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

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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 intracel-*

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.

lulare 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
<i>Mycobacterium abscessus</i>	JATA 63-01 (ATCC 19977)	AB087684	–	–
<i>Mycobacterium africanum</i>	KK 13-02 (ATCC 25420)	AB087685	–	–
<i>Mycobacterium avium</i>	JATA 51-01 (ATCC 25291)	AB087686	–	–
	Clinical isolate 22 strains			
<i>Mycobacterium bovis</i>	JATA 12-01 (ATCC 19210)	AB087687	–	–
<i>Mycobacterium chelonae</i>	JATA 62-01 (ATCC 35752)	AB087688	–	–
<i>Mycobacterium fortuitum</i>	JATA 61-01 (ATCC 6841)	AB087689	–	–
<i>Mycobacterium gastri</i>	KK 44-02 (ATCC 15754)	AB087690	–	+
<i>Mycobacterium goodii</i>	JATA 33-01 (ATCC 14470)	AB087691	–	–
<i>Mycobacterium intracellulare</i>	JATA 52-01 (ATCC 13950)	AB087692	–	–
	Clinical isolate 17 strains			
<i>Mycobacterium kansasii</i>	KK 21-01 (ATCC 12478)	AB087693	+	–
	Clinical isolate 10 strains		+	–
<i>Mycobacterium leprae</i>	Thai-53	AB087694	–	–
<i>Mycobacterium mageritense</i>	JATA 47-01 (ATCC 29571)	AB087695	–	–
<i>Mycobacterium marinum</i>	JATA 22-01 (ATCC 927)	AB087696	–	–
<i>Mycobacterium microti</i>	KK 14-01 (ATCC 19422)	AB087697	–	–
<i>Mycobacterium nonchromogenicum</i>	JATA 45-01 (ATCC 19530)	AB087698	–	–
<i>Mycobacterium parafortuitum</i>	ATCC 25807	AB087699	–	–
<i>Mycobacterium phlei</i>	ATCC 19249	AB087700	–	–
<i>Mycobacterium scrofulaceum</i>	JATA 31-01 (ATCC 19981)	AB087701	–	–
<i>Mycobacterium simiae</i>	KK 23-08 (ATCC 25275)	AB087702	–	–
<i>Mycobacterium smegmatis</i>	JATA 64-01	AB087703	–	–
<i>Mycobacterium szulgai</i>	JATA 32-01	AB087704	–	–
<i>Mycobacterium terrae</i>	KK 46-01 (ATCC 15755)	AB087705	–	–
<i>Mycobacterium triviale</i>	KK 50-02 (ATCC 23292)	AB087706	–	–
<i>Mycobacterium tuberculosis</i>	JATA 11-01 (H37Rv)	AB087707	–	–
<i>Mycobacterium ulcerans</i>	KK 43-01	AB087708	–	–
<i>Mycobacterium vaccae</i>	KK 66-01	AB087709	–	–
<i>Mycobacterium xenopi</i>	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 MgCl₂ (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

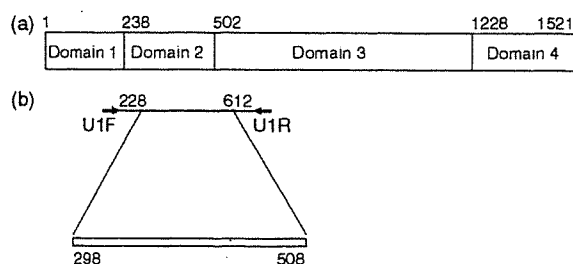


Fig. 1. Schematic representation of the DnaA protein and primer design for the amplification of the partial mycobacterial *dnaA* gene. Number indicates the nucleotide position of *Mycobacterium tuberculosis*, GenBank accession number AL021427. (a) The DnaA protein from *M. tuberculosis* contains four 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).

performed in accordance with the instruction of the manufacturer. Sequencing products were purified with a Centriseq 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).

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 (Fujitsu, 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 MgSO₄, 10 mM (NH₄)₂SO₄, 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.

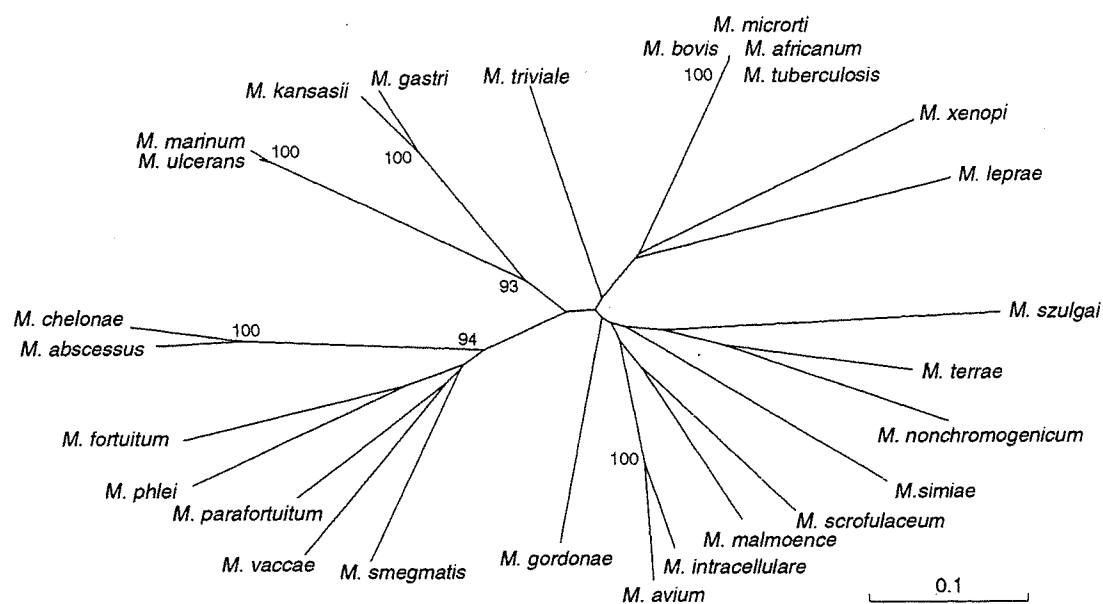


Fig. 2. Phylogenetic relationship of 27 *Mycobacterium* species. Unrooted tree based on the *dnaA* sequences. The tree was generated from DNASpace (Hitachi Software Engineering) with the Clustal W algorithm. The numbers on the dendrogram indicate the percentages of occurrence in 1000 bootstrapped trees;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

