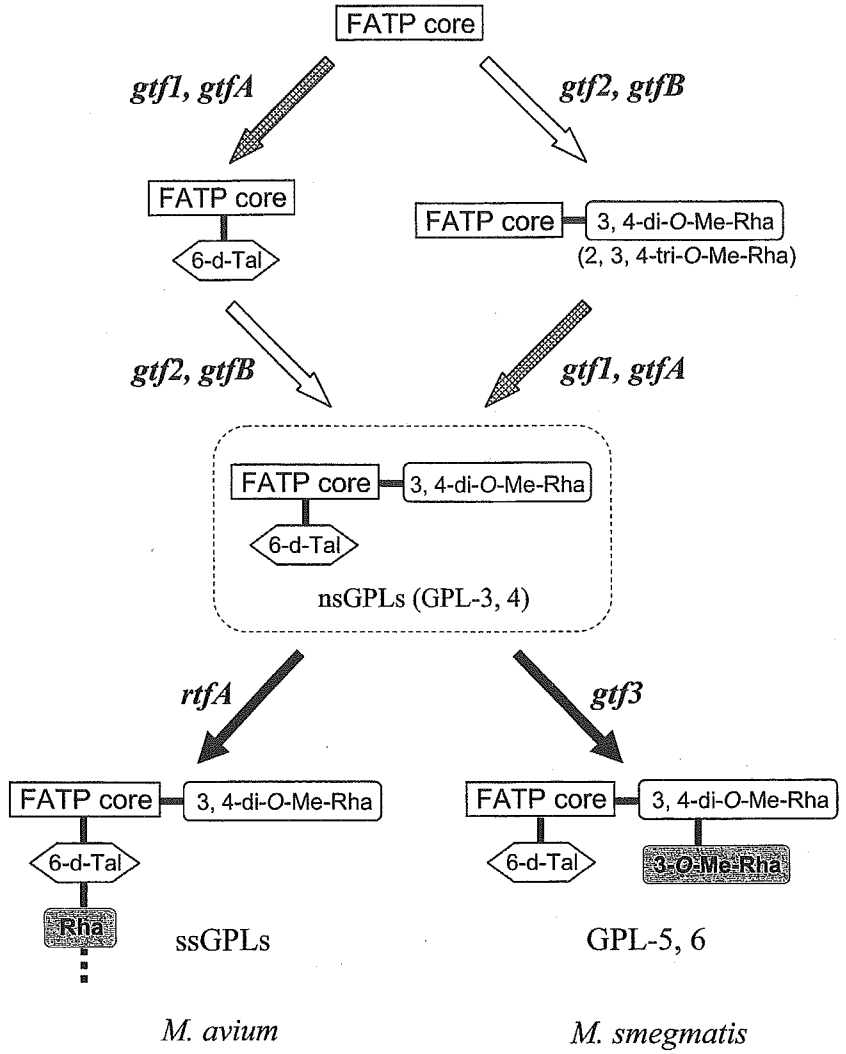

FIG. 9. Proposed biosynthetic pathways for GPLs of *M. smegmatis* and *M. avium*. FATP core, fatty acyl-tetrapeptide core.

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

			Primer set	
Species	Strains	Accession number	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).

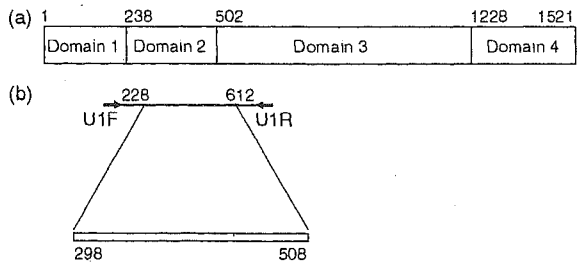

Fig. 1. Schematic representation of the DnaA protein and primer design for the amplification of the partial mycobacterial *dnaA* gene. Number indicates the nucleotide position of *Mycobacterium tuberculosis*, GenBank accession number AL021427. (a) The DnaA protein from *M. tuberculosis* contains four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

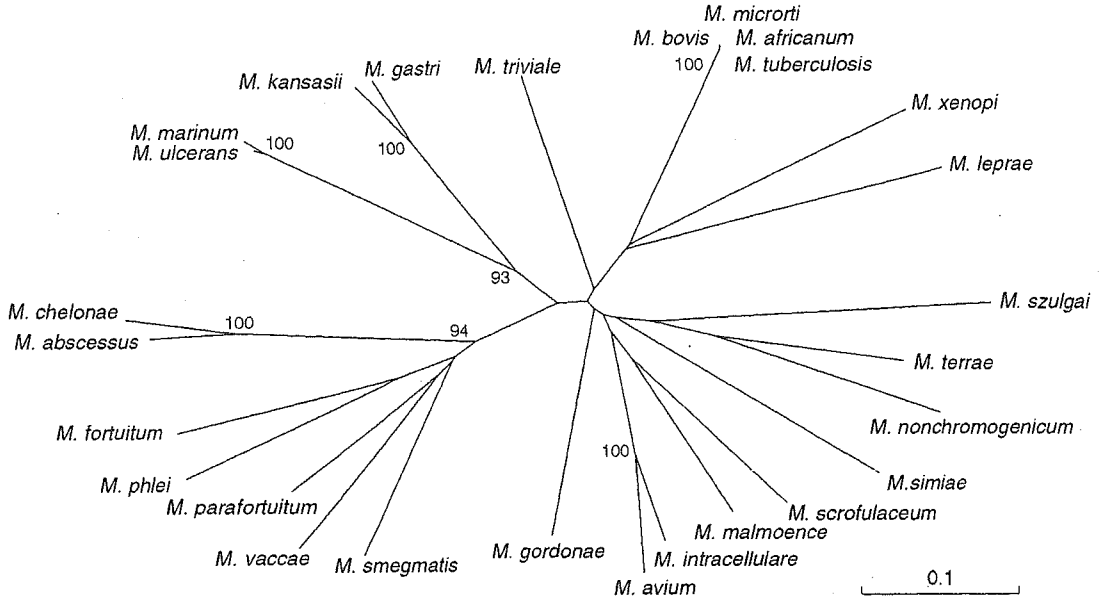

Fig. 2. Phylogenetic relationship of 27 *Mycobacterium* species. Unrooted tree based on the *dnaA* sequences. The tree was generated from DNASpace (Hitachi Software Engineering) with the Clustal W algorithm. The numbers on the dendrogram indicate the percentages of occurrence in 1000 bootstrapped trees;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

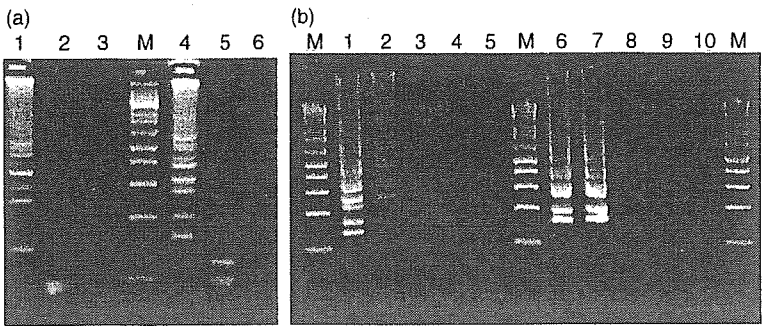

Fig. 4. (a) Four percent agarose gel electrophoresis and restriction enzyme analysis of loop-mediated isothermal amplification (LAMP) products of partial *dnaA* gene of *Mycobacterium kansasii* and *Mycobacterium gastri*. Lanes: M, 100 bp DNA ladder; lanes 1–3, LAMP carried out with *M. kansasii* primer, 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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DNA Augments Antigenicity of Mycobacterial DNA-Binding Protein 1 and Confers Protection against *Mycobacterium tuberculosis* Infection in Mice[1]

