Phenotypic and genetic characterization of vancomycin-resistant enterococci from hospitalized humans and from poultry in Korea

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Abstract

Vancomycin resistant enterococci (VRE) isolates from humans (23 isolates) and poultry (20 isolates) were characterized by antibiotic susceptibility, vancomycin resistance transferability, pulsed-field gel electrophoresis (PFGE), and structural analysis of Tn*1546*-like elements. VRE isolates from humans and poultry showed different resistance patterns, transferability, and transfer rate. In addition to these phenotypic differences between humans and poultry VRE, PFGE and the structure of Tn*1546*-like elements were also distinct. Most poultry isolates (16/20) were identical to the prototype *vanA* transposon, Tn*1546*, while most human isolates (21/23) had multiple integrations of insertion sequence. The transmission of VRE and vancomycin resistance determinant between humans and poultry could not be demonstrated in this study.

Introduction

Vancomycin resistant enterococci (VRE) that are resistant to high level of vancomycin and teicoplanin cause serious illness resulting in limited therapeutic options in hospitalized patients. The VanA phenotype of VRE, which is the most common one found in humans as well as animals, is mediated by Tn*1546*-like elements. In Korea, avoparcin was used as a growth promoter in poultry and pig production from 1983 to 1997 as in Europe, and glycopeptide antibiotics, such as vancomycin and teicoplanin, were also widely used in human medicine, as in the United States (Kirst *et al.*, 1998; Shin *et al.*, 2003). Since VRE were first detected in human patients in 1992, the percentage of VRE increased from 4% in 1997 to 16% in 2002 according to the Korean Nationwide Surveillance of Antimicrobial Resistance Study (Lee *et al.*, 2004). However, there was only limited information on the correlation between VRE isolated from humans and animals in Korea (Yu *et al.*, 2003; Seo *et al.*, 2004).

In this study, the phenotypic characteristics of VRE from humans and animals were defined by minimum inhibitory concentrations (MICs) against various antibiotics and vancomycin resistance transferability by filter matings. In addition, whole genomic DNA and Tn*1546*-like elements of VRE were also analyzed by pulsed-field gel electrophoresis (PFGE), overlapping PCR, and sequencing analysis to determine the genetic correlation of VRE from humans and animals.

Materials and methods

Bacterial strains

A total of 43 *vanA* positive VRE, composed of 23 human and 20 poultry isolates from 1997 to 2000 in Korea, were investigated as shown in Table 1. CCARM strains from poultry were isolated from poultry farms in Gyeong-gi, 2000 and SN strains from poultry farms in Gyeong-gi, 1999. AJ strains from human were isolated from Ajou University Hospital in Gyeong-gi, 2000, KS strains from different university hospitals in Seoul and Gyeong-gi from 1997 to 1999, and CCARM strains from different university hospitals in Seoul and Gyeong-gi, 1999. All poultry strains were isolated from feces whereas all human isolates were from hospitalized patients. The *vanA* gene was confirmed by PCR according to the previous study (Dutka-Malen *et al.*, 1995).

DNA preparation and PCR amplification

The chromosomal DNA of the isolate was extracted by the guanidinium thiocyanated method. Amplification of DNA was performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany) using a *Taq* polymerase (Takara, Tokyo, Japan). The positions and sequences of PCR primers used in this study are described in Table 2. Previously characterized *vanA*-containing *Enterococcus faecium* BM4147 was served as a reference strain (Arthur *et al.*, 1993).

Antibiotic susceptibility test

The MICs of the antibiotics were determined by the agar dilution method according to the criteria of the National Committee for Clinical Laboratory Standards (NCCLS, now renamed the Clinical and Laboratory Standards Institute, CLSI, 2000). The tested drugs were as follows: vancomycin, teicoplanin, ampicillin, chloramphenicol, ciprofloxacin, erythromycin, penicillin, and tetracycline. High-level resistance to gentamicin was determined on the Mueller–Hinton agar (Becton Dickinson & Co., Sparks, MD) supplemented with gentamicin (500 $\mu g\,mL^{-1}$). *Enterococcus faecalis* ATCC 29212 was used as a quality control for antibiotic susceptibility.

Mating procedures

Filter matings were performed with a donor/recipient ratio of 1:4 as described previously (Tomita *et al.*, 2002). *Enterococcus faecium* BM4105RF, which is resistant to rifampin and fusidic acid, was used as a recipient strain in the conjugation experiments (Carlier & Courvalin, 1990). At the second conjugation from transconjugants, *E. faecium* BM4105SS, which is resistant to spectinomycin and streptomycin, was used as a recipient strain. All of the *vanA* isolates were susceptible to rifampin and fusidic acid, and all of the *vanA* transconjugants with *E. faecium* BM4105RF were additionally susceptible to spectinomycin and streptomycin.

Statistical analysis

Statistical analysis was performed by the Analyse-it program (Analyse-it Software, Ltd., Leeds, UK). χ^2-statistic and the one-tailed *P*-value by the two-tailed probability by the Fisher's Exact test were applied to analyze significant differences between the observed antibiotic susceptibility percentages of VRE isolates from different origins. The transfer rates of human and poultry VRE isolates were compared using an *F*-test.

