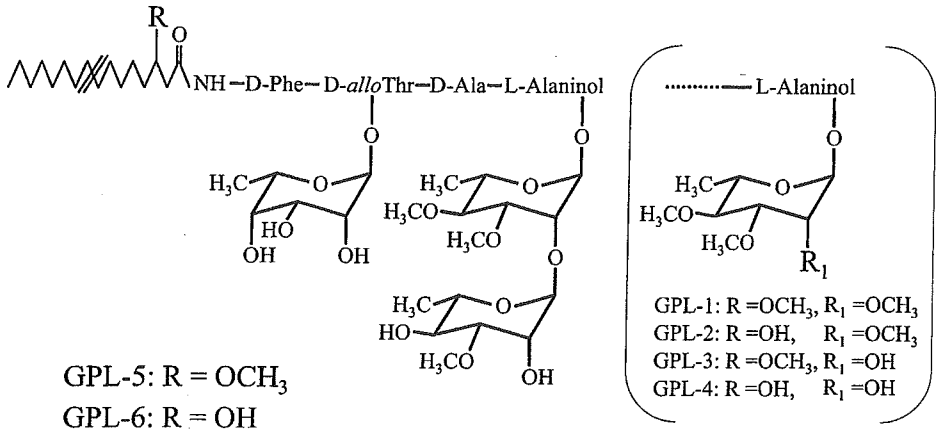

FIG. 6. Proposed structures of GPL-5 and GPL-6. Figure in parentheses shows the structure of GPL-1, GPL-2, GPL-3, and GPL-4, which were characterized in previous studies (10, 16, 25).

which contained two units of 3,4-di-*O*-Me-Rha at L-alaninol of the fatty acyl-tetrapeptide core with no 3-*O*-Me-Rha at any other position when cultured in carbon-limited medium (23, 24). However, the reason for not being able to detect 3-*O*-Me-Rha remains unknown.

In the *gtf3*-overexpressed strain Wt/pMVgtf3, the productivities of GPL-5 and GPL-6 were much higher than those of other GPLs (Fig. 7). So, we can speculate that the expression level of *gtf3* is usually repressed and could be regulated by some environmental factors, such as the nutrient condition or the gene encoding sigma factor (23, 24). GC/MS analyses showed that GPL-5 and GPL-6 have the structures in which 3-*O*-Me-Rha is linked to GPL-3 and GPL-4. These results suggest that GPL-3 and GPL-4 could be the precursors of GPL-5 and GPL-6, respectively, and in Wt/pMVgtf3, overexpression of *gtf3* resulted in 2-*O*-rhamnosylation of 3,4-di-*O*-Me-Rha in GPL-3 and GPL-4 instead of 2-*O*-methylation for converting to GPL-1 and GPL-2, so that GPL-5 and GPL-6 were synthesized.

Figure 9 represents proposed glycosylation steps related to *M. smegmatis* and *M. avium*. We showed that the functions of *gtf1* and *gtf2* corresponded to those of *gtfA* and *gtfB*, respectively. This finding demonstrates that the biosynthetic pathway for nsGPLs, which is the glycosylation of the fatty acyl-tetrapeptide core with the 6-d-Tal and Rha residues, is common between *M. smegmatis* and *M. avium*. Moreover, the biochemical characterization of Δgtf2 and Δgtf1 suggested that the glycosylation pathways for nsGPLs might not be stringent. On the other hand, it has been shown that the *rtfA* gene of *M. avium* triggers the biosynthesis of ssGPLs by transfer of Rha to 6-d-Tal of nsGPLs (12). In *M. smegmatis*, our results indicated that the *gtf3* gene plays a role in synthesis of 3-*O*-Me-rhamno-

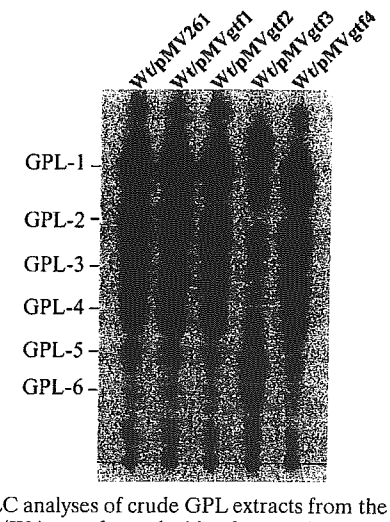

FIG. 7. TLC analyses of crude GPL extracts from the *M. smegmatis* mc^2155 strain (Wt) transformed with *gtf* expression vectors. Total lipid fraction after mild alkaline hydrolysis was spotted on plates and developed in $CHCl_3$-CH_3OH (9:1 [vol/vol]). GPLs were visualized by spraying with 10% H_2SO_4 and charring. Each total lipid fraction was extracted from an equal weight of harvested cells.

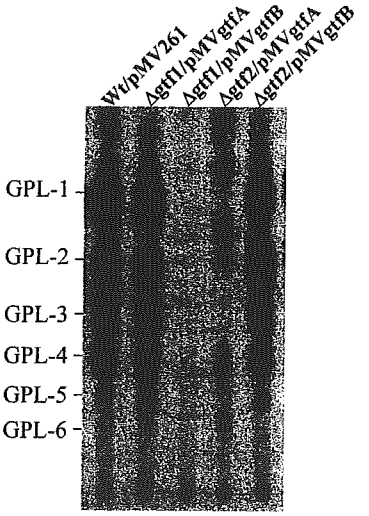

FIG. 8. TLC analyses of crude GPL extracts from the *M. smegmatis* mc^2155 strain (Wt) and its gene disruptants transformed with *M. avium* *gtfA* and *gtfB*. Total lipid fraction after mild alkaline hydrolysis was spotted on plates and developed in $CHCl_3$-CH_3OH (9:1 [vol/vol]). GPLs were visualized by spraying with 10% H_2SO_4 and charring. Each total lipid fraction was extracted from an equal weight of harvested cells.

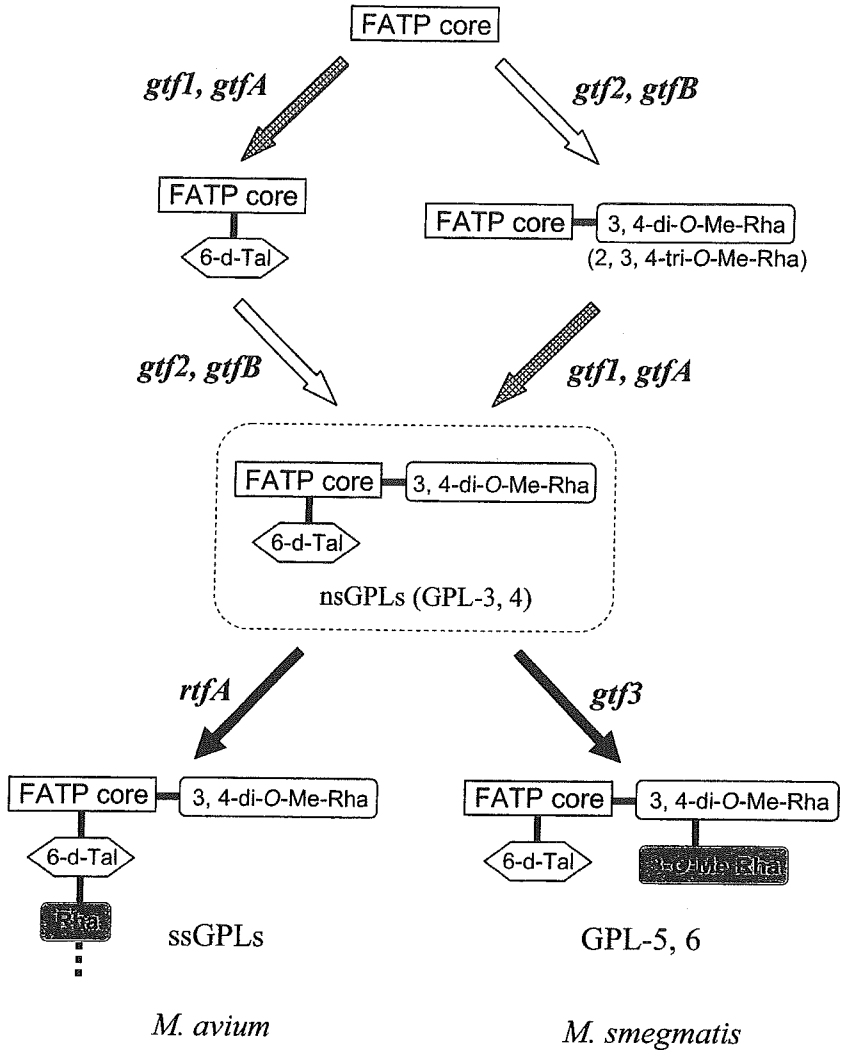

FIG. 9. Proposed biosynthetic pathways for GPLs of *M. smegmatis* and *M. avium*. FATP core, fatty acyl-tetrapeptide core.

syl-(1→2)-3,4-di-*O*-Me-Rha linked to L-alaninol of the fatty acyl-tetrapeptide core by transfer of an extra Rha residue to nsGPLs. Thus, the *rtfA* and *gtf3* genes have the ability to confer the biosynthetic differences between *M. avium* and *M. smegmatis*, suggesting that these genes may be responsible for the phylogenetic distinctions in the two species of mycobacteria.