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Mycobacterium **consists up to 7% of mycobacterial DNA-binding protein 1 (MDP1) in total cellular proteins. Host immune responses to MDP1 were studied in mice to explore the antigenic properties of this protein. Anti-MDP1 IgG was produced after infection with either bacillus Calmette-Guérin or *Mycobacterium tuberculosis* in C3H/HeJ mice. However, the level of Ab was remarkably low when purified MDP1 was injected. MDP1 is considered to be associated with DNA in nucleoid, which contains immunostimulatory CpG motif. Therefore, we examined coadministration of MDP1 and DNA derived from *M. tuberculosis*. Consequently, this procedure significantly enhanced the production of MDP1-specific IgG. Five nanograms of DNA was enough to enhance MDP1-specific IgG production in the administration of 5 μg of MDP1 into mice. Strong immune stimulation by such a small amount of DNA is noteworthy, because >1,000- to 100,000-fold doses of CpG DNAs are used for immune activation. A synthetic peptide-based study showed that B cell epitopes were different between mice administered MDP1 alone and those given a mixture of MDP1 and DNA, suggesting that DNA alters the three-dimensional structure of MDP1. Coadministration of DNA also enhanced MDP1-specific IFN-γ production and reduced the bacterial burden of a following challenge of *M. tuberculosis*, showing that MDP1 is a novel vaccine target. Finally, we found that MDP1 remarkably enhanced TLR9-dependent immune stimulation by unmethylated CpG oligo DNA in vitro. To our knowledge, MDP1 is the first protein discovered that remarkably augments the CpG-mediated immune response and is a potential adjuvant for CpG DNA-based immune therapies.** ***The Journal of Immunology,*** **2005, 175: 441–449.**

Tuberculosis is a disease caused by infection with *Mycobacterium tuberculosis* and remains a serious threat to health in the world. Annually, 8 million people contract tuberculosis, and nearly 2 million people die from the disease. Worldwide, 32% of the population is persistently infected with *M. tuberculosis*, and some of these bacteria are thought to be in a nonreplicating dormant state (1). The majority of the disease arises from reactivation of persisting, previously implanted bacteria (2–5).

Bacillus Calmette-Guérin (BCG)[3] is an attenuated live vaccine against tuberculosis and has been given to >2 billion individuals to date. BCG is safe, inexpensive, and effective against both meningitis and miliary tuberculosis in infants, but frequently fails to protect from the most prevalent form of the disease, adult pulmonary tuberculosis (6–9). In addition, there is the possibility of causing opportunistic disease in immunocompromised hosts, such as AIDS patients, because BCG is a live vaccine and can survive in the hosts. Accordingly, there is an urgent need to develop a more effective and safer vaccine than BCG. Extensive studies to date have evaluated possible vaccine candidate proteins, such as a 6-kDa early secretory antigenic target (10); Ag 85 complexes A, B, and C (11); MTB39 and MTB48 (12); and heat shock protein 60 (13).

Mycobacterial DNA-binding protein 1 (MDP1) is produced by the genus *Mycobacterium* and is a major cellular protein, consisting of up to 7% of the total cellular protein (14). MDP1 has nucleic acid-binding activity mediated through interaction with guanine and cytosine residues in DNA (14, 15). Thus, MDP1 is presumed to be a component of the mycobacterial nucleoid and has been shown to localize to the 50S ribosomal subunit and on the bacterial surface (14, 16). The cellular content of MDP1 is increased in the stationary growth phase of mycobacteria relative to the exponential growth phase (14). Dick et al. (17) found that histone-like protein (HLP), the homologue of MDP1, was substantially upregulated in the dormant state of *Mycobacterium smegmatis*. Our previous study showed that MDP1 inhibited macromolecular biosyntheses in vitro and substantially suppressed bacterial growth (18). Taken together, it is conceivable that MDP1 has fundamental

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[3] Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; Ag85B, Ag 85 complex B; HLP, histone-like protein; HLP*Mt*, histone-like protein of *Mycobacterium tuberculosis*; HrpA, heat stress-induced ribosome-binding protein A; KO, knockout; LBP-21, laminin-binding protein of 21 kDa; Me-oligo B, synthetic oligo DNA containing methylated CpG sequence; MDP1, mycobacterial DNA-binding protein 1; ODN, synthetic oligodeoxynucleotide; PPD, purified protein derivative; RIB, RIBI adjuvant system; rMDP1, recombinant histidine-tagged MDP1.

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roles in the suppression of growth from both stationary and dormant phases of mycobacteria.

Of interest, MDP1 localizes on the bacterial surface as well as intracellularly (14, 16, 19, 20). During host-bacterium interaction, MDP1 may play a role as an adhesin. Shimoji et al. (20) found that a 21-kDa protein could bind to laminin-2, which is thought to be an *Mycobacterium leprae* receptor involved in attachment to Schwann cells (21). They designated this protein as laminin-binding protein of 21 kDa (LBP-21) and showed it to be a homologue of MDP1 in *M. leprae*, although it was deficient for DNA-binding activity (20). Thus, LBP-21 may have a role in the invasion of *M. leprae* into peripheral nerves, presumably cooperating with another adhesion molecule, phenolic glycolipid-1 (22). In addition to laminin, we recently found that MDP1 binds to glycosaminoglycans (16), which are a major component of the extracellular matrix. Glycosaminoglycans are important in the attachment of mycobacteria, especially in the interaction with nonphagocytic cells such as fibroblasts and epithelial cells (23), which are possible reservoirs of persisting *M. tuberculosis* in healthy humans (24).

Prasad et al. (25) used T cell blot assay to identify an immunodominant protein in healthy contacts with tuberculosis patients. They designated that protein as histone-like protein of *M. tuberculosis* (HLP*Mt*), which is the same molecule as MDP1. Both humoral and lymphoproliferative responses against recombinant HLP*Mt*/MDP1 were greater in healthy tuberculin reactors than in nonreactors or tuberculosis patients (25). This suggests that HLP*Mt*/MDP1 is an immunodominant Ag that may have an important role in host defense.

In this study we report a series of studies that analyze the antigenicity of MDP1 in a mouse model. We show that both humoral and cellular immune responses to MDP1 are stimulated by the presence of bacterial DNA that contains immunostimulatory CpG motifs (26, 27) that initiate immune responses through TLR9 (28). Simultaneous immunization with MDP1 and DNA, but not MDP1 alone, promotes protection against an *M. tuberculosis* challenge. An in vitro study demonstrated that a complex of MDP1 and CpG DNA markedly stimulates the production of proinflammatory cytokines in a TLR9-dependent manner. Proteins produced by pathogenic organisms are major targets of host immune responses that lead to protective immunity. Our data demonstrate that immunostimulatory cellular components that interact with these proteins have significant effects on protein recognition by the host and the subsequent development of protective immunity.

Materials and Methods

Mice

Female A/J, BALB/c, C3H/HeJ, and C57BL/6 mice were purchased from Japan SLC at 5–7 wk of age. TLR9 knockout (KO) mice (B6 129F2 background) were supplied by Dr. S. Akira (Osaka University, Osaka, Japan) (28). All mice were kept under specific pathogen-free conditions.

Bacterial strains and culture

BCG (strain Tokyo) was grown at 37°C in Middlebrook 7H9 media (Difco) supplemented with 10% albumin, dextrose, and catalase enrichment (Difco) and 0.05% Tween 80. When the OD at 630 nm was ~0.5, bacteria were collected by centrifugation and suspended in sterilized water to adjust for an OD of 1.0. Mice were infected i.p. with 5–10 × 10^6 CFU of BCG in 0.2 ml of normal saline. Two weeks later, mice were boosted with the same dose of BCG i.p. The bacterial dose was determined by counting CFUs 3 wk after plating serial 10-fold dilutions of suspension onto Middlebrook 7H11 agar containing oleic acid, dextrose, albumin, and catalase enrichment (Difco; 7H11-OADC agar).