(a) Kan 32

101 150 200
 GACGAGGGTG CGCAGCCG93C **CGATGATTC** **GGCCTGGAAA** **TGTCACGGGA** **AACGTCAACC** GAAACCCCGG AAGCCCCCGG AGACACCGAC GACGCCGACG
 CTGCTCCAC GCGTCGGCGG GCTACTAAGG CGGACCTTT ACAGTCCCT TTGCAGTGGG CTTTGGGGG TCGGGGGG TCTGTGGCTG **CTGGGCTGC**
 201 **Nae I** B1c 250 F1c 300
 AGACCGCGG CGGCCCTGA **CCCGTTGGC** **CCACCTACTT** CACCAAGCGC CGTCCGGCA CGCCGATAC GGTGCTGCGC ACCGGCGAA CCAGCCTCA
 TCTGGCGGC GCGCGAGCT GGGCCACCG GGTGATGAA GTGTTCCGG GGCAGCCCGT GCGGGTATG **CCAGCGACGG** TGCCGCCCTT **GGTCGGATT**
 301 351 B2 B3 400
 CGCCGCTAC ACCTTGACA CCTTCGTGAT CGCGCCTCC AATCGTTCG CGCACCGCGC CACCTGGCC ATCGCCGAAG CACTGCGCG CCCTACAC
 GCGCGGATG TGCAAGCTGT GGAAGCACTA GCGCGGAGG TTAGCCACGC GGTGCGCGG GTGGACCGG TAGCGCTTC GTGACCGGC GCGGATGTTG

Gas 583

101 150 200
 GACGAGGGTG CTACGCCG93C **CGATGAGCC** **GGCCTGGAAA** **TCTCCCGGA** **ACCCGAAACC** **ATCGGAGACA** ACGAAGACGC CGACGAGAT GCGCGCAGCC
 CTGCTCTGC GAGTCGGCGG GCTACTCGGG CGGACCTTT AGAGGCCCT TGGGCTTGG TAGCCCTCTT TCTGCTGCG GCTGCTCTTA CGCCCTGG
 201 Hae II B1c 250 300
 CCGACCCAA TTGGCCCLCC TACTTACCA **AGCGCCGTC** **GGGCACCGAT** **ACGGTCGCG** CCGCCGCTGG AACCGCCTC AACCGCGCT ACACCTTGA
 GGGCTGGCTT **AACCGGCTG** ATGAAGTGT TCGCGGCGG CCGTGGCTA TGCCAGCGC GGTGGCCAC TTGCTGGAG TTGGCGCGA **TGTGGAAGCT**
 301 F1c 350 388 B2
 CACTTGGTT ATCGCGCCT CCAATG35TT CGCACCGCC GGCACCTCG CCAATCGCGA AGCACCTGCG CGCGCTACA ACCCGCTC
 GTGAAGCAA TAGCCCGGA **GTTAGCCAA** **CGGTGTGCG** CGTGGGAGC GGTAGCGCT TGTGCGCGC GCGCGATGT TGGGGG

B3

(b) Kan 32

F3 **CGATGATTCGCGCCTGGA**
 B3 **GTTGAGGCTGGTTCCGC**
 FIP **TCTCGTCGGCGTCGTCGGTATGTCA CGGAAACGTCAC**
 BIP **GACCCGGTTGGCCACCTAGCAGCGACCGTATCGGC**

Gas 583

F3 **AGCCCGCCTGGAAAT**
 B3 **GTGCGAACCGATTGGAGG**
 FIP **TGGCCCAATTGGGTGCGGGCCGGGAACCCGAAACCATC**
 BIP **TCCGGCACCGATACGGTCGGAAGGTGTCGAAGGTGTAGC**

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. *NaeI* cuts between F1 and B1c for the *M. kansasii* amplicon; *HaeII* 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 *NaeI* digestion, and 123 and 98 bp by *HaeII* 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

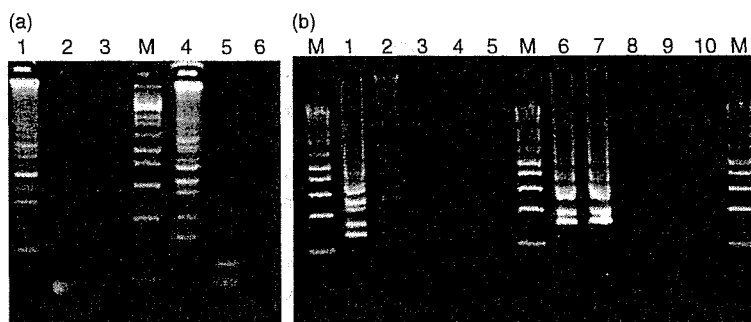


Fig. 4. (a) Four percent agarose gel electrophoresis and restriction enzyme analysis of loop-mediated isothermal amplification (LAMP) products of partial *dnaA* gene of *Mycobacterium kansasii* and *Mycobacterium gastri*. Lanes: M, 100 bp DNA ladder; lanes 1–3, LAMP carried out with *M. kansasii* primer, 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 *dnaA* 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 divergency 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.

Acknowledgements

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Impaired maturation and function of dendritic cells by mycobacteria through IL-1 β

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Dendritic cells (DC) are pivotal for initiation and regulation of innate and adaptive immune responses evoked by vaccination and natural infection. After infection, mycobacterial pathogens first encounter monocytes, which produce pro-inflammatory cytokines, including IL-1 β , TNF- α and IL-6. The role of these cytokines in DC maturation remains incompletely understood. Here, we show that maturation of DC from monocytes was impaired by pretreatment of monocytes with low doses of IL-1 β . Under these conditions, *Mycobacterium leprae*-infected DC failed to stimulate antigen-specific T cell responses. Expression of CD86 and CD83 and production of IL-12 in response to lipopolysaccharide and peptidoglycan were diminished. In contrast, these DC functions were not impaired by pretreatment with TNF- α , IL-6 or IL-10. When monocytes were infected with *M. bovis* Bacillus Calmette-Guérin, and subsequently differentiated to DC, the activity of these DC was suppressed as well. Thus, IL-1 β acts at early stages of differentiation of DC and impairs biological functions of DC at later stages. Therefore, production of IL-1 β by mycobacteria-infected antigen-presenting cells counteracts effective stimulation of innate and adaptive immune responses.