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Identification of *Mycobacterium* species by comparative analysis of the *dnaA* gene

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Mycobacterium spp.; *dnaA* gene; differential diagnosis; LAMP assay.

Abstract

For the establishment of a diagnostic tool for mycobacterial species, a part of the *dnaA* gene was amplified and sequenced from clinically relevant 27 mycobacterial species as well as 49 clinical isolates. Sequence variability in the amplified segment of the *dnaA* gene allowed the differentiation of all species except for *Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium microti*, which had identical sequences. Partial sequences of *dnaA* from clinical isolates belonging to three frequently isolated species revealed a very high intraspecies similarity, with a range of 96.0–100%. Based on the *dnaA* sequences, a species-specific primer set for *Mycobacterium kansasii* and *Mycobacterium gastri* was successfully designed for a simple loop-mediated isothermal amplification method. These results demonstrate that the variable sequences in the *dnaA* gene were species specific and were sufficient for the development of an accurate and rapid diagnosis of *Mycobacterium* species.

Introduction

Increasing reports of opportunistic infection by nontuberculous mycobacteria (NTM) in immunocompromised patients such as AIDS patients and elderly people are a matter of serious concern to public health (Horsburg, 1991; Montessori *et al.*, 1996; Primm *et al.*, 2004). The routine diagnosis of mycobacteriosis relies primarily on the detection of acid-fast-stained bacilli in the samples by microscopic observation, and the infecting mycobacterial species can be identified with conventional tests including observation of colony morphology and pigmentation, growth rate, and biochemical characteristics (Cernoch *et al.*, 1994; Metchock *et al.*, 1999). Disadvantages of this approach include the time taken to provide clinically relevant information. The clinician must initiate therapy for *Mycobacterium tuberculosis* against NTM infection several weeks before species identification (Montessori *et al.*, 1996), which may increase health care costs, and may reduce the social activity of the patients. Therefore rapid detection and identification of the species level of mycobacteria is required, both to decide whether measures are needed to prevent the spread of the disease and for adequate therapy (American Thoracic Society, 1997).

The mycobacterium species often implicated in NTM infection are *Mycobacterium avium–Mycobacterium intracellulare* complex (MAC), *Mycobacterium kansasii*, *Mycobacterium chelonae*, *Mycobacterium abscessus*, and *Mycobacterium xenopi* (Wayne & Sramek, 1992; Metchock *et al.*, 1999; Primm *et al.*, 2004). *Mycobacterium gordonae*, *Mycobacterium gastri*, or most of the rapidly growing species are rarely pathogenic, but are often encountered as contaminant in clinical samples. Therefore, the discrimination of these species from pathogenic ones is an important diagnostic issue (Primm *et al.*, 2004).

Several studies have been conducted to develop rapid methods based on molecular technique for identifying mycobacterial species in recent years. The DNA sequences reported for such usage are those of 16S rRNA gene (Kirschner *et al.*, 1993; De Beenhouwer *et al.*, 1995; Cloud *et al.*, 2002), *recA* (Blackwood *et al.*, 2000), *rpoB* (Kim *et al.*, 1999), *gyrB* (Kasai *et al.*, 2000), *hsp65* (Plikaytis *et al.*, 1992; Brunello *et al.*, 2001), or 16S–23S internal transcribed spacer (ITS) (De Smet *et al.*, 1995; Roth *et al.*, 1998). The 16S rRNA gene and ITS-based methods are currently widely accepted as rapid and accurate for identifying mycobacteria (Plikaytis *et al.*, 1992; De Smet *et al.*, 1995; Park *et al.*, 2000; Turenne *et al.*, 2001). However, some species have the same sequence or a very high similarity (Kim *et al.*, 1999; Kasai *et al.*, 2000). This fact indicates the need to develop more reliable and user-friendly molecule-based diagnostic tools.

Recently, Notomi *et al.* (2000) have reported a novel nucleic acid amplification method, termed loop-mediated

isothermal amplification (LAMP), that amplifies DNA with high specificity, efficacy, and rapidity under isothermal conditions. The LAMP reaction requires a *Bst* DNA polymerase with strand displacement activity and a set of four specially designed primers that recognize six distinct sequences on the target DNA, the specificity of which should be extremely high. The amplification products are stem-loop DNA structures with several inverted repeats of the target. The advantage of the LAMP method is that the reaction is performed under isothermal conditions of between 60 and 65 °C. As a result, it requires only simple and cost-effective reaction equipment. The LAMP method has emerged as a powerful tool to facilitate genetic testing for various infectious diseases (Enosawa *et al.*, 2003; Iwamoto *et al.*, 2003; Kuboki *et al.*, 2003; Ihira *et al.*, 2004; Parida *et al.*, 2004; Thai *et al.*, 2004).

The purpose of our work is to identify a species-specific region of *Mycobacterium* sp., and to develop a LAMP assay that can differentiate clinically relevant species.

Materials and methods

Bacterial strains and preparation of genomic DNA

The bacteria used in this study comprised 27 strains and 49 clinical isolates as shown in Table 1. All strains except for *Mycobacterium leprae* were cultured on 1% Ogawa medium (Nissui, Tokyo, Japan) at 37 °C. *Mycobacterium leprae* was prepared from infected nude mouse food pad (Shepard, 1960). Genomic DNA was extracted from mycobacterial strains as follows. Mycobacterial cells were resuspended in 1.8 mL of sterile phosphate-buffered saline (PBS) containing 0.1 mm diameter zirconia/silica beads (BioSpec Products Inc., Bartlesville, OK). The mixture was beaded for 20 s with a Beads Homogenizer Model BC-20 (Central Scientific Commerce, Tokyo, Japan), transferred to a 1.5 mL microcentrifuge tube, and the genomic DNA was purified with proteinase K treatment and phenol/chloroform extraction followed by ethanol precipitation, then suspended in 100 μL distilled water.

Table 1. *Mycobacterium* species and strains used in this study and results of the loop-mediated isothermal amplification assay

Species	Strains	Accession number	Primer set	
			Kan32	Gas583
Mycobacterium abscessus	JATA 63-01 (ATCC 19977)	AB087684	–	–
Mycobacterium africanum	KK 13-02 (ATCC 25420)	AB087685	–	–
Mycobacterium avium	JATA 51-01 (ATCC 25291)	AB087686	–	–
	Clinical isolate 22 strains			
Mycobacterium bovis	JATA 12-01 (ATCC 19210)	AB087687	–	–
Mycobacterium chelonae	JATA 62-01 (ATCC 35752)	AB087688	–	–
Mycobacterium fortuitum	JATA 61-01 (ATCC 6841)	AB087689	–	–
Mycobacterium gastri	KK 44-02 (ATCC 15754)	AB087690	–	+
Mycobacterium gordonae	JATA 33-01 (ATCC 14470)	AB087691	–	–
Mycobacterium intracellulare	JATA 52-01 (ATCC 13950)	AB087692	–	–
	Clinical isolate 17 strains			
Mycobacterium kansasii	KK 21-01 (ATCC 12478)	AB087693	+	–
	Clinical isolate 10 strains		+	–
Mycobacterium leprae	Thai-53	AB087694	–	–
Mycobacterium malmoense	JATA 47-01 (ATCC 29571)	AB087695	–	–
Mycobacterium marinum	JATA 22-01 (ATCC 927)	AB087696	–	–
Mycobacterium microti	KK 14-01 (ATCC 19422)	AB087697	–	–
Mycobacterium nonchromogenicum	JATA 45-01 (ATCC 19530)	AB087698	–	–
Mycobacterium parafortuitum	ATCC 25807	AB087699	–	–
Mycobacterium phlei	ATCC 19249	AB087700	–	–
Mycobacterium scrofulaceum	JATA 31-01 (ATCC 19981)	AB087701	–	–
Mycobacterium simiae	KK 23-08 (ATCC 25275)	AB087702	–	–
Mycobacterium smegmatis	JATA 64-01	AB087703	–	–
Mycobacterium szulgai	JATA 32-01	AB087704	–	–
Mycobacterium terrae	KK 46-01 (ATCC 15755)	AB087705	–	–
Mycobacterium triviale	KK 50-02 (ATCC 23292)	AB087706	–	–
Mycobacterium tuberculosis	JATA 11-01 (H37Rv)	AB087707	–	–
Mycobacterium ulcerans	KK 43-01	AB087708	–	–
Mycobacterium vaccae	KK 66-01	AB087709	–	–
Mycobacterium xenopi	KK 42-01 (ATCC 19250)	AB087710	–	–

All strains were kindly donated by Dr Kashiwabara, NIID.