PFGE of genomic DNA

Whole cell DNA in agarose plugs was digested with 20 U of *Sma*I (Roche Molecular Biochemicals, Mannheim, Germany) for 24 h. The gels were electrophoresed with a clamped homogeneous electric field (CHEF-DR-III, Bio-Rad, Richmond, CA). The total run time, switch time, and the voltage for the run were 20 h, 1.0–21.0 s, and 6 $V\,cm^{-1}$, respectively. The band patterns were analyzed using Quantity One ver. 4.6.0. (Bio-Rad).

DNA sequence analysis

PCR amplicons larger than those of the prototype *vanA* gene cluster of all isolates were sequenced (Bionics Co. Ltd, Seoul, Korea). The complete nucleotide sequence of IS*1216V*-IS*3*-like variant can be found in GenBank under accession no. AY916786.

Results and discussion

Antibiotic resistance and transferability of VRE

The antibiotic resistance patterns of the isolates are summarized in Table 1. The MICs of all the *vanA* isolates to vancomycin and teicoplanin were ≥ 512 and $\geq 32\,\mu g\,mL^{-1}$, respectively, indicating the VanA phenotype. Moreover, VRE had multidrug resistant characteristics (resistance to more than three antibiotics). In general, human and poultry isolates were resistant to ciprofloxacin (78% vs. 85%), erythromycin (91% vs. 65%), penicillin (87% vs. 75%), as well as tetracycline (78% vs. 95%), but they were susceptible to chloramphenicol (74% vs. 85%). However, a higher proportion of human isolates showed resistance to ampicillin (83%) and high level of gentamicin (70%) compared with animal isolates (25% and 20%, respectively) ($P < 0.01$). VRE isolates from humans showed a higher rate of multidrug resistance because of their stronger resistance to ampicillin and high-level resistance to gentamicin (Table 1).

Table 1. Genetic and phenotypic characteristics of *vanA* enterococci from humans and poultry

Strain number*/species	PFGE type	Tn*1546* type	No. of transconjugants per donor	MIC (μg mL^{-1}) Vm	Tc	Resistance profile
Poultry isolates						
CCARM5028/*E. faecium*	10	I	–[†]	≥1024	256	Vm, Tc, Cp, Em, Pn, Te
CCARM5030/*E. faecium*	9	I	–	≥1024	256	Vm, Tc, Cp, Em, Pn, Te
CCARM5041/*E. faecium*	7	I	–	≥1024	256	Vm, Tc, Cp, Em, Pn, Te
CCARM5043/*E. faecium*	7	I	–	≥1024	256	Vm, Tc, Cp, Em, Pn, Te
CCARM5044/*E. faecium*	9	I	–	≥1024	256	Vm, Tc, Cm, Cp, Em, Gm, Te
CCARM5046/*E. faecium*	10	I	–	≥1024	256	Vm, Tc, Cp, Em, Pn, Te
CCARM5048/*E. faecium*	14	I	–	≥1024	256	Vm, Tc, Cp, Em, Pn, Te
CCARM5049/*E. faecium*	10	I	–	≥1024	256	Vm, Tc, Cp, Em, Pn, Te
CCARM5050/*E. faecium*	14	I	5.8×10^{-4}	≥1024	256	Vm, Tc, Ap, Cp, Em, Pn, Te
CCARM5052/*E. faecium*	7	I	–	≥1024	256	Vm, Tc, Cp, Em, Pn, Te
CCARM5055/*E. faecium*	10	I	–	≥1024	256	Vm, Tc, Cp, Em, Pn, Te
CCARM5058/*E. faecium*	6	I	–	≥1024	128	Vm, Tc, Ap, Cp, Pn, Te
CCARM5060/*E. faecium*	18	I	–	≥1024	256	Vm, Tc, Ap, Cp, Pn, Te
CCARM5076/*E. faecium*	6	I	–	≥1024	256	Vm, Tc, Ap, Cp, Pn, Te
SN89-4/*E. faecium*	17	II	3.7×10^{-4}	≥1024	32	Vm, Tc, Cp, Gm, Pn, Te
SN100-1/*E. faecium*	17	I	5.6×10^{-5}	≥1024	32	Vm, Tc, Te
SN100-2/*E. faecium*	17	I	–	≥1024	32	Vm, Tc, Te
SN119-1/*E. faecium*	1	IVa	–	≥1024	32	Vm, Tc, Cm, Cp, Em, Gm, Te
SN128-1/*E. faecalis*	–[‡]	IVa	–	≥1024	32	Vm, Tc, Cm, Em, Gm
SN154-1/*E. faecium*	18	III	–	≥1024	128	Vm, Tc, Ap, Cp, Em, Pn, Te
Human isolates						
AJ22/*E. faecium*	11	VIII	1.2×10^{-3}	512	512	Vm, Tc, Ap, Cm, Cp, Em, Gm, Pn, Te
AJ38/*E. faecium*	11	IX	1.6×10^{-4}	512	512	Vm, Tc, Ap, Cp, Gm, Pn
AJ43/*E. faecium*	13	VIIc	4.9×10^{-3}	512	512	Vm, Tc, Ap, Cp, Em, Gm, Pn
AJ48/*E. faecalis*	–	VIII	–	512	256	Vm, Tc, Ap, Cp, Em, Gm, Pn
AJ59/*E. faecium*	5	VIc	7.4×10^{-3}	512	512	Vm, Tc, Ap, Cp, Em, Gm, Pn
AJ64/*E. faecalis*	–	VIb	–	512	512	Vm, Tc, Cp, Em, Gm, Te
KS1/*E. faecium*	19	VIIa	2.4×10^{-3}	≥1024	256	Vm, Tc, Ap, Cp, Em, Pn, Te
KS2/*E. faecium*	19	V	1.1×10^{-4}	≥1024	256	Vm, Tc, Ap, Cp, Em, Pn, Te
KS3/*E. faecium*	4	VIIa	6.1×10^{-3}	512	256	Vm, Tc, Ap, Cp, Em, Gm, Pn, Te
KS4/*E. faecium*	15	VIIa	7.2×10^{-5}	≥1024	256	Vm, Tc, Ap, Cp, Em, Gm, Pn, Te
KS5/*E. faecium*	15	VIIa	4.3×10^{-5}	≥1024	256	Vm, Tc, Ap, Cp, Em, Gm, Pn, Te
KS6/*E. faecium*	15	VIIa	3.3×10^{-3}	≥1024	256	Vm, Tc, Ap, Em, Gm, Pn, Te
KS7/*E. faecium*	2	VIIb	8.6×10^{-3}	≥1024	512	Vm, Tc, Ap, Cm, Cp, Em, Gm, Pn, Te
KS8/*E. faecium*	14	VIIa	1.0×10^{-3}	≥1024	512	Vm, Tc, Ap, Cp, Gm, Pn, Te
CCARM5002/*E. faecium*	8	I	–	≥1024	256	Vm, Tc, Ap, Cp, Em, Pn, Te
CCARM5003/*E. faecium*	16	VIIa	7.1×10^{-1}	≥1024	256	Vm, Tc, Ap, Em, Gm, Pn, Te
CCARM5005/*E. faecium*	3	IVb	–	≥1024	256	Vm, Tc, Ap, Em, Pn, Te
CCARM5007/*E. faecium*	13	VIIa	0.9×10^{-3}	≥1024	256	Vm, Tc, Ap, Cp, Em, Pn
CCARM5008/*E. faecium*	12	VIIa	8.7×10^{-3}	≥1024	256	Vm, Tc, Ap, Cp, Em, Gm, Pn, Te
CCARM5009/*E. faecalis*	–	IVa	–	≥1024	256	Vm, Tc, Cp, Em, Pn, Te
CCARM5010/*E. faecium*	5	VIa	2.1×10^{-3}	≥1024	256	Vm, Tc, Ap, Cp, Em, Gm, Pn, Te
CCARM5011/*E. faecalis*	–	VIIa	3.0×10^{-5}	≥1024	512	Vm, Tc, Em, Gm, Te
CCARM5024/*E. faecalis*	–	I	4.6×10^{-3}	≥1024	256	Vm, Tc, Em, Te