Antigens

Recombinant histidine-tagged MDP1 (rMDP1) was purified from *Escherichia coli* transfected with pET21b$^+$-*mdp1* by methods described previously (16). Native MDP1 was purified from BCG (Tokyo strain) using the method described previously (14). Ag 85 complex B (Ag85B) purified from *M. tuberculosis* H37Rv was a gift from Dr. S. Nagai (29). Heat stress-induced ribosome-binding protein A (HrpA), purified as a recombinant protein (30), was supplied by Drs. N. Ohara and T. Tabira (Nagasaki University, Nagasaki, Japan). Bovine histone H1, histone H2A, and histone H3 were purchased from Roche. Bacterial DNA was purified from *M. tuberculosis* H37Rv by phenol-chloroform extraction (31). Briefly, 5 g of *M. tuberculosis* H37Rv (wet weight) was suspended in 5 ml of 10 mM Tris-HCl and 1 mM EDTA (pH 7.5; TE buffer), mixed with the same volume of chloroform/methanol (2/1), and incubated for 5 min to remove lipids. The suspension was centrifuged at 2,500 × *g* for 20 min, and both organic and aqueous layers were decanted to leave a packed bacterial band. Delipidated bacteria were incubated at 55°C for 20 min to remove traces of organic solvents and were resuspended in 5 ml of TE buffer and 0.5 ml of 1 M Tris-HCl (pH 9.6). Lysozyme (Sigma-Aldrich) was added to a final concentration of 100 μg/ml and incubated for 2 h. Then 0.1 vol of 10% SDS and 0.01 vol of proteinase K (Sigma-Aldrich) were added and additionally incubated overnight. To remove contaminating proteins, the same volume of phenol was added, gently mixed for 20 min, and centrifuged at 12,000 × *g* for 20 min. The aqueous layer was transferred to the fresh tube, and the protein-removing step was repeated again. Then the same volume of chloroform/isoamyl alcohol (24/1) was added and gently mixed for 10 min. The tube was centrifuged at 12,000 × *g* for 10 min, then the supernatant was transferred to new tube. DNA was precipitated by gently mixing after adding 0.1 vol of 3 M sodium acetate (pH 5.2) and 2.5 vol of ethanol. The tube was then centrifuged at 12,000 × *g* for 10 min, and the DNA pellet was rinsed with 70% ethanol. The pellet was resolved in pure water, and the concentration was determined by the absorbance at 260 nm. The endotoxin level of Ags was <50 pg/100 μM, as determined by a *Limulus* test.

Immunization of mice with protein Ags and BCG

Protein Ags were emulsified using the RIBI adjuvant system (RIB; Corixia), which consists of synthetic trehalose dicorynomycolate and monophosphoryl lipid A, or by IFA (Difco). In some cases, Ags were mixed with various amounts of DNA for 10 min at 37°C and then emulsified. Five micrograms of protein with or without DNA was injected i.p. Three weeks later, mice were boosted using the same method as the primary immunization. The same protocol was used for BCG immunization. Five to 10 × 10^6 CFU of BCG was i.p. injected per mouse. Three weeks after the boost, peripheral blood was obtained from the retro-orbital plexus of anesthetized mice, and sera were isolated and stored at −80°C until the assays.

Western blot

One microgram of purified MDP1 was fractionated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and reacted with antisera diluted 1/200.

ELISA

Ninety-six-well ELISA plates (Sumitomo) were coated with individual protein Ags, such as MDP1, HrpA, Ag85B, histone H1, histone H2A, and histone H3, by overnight incubation in carbonate buffer (pH 9.6) at 4°C. Wells were then blocked by PBS containing 3% BSA for 2 h at room temperature. Equal volumes of sera from at least five mice were mixed in each experimental group. Sera were serially diluted in PBS containing 1% BSA, added to wells, and incubated overnight at 4°C. The wells were washed four times with PBS containing 0.05% Tween 20, and HRP-conjugated goat anti-mouse IgG (DakoCytomation), IgG1, IgG2a, IgG2b, IgG3 (Santa Cruz Biotechnology), or IgG2c (Bethyl) diluted in PBS containing 1% BSA was added and incubated for 2 h at room temperature. After washing as before, 100 μl of 80 mM citrate-phosphate buffer (pH 5.0) containing 0.4 μg/ml *o*-phenylendiamine dihydrochloride (Wako Pure Chemicals) was added to the wells, and absorbance at 492 nm was measured by an MTP-300 microplate reader (Corona Electronic).

To determine B cell epitopes, overlapping peptides covering the entire sequence of MDP1 were synthesized previously as 20-mer molecules with 10-aa overlaps with the neighboring peptides, with exception of the C-terminal (15). Each peptide was dissolved in PBS at a concentration of 10 μg/ml and immobilized onto type A ELISA plates (Sumitomo) after activation of the wells by 2% glutaraldehyde. Sera diluted 1/200 by PBS containing 0.05% Tween 20 was added and incubated at 4°C overnight. The ELISA procedure described above was performed, and B cell epitopes were defined by color development with *o*-phenylendiamine dihydrochloride.

Lymph node cell culture and stimulation

Mice were killed 3 wk after the booster injection of Ags, and mesenteric lymph node cells were prepared. Cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 25 mM HEPES, 2 mM L-glutamine, 5.5×10^{-5} M 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete RPMI medium) in the presence or the absence of 10 μg/ml, MDP1, Ag85B, or purified protein derivative (PPD; Kyowa) in a humidified incubator at 37°C under 5% CO_2. IFN-γ in the culture supernatant was measured with ELISA kits (Genzyme Techne).

Prior immunization and challenge with M. tuberculosis

C3H/HeJ or BALB/c mice were s.c. immunized with 5 μg of RIB-emulsified MDP1 with or without 5 ng of *M. tuberculosis* DNA, DNA alone (5 ng), or 5 μg of Ag85B. BCG Tokyo at a dose of 10^6 CFUs was inoculated using the same procedure without emulsification. After 3 wk, mice were boosted i.p. by the same Ags and were challenged 3 wk later i.v. with 1×10^6 CFU of *M. tuberculosis* Kurono strain (ATCC 35812; American Type Culture Collection). On days 14 and 28, lungs were removed and homogenized using an LS-50 homogenizer (Yamato). The lung homogenates were serially diluted and inoculated onto 7H11-OADC agars. Bacterial numbers were calculated and expressed as CFU.

Spleen cell culture and stimulation

Synthetic oligodeoxynucleotides (ODNs) of sequence GGGGGGAACGT TGGGGGGGGGGGGGGGGGG were purchased from Nisshinbo and designated oligo B. As a control, cytosine-methylated oligo B was synthesized (Me-oligo B). The endotoxin level was <50 pg/100 μM, as determined by a *Limulus* test. Spleens obtained from C57BL/6 and TLR9 KO mice were cut into small pieces and homogenized. These cell suspensions were depleted of erythrocytes using a Ficoll gradient (Lympholyte-M; Cedarlane Laboratories) and centrifuged for 20 min at 1000 × *g* at room temperature. Spleen cells (1×10^6 cells/well) were cultured in the presence or the absence of MDP1 and ODNs at final concentrations of 0.5 and 1 μM, respectively. After 10-min incubation at 37°C, the MDP1-ODN mixture was added to the cell cultures and incubated for 24 h. As a control, cells were also stimulated with LPS derived from *E. coli* 0111 134 (Difco) at concentration of 100 ng/ml. The amounts of TNF-α and IL-6 in the culture supernatants were measured with ELISA kits (Genzyme Techne).

Statistical analyses

Statistical analysis was conducted with a Power Macintosh G4 using StatView 5.0 (SAS Institute). ANOVA was used to determine the significance of differences in means between multiple experimental groups. The significance level of the test was <5%.

Results

Anti-MDP1 IgG production in mice

To explore the antigenicity of MDP1, we first analyzed the humoral immune response to MDP1 in mice. BCG was inoculated into three strains of mice, including A/J, BALB/c, and C3H/He. Western blot analysis showed that MDP1 elicited a humoral immune response in all strains (Fig. 1*A*). Sera from nonimmunized mice did not react with MDP1 (data not shown). Additionally, anti-MDP1 IgG was produced in C3H/He and BALB/c mice challenged with *M. tuberculosis* H37Rv (data not shown).