Clinical isolates were identified by Amplicore *Mycobacterium* kit (Roche Pharma, Basel, Switzerland) or conventional biochemical test (Jamal *et al.*, 2000).

Amplification of the region within *dnaA* gene

Highly polymorphic regions flanked by conserved regions were identified by aligning the *Mycobacterium* spp. *dnaA* sequences, which were available in GenBank at the time this study was initiated. These regions were used to design a pair of degenerate primers, U1F 5′-GTS CAR AAC GAR ATC GAR CG-3′ and U1R 5′-CCB GAY TCR CCC CAG ATG AA-3′. A schematic representation of the primer design is shown in Fig. 1a. PCR was performed in a TAKARA Thermal Cycler MP (TAKARA Biomedical, Otsu, Japan) with a reaction mixture consisting of 1 μL of genomic DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, each primer at a concentration of 0.4 μM, 1 × PCR buffer with 1.5 mM $MgCl_2$ (TAKARA Biomedical), and 1.25 U of ExTaq (TAKARA Biomedical), with 10 μL PCRX Enhancer System solution (Gibco BRL, Rockville, MD) in a total volume of 50 μL. The PCR thermocycles were 3 min at 94 °C, followed by 30 cycles of 94 °C for 10 s, 50 °C for 20 s, and 72 °C for 45 s, with a final extension step at 72 °C for 7 min. PCR products were visualized by UV illumination of an ethidium bromide-stained 1.5% agarose gel and cut out to purify with EASYTRAP Ver.2 (TAKARA Biomedical) according to the manufacturer's instruction.

DNA sequencing and sequencing analysis

The ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (PE Biosystems, Foster City, CA) was used for the sequencing of the PCR products. The same primers for amplification were used for sequencing. The sequencing reaction was performed in accordance with the instruction of the manufacturer. Sequencing products were purified with a Centrisep column (Princeton Separations, Adelphia, NJ).

The sequencing output was analyzed by using the DNA Sequence Analyzer computer software (PE Biosystems). The partial *dnaA* sequences were aligned using the Clustal W algorithm (Thompson *et al.*, 1994) of the software DNASpace ver. 3.5 (Hitachi Software Engineering, Yokohama, Japan), and the alignment was manually corrected. A phylogenetic tree was generated by DNASpace ver. 3.5 (Hitachi Software Engineering) with a total of 1000 bootstraps. Pairwise similarity of the partial *dnaA* sequences was determined by using DNASIS package (Hitachi Software Engineering).

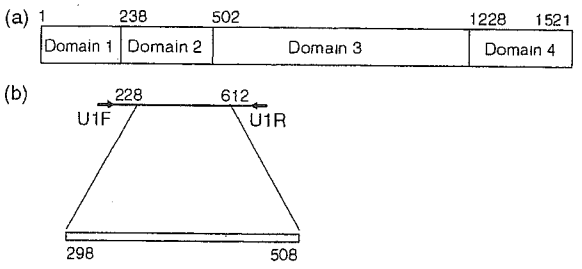


Fig. 1. Schematic representation of the DnaA protein and primer design for the amplification of the partial mycobacterial *dnaA* gene. Number indicates the nucleotide position of *Mycobacterium tuberculosis*, GenBank accession number AL021427. (a) The DnaA protein from *M. tuberculosis* contains four domains. Domain 1 is involved in interaction with DnaB. Domain 2 constitutes a flexible loop. DNA unwinding required Domain 3. Domain 4 is sufficient for specific binding to DNA. Primers U1F and U1R were used to generate about 400 bp fragment from *dnaA* of 27 mycobacterial spp. (b) Analysis and comparison region used in this study are indicated by a bar (298–508 bp).

Species-specific LAMP assay for *Mycobacterium kansasii* and *Mycobacterium gastri*

A set of four primers comprising two inner primers and two outer primers that recognized six distinct regions on the target sequence were designed with PrimerExplorer Ver.3 (Fujitu, Tokyo, Japan). The detailed sequences of the primers are shown in Fig. 3. The two inner primers are called the forward inner primer (FIP) and the backward inner primer (BIP), and each contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in late stages. FIP contains the sequence complementary F1 (F1c) and F2. BIP contains the complementary B1 (B1c) and B2. The two outer primers consist of F3 and B3.

The LAMP reaction was carried out in 25 μL of reaction mixture by using the Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tochigi, Japan) containing 2.4 μM (each) FIP and BIP, 0.2 μM (each) of the outer primers, F3 and B3, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine, 1.4 mM (each) of dNTP, 8 U of *Bst* DNA polymerase (New England BioLabs, Beverly, MA), and the template DNA. Amplification was undertaken in 0.5 μL microtubes in a heatblock under isothermal conditions of 63 °C for 60 min, followed by 80 °C for 2 min to terminate the reaction. Positive and negative controls were included in each run, and precautions to prevent cross-contamination were observed. Two microliter aliquots of LAMP products were subjected to electrophoresis on a 4% agarose gel in Tris-borate-EDTA buffer followed by staining with ethidium bromide and were visualized on a UV transilluminator at 302 nm. The specificity of the LAMP-amplified products were further validated by restriction enzyme digestion with *Nae*I and *Hae*II for *M. kansasii* and *M. gastri*, respectively. The diluted genomic DNA was used for determining the sensitivity of the species-specific LAMP assay.

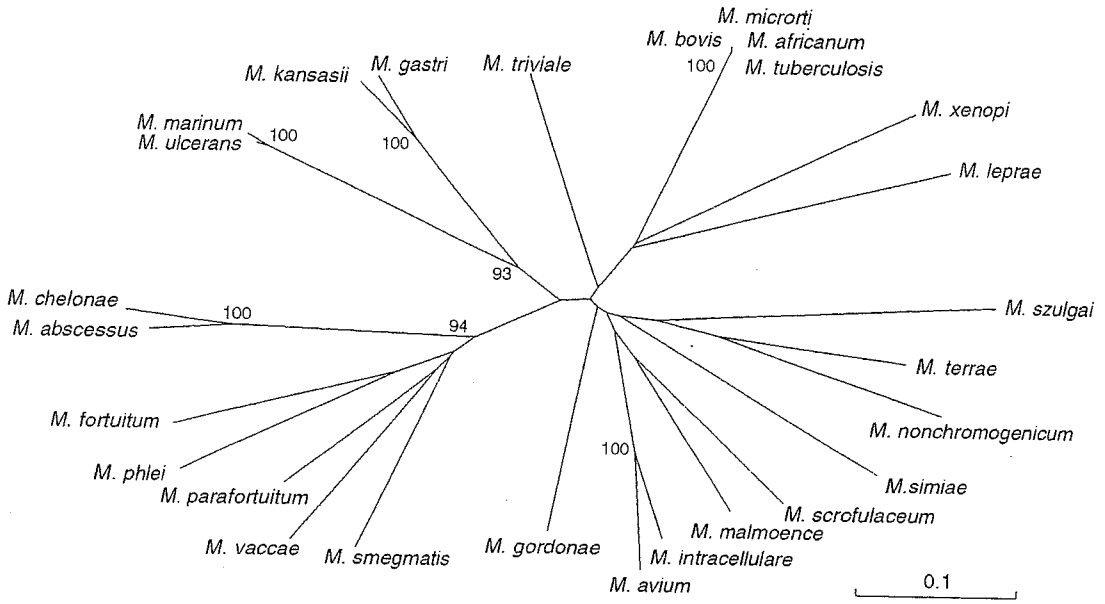


Fig. 2. Phylogenetic relationship of 27 *Mycobacterium* species. Unrooted tree based on the *dnaA* sequences. The tree was generated from DNASpace (Hitachi Software Engineering) with the Clustal W algorithm. The numbers on the dendrogram indicate the percentages of occurrence in 1000 bootstrapped trees; only values of > 90% are shown.