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Introduction

Infections by mycobacterial pathogens still cause major health problems globally. *Mycobacterium tuberculosis* kills more adults than any other infectious agent and one third of the world's population is considered infected with this pathogen [1, 2]. *M. leprae* affects skin and peripheral nerves, causing massive body deformation [3, 4]. These pathogenic mycobacteria persist *in vivo*

over long periods without being eradicated by the host immune system [5]. The current vaccine against tuberculosis, BCG has only limited protective effects, and no reliable vaccine has been developed against leprosy [6, 7].

Host defense against mycobacteria in human is primarily conducted by type 1 adaptive immune responses, and DC play a major role as APC [8, 9]. To elicit T cell immunity, activation of APC is critical and strongly influenced by pro-inflammatory cytokines [10–12]. Type 1 CD4⁺ T cells are stimulated by cognate interactions with APC and IL-12 costimulation [13]. IL-12 is preferentially produced by activated DC and its production is associated with the activation of NF- κ B [14, 15]. After appropriate stimulation, blood monocytes can mature to DC and 25% of the circulating inflammatory monocytes are estimated to differentiate to DC and 75% to tissue macrophages [16, 17]. Resting

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Abbreviations: MLC: *M. leprae*-derived cytosolic fraction · MMP: major membrane protein · PGN: peptidoglycan

macrophages serve as habitat for mycobacteria [18]. After infection with mycobacteria, mononuclear phagocytes rapidly produce cytokines including IL-1 β as first-line mediators of defense [5, 19].

IL-1 β is a 17-kDa prototypic pro-inflammatory cytokine. IL-1 β is multifunctional and acts on a wide variety of target cells [20]. It induces numerous genes regulated by IL-1 β -inducible transcription factors such as NF- κ B [21, 22]. Therefore, IL-1 β not only participates in the innate immune response, but also influences T cell activity. However, the effect of IL-1 β on monocytes undergoing differentiation into DC remains unknown especially with regard to the adaptive T cell response against mycobacteria.

Here, we show that IL-1 β in picomolar quantities markedly influences monocyte maturation to DC with profound consequences for subsequent T cell responses. Our findings that mycobacteria impair DC differentiation and function via IL-1 β can explain at least partially the persistence of mycobacteria in host cells.

Results

Effects of IL-1 β pretreatment of monocytes on DC-mediated T cell stimulation

Monocytes primed with BCG produced significant concentrations of IL-1 β at MOI \geq 0.06 (Table 1). Similarly, macrophages produced IL-1 β in response to priming with BCG although less efficiently than monocytes. These findings raised the question whether IL-1 β influences maturation of DC from monocytes.

To this end, we pretreated monocytes with IL-1 β and then allowed them to mature into DC (Table 2). DC derived from untreated monocytes stimulated CD4⁺ T cells after a pulse with *M. leprae* (MOI 20). In contrast, CD4⁺ T cell stimulation by DC differentiated from IL-1 β pretreated monocytes was markedly impaired. Inhibition depended on the IL-1 β concentration used for pretreatment of monocytes. Significant inhibition was achieved at \geq 100 pg/mL IL-1 β . Similarly, IL-1 β inhibited T cell stimulation when other Ag, including

heat-killed *M. leprae* or major membrane protein (MMP)-II were used to pulse DC. IL-4 or IL-10 were not produced by CD4⁺ T cells under these conditions (data not shown). IL-1 α impaired APC functions of DC in a similar manner as IL-1 β , whereas other pro-inflammatory cytokines, including TNF- α and IL-6 did not affect Ag-presenting functions of DC.

Phenotype of DC derived from IL-1 β -pretreated monocytes

In an attempt to characterize DC derived from IL-1 β -pretreated monocytes, we determined surface markers of immature DC (Fig. 1). Immature DC derived from monocytes, which had been pretreated with IL-1 β or not, expressed similar or mildly reduced cell surface levels of HLA-ABC, HLA-DR and CD1a, and were devoid of the macrophage marker CD14. Similarly, the ability of immature DC to engulf mycobacteria as assessed by uptake of GFP-expressing BCG did not differ significantly (GFP-expressing *M. leprae* could not be prepared because of the inability of this obligate intracellular pathogen to grow *in vitro*). Subsequently, we determined surface markers of mature DC generated by LPS stimulation (Fig. 2). Expression of HLA-ABC and HLA-DR was not significantly altered by IL-1 β pretreatment of monocytes. In contrast, CD86 and CD83 were down-regulated by IL-1 β pretreatment, both with respect to surface expression and percent of positive cell numbers (Fig. 2A). These phenotypic alterations were also observed with *M. leprae*-infected mature DC (data not shown). IL-1 β did not influence the surface expression of MMP-II, one of the dominant Ag of *M. leprae* [23], on *M. leprae*-pulsed mature DC (Fig. 2B). In addition, IL-1 β or BCG pretreatment of monocytes did not alter the expression of TLR2 and TLR4 (Fig. 2C). Finally, IL-1 β pretreatment of monocytes did not cause apoptosis of DC (data not shown). Thus, DC differentiated from IL-1 β pretreated monocytes were only partially activated.

Table 1. IL-1 β production after stimulation of mononuclear phagocytes with BCG^{a)}

Cell type	IL-1 β (pg/mL) production after stimulation with BCG at MOI:			
	0	0.0625	0.25	1.0
Monocytes	2.2 \pm 0.2	23.7 \pm 4.6 ^{b)}	229.4 \pm 19.1 ^{b)}	861.4 \pm 22.3 ^{***b)}
Macrophages	3.3 \pm 0.4	4.0 \pm 1.1	34.6 \pm 2.8 ^{**}	163.3 \pm 20.1 ^{****b)}

^{a)} Plastic adherent monocytes, and M-CSF (5 ng/mL)-treated macrophages (1×10^5 /well) were stimulated for 24 h with BCG at the indicated MOI. Assays were done in triplicate, and results are expressed as mean \pm SD.

^{b)} ^{*}*p* < 0.05 vs. control (MOI 0), ^{**}*p* < 0.005 vs. control (MOI 0), ^{***}*p* < 0.0005 vs. control (MOI 0), ^{****}*p* < 0.01 vs. control (MOI 0).