*Strain sources are as follows: CCARM, Culture Collection of Antibiotic Resistant Microbes, Seoul, Korea; SN, Seoul National University, Seoul, Korea (Seo *et al.*, 2004); AJ, Ajou University School of Medicine, Suwon, Korea; KS, National Institute of Health, Seoul, Korea.

[†]Not transferable.

[‡]*Enterococcus faecalis* were not included in PFGE analysis.

Vm, Vancomycin; Tc, teicoplanin; Ap, ampicillin; Cm, chloramphenicol; Cp, ciprofloxacin; Em, erythromycin; Gm, high-level resistance to gentamicin; Pn, penicillin; Te, tetracycline.; *E. faecium, Enterococcus faecium*; PFGE, pulsed-field gel electrophoresis; MIC, minimum inhibitory concentration.

These findings probably reflect the differences in the selective pressure exerted by antibiotic usage in different environments.

Moreover, the transferability of vancomycin resistance between enterococci was confirmed in most human isolates (18/23) via mating experiments whereas only three poultry

Table 2. Primers used in this study

Primer	Nucleotide sequence (5′ → 3′)	Position*	Reference
Tn*1546* primers			
orf1 F1	ACGTTAAGAAAGTTTTAGTGG	72–92	This study
orf1 R3	ACGCACCATACAGCATCA	324–307	This study
orf1 R2	GACACTGCCGGTTACACT	773–756	This study
orf1 F2	CATACATGCGCCATTGAGATA	1085–1105	This study
orf1 R1	GCCCTTTTAGGAATGG	1190–1175	This study
orf2 F1	CTTGCTTCCCACACCATT	2524–2541	This study
orf2 R1	GTTAGTCCATCCTCGCTTGAT	2780–2760	This study
orf2 F2	GCCATTCTGTATTCCGCTAA	3762–3781	This study
vanR F1	ATGAGCGATAAAATACTT	3976–3993	This study
vanR R1	GGCAATTTCATGTTCATCATC	4020–4000	This study
vanS F1	TTGGTTATAAAATTGAAAAATAA	4649–4671	This study
vanS R1	TTAGGACCTCCTTTTATC	5803–5786	This study
vanH F1	ATGAATAACATCGGCATTAC	6018–6037	This study
vanH R1	CTATTCATGCTCCTGTCT	6986–6968	This study
vanA F	GGGAAAACGACAATTGC	7153–7170	Dutka-Malen *et al.* (1995)
vanA R	GTACAATGCGGCCGTTA	7885–7869	Dutka-Malen *et al.* (1995)
vanX F1	ATGGAAATAGGATTTACTTT	8016–8035	This study
vanX F2	CGAATGGTGGCACTATGT	8555–8572	This study
vanX R1	TTATTTAACGGGGAAATC	8624–8607	This study
vanY F1	ATGAAGAAGTTGTTTTTTTTA	9052–9072	This study
vanY R1	TTACCTCCTTGAATTAGTAT	9963–9944	This study
vanZ R1	CTTACACGTAATTTATTC	10602–10585	This study
IS*1216V*-IS*3*-like primers			
IS1216V F6	ACGACTACCAGCAACCAAG	68–86	This study
IS1216V F1	GCCGTGGGCTACTATCTT	287–304	This study
IS1216V F2	TTATTTGTATCGAGCCATC	499–517	This study
IS1216V R1	GGTAGAGGCAGTGCGTAA	802–785	This study
IS1216V F4	TGGAAGCCATTCGAGGA	816–832	This study
IS1216V R4	ATTCCACTTCTTGTCTAACCC	1195–1174	This study
IS1216V F5	AAAATCAAGGCATACATCGC	1948–1968	This study
IS*1542* primer			
IS1542 F2	AACGTCCTCCTGGGTATG	98–81	This study
IS*19* primer			
IS19 F3	CCTCATTTCTTGGGAGTTT	103–85	This study