Results

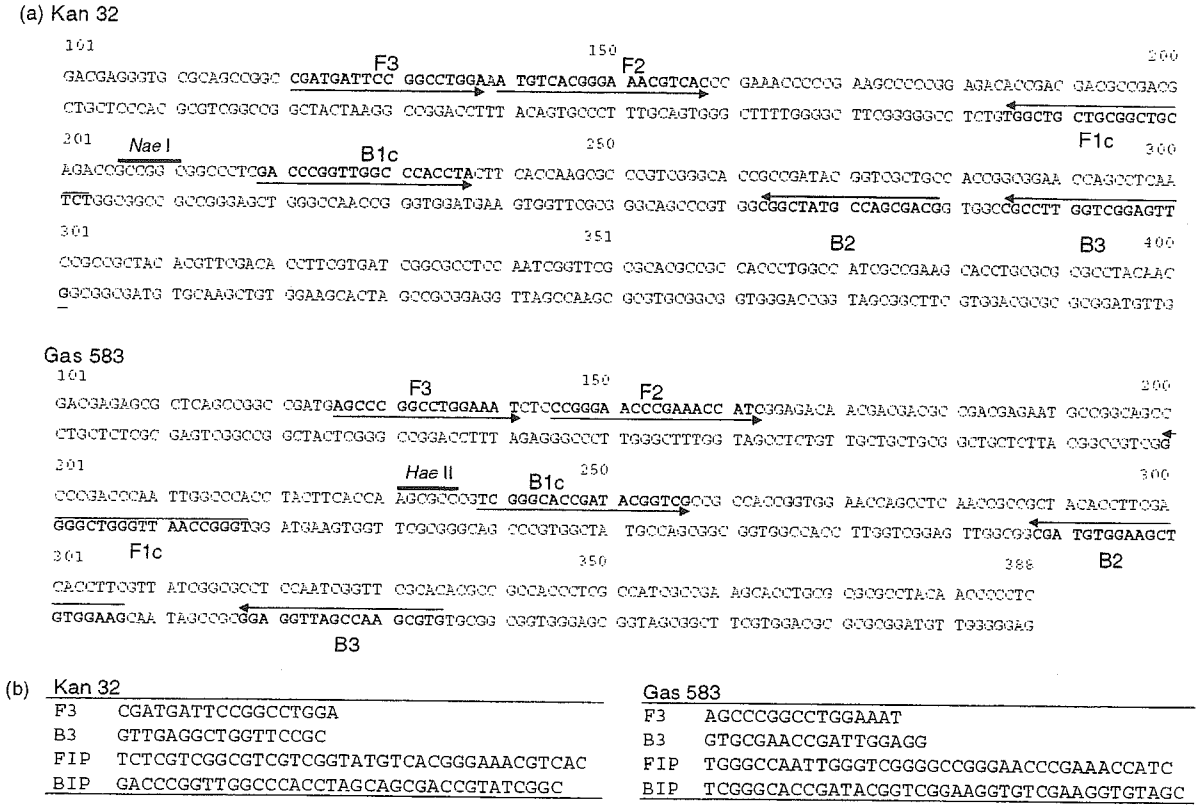
Comparison of partial *dnaA* sequence to identify the *Mycobacterium* species

For the species identification of mycobacterial species, we analyzed some possible variable regions of mycobacterial sequences deposited in the GenBank, and found the 5′ part of the *dnaA* gene as a candidate target for PCR amplification. The PCR products with U1F and U1R, from 27 mycobacterial species, showed the ragged pattern around 400 bp in size (data not shown). Therefore, we determined nucleotide sequences, corresponding to position 228–612 bp of *Mycobacterium tuberculosis*, of all 27 species (Fig. 1a). The alignment of the sequence shows that the region (298–508 bp) in the amplified products had the highest species-specific variability (Fig. 1b). The size of the variable fragment in *dnaA* ranged from 154 bp in *M. triviale* to 232 bp in *M. kansasii*. The variable region exhibits a reasonable number of nucleotide substitution and insertion or deletion sites, which is important for the development of a differential diagnostic tool. The lowest interspecies similarity was 28.2% in *M. leprae* versus *M. vaccae*. The similarity between *M. avium* and *M. intracellulare* was 78.3% and that between *M. marinum* and *M. ulcerans* was 97.7%. Pathogenic *M. kansasii* were easily differentiated from nonpathogenic *M. gastri* (83.6%). The sequences of *M. tuberculosis*, *M. microti*, *M. africanum*, and *M. bovis* were found to be identical, except for one nucleotide substitution that occurred in *M. bovis*. When clinical isolates from clinically relevant mycobacterial strains were analyzed, the following minor variation was found among each species: 97.7–100% (*M. avium*) and 96.0–100% (*M. intracellulare*). We did not find any intraspecies variation in 10 clinical isolates and the standard strain of *M. kansasii*. Because other reports using different systems revealed the existence of more than one sequevar (Yang *et al.*, 1993; Alcaide *et al.*, 1997), we may need to examine a bigger number of clinical isolates.

The unrooted phylogenetic tree showed that the 27 mycobacterial species were resolved by the variable region in the *dnaA* sequence (Fig. 2). All rapidly growing species, *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. parafortuitum*, *M. phlei*, *M. vaccae*, and *M. smegmatis*, made a cluster that was clearly separated from those of the other species so far examined. On the other hand, *M. kansasii*, *M. gastri*, *M. avium*, and *M. intracellulare* are clinically relevant species; however, the branch of the former two species was obviously segregated from one of the later two species, which was supported by high bootstrap values. The results indicated that the partial *dnaA* sequence could be useful for the differentiation of NTM (Fig. 2).

Identification of mycobacteria by *dnaA* sequence-targeted species-specific LAMP assay

Several sets of primers designed from the *dnaA* sequence were evaluated for their specificity and sensitivity by the LAMP method. One set of primers named Kan-32 for *M. kansasii* and Gas-583 for *M. gastri* was selected (Fig. 3), and



(b)

Kan 32	
F3	CGATGATTCCGGCCTGGA
B3	GTTGAGGCTGGTTCCGC
FIP	TCTCGTCGGCGTCGTCGGTATGTCACGGGAAACGTCAC
BIP	GACCCGGTTGGCCCACCTAGCAGCGACCGTATCGGC

Gas 583	
F3	AGCCCGGCCTGGAAAT
B3	GTGCGAACCGATTGGAGG
FIP	TGGGCCAATTGGGTCGGGGCCGGGAACCCGAAACCATC
BIP	TCGGGCACCGATACGGTCGGAAGGTGTCGAAGGTGTAGC

Fig. 3. Location of oligonucleotide primer sets Kan 32 and Gas 583, used for the loop-mediated isothermal amplification method. For *Mycobacterium kansasii* partial *dnaA* gene (GenBank accession number AB087693) and for *Mycobacterium gastri* partial *dnaA* gene (GenBank accession number AB087690). A right arrow indicates the sense sequence which is used as the primer. A left arrow indicates that a complementary sequence is used as the primer. The unique restriction enzyme recognition sites in the amplified product are shown with a bold bar. (b) List of each primer sequence.

by using these primer sets, a successful LAMP product appeared as a ladder of multiple bands (Fig. 3a).

The species specificity and intraspecies stability of each primer set were examined with purified DNA from 27 mycobacterial species and 10 clinical isolates of *M. kansasii*. We subjected each sample to amplification using Kan-32 or Gas-583 primer set. The results obtained by electrophoretic examination are summarized in Table 1. Although 200 pg of nontargeted species DNA were not amplified, significant amplification of targeted respective isolates was observed after a 60 min incubation at 63 °C. To confirm that the amplification products had corresponding DNA structures, the amplified products were digested with restriction enzymes and the size of the fragments was analyzed by electrophoresis. *Nae*I cuts between F1 and B1c for the *M. kansasii* amplicon; *Hae*II was used for the *M. gastri* amplicons. The sizes of the fragments generated after digestion were in good agreement with sizes predicted theoretically from the expected DNA structure: 100 and 93 bp by *Nae*I digestion, and 123 and 98 bp by *Hae*II digestion (Fig. 4a). Thus, we concluded that each primer set was species specific.

We next assessed the sensitivity of the assay. Serially diluted *M. kansasii* or *M. gastri* genomic DNA was used. The results of a typical experiment are shown in Fig. 4b. Amplified DNA was readily visible when 500 copies of genomic DNA were present in a 60 min incubation assay. The detection limit did not change with a longer incubation period (data not shown).