Table 2. *M. leprae*-specific IFN- γ production by CD4⁺ T cells stimulated by *M. leprae*-pulsed DC derived from IL-1 β pretreated monocytes^{a)}

IL-1 β pretreatment of monocytes (pg/mL)	<i>M. leprae</i> infection of immature DC (MOI)	IFN- γ (pg/mL) secretion by CD4 ⁺ T cells after stimulation with DC at ratio (T: DC):	
		20:1	40:1
0	0	0.7 \pm 0.0	0.2 \pm 0.0
0	20	206.7 \pm 15.1	50.7 \pm 4.9
30	0	0.4 \pm 0.0	0.3 \pm 0.1
30	20	192.8 \pm 19.3	24.9 \pm 11.2
100	0	0.3 \pm 0.1	0.6 \pm 0.2
100	20	50.9 \pm 6.1 ^{b)}	13.4 \pm 0.3 [*]
300	0	0.7 \pm 0.4	0.8 \pm 0.7
300	20	30.3 \pm 2.6 [*]	9.7 \pm 1.9 ^{**b)}
1000	0	0.3 \pm 0.1	0.4 \pm 0.2
1000	20	9.5 \pm 2.1 [*]	3.0 \pm 0.5 [*]

^{a)} CD4⁺ T cells (1×10^5 /well) were stimulated for 4 days with autologous DC at the indicated T cell:DC ratio. Immature DC differentiated from untreated or IL-1 β -treated monocytes were pulsed with *M. leprae* on day 3, treated with LPS (25 ng/mL) on day 4, and were used as APC on day 5. Representative data of three separate experiments are shown. Assays were done in triplicate, and results are expressed as mean \pm SD.

^{b)} * $p < 0.0001$ vs. control (IL-1 β 0 pg/mL, *M. leprae* (MOI 20)), ** $p < 0.0005$ vs. control (IL-1 β 0 pg/mL, *M. leprae* (MOI 20)).

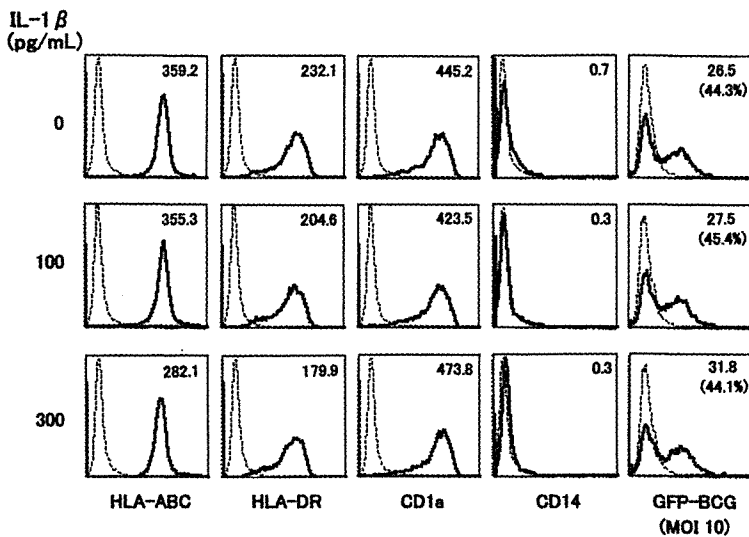


Figure 1. Phenotype and phagocytic activity of immature DC differentiated from IL-1 β pretreated monocytes. Plastic adherent monocytes were pretreated with the indicated doses of IL-1 β and were subsequently differentiated into DC by 3-day culture with rGM-CSF and rIL-4. For analysis of phagocytic activity of DC, the immature DC (cultured for 3 days) were pulsed with GFP-expressing BCG (MOI 10) and expression of GFP was assessed on day 4 of culture. Dashed lines, isotype-matched control IgG; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dotted and solid lines. The number in parentheses indicates percent positive cell number. Representative data of three independent experiments are shown.

Effects of IL-1 β pretreatment of monocytes on IL-12 production by DC

In order to determine whether DC differentiated from IL-1 β pretreated monocytes can be adequately activated, we determined IL-12p70 production by DC in response to TLR2 and TLR4 signaling. DC obtained on day 4 of

culture were found optimal for stimulation by the TLR4 ligand LPS and the TLR2 ligand peptidoglycan (PGN) (data not shown). DC derived from untreated monocytes produced IL-12p70 in response to LPS in a dose-dependent manner, whereas DC differentiated from monocytes pretreated with IL-1 β failed to produce significant amounts of IL-12 (Fig. 3A). Similar results