*The positions of the Tn*1546*, IS*1216*V-IS*3*-like, IS*1542*, and IS*19* primers are based on the sequence of Tn*1546*, IS*1216*V-IS*3*-like, IS*1542*, and IS*19* (GenBank accession nos M97297, L40841, AF114715, and AF169285, respectively).

isolates showed transferability with a significantly low transfer rate ($P < 0.05$). This result suggests that vancomycin resistance determinant transmission from poultry might rarely happen.

PFGE profiles

A total of 37 *vanA E. faecium* from humans (18 isolates) and poultry (19 isolates) were genotyped by PFGE (Fig. 1). PFGE analysis using Quantity One ver. 4.6.0. revealed extensive heterogeneity with no PFGE-deduced genetic overlap between human and poultry isolates and differentiated all isolates into 19 types with less than 60% similarity (Fig. 1). There was no prevalent type, even among the same sources. The highest similarity value shown between poultry isolate CCARM5050 and human isolate KS8 was 63%.

Structural analysis of Tn*1546*-like elements

All isolates were divided into nine main types on the basis of the patterns of ISs inserted into Tn*1546*-like elements. Overall, human and poultry isolates belonged to different types; most poultry isolates (16/20, 80%) were identical to the prototype *vanA* transposon, Tn*1546*, while most human isolates (21/23, 91%) had multiple integrations of ISs (Fig. 2).

The majority of IS*1216V* inserted in the *vanX-vanY* intergenic region of isolates (AJ22, AJ37, AJ38, AJ48, KS1, KS2, KS4, KS5, KS6, KS8, CCARM5003, CCARM5007, CCARM5008, CCARM5009, CCARM5010, and CCARM5011) had a synonymous point mutation (T → C) at position 826 based on the sequence of IS*1216V* (GenBank accession no. L40841), which is an identical observation to the previous study (Willems *et al.*, 1999). All isolates with IS*1216V* insertions in the *vanX-vanY* intergenic region, in