Discussion and conclusions

For the identification of species, a target gene must be conserved among strains and species. As the DnaA protein is generally conserved among microbial organisms (Mizrahi *et al.*, 2000), this coding region could be used for the target analysis. Four functional domains of the DnaA protein have been defined (Messer *et al.*, 1998). Domain 1 is involved in oligomerization and interaction with DnaB, Domain 2 constitutes a flexible loop, Domain 3 has ATPase function, and Domain 4 is sufficient for specific binding to DNA. The variable region that we identified in the *dnaA* sequence was equivalent to the Domain 2 coding nucleotide sequence

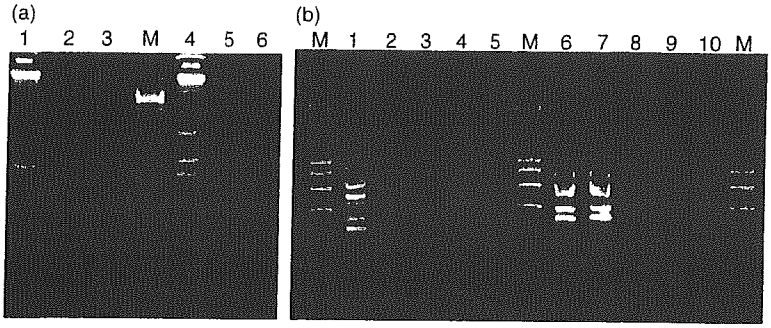


Fig. 4. (a) Four percent agarose gel electrophoresis and restriction enzyme analysis of loop-mediated isothermal amplification (LAMP) products of partial *dnaA* gene of *Mycobacterium kansasii* and *Mycobacterium gastri*. Lanes: M, 100 bp DNA ladder; lanes 1–3, LAMP carried out with *M. kansasii* primer, Kan 32, in the presence of genomic DNA from *M. kansasii* (lanes 1 and 2) and *M. gastri* (lane 3); lane 2, LAMP product from lane 1 after digestion with *Nae* I; lanes 4–6, LAMP carried out with *M. gastri* primer, Gas 583, in the presence of genomic DNA from *M. gastri* (lanes 4 and 5) and *M. kansasii* (lane 6). lane 5, LAMP product from lane 4 after digestion with *Hae* II. (b) Serial dilution of purified *M. kansasii* or *M. gastri* genomic DNA was amplified to determine the sensitivities by LAMP. Lanes: M, 100 bp DNA ladder; lanes 1–5 LAMP carried out with Kan 32 primer set in the presence of genomic DNA of *M. kansasii*, lane 1, 1000 copies; lane 2, 500 copies; lane 3, 100 copies; lane 4, 10 copy; lane 5, distilled water. lanes 6–10 LAMP carried out with gas 583 primer set in the presence of genomic DNA of *M. gastri*, lane 6, 1000 copies; lane 7, 300 copies; lane 8, 100 copies; lanes 9, 10 copy; lane 10, distilled water.

(Fig. 1). This domain is the least conserved region in the *dna A* gene with respect to sequence and length among *M. smegmatis*, *M. tuberculosis*, and *M. leprae* (Fsihi *et al.*, 1996). However, comparative studies of this region using 27 mycobacteria have not been reported and, as far as we know, this is the first report indicating the usefulness of the *dnaA* Domain 2 sequence as a differential diagnostic tool.

An accurate and rapid bacterial identification greatly contributes to this field of medication. Several methods based on molecular biological techniques have been reported. The sequences that have been reported include *hsp65*, 16S rRNA gene, and ITS (Plikaytis *et al.*, 1992; De Smet *et al.*, 1995; Springer *et al.*, 1996; Messer & Weigel, 1997; Roth *et al.*, 1998; Brunello *et al.*, 2001). Each gene has several advantages and disadvantages. An excessive degree of variability is found in the *hsp65* gene (Telenti *et al.*, 1993), which may hinder the development of reliable probes. While 16s rRNA gene sequence is identical in *M. kansasii* and *M. gastri* and shows narrow divergenicity within species (Taylor *et al.*, 1997), ITS sequence can be used to distinguish between *M. kansasii* and *M. gastri* (Roth *et al.*, 1998). While *M. kansasii* is a representative pathogenic mycobacteria, *M. gastri* does not induce an apparent disease. The discrimination between these mycobacteria provides useful information to select the appropriate therapy. The percent similarity of ITS between two species was 93% (Roth *et al.*, 1998), and that of the *dnaA* variable region was found to be 83.6%. These observations may indicate the usefulness of the *dnaA* gene for discrimination of these species, at least in complement with ITS.

The recent trend in genetic testing is to make systems fully automatic with high-throughput analysis. Although this may be an ideal approach, it requires expensive equipment as well as a well-trained person in diagnostic laboratories. The LAMP method could be conducted under isothermal conditions ranging from 60 to 65 °C by a single enzyme. The only equipment needed for LAMP reaction is a regular laboratory water bath or a heat block that furnishes a constant temperature around 63 °C. LAMP does not require a thermal cycling step, and an isothermal reaction for a short time (60 min) is enough to amplify the target DNA to a detectable level. As PCR and other molecular biological techniques are conducted in well-equipped laboratories, these methodologies are often impracticable under a field diagnosis.

In this paper, we demonstrated that the *dnaA* region could be an effective new nucleotide region for the diagnosis of NTM infection and that the LAMP method could be applied for a *dnaA* gene-based differential diagnostic tool.

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臨床免疫，43(4)：392-399，2005

特集Ⅰ 微生物の免疫回避機序

らい菌による免疫抑制の機序*

福富康夫**

Key Words：*M. leprae*, Hansen's disease, immunological spectrum, cytokine, macrophage

はじめに

ハンセン病は抗酸菌症のひとつであり，らい菌によってひき起こされる慢性の肉芽腫性炎症である．らい菌はシュワン細胞やマクロファージ内で増殖する細胞内寄生菌であり，主として末梢神経と皮膚を侵し，病勢が進行した場合，眼，上気道，精巣なども侵される．らい反応(後述)や神経炎が起こってしまった場合，身体障害を生み，それが差別や偏見の一因となった．すなわち，末梢神経障害により発汗作用が傷害されるために皮膚は乾燥し傷を受けやすくなり，血管などの神経反射作用が失われると防御作用や炎症の治癒機転の障害につながる．さらに運動機能障害によって，たとえば，顔面神経麻痺から顔瞼と周囲の筋力が低下して兎眼を起こす．四肢の麻痺からは手の鷲手変形と足の垂足という特徴的な変形が起こり，これに，知覚障害による2次的損傷が加わり，皮膚または関節の拘縮，骨吸収や破壊なども起こって複雑な変形となる(図1)．世界的にみると，登録患者数は1980年代半ばには500万人以上であった．いくつかの抗らい菌剤を処方する多剤併用療法(MDT)が功を奏して患者数はその後著しく減少したが，途上国を中心にしていまだに年間50万人以上の新患発生をみる(2003年WHO統計)．本稿のテーマは「らい菌による免疫抑制の機序」である．この免疫抑制とは細胞内寄生菌であるらい菌がマクロファージ内で増殖する宿主の免疫状態であり，

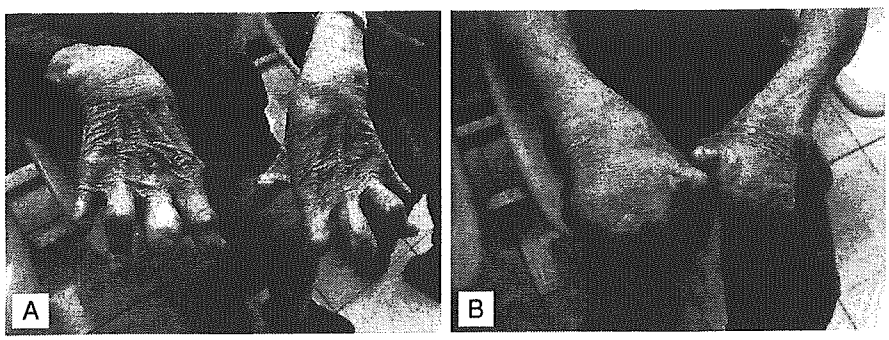

図1 ハンセン病における手の変形
神経障害によって生じた鷲手変形(A)と骨吸収などが起こりさらに進行した症例(B)．

* Mechanisms of immunosuppression by *M. leprae* in Hansen's disease.