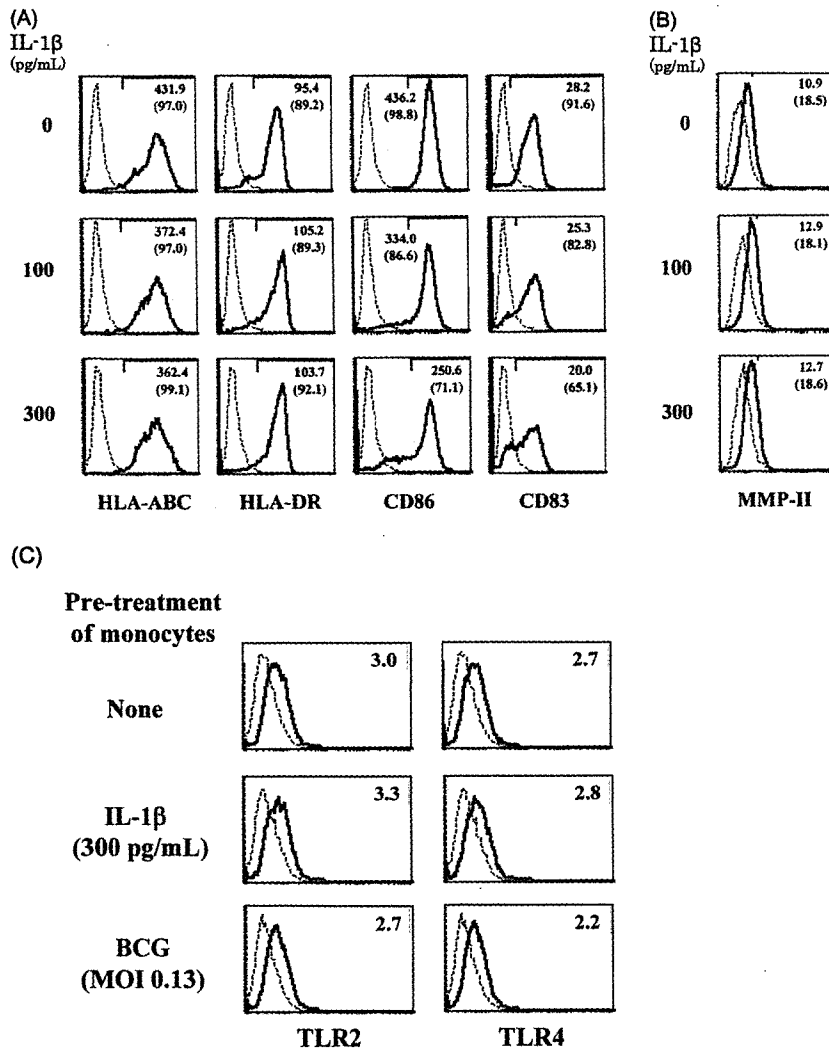


Figure 2. (A) Phenotype of mature DC differentiated from monocytes pretreated with IL-1 β . Plastic adherent monocytes were pretreated with the indicated doses of IL-1 β and were subsequently differentiated into DC by 5-day culture with rGM-CSF and rIL-4. Cells were treated with LPS (25 ng/mL) on day 4 and analyzed on day 5. Dashed lines, isotype-matched control IgG; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dotted and solid lines. The number in parentheses indicates percent positive cell number. Representative data of three independent experiments are shown. (B) MMP-II expression on mature DC obtained as in (A), except that DC were infected with *M. leprae* (MOI 20) on day 3. Dashed lines, isotype-matched control IgG; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dotted and solid lines. The number in parentheses indicates percent positive cell number. Representative data of three independent experiments are shown. (C) Expression of TLR on mature DC differentiated from monocytes pretreated with IL-1 β or BCG. Plastic adherent monocytes were pretreated with the indicated doses of IL-1 β or BCG and were subsequently differentiated into mature DC as in (A). Dashed lines, isotype-matched control IgG; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dotted and solid lines. Representative data of three independent experiments are shown.

were obtained for TNF- α production by DC (data not shown). Moreover, untreated DC produced IL-12p70 in response to PGN in a dose-dependent manner, whereas IL-1 β pretreatment of monocytes significantly impaired IL-12p70 production (Fig. 3B) although expression of TLR2 on the surface of DC was not altered under these conditions (data not shown). IL-12p70 production in response to MMP-II, which ligates to TLR2, was similarly affected by pretreatment of monocytes with IL-1 β (Fig. 3C). Also, both rIL-1 β (Fig. 3D) and BCG (Fig. 3E) induced IL-12p70 production only in DC derived from IL-1 β untreated-monocytes. The failure to produce IL-12p70 was long lasting as DC differentiated from IL-1 β pretreated monocytes in the presence of GM-CSF and IL-4 did not produce IL-12p70 in response to LPS for up to 7 days (data not shown). Next, we examined whether other cytokines produced by monocytes infected with BCG inhibited IL-12 production by DC. Monocytes were pretreated with 100 pg/mL of TNF- α , IL-6 or IL-10, differentiated to DC, which were then stimulated with LPS. None of these cytokines

impaired IL-12p70 secretion by DC (Table 3). Thus, IL-1 β signaling in monocytes but not IL-6, TNF- α or IL-10 signaling, inhibited IL-12 secretion by DC. Subsequently, IL-1 β sensitivity of monocytes undergoing DC maturation was determined. Monocytes were cultured for 3 days in the presence of rGM-CSF and rIL-4 and then treated with IL-1 β or other cytokines and 24 h later stimulated with LPS. Cells treated with TNF- α or IL-6 produced similar concentrations of IL-12p70 as compared to untreated DC in response to LPS. In contrast, IL-1 β treatment significantly reduced IL-12p70 production (Table 4).

Effects of endogenous IL-1 β on IL-12p70 production by DC

We assessed the influence of endogenously produced IL-1 β on IL-12p70 production by DC (Table 5). The *M. leprae*-derived cytosolic protein (MLC preparation), which represents an antigenic fraction, did not induce IL-1 β production in monocytes at concentrations up to