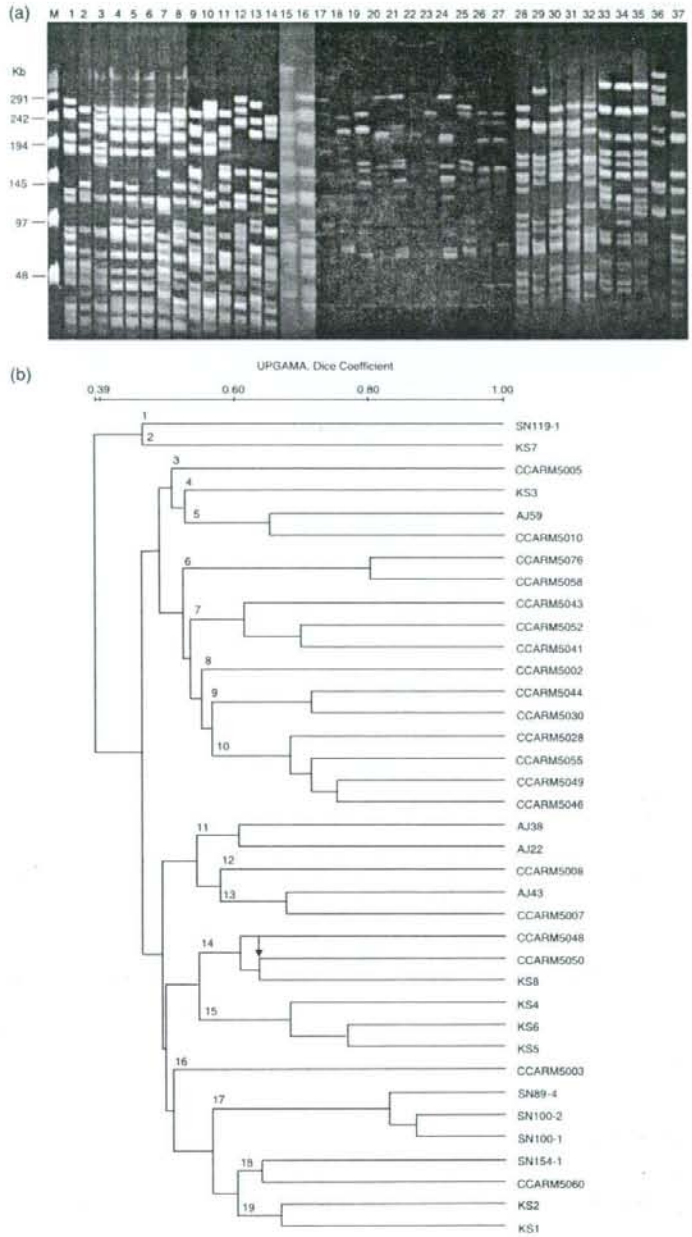

Fig. 1. Pulsed-field gel electrophoresis and dendrogram analysis of *Sma*I-digested whole genomic DNAs of 37 *vanA Enterococcus faecium* from hospitalized patients (18) and poultry (19). (a) PFGE: Lane M, molecular weight marker; lanes 1–8, *E. faecium* KS1–KS8; lane 9, *E. faecium* CCARM5002; lane 10, *E. faecium* CCARM5003; lane 11, *E. faecium* CCARM5005; lane 12, *E. faecium* CCARM5007; lane 13, *E. faecium* CCARM5008; lane 14, *E. faecium* CCARM5010; lane 15, *E. faecium* AJ22; lane 16, *E. faecium* AJ38; lane 17, *E. faecium* AJ43; lane 18, *E. faecium* AJ59; lane 19, *E. faecium* CCARM5028; lane 20, *E. faecium* CCARM5041; lane 21, *E. faecium* CCARM5043; lane 22, *E. faecium* CCARM5046; lane 23, *E. faecium* CCARM5049; lane 24, *E. faecium* CCARM5052; lane 25, *E. faecium* CCARM5055; lane 26, *E. faecium* CCARM5058; lane 27, *E. faecium* CCARM5076; lane 28, *E. faecium* CCARM5030; lane 29, *E. faecium* CCARM5044; lane 30, *E. faecium* CCARM5048; lane 31, *E. faecium* CCARM5050; lane 32, *E. faecium* CCARM5060; lane 33, *E. faecium* SN89-4; lane 34, *E. faecium* SN100-1; lane 35, *E. faecium* SN100-2; lane 36, *E. faecium* SN119-1; lane 37, *E. faecium* SN154-1. Strains KS were from Korea Center for Disease Control and Prevention, CCARM from Culture Collection of Antibiotic Resistant Microbes, AJ from Ajou University School of Medicine, and SN from Seoul National University. (b) Dendrogram of PFGE analysis: PFGE patterns were analyzed using Quantity One ver. 4.6.0. by the unweighted pair group method with average linkages (UPGAMA) (Bio-rad). Nineteen groups were made on the basis of dice coefficient, 0.6. The PFGE type (1–19) is shown as bold letter on dendrogram. The arrow indicates the highest level of similarity between *vanA E. faecium* from poultry and hospitalized humans (CCARM5050 vs. KS8).

which the IS insertions were accompanied by adjacent deletions, had an 8 bp duplication of the target sequence, CTTCCAAC in type IVa and CCCATTGT in types IVb, VIa, VIb, VIIa, and VIIb. Interestingly, all of the IS*1216V* detected in the *vanX-vanY* intergenic region were inserted reversely, which is commonly reported in Europe unlike other Korean studies where IS*1216V* was found to be inserted directly in the *orf1* region, *orf2-vanR* and *vanX-vanY* intergenic region (Willems *et al.*, 1999; Huh *et al.*, 2004).