We next assessed the antigenicity of purified MDP1. Five micrograms of MDP1 was emulsified in RIB and injected into C3H/He mice. In contrast to inoculation of BCG, we could not detect a significant level of anti-MDP1 IgG (Fig. 1*A*). MDP1 presumably binds to DNA, which includes immunostimulatory CpG motifs (27). Therefore, we tested the simultaneous administration of MDP1 and DNA. Five micrograms of MDP1 was incubated with 0.5 μg of DNA derived from *M. tuberculosis* H37Rv, and the mixture was injected into C3H/He mice. Western blot analysis showed that a combination of MDP1 and DNA elicited MDP1-specific IgG production, whereas MDP1 or DNA alone did not (Fig. 1*A*).

We next determined the optimal dose of DNA that could enhance anti-MDP1 IgG production. Using RIB, 5 μg of MDP1 was administered to C3H/He mice with or without 10-fold serial dilutions of DNAs ranging from 5 μg to 5 ng. Three weeks after the booster injection, the level of anti-MDP1 IgG was measured by ELISA (Fig. 1*B*). The production of IgG was dependent on the amount of DNA; interestingly, 5 ng of DNA most efficiently stimulated IgG production against MDP1. We observed enhanced anti-MDP1 IgG production by coadministration of DNA and MDP1 in the presence of IFA (Fig. 1*C*), and the result was similar to that

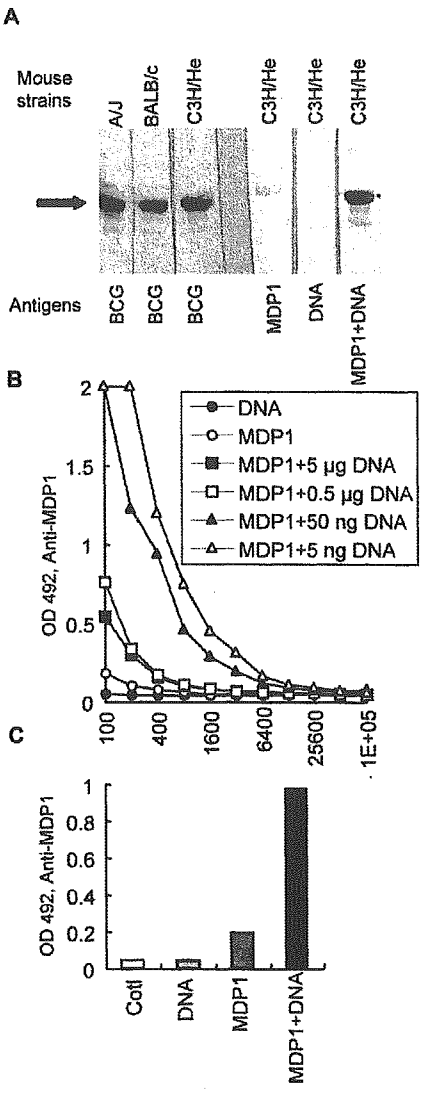

FIGURE 1. Humoral immune responses to MDP1 in mice. *A*, Western blot analysis. Purified MDP1 was blotted onto the membrane and incubated with 200/1 diluted antisera. Mouse strains are indicated along the *top* of the panel. The injected Ags, such as BCG, MDP1 alone (MDP1), *M. tuberculosis* DNA alone (DNA), and MDP1 plus DNA (MDP1+DNA) are shown along the *bottom*. *B*, DNA dose effects on anti-MDP1 IgG production. C3H/He mice were immunized with MDP1 (5 μg/mouse) with or without various amounts of DNA (5 μg to 5 ng) emulsified in RIB adjuvant, and levels of anti-MDP1 IgG were determined by ELISA. The horizontal axis shows dilution factors of antisera. *C*, Immunization with MDP1 plus DNA emulsified in IFA augmented anti-MDP1 IgG production. C3H/He mice were immunized with Ags emulsified in IFA. Immunized Ags are described *below* the horizontal axis. Cotl, IFA alone. The sera from at least five mice of each experimental group were mixed and diluted to 1/400, and the levels of anti-MDP1 IgG were determined by ELISA.

observed using RIB adjuvant, suggesting that the immunostimulatory effect of DNA on anti-MDP1 IgG production is not restricted to RIB adjuvant. The results prompted us to explore whether DNA-dependent IgG production varies between mouse strains. The same immunization procedure using RIB as an adjuvant was performed in other mouse strains, including A/J, BALB/c, and C57BL/6. The results revealed that simultaneous inoculation of MDP1 and DNA augmented the production of IgG against MDP1 in all tested strains (Fig. 2).

To determine whether DNA-mediated enhancement of anti-MDP1 IgG production is restricted to the particular IgG isotype, we analyzed the distribution of subclasses of IgG by ELISA. As shown in Fig. 3, each mouse strain possessed a specific pattern of MDP1-specific IgG isotypes, but DNA enhanced only IgG subclasses produced in mice immunized with MDP1 alone. Thus, a small dose of DNA augments the humoral response to MDP1 without altering the pattern of IgG isotypes.

MDP1-specific, DNA-dependent stimulation of IgG production

Our data showed that a small amount of DNA (5 ng) magnified anti-MDP1 IgG production (Fig. 1*B*). In contrast, 1,000- to 100,000-fold higher amounts (5–500 μg) of bacterial DNA and CpG ODNs have been applied as adjuvants in immunization with foreign Ags (32–34) or immunotherapeutic treatments (34–38). Therefore, we next examined whether 5 ng of DNA stimulated Ab production against other immunogenic mycobacterial proteins such as HrpA (39) and Ag85B (11). These Ags did not bind to DNA, as determined by gel retardation assay (data not shown). Five micrograms of each Ag was injected into BALB/c, C3H/He, and C57BL/6 mice, with or without 5 ng of DNA. We could not detect enhanced Ab production by coadministration of DNA in any of the three mouse strains (Fig. 4, *A* and *B*).

Next we examined whether DNA combined with DNA-binding proteins other than MDP1 stimulates IgG production. Bovine histone H1, histone H2A, and histone H3 were injected into three strains of mouse (BALB/c, C3H/He, and C57BL/6) with or without 5 ng of DNA. We could not detect the production of IgG against both histones H2A and H3 in any of mouse strains tested (data not shown). In contrast, anti-histone H1 Ab was detectable in all mouse strains, but DNA alone did not stimulate anti-histone H1 IgG production (Fig. 4*C*). Although we have not tested all DNA-binding proteins, these results imply that enhanced Ab production by a very small amount of bacterial DNA is a unique feature of MDP1.

DNA alters B cell epitopes of MDP1

To examine humoral immune responses against MDP1 more precisely, we defined the region(s) recognized by anti-MDP1 IgG. B cell epitope mapping was conducted by ELISA using synthetic 20-mer peptides covering the entire MDP1 sequence. Antisera were obtained from four strains of mice, including A/J, BALB/c, C3H/He, and C57BL/6, immunized with MDP1 alone or with 5 ng of DNA and were reacted with each peptide. In A/J mice, IgG from animals immunized with MDP1 alone did not react with peptides, although it was bound to MDP1, suggesting that IgG in these mice recognized the conformational structure of MDP1 (Fig. 5*A*). In contrast, two peptides corresponding to aa 61–80 and 71–90 of MDP1 were recognized by anti-MDP1 IgG in mice immunized with MDP1 plus DNA (Fig. 5*A*). In BALB/c mice, anti-MDP1 IgG induced by injection of both MDP1 alone and MDP1 plus DNA reacted with the peptide corresponding to 51–70 of MDP1 (Fig. 5*B*). In C3H/He mice, the level of anti-MDP1 IgG was insignificant when MDP1 alone was used (Fig. 5*C*). In contrast, anti-MDP1 IgG was produced in animals immunized with MDP1 plus DNA and reacted with peptides corresponding to 141–160 and 151–170 (Fig. 5*C*). Thus, the epitope was likely to be the 151–160 region of MDP1. In C57BL/6 mice, Abs from mice immunized with MDP1 alone and MDP1 plus DNA reacted with the 61–80 and 1–20 regions, respectively (Fig. 5*D*).