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マクロファージの各種殺菌機構からエスケープして，かつ細胞性免疫(Th1型反応)が発動されずマクロファージの抗菌活性が増強されない状態としてとらえることができる．しかし，ハンセン病にはTh1型反応もあり，それが原因で著明な神経障害を生じるなど宿主側の多彩な免疫反応を特徴とする疾患である．本特集の趣旨から逸脱するかもしれないが，ハンセン病の免疫全般を概説することにより，そのなかで，らい菌側と宿主側それぞれに起因すると思われる免疫抑制現象についてご理解頂ければ幸いである．

ハンセン病の病形と免疫反応

ハンセン病は，免疫学的にみて，きわめて特異な臨床症状を呈する疾患である．病理組織学的特徴からLL型(らい腫型)lepromatous type, TT型(類結核型)tuberculoid type, BB(境界群)borderline group, その中間型であるBL型，BT型，およびI群(未定型群)indeterminate groupに分類される[1](表1)．LL型ではマクロファージの殺菌作用が進まず，マクロファージ(らい細胞)内で増殖したらい菌がグロビー(globi)を形成し紡錘形細胞になるか陳旧化してらい菌脂質が貯留した泡沫細胞となる．その結果，大量に存在する菌抗原に対し液性免疫反応が成立し抗体が産生される．らい菌など抗酸菌の細胞壁の構造は複雑でアラビノガラクタンやミコール酸などが存在しており，らい菌に特異的な糖脂質成分として，抗原性の非常に強い3糖を含有する脂質phenolic glycolipid-I (PGL-I)が存在し，未治療LL型患者血清中には高値の抗PGL-I抗体価が認められる[2]．Lipoarabinomannan (LAM)もまた抗原性が強く抗体が作られる(図2)．熱ショック蛋白質やファイブロネクチン結合性85複合体蛋白質，MBP51, MMP-IIなどの各種らい菌蛋白質に対する抗体も存在する．一方，細胞性免疫が成立したTT型ではマクロファージの活性化に伴って殺菌反応が起こるため組織内の菌は少ないかまったくみられず，液性免疫応答は一般に低くマクロファージは類上皮細胞となり巨細胞を形成する(図3)．境界群はTh1反応とTh2反応が混在しており免疫学的に不安定な状態で境界群反応を生じやすい(後述)．現在ではほとんど用いられることがなくなったが，ハンセン病の病型分類，患者の予後判定に用いられる皮内反応をレプロミン反応と呼ぶ．結核のツベルクリン反応にヒントを得て開発されたものでありらい菌の菌体を用いるが，らい菌は人工培養できないのでらい腫から得る．本反応は光田により提唱され，その後，林，Wadeらにより改良が加えられた[3]．レプロミン反応の名称については注射後24ないし48時間後に最大となる遅延型過敏症反応を早期反応(Fernandez反応)と呼び，4週後の硬結と潰瘍を伴う晩期反応を光田反応と呼んで区別し，この両反応を総称してレプロミン反応と呼んでいる．TT型患者では強陽性でLL型に移行するに従って漸次陰性となる．LL型における陰性反応がらい菌に対する特異的な細胞性免疫低下の状態，つまりらい菌に対する特異的な免疫抑制として長年理解されてきたのである．ちなみに，

表1　ハンセン病の病型分類

	LL	BL	BB	BT	TT
皮疹	びまん性	びまん性	びまん性	多数限局性	単一限局性
神経病巣	全身性	全身性	多数	限局性	限局性
類上皮細胞	−	±〜−	++	++	++
組織球/泡沫細胞	++	++	−	−	−
リンパ球	±	+	++	+++	++++
らい菌	++++	+++	++	+	±
抗体産生	++++	+++	++	+	+〜−
レプロミン反応	−	−	−	+	+
リンパ球芽球化試験	0.4/0.6	0.9	2.8	6	15
免疫学的安定性	++/+	±	−	±	++
リバーサル反応(1型)	−〜±	+	++	+	−
ENL(2型)	++	±	−	−	−

(文献[1]より引用)

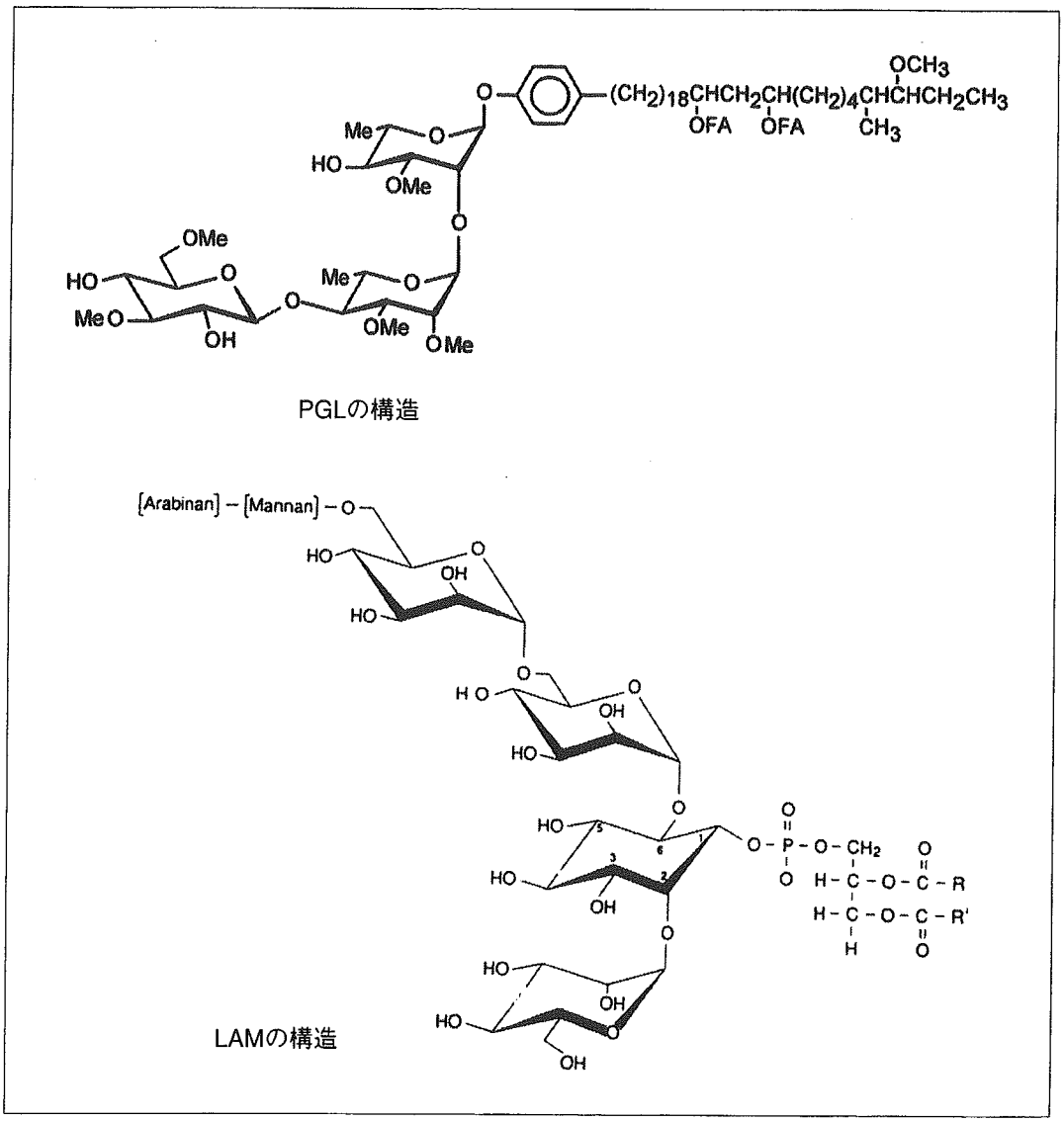

図2　フェノリックグリコリピドPGL-Iとリポアラビノマンナン LAMの構造
PGL-Iの構造中のFAは脂肪酸.　（文献[24]より引用）

日本におけるLL型患者のPPD反応は陽性である.