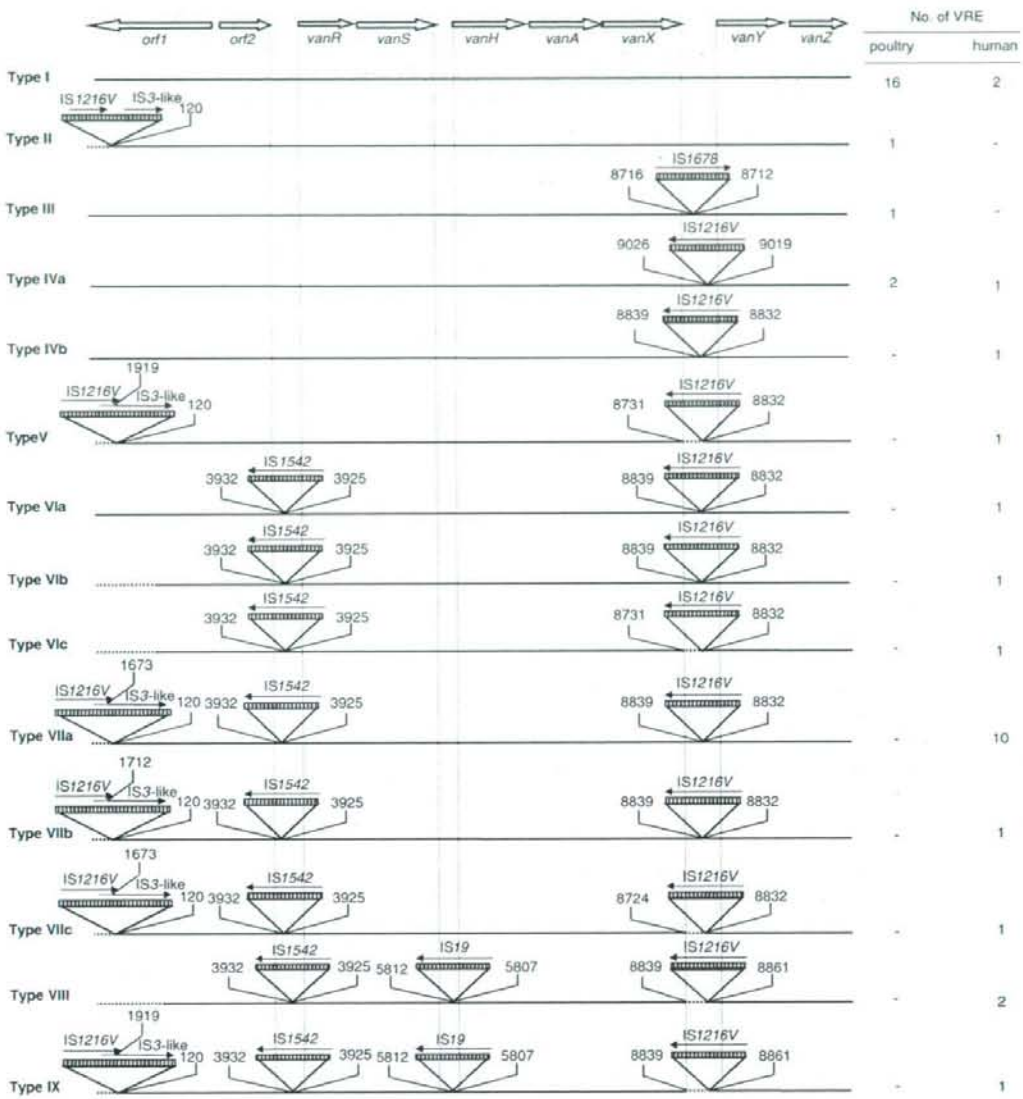

Fig. 2. Genetic maps of the Tn*1546*-like elements of VRE isolates from humans and poultry. Open arrows indicate the positions and transcription directions of genes and *orf*. Lined boxes indicate IS elements. The positions of the first nucleotide upstream and the first nucleotide downstream from the IS insertion sites are depicted. Lined arrows upside the ISs indicate the transcriptional orientation of the inserted IS elements. Deletion in Tn*1546*-like elements is indicated by dotted lines. The vertical dotted lines indicate the boundaries of the different *orf* of Tn*1546*-like elements.

The insertion of IS*1542* in the *orf2-vanR* intergenic region induced the 8 bp duplication of the target sequence (CTA-TAATC), and the insertion of IS*1678* in the *vanX-vanY* intergenic region in Type III also induced the 5 bp duplication (ATATA). A single nucleotide change (G → T) at position 8234 in *vanX* gene was found in only three poultry isolates, SN89-4, SN100-1, and SN100-2, but not in human isolates.

We found variations of Tn*1546*-like elements by sequence analysis, which may reflect the unique characteristics of VRE

in Korea. First, the 5′ end of IS3-like located at the left end of Tn*1546* (Type V, VII, and IX) had the deletions at various positions (Fig. 2), mutations (GA → AG) at positions 1831 and 1832 of Type II and VII, and additional single nucleotide insertion (C) at position 2014, unlike the sequence of IS*1216V*-IS3-like (GenBank accession no. L40841). Moreover, Type II had 310-bp of additional sequences between IS*1216V* and IS3-like (GenBank accession no. AY916786). Secondly, all of the IS*19* insertion in the *vanS-vanH* intergenic region (Type VIII and IX) also contained C to T, G to A, and A to G point mutations at positions 15, 568, and 782, respectively, and a slightly modified-single nucleotide mutation at the duplication region of integration site (GATG-TAT → TATGTAT) compared with the sequence of IS*19* (GenBank accession no. AF169285) and the first study reporting IS*19* integration (Huh *et al.*, 2004). Finally, we found a new IS-like element, IS*1678*, in the *vanX-vanY* intergenic region of the poultry isolate (Type III, SN154-1) (Jung *et al.*, 2005).

The characteristics of human isolates, including a heterogeneity of the PFGE pattern, the predominance of the Tn*1546*-like element Type VIIa, and the transferability of most isolates (18/23, 78%), suggest that the main contributory factor of the prevalence of VRE in humans might be the horizontal dissemination of the Tn*1546*-like element in hospitals rather than the clonal spread of VRE itself. The human (KS8) and poultry (CCARM5050) isolates which showed the highest similarity (63%) in PFGE analysis belonged to different transposon types, and their antibiotic resistance patterns were also different (Table 1). These findings strongly suggest that they may have been independent strains.

In conclusion, this study reveals a degree of diversity between human and poultry VRE in Korea. Although a small number of VRE were analysed in this study, our findings suggest that the occurrence of VRE in humans and poultry developed independently in Korea. Further investigations using more VRE recently isolated from both origins are underway to provide more information on epidemiological and evolutionary events of VRE dissemination in Korea.

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Author contribution

The authors W.K.J. and S.K.H. contributed equally to this study.

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

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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).