Although the anti-MDP1 Ab titer was higher in BALB/c mice injected with MDP1 plus DNA than in mice immunized with

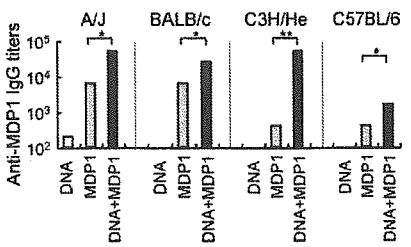

FIGURE 2. DNA stimulates the production of anti-MDP1-IgG in mice. Four strains of mice, including A/J, BALB/c, C3H/He, and C57BL/6, were immunized with DNA alone (□), MDP1 (5 μg/mouse) alone (▨), or MDP1 plus DNA (■). The titer of anti-MDP1-IgG was determined by ELISA. *, $p < 0.05$; **, $p < 0.01$ (by ANOVA).

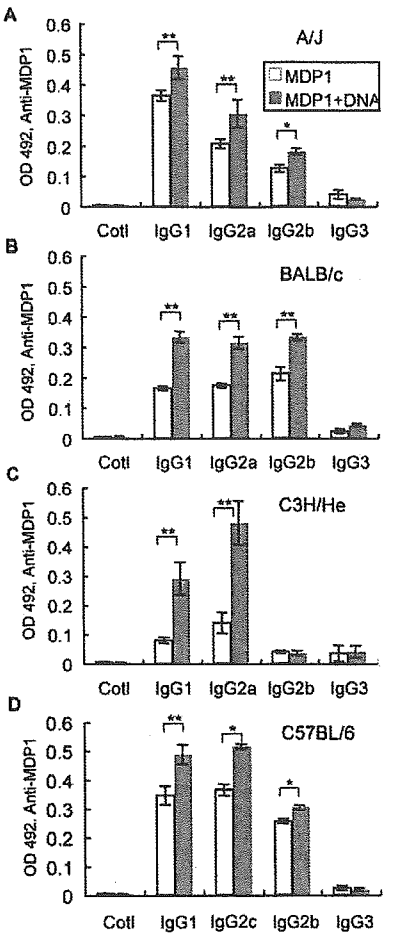

FIGURE 3. Isotypes of anti-MDP1 IgG. The levels of IgG subclasses were measured using isotype-specific Abs against IgG1, IgG2a, IgG2b, IgG2c, and IgG3. Cotl, Controls without secondary Ab. □, Immunization with MDP1 alone; ▨, coadministration of MDP1 and DNA. Antisera were diluted 1/100 (*A–C*) or 1/50 (*D*). *, $p < 0.05$; **, $p < 0.01$ (by ANOVA).

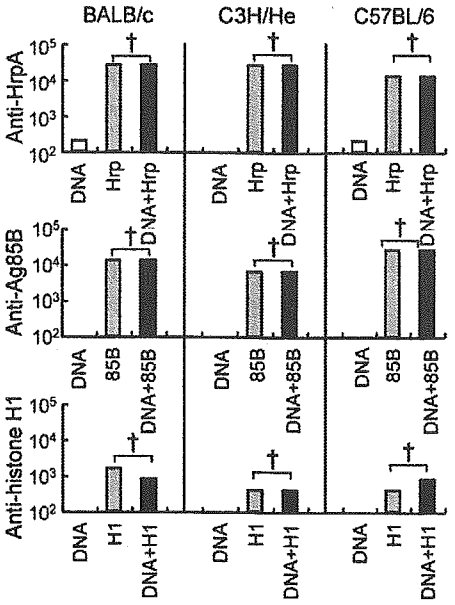

FIGURE 4. DNA fails to stimulate production of IgGs against mycobacterial Ags, HrpA and Ag85B, and a DNA-binding protein, histone H1. BALB/c, C3H/He, and C57BL/6 mice were immunized with 5 μg of HrpA (Hrp), Ag85B (85B), and histone H1 (H1) with (■) or without (▨) 5 ng of DNA. □, Immunization with DNA alone. The titer of Ag-specific IgG was measured by ELISA. †, $p > 0.1$ (by ANOVA).

MDP1 alone, the level of anti-MDP1 IgG against the defined epitope (aa 51–70) was reversed (Fig. 5*B*). This suggests that anti-MDP1 IgG recognizes mainly conformational epitopes in mice immunized with a mixture of MDP1 and DNA. To examine this possibility, inhibition assays were performed. The interaction between MDP1 and IgG from mice immunized with MDP1 alone (Fig. 6*A*), but not with MDP1-DNA (Fig. 6*B*), was inhibited by exogenously added peptide corresponding to aa 51–70 of MDP1 (Fig. 6, *A* and *B*). In contrast, the same molar amount of exogenously added MDP1 alone inhibited both reactions (Fig. 6, *A* and *B*). These data indicate that in BALB/c mice, administration of MDP1 alone produces IgG that recognizes only the 51–70 region. In contrast, administration of MDP1 plus DNA induces anti-MDP1 IgG targeting conformational epitopes on MDP1 in addition to the 51–70 region.

Similar inhibition experiments were conducted using sera from BALB/c mice injected with live BCG. The 51–70 peptide failed to abrogate the IgG-MDP1 interaction (Fig. 6*C*), although MDP1 itself did. This suggests that MDP1 is actually binding to DNA in vivo and is targeted by the host immune response.

MDP1 stimulates IFN-γ production

Protective immunity against *M. tuberculosis* infection is mediated primarily by Th1-type cell-mediated immunity (40, 41). IFN-γ triggers Th1-type cell-mediated immune responses and plays a critical role in host defense against *M. tuberculosis* infection in mice (42, 43). To investigate whether MDP1 participates in BCG-mediated protection against tuberculosis, we examined IFN-γ production induced by MDP1 stimulation. Lymph node cells from C3H/He mice immunized with BCG were cultured in the presence or the absence of MDP1, and the level of IFN-γ in culture supernatants was measured by ELISA. The results show that MDP1 stimulated IFN-γ production in a manner similar to Ag85B and PPD (Fig. 7*A*). We next examined isotypes of anti-MDP1 IgG in