ハンセン病の免疫組織的所見は以下のとおりである(図4)[4]. TT型ではCD8⁺細胞とB細胞が病巣周囲を取り囲み，そのなかに類上皮細胞とCD4⁺細胞が存在するが，LL型ではCD4⁺とCD8⁺が混在してびまん性に広がっている．そして，CD4⁺/CD8⁺比はTT型で大きく，LL型では小さい．病巣でのサイトカイン発現についてはTT型ではIL-2とIFNγが強く発現し，また，肉芽腫形成に関係するTNFもみられる．LL型ではB細胞の増殖・分化に関係するIL-4, IL-5, IL-10が強く発現されることが見出されている．IL-10はTNF, GMCSF, IFNγの産生を抑制する．TT型，LL型においてそれぞれTh1型，Th2型サイトカインが優位である．また，マクロファージが産生の主体となるIL-1β, TNF-α, GM-CSF, TGF-β1, IL-6といったサイトカインはTT型に多く発現している．IL-12はT細胞やNK細胞を刺激してIFNγを誘導するTh1型サイトカインとして知られており，TT型では強く発現している．IL-12はTh2型サイトカインであるIL-10産生を抑制することが判明している．LL型病巣では，*M. leprae*刺激によりマクロファージからIL-10が産生されているが，TT型病巣ではTh1型T細胞よりIFNγが供給されるためにIL-12産生が誘導され，その結果マクロファージのIL-10産生抑制状態にあるとも考えられる[5]. 興味深い現象としてHIV感染を伴ったハンセン病患者の末梢血中のCD4⁺T細胞は減少するが，LL

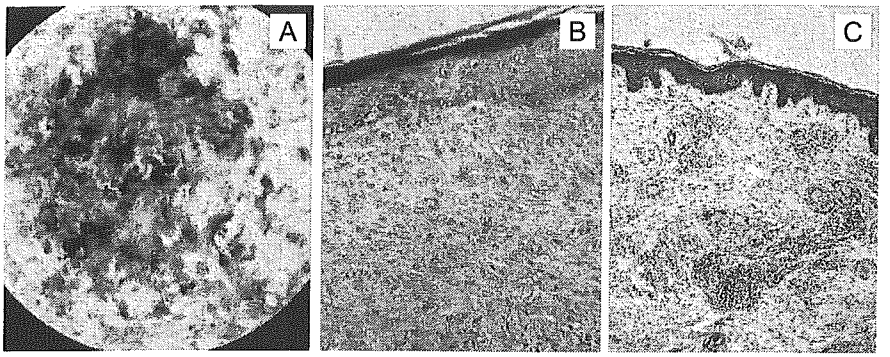

図3　ハンセン病の皮膚塗抹標本と病理組織像
皮疹部位から得られた塗抹標本を抗酸菌染色して顕微鏡(1,000倍)で観察した像(A)．多数のらい菌がみられる．HE染色した皮膚生検の病理組織像(B, C)．泡沫細胞のみられるBL型(B：400倍)とランゲルハンス巨細胞のみられるBT型(C：200倍)症例．

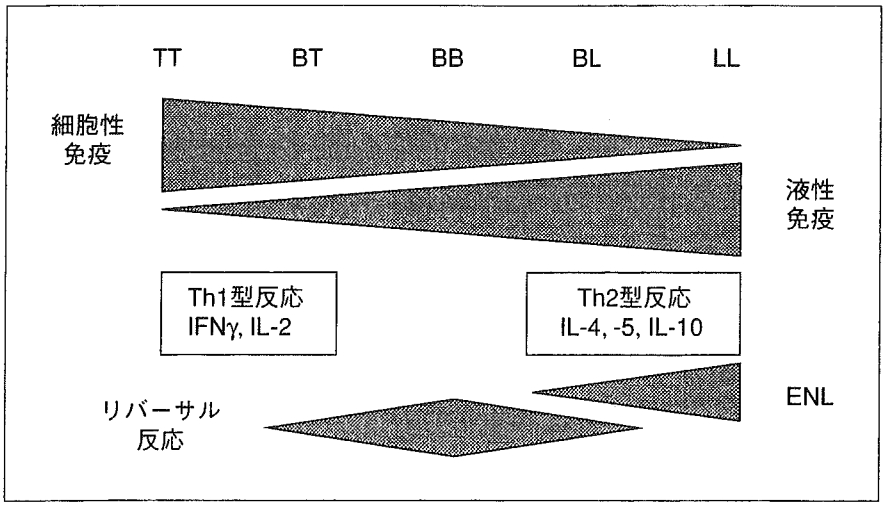

図4　ハンセン病の免疫病理

型，TT型とも組織学的，臨床的には病型の変化は認められず，肉芽腫形成などらい菌に対する反応性には影響がないことが示されている[6]．

らい反応

ハンセン病においてはらい反応と呼ばれる特徴的な炎症反応が観察される．病型により異なる次の2種がみられる．①Ⅰ型らい反応，境界群反応あるいはリバーサル反応と呼ばれるもので，組織学的にはリンパ球の急性浸潤，巨細胞の出現などが起こるので，Ⅳ型の遅延型アレルギー反応に相当する．境界群を中心としてみられる現象である．②Ⅱ型らい反応あるいはらい性結節性紅斑erythema nodosum leprosum(ENL)と呼ばれるもので，LL型において治療が進んで菌が破壊されると，血流中に遊出した菌体抗原と抗体との免疫複合体が形成されてArthus型反応が起こり，高熱と結節性紅斑類似の皮疹が現れる．組織学的には病巣に多型核白血球の浸潤がみられる．らい反応を起こしている患者血清中にはIL-1やTNFといった炎症性サイトカインがみられるがENL発症時においてはとくにTNF値が高く，BL型ではIL-1が多い[7]．Ⅰ型らい反応ではTh1型サイトカインの発現が強くみられ，Ⅱ型らい反応ではTh2型サイトカインが優位と報告されているが，両型のサイトカインが混在してみられる場合がありいわゆるTh0型の反応もある．皮膚組織中のγδT細胞の占める割合は通常5％程度と少なく，ハンセン病病巣においても，LL型，または慢性状態で病態が安定している場合には同様に少ないが，リバーサル反応時やレプロミン注射をした皮内局所では25～35％に増加する．IFNγ

を産生するなどして感染初期防御に寄与しているものと考えられているほか，肉芽組織形成を促進する働きもしている．

抗原提示

一般的に，T細胞は抗原提示細胞上のMHCクラスⅠ分子やクラスⅡ分子を介して外来ペプチド抗原を認識する．しかし，結核やハンセン病では糖脂質や脂質が抗原としてCD1分子を通じてT細胞に提示されうることが報告されている[8]．CD1分子は抗原提示細胞や樹状細胞上に存在しCD1a, -b, -c, -dの4種が知られている．ハンセン病患者の末梢血中にはαβ型T細胞のうち$CD4^-$ $CD8^-$のダブルネガティブ細胞（αβDN細胞）は1％に満たないが，皮膚病巣では8％にも増加している．LAMは抗原提示細胞のCD14やマンノースレセプターを介してearly endosomeに取り込まれ，次にlate endosome中でCD1分子と会合し細胞表面に現れる．αβDN細胞はLAMにより刺激されるとIFNγを産生しTh1型反応に深くかかわる細胞であると思われる．病変を観察するとTT型を代表とする小菌型ハンセン病患者皮膚病変には類上皮肉芽腫性病変を構成する細胞集団の中にCD1a陽性の樹状細胞が存在するが，LL型のような多菌型患者の病変中には存在しない．樹状細胞は抗原提示やTh1型を誘導するIL-12を産生するなど細胞性免疫反応の活性化に重要な働きを果たしていることが示唆される[9]．

ハンセン病の病型と遺伝学的解析

抗原提示に関しては近年，らい菌ペプチド抗原のHLA分子に対する親和性が調べられており，ハンセン病の罹りやすさや患者の病型を決定する素因のひとつとして注目されている．また，日本を含むアジア，メキシコではハンセン病患者にHLA-DR2が多く，とくに日本ではTT型においてHLA-MT1が有意に高い．インドでの調査では，家族内TT型罹患とDR2に相関がみられる．一方，ベネズエラ，スリナムではDR3との相関が強く，大陸間での差が認められている．そして，HLA-DR3がTT型に多くHLA-DQ1がLL型に多いという統計結果から，前者が細胞性免疫反応誘導に関与し，後者がその反応を抑制する細胞を誘導する可能性が示唆されている．さらにHLADR2とDR3のアリルがTT型発症と関係しHLADQ1はLL型発症につながる[10]．NRAMP1遺伝子についてはその多型性がアフリカにおける多菌型発症に関係している．この遺伝子はらい菌に対する細胞性免疫とも関係しており，ヴェトナムの患者における光田反応とNRAMP1との関連性が指摘されている[11]．サイトカインとの関連では，TNFとIL-10遺伝子のプロモーター領域の多型性がハンセン病発症と関係し，とくに，TNFのプロモーターの多型性は多菌型発症と関係するといわれる．また，IL-12レセプター（IL-12R）はβ_1鎖とβ_2鎖からなるヘテロダイマーであり，β_2鎖のチロシン残基がSTAT4分子の活性化に関与することからβ_2鎖の発現量の違いがTh1/Th2細胞の分化誘導において重要な役割を担うことが示唆され，実際に*IL-12RB2*の発現量の違いがハンセン病患者の病型成立に関与することが報告されており，LL型患者の病巣やらい菌刺激時の末梢血単核球の*IL-12RB2*遺伝子の発現は少ないことが示されている．さらに，LL型患者の*IL-12RB2*制御領域にSNPsが有意に高いことがわかっていてIL-12に対するT細胞応答性の個体差をIL-12RB2の制御領域の多型性によって説明でき興味深い[12]．TLR2の突然変異がLL型発症につながることも認められている[13]．