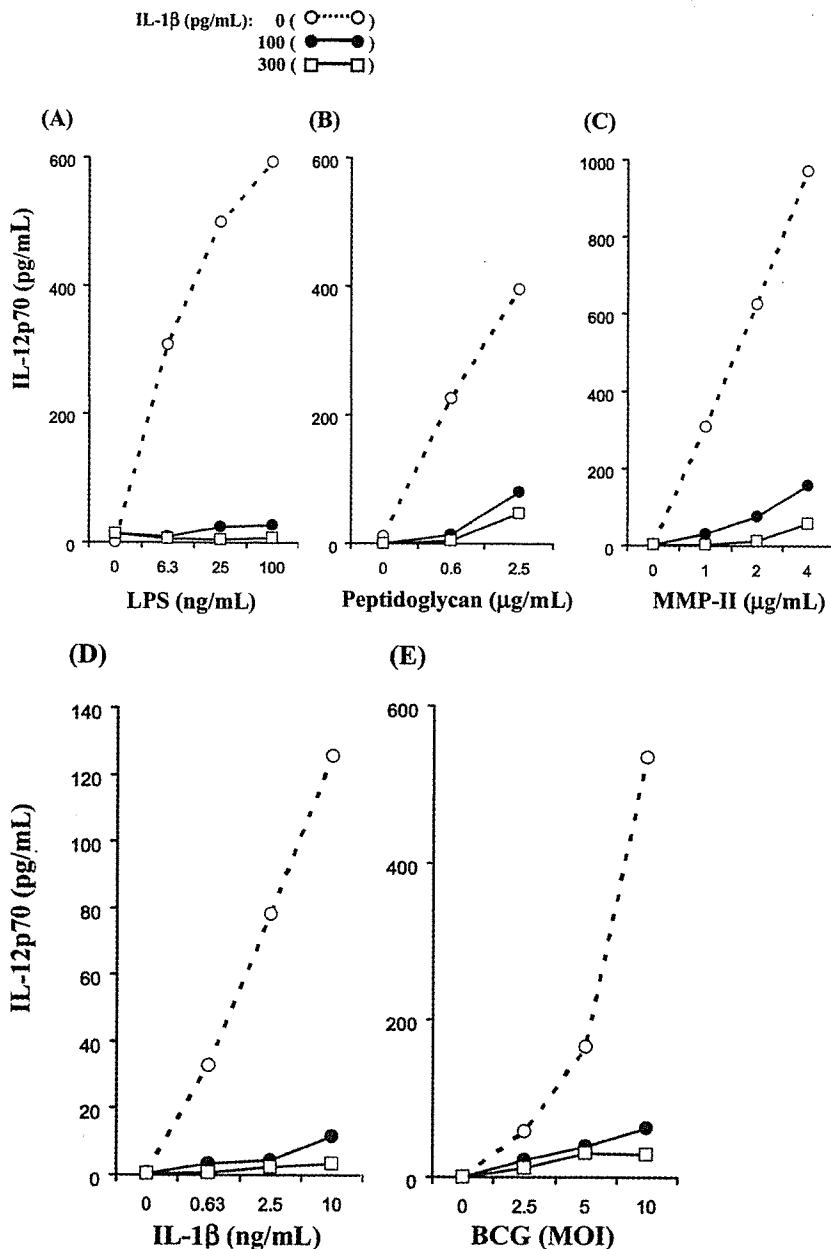


Figure 3. IL-12p70 production by stimulated mature DC. DC were differentiated by 4-day culture with rGM-CSF and rIL-4 from monocytes. Monocytes were treated with the indicated doses of rIL-1β (○---○: IL-1β 0, ●---●: IL-1β 100 and □---□: IL-1β 300 pg/mL), and were stimulated for 24 h with: (A) LPS, (B) PGN, (C) MMP-II, (D) IL-1β and (E) BCG at indicated doses. IL-12p70 concentrations were measured by ELISA. Representative data of three independent experiments are shown.

1 μg/mL, whereas 1 pg/mL LPS stimulated IL-1β secretion in monocytes. Accordingly, DC derived from monocytes pretreated with MLC produced equal concentrations of IL-12p70 as untreated DC did, whereas DC derived from LPS pretreated monocytes failed to secrete significant concentrations of IL-12p70. Monocytes infected with BCG at MOI <0.03 did not produce significant levels of IL-1β and accordingly were able to produce IL-12p70 after differentiation into DC. In contrast, BCG at MOI 0.13 stimulated IL-1β production from monocytes and concomitantly inhibited IL-12p70 secretion by DC. BCG infection did not cause apoptosis under these conditions (data not shown). To directly determine the role of endogenous IL-1β on IL-12 production by DC, antibody neutralization experiments

were performed (Table 6). DC derived from BCG-pretreated monocytes were significantly impaired in IL-12p70 production and this impairment was abrogated when neutralizing IL-1β mAb were present during BCG pretreatment. Control mAb showed no neutralizing effects. In addition, impaired IL-12p70 production by LPS pretreated monocytes was abrogated by neutralizing mAb to IL-1β (not shown). Thus, BCG inhibited IL-12 production by DC via IL-1β signaling in monocytes.

Discussion

Host defense against mycobacterial pathogens is mediated by innate and adaptive immune responses.

Table 3. IL-12p70 production by DC differentiated from monocytes pretreated with various cytokines^{a)}

Cytokine pretreatment of monocytes (100 pg/mL)	IL-12p70 (pg/mL) production by DC after stimulation with LPS at dose:		
	0	6.3 ng/mL	25 ng/mL
None	4.7 ± 0.8	363.4 ± 6.2 ^{b)}	546.7 ± 8.7*
IL-1 β	3.4 ± 1.0	18.0 ± 2.2*	48.0 ± 5.1*
TNF- α	3.8 ± 0.8	352.8 ± 5.8	531.5 ± 7.3
IL-6	4.0 ± 0.4	375.2 ± 9.8	564.4 ± 11.8
IL-10	1.3 ± 0.1	400.3 ± 11.9	895.8 ± 29.9

^{a)} DC (1×10^5 /well) were differentiated from monocytes pretreated with either of the above cytokines on day 0 of culture. On day 4 of culture, DC were stimulated with LPS for 24 h. Representative data of three separate experiments are shown. Assays were done in triplicate, and results are expressed as mean ± SD.

^{b)} * $p < 0.0001$ vs. control (LPS 0 ng/mL).

Table 4. Effects of cytokine pretreatment on IL-12p70 production by DC^{a)}

Cytokine pretreatment of immature DC (100 pg/mL)	IL-12p70 production (pg/mL) by DC after stimulation with LPS at dose:		
	0	6.3 ng/mL	25 ng/mL
None	0.2 ± 0.0	333.9 ± 11.0 ^{b)}	539.2 ± 20.3*
IL-1 β	0.3 ± 0.0	68.5 ± 9.8*	219.1 ± 10.7*
TNF- α	8.4 ± 1.9	330.7 ± 12.8	534.1 ± 30.0
IL-6	3.6 ± 0.8	331.8 ± 13.4	535.9 ± 29.2

^{a)} Monocytes cultured for 3 days in the presence of rGM-CSF and rIL-4 (1×10^5 /well) were pretreated with cytokines and on day 4 cells were further stimulated with LPS for 24 h. Representative data of three separate experiments are shown. Assays were done in triplicate, and results are expressed as mean ± SD.

^{b)} * $p < 0.0005$ vs. control (LPS 0 ng/mL).

Table 5. IL-12p70 production by DC derived from Ag-pulsed monocytes^{a)}

Exp.	Monocyte treatment	IL-1 β production (pg/mL) by monocytes ^{b)}	IL-12p70 production (pg/mL) by DC after stimulation with LPS at dose:		
			0	6.3 ng/mL	25 ng/mL
Exp. 1	None	1.3 ± 0.1	11.8 ± 2.1	250.8 ± 18.7	489.9 ± 21.0
	MLC ^{c)} (1 μ g/mL)	1.6 ± 0.2	13.3 ± 1.8	224.1 ± 17.9	360.9 ± 15.4
	LPS (1 pg/mL)	150.3 ± 3.9	4.0 ± 0.9	1.8 ± 0.3 ^{d)}	1.5 ± 0.1**
	MLC ^{c)} (1 μ g/mL) + LPS (1 pg/mL)	160.8 ± 4.3	4.4 ± 0.8	0.9 ± 0.2*	0.0 ± 0.0 ^{d)}
Exp. 2	None	0.0 ± 0.1	0.0 ± 0.0	309.6 ± 20.5	499.3 ± 23.7
	BCG (MOI 0.03)	6.8 ± 0.7	14.6 ± 2.6	264.7 ± 21.8	493.2 ± 21.8
	BCG (MOI 0.13)	148.7 ± 9.6	5.0 ± 1.1	44.6 ± 3.9*	91.8 ± 12.3**

^{a)} DC (1×10^5 /well) were derived from monocytes pulsed with Ag or LPS. DC were then stimulated for 24 h with LPS. Representative data of three separate experiments are shown. Assays were done in triplicate, and results are expressed as mean ± SD.