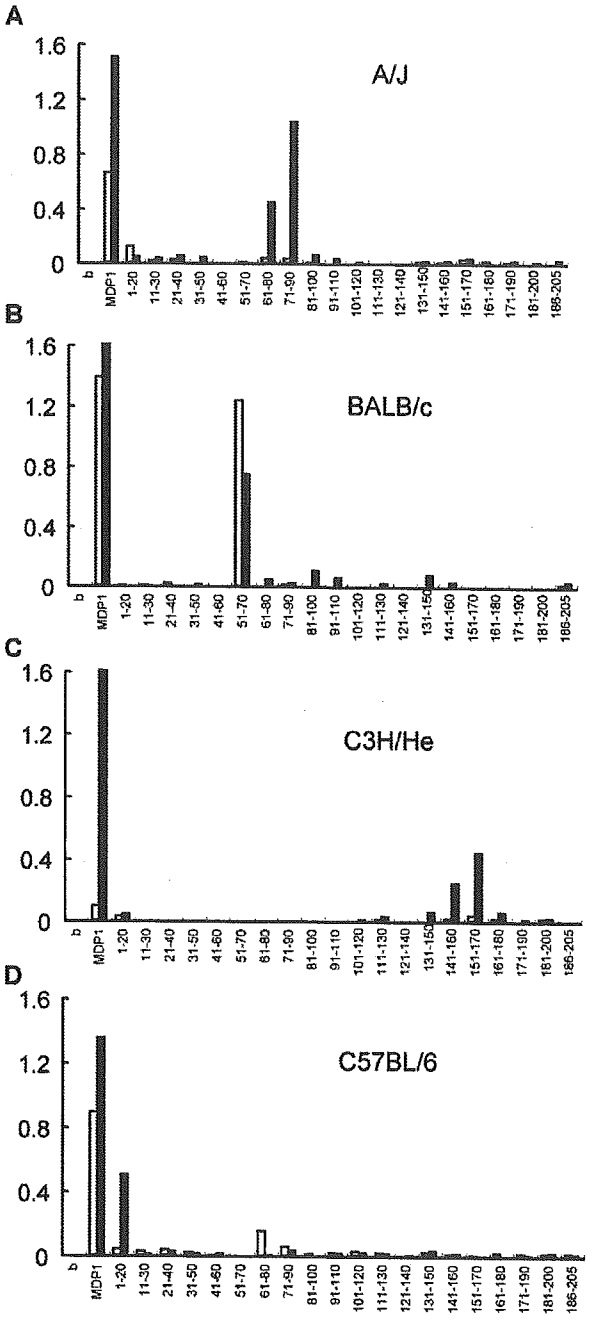

FIGURE 5. B cell epitope mapping of anti-MDP1 IgG. Epitopes of anti-MDP1 IgGs were determined by ELISA. MDP1 or 20-mer synthetic peptides covering the entire MDP1 sequence were coated on the ELISA plate as indicted at the *bottom* of each graph. b, Blank well without Ag coating. The same antisera as those described in Fig. 2 were diluted 1/200 and applied to the wells. *A–D*, Analysis of antisera derived from A/J, BALB/c, C3H/He, and C57BL/6 mice, respectively. □, Antisera from mice inoculated with MDP1 alone; ■, antisera from mice inoculated with MDP1 plus DNA. The ELISA units represent the average of duplicate samples.

BCG-immunized C3H/He mice. BCG inoculation stimulated the production of MDP1-specific IgG1 and IgG2a, but not IgG2b or IgG3 (Fig. 7*B*). Interestingly, the pattern of IgG isotypes was similar to that observed in the same mouse strain immunized with both

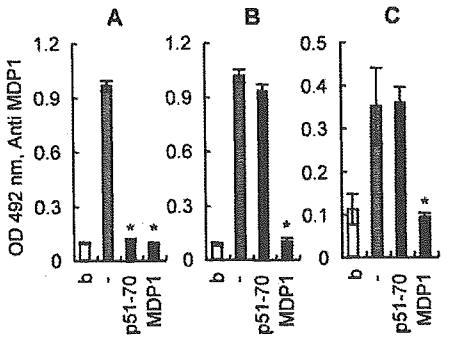

FIGURE 6. Production of anti-MDP1 IgG-targeting conformational epitopes in BALB/c mice immunized with MDP1 plus DNA or BCG, but not with MDP1 alone. Antisera from BALB/c mice immunized with MDP1 alone (*A*), MDP1 plus DNA (*B*), and BCG (*C*) were reacted with immobilized MDP1 on ELISA plates with or without exogenously added peptide corresponding to the 51–70 region of MDP1 (p51–70) or MDP1 (MDP1). b, Blank without Ag coating; −, positive controls without inhibitors. *, $p < 0.05$ (by ANOVA, vs controls without inhibitors (−)).

MDP1 and DNA (Fig. 3*C*). IFN-γ induces IgG2a production (44), whereas both Th1-related and Th2 cytokines stimulate IgG1 production (45, 46). The predominant production of IgG2a implies that the immune response to MDP1 is polarized toward the Th1 type. It is likely that MDP1 is one of the Ags that induce protective immunity after BCG immunization in C3H/He mice.

Next we examined whether the administration of purified MDP1 induces IFN-γ production. C3H/He mice were immunized with MDP1 alone or with MDP1 plus DNA. As controls, RIB and DNA alone were administered to mice as well. Lymph node cells were cultured with or without 10 μg/ml MDP1, and the production of IFN-γ was assessed. The results showed that MDP1 stimulates IFN-γ production (Fig. 7*C*). However, immunization with MDP1 mixed with DNA produced much more IFN-γ than that with MDP1 alone, demonstrating that DNA augments cell-mediated immune responses to MDP1 (Fig. 7*C*).

Simultaneous administration of MDP1 and DNA confers protection against M. tuberculosis *infection in mice*

The ability to produce IFN-γ by MDP1 prompted us to explore whether MDP1 could induce protection in vivo against challenge with a virulent strain of *M. tuberculosis*. C3H/He mice were immunized with MDP1 alone or with MDP1 plus 5 ng of DNA. As controls, mice were given RIB alone, DNA alone, BCG, or Ag85B, which is a major vaccine candidate (11). After a 3-wk interval, mice were boosted with the same Ag; 3 wk later, mice were challenged with *M. tuberculosis* Kurono. After 14 and 28 days, mice were killed, and the numbers of bacteria in the lungs and spleens were determined. These data showed that immunization with Ag85B, DNA, and MDP1 failed to protect (Fig. 8, *A–C*). In contrast, BCG and coadministration of MDP1 and DNA significantly reduced the bacterial load in the lungs ($p < 0.005$ and $p = 0.0119$ on day 14, and $p = 0.008$ and $p = 0.0316$ on day 28, respectively). A protective effect of immunization of MDP1 plus DNA, but not MDP1 alone, was also observed in the spleens ($p = 0.021$; Fig. 8*C*). As shown in Fig. 8*D*, immunization with both MDP1 and DNA resulted in a modest, but significant, decrease in bacterial burden in BALB/c mice as well ($p < 0.005$). Although the effect was less than that of BCG, MDP1 confers substantial protection against *M. tuberculosis* challenge only when it is administered with DNA.

MDP1 augments TLR9-dependent immunostimulation by CpG DNA

Immunostimulatory effects of DNA are dependent on unmethylated CpG motifs (27) that signal via TLR9 (28). Our data revealed that a very small amount of DNA stimulates immune responses against MDP1, in contrast with previous reports (32–34). Therefore, we hypothesized that MDP1 might enhance the immunostimulatory activity of CpG DNA. To test this hypothesis, we evaluated the effect of MDP1 on CpG-ODN-mediated immune activation in vitro. Spleen cells from both C57BL/6 and TLR9 KO C57BL/6 mice were stimulated with oligo B containing CpG DNA sequence in the presence or the absence of rMDP1. Me-oligo B, which has the same structure, except that its cytosine is methylated, and LPS, which signals via TLR4 (47, 48), were used as controls. After 24 h, levels of the proinflammatory cytokines TNF-α and IL-6 in the culture supernatants were determined by ELISA. Oligo B alone (1 μM) did not induce the production of TNF-α (Fig. 9). In contrast, the mixture of rMDP1 and oligo B dramatically stimulated TNF-α production (Fig. 9). This effect was undetectable in splenocytes from TLR9 KO mice or with the combination of Me-oligo B and rMDP1. Similar results were seen for