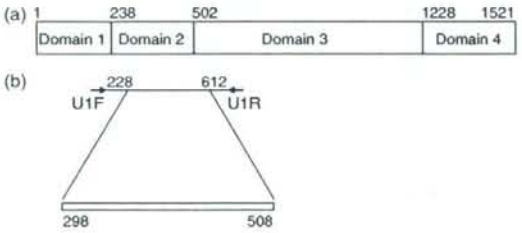

Fig. 1. Schematic representation of the DnaA protein and primer design for the amplification of the partial mycobacterial *dnaA* gene. Number indicates the nucleotide position of *Mycobacterium tuberculosis*, GenBank accession number AL021427. (a) The DnaA protein from *M. tuberculosis* contains four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

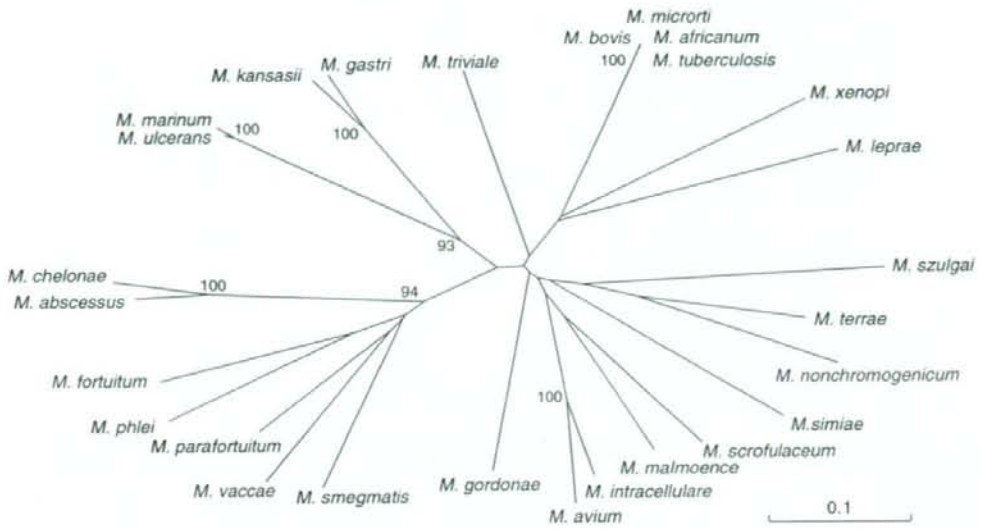

Fig. 2. Phylogenetic relationship of 27 *Mycobacterium* species. Unrooted tree based on the *dnaA* sequences. The tree was generated from DNASpace (Hitachi Software Engineering) with the Clustal W algorithm. The numbers on the dendrogram indicate the percentages of occurrence in 1000 bootstrapped trees; 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		Gas 583	
F3	CGATGATTCCGGCCTGGA	F3	AGCCCGGCCTGGAAAT
B3	GTTGAGGCTGGTTCCGC	B3	GTGCGAACCGATTGGAGG
FIP	TCTCGTCGGCGTCGTCGGTATGTCACGGGAAACGTCAC	FIP	TGGGCCAATTGGGTCGGGGCCGGGAACCCGAAACCATC
BIP	GACCCGGTTGGCCCACCTAGCAGCGACCGTATCGGC	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

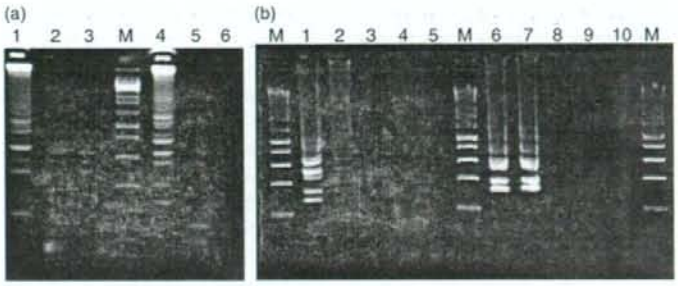

Fig. 4. (a) Four percent agarose gel electrophoresis and restriction enzyme analysis of loop-mediated isothermal amplification (LAMP) products of partial *dnaA* gene of *Mycobacterium kansasii* and *Mycobacterium gastri*. Lanes: M, 100 bp DNA ladder; lanes 1–3, LAMP carried out with *M. kansasii* primer,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

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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-1β, 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-1β. 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-1β acts at early stages of differentiation of DC and impairs biological functions of DC at later stages. Therefore, production of IL-1β 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-κB [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-1β as first-line mediators of defense [5, 19].

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

Here, we show that IL-1β 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-1β can explain at least partially the persistence of mycobacteria in host cells.

Results

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

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

To this end, we pretreated monocytes with IL-1β 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-1β pretreated monocytes was markedly impaired. Inhibition depended on the IL-1β concentration used for pretreatment of monocytes. Significant inhibition was achieved at ≥ 100 pg/mL IL-1β. Similarly, IL-1β 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-1α impaired APC functions of DC in a similar manner as IL-1β, 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-1β-pretreated monocytes

In an attempt to characterize DC derived from IL-1β-pretreated monocytes, we determined surface markers of immature DC (Fig. 1). Immature DC derived from monocytes, which had been pretreated with IL-1β 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-1β pretreatment of monocytes. In contrast, CD86 and CD83 were down-regulated by IL-1β 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-1β 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-1β or BCG pretreatment of monocytes did not alter the expression of TLR2 and TLR4 (Fig. 2C). Finally, IL-1β pretreatment of monocytes did not cause apoptosis of DC (data not shown). Thus, DC differentiated from IL-1β pretreated monocytes were only partially activated.