らい菌のマクロファージ内生存・増殖とらい菌に対するマクロファージの反応・殺菌作用

細胞内寄生菌であるらい菌はマクロファージやシュワン細胞に侵入しそのなかで増殖する．らい菌は貪食作用により受動的にマクロファージに取り込まれるが，シュワン細胞への侵入はファイブロネクチンレセプターを介しているといわれる．また，らい菌の神経細胞への侵入に細胞接着性蛋白質の一種であるラミニンが関与することが明らかとなっている[14]．通常，マクロファージの貪食による取込みではファゴゾームが形成されそのなかに菌が存在するが，らい菌感受性動物であるアルマジロでは心筋細胞など非貪食系細胞の細胞質中にファゴゾーム形成をみずにらい菌が見出される．マクロファージの

らい菌貪食にはいくつかの因子がかかわっており，ヒトマクロファージにおいては補体レセプターやIgGFcレセプターを介するオプソニンが貪食効率を高める[15]．その過程でC3とPGL-Ⅰの相互作用がみられる．マクロファージ上のマンノースレセプターに菌が結合して貪食を高めることもわかっている．

らい菌を貪食したマクロファージからはTNFやIL-1，さらにはIL-10が産生される．しかし，BCGなどほかの抗酸菌と比較するとTNFの産生量は低く，その原因はらい菌に含まれる脂質成分による抑制作用と考えられており，ダルメンドラ抗原と呼ばれる脱脂らい菌菌体ではサイトカインが強く誘導され，T細胞も刺激を受けやすくなる．抗酸菌のLAMには菌株によりマクロファージのTNFを誘導する構造のもの(アラビナンキャップ型)と誘導しない構造(マンナンキャップ型)のものがあるが，らい菌のLAMはマンナンタイプといわれており，面白いことにらい菌由来LAMで前処理したマクロファージの活性化は抑制される．しかしながら，らい菌由来のLAMについてはTNF産生を誘導するという報告と誘導しないという報告があり，その相反する結果がLAMの精製方法の違いによるのかなどさらなる解析が必要であろう．いずれにしろ，LAMによる宿主免疫系へのなんらかの影響が考えられる．ちなみにマクロファージはHLA-DR(Ia)を通じた抗原提示能を有しており，らい菌や抗酸菌に感染したマクロファージのIa発現低下がみられて抗原提示機能の低下が示唆され[16]，LAMはIaの発現を低下させる．LAMなど抗酸菌菌体成分による宿主マクロファージのサイトカイン誘導については最近，TLRを介した刺激伝達機構があることがわかってきた[17]．

マクロファージはIL-10やTGFβなど抑制性のサイトカインも産生するが，らい菌に反応して免疫反応抑制因子を産生する．また，ヒトモノサイトがらい菌を貪食すると，BCGと比較してIL-1，IL-6，TNFの産生が低い一方で，抑制因子のひとつであるIL-1Rアンタゴニストを多く産生する．これら抑制因子がLL型病変部における殺菌能低下に関与しているものと考えられる[18]．IL-10はTh1型サイトカイン産生を抑制する作用をもっているほか，マクロファージの殺菌能を抑制することが知られており，ハンセン病においてもらい菌の増殖するLL型病巣部ではIL-10の発現がみられ増菌の一因となる．以前よりPGE$_2$はT細胞やマクロファージに対して免疫抑制的に作用するプロスタノイドとして知られていた．ハンセン病においてもPGE$_2$を誘導するシクロオキシゲナーゼ-2がLL型病巣で多く発現している．われわれはマウスマクロファージにおいてPGE$_2$がマクロファージからのIL-10産生に必要であることを見出した．また，*in vitro*でらい菌感染マクロファージを持続的にIL-10を添加した中で培養すると，らい菌の代謝活性が長期温存され菌体の伸長現象が観察される(図5)[19]．

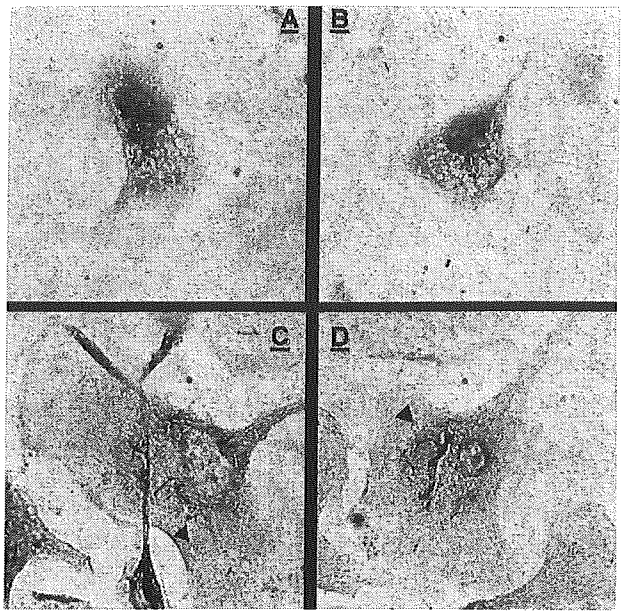

図5　マクロファージ内らい菌の伸長現象
マウスマクロファージにヌードマウス由来らい菌を感染させてIL-10存在下で1か月間*in vitro*培養を行った．培養開始時(A)，IL-10非存在下1か月培養後(B)，IL-10存在下1か月培養後(C, D)．CとDではらい菌菌体が伸長している(矢印)．　(文献[19]より引用)

らい菌は培養できないためマクロファージのらい菌に対する殺菌作用の解析は困難であったが，現在，らい菌はヌードマウス足しょやアルマジロに接種して*in vitro*増殖したものが得られ，また，菌のATPやPGL量を測定したり，放射性同位元素標識パルミチン酸の代謝量を測定する方法でらい菌の生存率をより正確に定量する方法が開発されている．この方法を用いてマクロファージの抗らい菌活性が調べられ，細胞性免疫の主

役を担っているIFNγがTNFと共同してマウスマクロファージを活性化し抗らい菌活性を発現することが証明されている[20]．ハンセン病におけるマクロファージ機能低下については，LL型マクロファージのIL-1産生低下や，抗原提示機能低下が見出されている．しかし，末梢血中のモノサイトの機能は正常であると報告されており，病態とマクロファージの関係を論じるためには病巣における機能変化に着目すべきである．らい菌の宿主細胞による殺菌作用からのエスケープにはらい菌表面にある成分によるファゴゾームとリソゾームの融合阻害が関与している[21]．Sibleyらはらい菌そのものが大量に感染しているとマクロファージのトキソプラズマを殺す活性が抑制され，LL型におけるらい菌を大量に含んだマクロファージの機能欠損を示した．さらに，スーパーオキサイドディスムターゼやパーオキシダーゼがアルマジロ由来のらい菌から精製され，らい菌中に宿主細胞の殺菌作用に抵抗するスカベンジャーの存在が示されている．また，PGLはマクロファージの酸化反応をも抑制し，抗菌活性発現を抑制することが判明している．マクロファージが産生するH_2O_2は結核菌と同様，らい菌に対して殺傷活性があり，ヒトのモノサイト，マウスのマクロファージともにBCGには反応してO_2^-を産生するが，らい菌に対してはO_2^-放出抑制がみられる．放出しない原因はPGLによるO_2^-産生抑制であることが示されている[22]．殺菌分子については近年，窒素酸化物の生理活性が重要視され，マクロファージの腫瘍細胞を殺傷する機構を解析する過程で，窒素酸化物の関与が証明された．同様に細胞内寄生体に対する障害際作用がいくつも報告されており，らい菌に対する殺菌活性においても窒素酸化物の関与がみられる[20]．これらはいずれもマウスの系であり，ヒトでは窒素酸化物による殺菌作用は*in vitro*では明確には証明されていないが，ハンセン病において免疫組織化学染色によりiNOS蛋白の存在が証明されている[23]．TT型やリバーサル反応時に病巣において酸化窒素合成酵素の発現が起こっており，今後，ヒトでの*M. lepra*eに対する宿主細胞の殺菌作用，ならびに*M. lepra*eが殺菌作用を逃れて増殖する機構についてより解明が進むであろう．

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