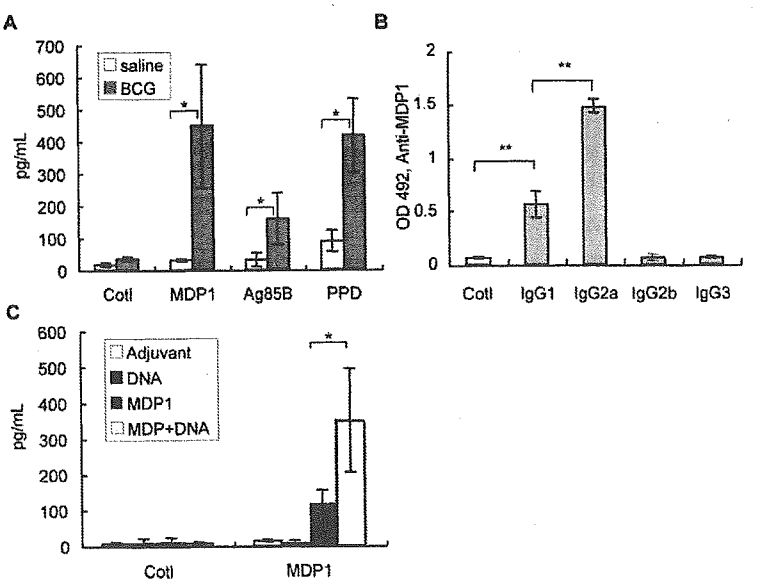

FIGURE 7. Development of Th1-type immune responses against MDP1 after challenge with BCG (*A* and *B*) or MDP1 (*C*). *A*, Amounts of IFN-γ in culture supernatants from lymph node cells were determined by ELISA. Lymph nodes were derived from C3H/He mice immunized with saline (□) or BCG (▨) and incubated for 5 days with 10 μg/ml MDP1, Ag85B, and PPD as indicated. Cotl, without Ag stimulation. The production of IFN-γ was measured by ELISA. *B*, MDP1-specific IgG isotypes (IgG1, IgG2a, IgG2b, and IgG3) in sera of C3H/He mice immunized with BCG detected by ELISA. Cotl, controls without secondary Ab. *C*, Lymph node cells derived from C3H/He mice immunized with adjuvant alone (□), DNA (■), MDP1 (▧), and MDP1 plus DNA (▨) were cultured in the presence (MDP1) or the absence (Cotl) of 10 μg/ml MDP1 for 5 days, and the amounts of IFN-γ in the culture supernatants were determined by ELISA. *, $p < 0.05$; **, $p < 0.01$ (by ANOVA).

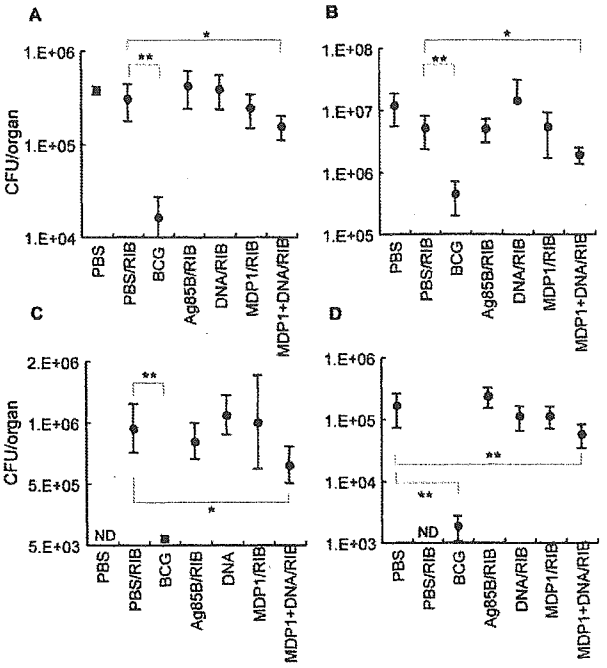

FIGURE 8. Coadministration of MDP1 and DNA confers protection against *M. tuberculosis* challenge. C3H/HeJ (*A–C*) and BALB/c (*D*) mice were immunized with Ags, as indicated below the horizontal axis, and challenged i.v. with 10^6 CFU of *M. tuberculosis* Kurono strain. Fourteen (*A*) and 28 (*B–D*) days after the challenge, bacterial numbers in lung (*A*, *B*, and *D*) and spleen (*C*) were determined by counting CFUs. *, $p < 0.05$; **, $p < 0.005$ (by ANOVA).

IL-6 production, although 0.5 μM rMDP1 itself induced a low level of IL-6 production (Fig. 9). Similar data were obtained when natural MDP1 was used under the same conditions (data not shown). These data clearly demonstrate that MDP1 activates TLR9-dependent immunostimulation by CpG ODN.

Discussion

In the present study we have evaluated the antigenicity of MDP1, a DNA-binding protein specific to mycobacteria. Anti-MDP1 IgG

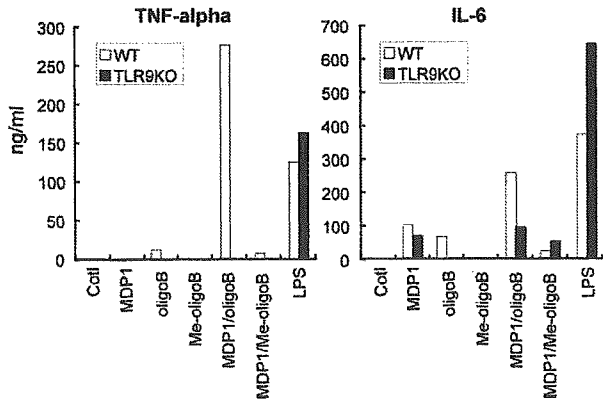

FIGURE 9. MDP1 enhances CpG-mediated production of proinflammatory cytokines in vitro. Splenocytes were stimulated with 0.5 μM MDP1 alone, a mixture of MDP1 and ODNs (1 μM), or *E. coli* LPS (100 ng/ml) for 24 h in vitro. Levels of TNF-α (*right*) and IL-6 (*left*) were determined by ELISA. The ELISA units represent the average of duplicate samples and are representative of two experiments performed.

was produced in C3H/He mice challenged with either BCG (Fig. 1*A*) or *M. tuberculosis* (data not shown). Marked cell proliferation occurred when splenocytes from *M. tuberculosis*-infected mice were stimulated with 10 μg/ml MDP1 in vitro. Uptake of [^{3}H]thymidine was higher compared with stimulation with the gold standard, PPD (our unpublished observations). Thus, in agreement with a human study (25), MDP1 is also highly immunogenic in mice.

However, administration of purified MDP1 failed to produce anti-MDP1-IgG in C3H/He mice (Fig. 1). This lack of antigenicity was reversed by adding mycobacterial DNA when immunizing with MDP1 (Fig. 1). Similarly, DNA enhanced the production of MDP1-specific IgG in other mouse strains (Fig. 2). B cell epitope mapping (Fig. 5*B*) and Ab reaction-inhibition assay (Fig. 6) implied association of MDP1 with genomic DNA in live BCG. These results suggest that the strong immunogenicity of MDP1 in mycobacterial infection is responsible for colocalization of DNA.

Studies to determine the optimal dose of DNA showed that 5 ng of DNA was enough to activate MDP1-specific IgG production (Fig. 1*B*). This dose is unusually low compared with other studies in which 5–500 μg of DNA or ODN/mouse was used for immune activation (32–34, 36–38). We confirmed that 5 ng of DNA did not enhance the production of IgG against other proteins, including DNA-binding proteins (Fig. 4). Thus, a very small amount of DNA-stimulated Ig production appears to be a specific feature of MDP1.

We determined B cell epitopes on MDP1 by using synthetic peptides. B cell epitopes differed among mouse strains. Surprisingly, the epitopes were different when DNA was coadministered with MDP1, even within the same mouse strain (Fig. 5). Thus, DNA not only stimulates MDP1-specific IgG production, but also modifies the recognition site of IgG. This suggests that the three-dimensional structure of MDP1 differs depending on whether DNA is present or absent, and this difference is recognized by the immune system of the host. This conformational change might be involved in the disparate antigenicities of this protein.

To investigate the role of MDP1 in host protection, we examined the activity of MDP1 in the induction of IFN-γ that is critical for host defense against *M. tuberculosis* infection in mice (42, 43). When stimulated in vitro with 10 μg/ml MDP1, lymph node cells derived from BCG-immunized C3H/He (Fig. 7*A*) and C57BL/6 (data not shown) mice produced a significant amount of IFN-γ. Analysis of IgG isotype in BCG-immunized mice revealed the production of MDP1-specific IgG2a, which was indicative of a Th1-type immune response (Fig. 7*B*) (44). Administration of purified MDP1 also expanded the population of IFN-γ-producing cells (Fig. 7*C*) and stimulated Th1-associated IgG2a production (Fig. 3). Again, simultaneous injection of MDP1 and DNA stimulated adaptive immunity and enhanced IFN-γ production (Fig. 7*C*). This was confirmed when mice were infected with *M. tuberculosis*, and MDP1 was found to decrease bacterial load only when coadministered with DNA (Fig. 8). Thus, MDP1 can be a novel vaccine target, although it is effective only when administered simultaneously with DNA. Because *M. tuberculosis* is transmitted by the aerogenic route, future studies are needed to explore the efficacy using the aerosol challenge model.