^{b)} The concentration of IL-1 β produced by monocytes after priming with indicated stimulator.

^{c)} MLC: *M. leprae*-derived cytosol fraction.

^{d)} * $p < 0.005$ vs. None (Monocyte not pretreated), ** $p < 0.001$ vs. None (Monocyte not pretreated).

Table 6. Effect of IL-1 β neutralization on IL-12p70 production by DC^{a)}

Monocyte pretreatment		IL-12p70 production (pg/mL) by DC after stimulation with LPS at dose:		
BCG (MOI)	mAb (2 μ g/mL)	0	6.3 ng/mL	25 ng/mL
0	None	10.1 \pm 0.9	72.4 \pm 12.1	301.0 \pm 20.0
0.13	None	11.3 \pm 0.8	21.5 \pm 6.5 ^{b)}	29.9 \pm 9.8 ^{b)}
0	IL-10	9.1 \pm 0.8	80.3 \pm 11.9	320.3 \pm 21.9
0	IL-1 β	12.3 \pm 2.1	70.8 \pm 9.5	298.0 \pm 10.4
0.13	IL-10	13.1 \pm 1.9	20.1 \pm 7.0 [*]	30.0 \pm 10.5 ^{**}
0.13	IL-1 β	10.8 \pm 1.3	81.3 \pm 9.9	270.3 \pm 13.1

a) Monocytes were pretreated with BCG (MOI 0.13) and/or neutralizing mAb to IL-1 β or IL-10 (control) (2 μ g/mL) and subsequently differentiated into DC by culture with rGM-CSF and rIL-4 for 4 days. DC were stimulated for 24 h with LPS. Representative data of three separate experiments are shown. Assays were done in triplicate, and results are expressed as mean \pm SD.

b) ^{*} $p < 0.005$ vs. control of each column (BCG MOI 0, mAb: none), ^{**} $p < 0.0005$ vs. control of each column (BCG MOI 0, mAb: none).

The two responses are intertwined and the innate immune response instructs the acquired immune response. Therefore, an effective vaccine should elicit both types of immunity in the most appropriate way [5, 24–27]. DC act as central stimulators of T cells and their maturation and activation is modulated by signals mediated by TLR that sense ligands from microbial pathogens. Subsequently, IL-12 promotes stimulation of protective T cells of Th1 phenotype [12, 28–31]. BCG, *M. tuberculosis* and *M. leprae* preferentially reside in mononuclear phagocytes in which they induce various cytokines including IL-1, TNF- α , IL-6 and IL-10 [32–34]. It is well established that IL-1 β is a multifunctional cytokine that plays a key role in the induction of innate and acquired immune responses [20]. In fact, both IL-1 β and BCG stimulated massive production of IL-12 from normal DC (Fig. 3). Yet, effects of this cytokine and BCG on maturation of monocytes to DC and subsequent DC activation remain elusive. As shown here, IL-1 β pretreatment of monocytes markedly impairs DC maturation and subsequent stimulation of antigen-specific T cells. Moreover, we show that these inhibitory effects are mediated both by exogenous and endogenously produced IL-1 β . Phenotype analysis of mature DC revealed that IL-1 β impaired surface expression of the costimulatory molecules CD86 and CD83, but had no apparent effect on the phenotype of monocytes and immature DC. Furthermore, secretion of IL-12p70 by mature DC was affected by treatment of monocytes with IL-1 β but was not affected by IL-1 β -non-inducers, such as LPS-free fraction of *M. leprae* (MLC) (Table 5). TLR signaling induced IL-12p70 production in DC, which, however, was markedly impaired by IL-1 β . This was observed when either monocytes or immature DC were treated with IL-1 β .

In contrast to IL-1 β , treatment of monocytes with TNF- α , IL-6 or IL-10 did not affect IL-12p70 secretion.

Moreover, neutralization of endogenously produced IL-1 β , but not of IL-10, reversed the inhibitory activity of cytokines produced by BCG-primed monocytes. Thus, IL-1 β acts prior to maturation of DC but affects downstream processes in activated DC. Moreover, our data demonstrate that infection of monocytes with BCG stimulates endogenous IL-1 β secretion. Thus, interactions between mycobacteria and monocytes affect the development of acquired immunity by impairing secretion of IL-12p70 by BCG-infected monocyte-derived mature DC.

The precise mechanisms underlying inhibition of antigen-specific T cell stimulation by DC via mycobacteria-induced IL-1 β remain to be established. It is possible that IL-1 β subverts the NF- κ B signaling pathway at early stages of DC maturation with downstream consequences on the function of mature DC. Precedences exist, showing that pathogens have developed strategies to circumvent or subvert the NF- κ B signaling pathway to promote successful invasion of, and persistence in, the host [35–37]. Moreover, some bacterial pathogens block differentiation of monocytes to DC *in vivo* [38]. An alternative possibility would be induction of apoptosis in DC, a strategy that is, for example, employed by measles virus [39]. However, in our experiments, no evidence for apoptosis of immature or mature DC via IL-1 β was obtained. Hence, we consider this possibility less likely. IL-10 is a well-known immunosuppressive cytokine and in HIV infection, IL-10 production severely affects DC maturation resulting in reduced IL-12p70 production and anergic T cell responses [40, 41]. In contrast, our experiments did not reveal evidence for a role of IL-10 in impaired IL-12p70 secretion by DC since neither exogenously added IL-10 on day 0 of monocytes culture nor neutralization of endogenously produced IL-10 caused any apparent effects (Table 6). Thus, IL-1 β effects on

monocytes is not dependent on IL-10 expression. One possible mechanism underlying impaired DC function is the attenuation of NF- κ B activity. Negative regulation of this pathway could proceed through the induction of negative regulators such as IRAK-M (IL-1 receptor-associated kinases) or other factors at the early stage of monocyte differentiation, which could in turn contribute to impaired TLR signaling by inducing a tolerant phenotype, which hinders production of NF- κ B dependent cytokines [42]. Although we consider this explanation likely, the exact mechanism by which IL-1 β impairs DC function remains to be elucidated.