Table 1. IL-1β production after stimulation of mononuclear phagocytes with BCG[a)]

Cell type	IL-1β (pg/mL) production after stimulation with BCG at MOI:			
	0	0.0625	0.25	1.0
Monocytes	2.2 ± 0.2	23.7 ± 4.6*b)	229.4 ± 19.1**b)	861.4 ± 22.3***b)
Macrophages	3.3 ± 0.4	4.0 ± 1.1	34.6 ± 2.8**	163.3 ± 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 ± 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-1β pretreated monocytes[a)]

IL-1β pretreatment of monocytes (pg/mL)	*M. leprae* 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 ± 0.0	0.2 ± 0.0
0	20	206.7 ± 15.1	50.7 ± 4.9
30	0	0.4 ± 0.0	0.3 ± 0.1
30	20	192.8 ± 19.3	24.9 ± 11.2
100	0	0.3 ± 0.1	0.6 ± 0.2
100	20	50.9 ± 6.1[*b)]	13.4 ± 0.3[*]
300	0	0.7 ± 0.4	0.8 ± 0.7
300	20	30.3 ± 2.6[*]	9.7 ± 1.9[**b)]
1000	0	0.3 ± 0.1	0.4 ± 0.2
1000	20	9.5 ± 2.1[*]	3.0 ± 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-1β-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 ± SD.

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

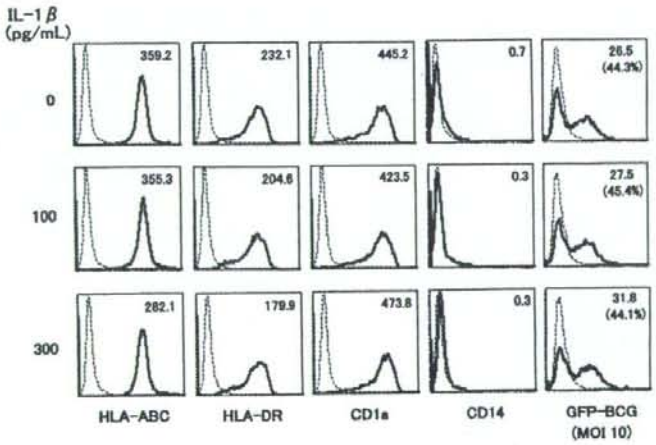

Figure 1. Phenotype and phagocytic activity of immature DC differentiated from IL-1β pretreated monocytes. Plastic adherent monocytes were pretreated with the indicated doses of IL-1β and were subsequently differentiated into DC by 3-day culture with rGM-CSF and rIL-4. For analysis of phagocytic activity 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-1β pretreatment of monocytes on IL-12 production by DC

In order to determine whether DC differentiated from IL-1β 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-1β failed to produce significant amounts of IL-12 (Fig. 3A). Similar results

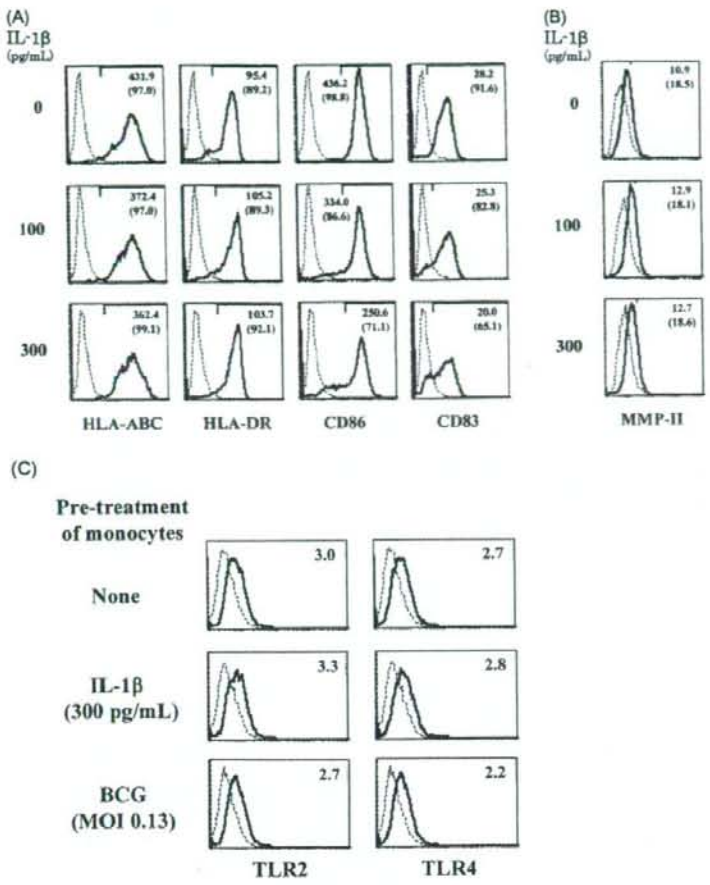

Figure 2. (A) Phenotype of mature DC differentiated from monocytes pretreated with IL-1β. Plastic adherent monocytes were pretreated with the indicated doses of IL-1β and were subsequently differentiated into DC by 5-day culture with rGM-CSF and rIL-4. Cells were treated with LPS (25 ng/mL) on day 4 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 IgM; 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-1β or BCG. Plastic adherent monocytes were pretreated with the indicated doses of IL-1β 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-1β 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-1β (Fig. 3C). Also, both rIL-1β (Fig. 3D) and BCG (Fig. 3E) induced IL-12p70 production only in DC derived from IL-1β untreated-monocytes. The failure to produce IL-12p70 was long lasting as DC differentiated from IL-1β 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-1β signaling in monocytes but not IL-6, TNF-α or IL-10 signaling, inhibited IL-12 secretion by DC. Subsequently, IL-1β 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-1β 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-1β treatment significantly reduced IL-12p70 production (Table 4).

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

We assessed the influence of endogenously produced IL-1β 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-1β production in monocytes at concentrations up to