As discussed above, our data show that MDP1 has a unique feature as an Ag, in that its antigenicity is profoundly enhanced by even a small amount of DNA. This raises an important question as to how this immune stimulation is coordinated. At least six nucleotides are necessary for immune activation by ODN (49). Because DNA is highly sensitive to degradation by DNases, a large amount of DNA is required for immune activation (50). We found that

MDP1 blocks degradation of DNA by DNases in vitro (unpublished observations), and this DNA-protective activity of MDP1 is one possible explanation.

Another possible explanation is the cell-binding activity of MDP1. To exert immunostimulatory activity, CpG DNA must attach to the macrophage surface and be internalized, with subsequent maturation of the phagosome (51). In our preliminary work, biotin-labeled ODN was more quickly bound to the macrophage surface and internalized when it was added with MDP1 (our unpublished observations). We have demonstrated that MDP1 binds to glycosaminoglycans and to A549 human lung epithelial cells through hyaluronic acid (16). After adding 0.5 μM MDP1, >95% of A549 cells became MDP1 positive in 60 min (16). In addition, it has been shown that HupB/MDP1 binds to C3 (52). Complement receptors are major receptors for *M. tuberculosis* on macrophages (53, 54). Collectively, it is reasonable to assume that MDP1 binds to macrophages through surface glycosaminoglycans or complement receptors. This cell-binding activity of MDP1 is advantageous for carrying DNA to/into macrophages, resulting in subsequent immunostimulation.

Immunization with MDP1 plus mycobacterial DNA significantly reduced the bacterial burden compared with treatment with Ag85B (Fig. 8). To develop effective vaccines against tuberculosis, additional studies are necessary to assess vaccine efficacy using MDP1 in conjunction with CpG-ODNs that can induce a Th1 response (32–34). Although the Ag85 complex has been widely studied as a major component of tuberculosis vaccines (11, 55), we did not observe a protective effect (Fig. 8). These conflicting results may be due to the mouse strains used in this experiment, because Ag85A and 85B induce protective immunity against mycobacterial infection in C57BL/6 mice (55, 56). The protective effect of Ag85B is conspicuous in guinea pigs as well (11). Guinea pigs are relatively susceptible to *M. tuberculosis* infection, whereas the mouse has low to moderate susceptibility (57, 58). In addition, guinea pigs, but not mice, develop cavitary lesions and caseous necrosis similar to human tuberculosis. It will be important to examine the protective effect of coadministration of MDP1 and DNA in a guinea pig model.

A key step in initiating adaptive immunity is the presentation of pathogen-derived peptides on class II MHC molecules by APCs. APC functions are up-regulated after recognition of pathogen-associated molecular patterns, including CpG DNA motifs (28). Therefore, we examined the effects of MDP1 on CpG ODN-mediated immune activation. We found that MDP1 magnified CpG-DNA effects, such as the production of the proinflammatory cytokines TNF-α and IL-6 (Fig. 9). As far as we know, MDP1 is the first protein identified that remarkably enhances CpG-mediated immune stimulation. Proinflammatory cytokines are critical for APC activation and promote the maturation of professional APCs. Immunostimulation induced by the interaction between MDP1 and CpG DNA might be involved in inducing strong adaptive immune responses against MDP1, which lead to protection (Fig. 8).

MyD88 is an adaptor molecule critical for the CpG-DNA-TLR9 signaling pathway (59, 60). Recently, it was shown that MyD88 KO mice are highly susceptible to *M. tuberculosis* (61) and *M. avium* (62), although mice with genetic mutations of TLR2 and TLR4 displayed comparable resistance as wild-type mice challenged with *M. tuberculosis* (63) and *M. avium* (62). These studies suggest that resistance to mycobacterial infection is regulated by multiple MyD88-dependent signals in addition to those attributed to TLR2 (64) or TLR4. As we show in this study, MDP1 stimulates TLR9-dependent immune responses by CpG ODN (Fig. 9), and the MDP1-DNA complex can induce protective immunity (Fig. 8). TLR9 signaling stimulated by MDP1-mycobacterial DNA complexes might be involved in MyD88-dependent antimycobacterial immunity.

The immunostimulatory activity of DNA was initially discovered in a DNA-rich fraction derived from BCG, referred to as MY1 (65, 66). Those studies demonstrated that the antitumor activity of MY1 was diminished by DNase treatment. MY1 is a mycobacterial nucleoid (65, 66). It is conceivable that MDP1 is involved in the activity of MY1.

The immunostimulatory activity of DNA has huge potential for immunotherapy against infectious, neoplastic, and allergic diseases (50, 67–69). To our knowledge, MDP1 is the first protein discovered that remarkably augments CpG-mediated immune stimulation (Fig. 9). MDP1 has great potential as an adjuvant for CpG-ODN-based immune interventions.

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Disclosures

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Mycobacterial trehalose 6,6′-dimycolate preferentially induces type 1 helper T cell responses through signal transducer and activator of transcription 4 protein

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Abstract

Mycobacterium tuberculosis is an intracellular pathogen of tuberculosis and its pathogenicity is related to the ability to escape killing by ingested macrophages and induce delayed-type hypersensitivity (DTH). A major component of the cell wall of *M. tuberculosis* is trehalose 6,6′-dimycolate (TDM), which has been implicated as a pathogenetic factor. The expression of DTH and cell-mediated immunity is dependent on the macrophage-cytokine-type 1 helper T (Th1) lymphocyte axis. Cytokines, interleukin-12 (IL-12) and interferon-γ (IFN-γ), play a critical role in the process and IL-12-activated signal transducer and activator of transcription (STAT) 4 is required for the development of fully functional Th1 cells. To clarify host responses to mycobacterial TDM, we have analyzed footpad reaction, histopathology and cytokine profile of experimental granulomatous lesions using STAT4-deficient mice. In the present study, we have demonstrated that mycobacterial TDM selectively induces the Th1 response through the STAT4 signaling pathway, because mice lacking STAT4 protein significantly reduced to develop DTH, hypersensitivity granulomas, and Th1 cytokine responses, when compared to BALB/c mice. These results shed light on the molecular pathogenesis of mycobacterial disease. Taken together with previous studies, TDM is a pleiotropic molecule against the host and participates in the pathogenesis.

Keywords: Trehalose 6,6′-dimycolate (TDM); *Mycobacterium tuberculosis*; STAT4; Granuloma; Delayed-type hypersensitivity (DTH)

1. Introduction

Mycobacterium tuberculosis is one of the most successful human pathogens and infects one-third of world population [1]. The pathogenesis of tuberculosis is concerned with both virulence of the pathogen, *M. tuberculosis*, and the host immune response to the pathogen [2]. *M. tuberculosis* is an intracellular pathogen and its pathogenicity is related to the ability to escape killing by ingested macrophages and induce delayed-type hypersensitivity (DTH) [3]. The macrophage–cytokine–T lymphocyte axis plays a critical role in both disease expression and defense against the infection.

There is much known but we still have a long way to understand the mechanism of *M. tuberculosis* pathogenicity. Mycobacteria are rich in lipids. Lipid components of the mycobacterial cell wall participate in the pathogenesis. Cord factor (trehalose 6,6′-dimycolate; TDM), a surface glycolipid, causes *M. tuberculosis* to grow in serpentine cords in vitro. Injection of cord factor/TDM into experimental animals induces both foreign-body and hypersensitivity granulomas [4,5]. Hypersensitivity lesions are the expression of DTH through induction of interleukin-12 (IL-12) and interferon γ (IFN-γ). TDM can mediate intracellular trafficking events, and influences macrophage

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