Our experiments reveal production of inhibitory IL-1 β by monocytes not only after LPS stimulation but also after mycobacterial infection. Our findings provide strong evidence for a critical role of IL-1 β in impaired immunity during mycobacterial infections. Hence, they add a guideline for the development of immunological intervention strategies against tuberculosis and leprosy. Strategies that inhibit IL-1 β induction or neutralize IL-1 β effects at the stage of monocyte maturation to DC could improve the ensuing protective T cell response.

Materials and methods

Preparation of cells and bacteria

Peripheral blood was obtained from healthy PPD-positive Japanese individuals under informed consent. In Japan, BCG vaccination is compulsory for children (approx. 0- to 4-year-old). PBMC were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described [43]. For preparation of peripheral monocytes, CD3⁺ T cells were removed from either freshly isolated heparinized blood, or cryopreserved PBMC using immunomagnetic beads coated with anti-CD3 mAb (Dynabeads 450, DYNAL, Oslo, Norway). The CD3⁻ PBMC fraction was plated on collagen-coated plates and the non-plastic adherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes [44]. Macrophages were differentiated by culturing monocytes in the presence of 20% FCS and 5 ng/mL of M-CSF (R and D Systems, Abingdon, UK), as described [18]. Monocyte-derived DC were differentiated as described [43, 45]. Briefly, monocytes were cultured in the presence of 50 ng of rGM-CSF (Pepro Tech, London, UK) and 10 ng of rIL-4 (Pepro Tech)/mL [45]. Additionally, monocytes were treated with rIL-1 β (R and D Systems), rIL-1 α (Genzyme, Cambridge, MA), TNF- α (Boehringer Mannheim Biochemica, Mannheim, Germany), rIL-6 (Strathmann Biotech GMBH, Hannover, Germany), or rIL-10 (R and D Systems), and subsequently differentiated into immature DC by culturing the monocytes for 3 days in the presence of rGM-CSF, rIL-4 and either of those cytokines. Mature DC were produced by culturing immature DC, which were unpulsed or infected with *M. leprae*, in the presence of LPS (*Escherichia coli* 0111: B4; Difco Laboratories,

Detroit, MI). *M. leprae* (Thai-53 strain) was isolated from the footpads of BALB/c-*nu/nu* mice [46]. The isolated bacteria were counted by Shepard's method [47]. The viability of *M. leprae* was assessed by using fluorescent diacetate/ethidium bromide test [48]. BCG (Pasteur strain) was cultured *in vitro* using Middlebrook 7H9 broth supplemented with 0.05% Tween 80 and albumin-dextrose-catalase. BCG expressing GFP was constructed as follows: GFP sequence was amplified from pEGFP-1 vector (CLONTECH, Palo Alto, CA), and cloned into pMV261 [49]. Transformants were selected on 7H10 plate containing 25 μ g/mL kanamycin. The uptake of BCG by DC after culture was determined using FACScalibur (Becton Dickinson Immunocytometry System, San Jose, CA). The MOI was determined based on the assumption that DC were equally susceptible to infection with *M. leprae* [12], and immature DC were infected with *M. leprae* at MOI 20. The MLC was prepared by fractionation of mycobacterial proteins according to previous reports [18, 28]. Briefly, the mycobacterial suspension was mixed with Zirconium beads and homogenized. The suspension was ultra-centrifuged and the resulting supernatant was used as MLC. The amount of LPS in the purified protein was quantitated by the Limulus Amebocyte Lysate assay (Whittaker Bioproducts, Walkersville, MD) and found to be <10 pg/mg protein.

Analysis of cell surface Ag

The expression of cell surface Ag on DC was analyzed using FACScalibur. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical, St. Louis, MO) and 1×10^4 live cells were analyzed. For analysis of cell surface Ag, the following mAb were used: FITC-conjugated mAb against HLA-ABC (G46-2.6, PharMingen, San Diego, CA), HLA-DR (L243, PharMingen), CD1a (OKT6, Ortho Diagnostic Systems, Raritan, NJ), CD14 (M5E2, BD Biosciences, San Jose, CA), CD86 (FUN-1, PharMingen), CD83 (HB15a, Immunotech, Marseille, France), TLR2 (TL2.3, Serotech, Oxford, UK), TLR4 (HTA125, Santa Cruz Biotech, Santa Cruz, CA).

The expression of MMP-II, which is one of the dominant antigenic entities of *M. leprae* [23], on *M. leprae*-infected DC was determined using the mAb (IgM, kappa) against MMP-II, followed by FITC-conjugated anti-mouse Ig Ab (Tago-immunologicals, Camarillo, CA).

APC functions of DC

The ability of *M. leprae*-infected DC to stimulate T cells was assessed using an autologous DC-T cell co-culture as previously described [12, 45]. Freshly thawed PBMC were depleted of MHC class II⁺ and CD8⁺ cells by using magnetic beads coated with mAb to MHC class II and CD8 (Dynabeads 450; DYNAL) [45]. The purity of CD4⁺ T cells was more than 98%. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates and DC were added to give the indicated DC:CD4⁺ T cell ratio. Supernatants of DC-T cell co-cultures were collected on day 4 and cytokines determined.

IL-12p70 production by DC

On day 4, DC were stimulated with the following reagents: LPS, PGN (*Staphylococcus aureus*, Fluka Production GmbH, Buchs, Switzerland), rIL-1 β , purified MMP-II, *M. bovis* BCG. After 24 h, IL-12p70 concentrations were measured.

Cytokine production

Levels of the following cytokines were measured; IFN- γ produced by CD4⁺ T cells, IL-12p70 produced by DC, and IL-1 β produced by monocytes and macrophages stimulated for 24 h with *M. leprae* or BCG. The mAb to IL-1 β with neutralizing activity was obtained from R & D Systems. The concentrations of IFN- γ , IL-12p70 and IL-1 β were quantified using the enzyme assay kits, Opt EIA Human ELISA Set (BD PharMingen International).

Statistical analysis

Student's *t*-test was applied to determine statistical differences.

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

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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 intracel-*

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.

lulare 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