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Recent advances in the molecular epidemiology of leprosy

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Recent advances in the molecular epidemiology of leprosy through genotyping of variable number tandem repeats (VNTRs) and single nucleotide polymorphisms (SNPs) are described. VNTRs with a broad range of diversity are useful genotyping tools for analyzing transmission in community areas, and SNPs and VNTRs with a small degree of variation are favorable for investigating the global transmission of leprosy. We expect that the transmission of leprosy can be fully analyzed by the application of these new methodologies.

Introduction

The number of newly detected cases of leprosy has been gradually decreasing around the world in recent years ¹⁾. Even so, some details of disease transmission remain to be elucidated. One of the important subjects still under dispute, and essential for establishing a better strategy for preventing new cases, is the transmission mode of leprosy including the infectious source. Phenotype, serotype, phage type and genotype are robust metrics for analyzing and tracing transmission in many infectious diseases ^{2,3)}. These useful tools were not available

for understanding the transmission of leprosy until two kinds of variable number tandem repeat (VNTR) were identified ^{4,5)}, followed by the discovery of single nucleotide polymorphisms (SNPs) in the *Mycobacterium leprae* genome ⁶⁾. Epidemiological studies of leprosy using these two assays have been gradually accumulating since 2004, and findings generated by these breakthroughs in molecular epidemiology are now taking the place of classical epidemiological methods. An international study group for investigating the transmission of leprosy through the application of advances in molecular biology, including genotyping, has been organized and collaborative studies have begun ⁷⁾. VNTRs with broad allelic diversity are useful for analyzing transmission patterns in the community ⁸⁻¹¹⁾. SNPs and VNTRs with a narrow range of diversity are practical for characterizing the global geographical distribution of different genotypes of *M. leprae*,

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and are beneficial in tracing the dissemination of leprosy in prehistoric times and following current global leprosy migration patterns¹²⁻¹⁶. This article describes recent advances in the molecular epidemiology of leprosy.

Application of VNTRs to Analyze the Transmission of Leprosy

It has long been believed that leprosy is transmitted by heavy contact with leprosy patients; however, there are some findings which contradict this premise. For instance, a study on the geographical distribution of responders to *M. leprae* antibodies showed a distribution of positive responders in houses remote from those with known patients¹⁷. The limited effect of chemoprophylaxis¹⁸ means that drugs prevent the progression of an active infection to overt disease¹⁹ but do not interrupt transmission. Attempts to curtail transmission and reduce new infections by chemoprophylaxis in the Federated States of Micronesia (FMS) failed. The number of new cases detected in FMS has increased again after 1999 and over the last several years has risen to nearly the same level as before execution of the chemoprophylaxis program¹¹. Many new cases without known contact with leprosy patients^{20, 21} have also been reported. These findings suggest gaps in the traditional concept of leprosy transmission and imply the existence of an infectious source of *M. leprae* other than leprosy patients.

PCR studies have shown that in areas with a high prevalence of leprosy, many individuals harbor *M. leprae* in their nasal mucus^{22, 23}. These findings indicate that people in such areas are frequently exposed to *M. leprae* infection. The hypothesis that the bacterial genotypes from household members or other contacts of patients should be identical if the bacilli are transmitted by contact

with the patient, was tested by a comparison of genotypes from the nasal mucus of patients and family members, and also from new patients and the supposed index case of a multifamily case⁸. Genotyping based on the copy number of a TTC repeat^{5, 8} and another genotyping method with higher discriminatory power were applied¹⁰. Results revealed that the genotype of *M. leprae* in the nasal mucus differed between the patient and contacts in a given household. The existence of *M. leprae* strains with allelic diversity in a multifamily case in China was also shown¹¹. On the other hand, a study conducted in India revealed no divergence among the three VNTRs from two patients in a multifamily case⁹. The lack of divergence among the patients of this case could be explained by the low discriminatory power of genotyping using only three polymorphic VNTRs. In another study, isolates determined as having identical genotypes using a few VNTRs were separated into different genotypes by a method with high resolution power using a combination of other appropriate VNTRs¹⁰. Thus, we theorize that the Indian clinical isolates with identical genotypes might be classified into different genotypes with the improved methodology. Although VNTRs seem to fluctuate in biopsy samples from the same patient^{24, 25}, co-infection of *M. leprae* strains with different VNTRs may occur. While the variation of VNTRs in a given patient is often by only one or two copies, the TTC copy number of the isolate from a supposed index case was 18 and the TTC copy number of the bacilli from his son was 10 in one multifamily case report⁸. It is clear that the genotypes of the bacilli from father and son were distinct and hence did not come from the same infectious source⁸. This suggests the presence of an alternate source of infection.

Water sources used daily by people in an endemic area are thought to be a potential source

of *M. leprae* infection, since many water-borne mycobacterial infectious diseases exist^{26, 27, 28)}. Water samples collected at 6 villages with a high prevalence of leprosy in Indonesia were assayed by PCR. Of 44 water samples, 21 were positive by PCR assay for a 65 kDa protein coding sequence²⁹⁾. The relative risk of leprosy infection was 3.24 for those using the PCR-positive water source for washing and bathing, but no difference was shown in risk for water used for drinking and cooking. Water is heated for drinking and cooking, and consequently bacilli in the water are inactivated²⁹⁾. PCR positivity does not prove the existence of live *M. leprae* in the water. However, reverse transcription-PCR (RT-PCR) targeting 16S rRNA did detect the presence of *M. leprae* organisms³⁰⁾. This method was used to analyze the water from wells in a village on the Indonesian island of North Sulawesi that has many newly detected patients every year. Of 51 well water samples, 17 were positive by RT-PCR³¹⁾. These results strongly suggest that water is an environmental reservoir of *M. leprae* and an infectious source of leprosy. Possible infection from the soil has also not been ruled out³²⁾.

Application of SNPs and VNTRs with a Small Range of Diversity to Analyze the Global Dissemination of Leprosy

Using a VNTR with either 3 or 4 copies of a 6-bp sequence, a pattern in the global geographical distribution of *M. leprae* has been found^{4, 12, 13, 15, 16)}. The 3-copy strain is overwhelmingly predominant in most countries around the world, while the 4-copy strain is predominantly found in East Asia, including the mainland of Japan, Korea and eastern parts of China, as well as western parts of Mexico^{4, 12-14)}. No 4-copy isolates have been obtained from the Okinawa and Amami Islands

of Japan. These islands are located at the southern end of Japan and have their own unique culture. The presence of different genotypes between these two regions of Japan is also found in other species of microorganisms^{33, 34)}. It is thought that the predominance of the 4-copy strain in East Asian countries was established in prehistoric times by the movement of Mongoloid people in the region. In Japan, it is likely that the characteristic distribution of each genotype was established by the movement of two ethnic groups which migrated to Japan about 30,000 years ago through the Okinawa Islands, and 2,800 to 1,700 years ago through the Korean peninsula³⁵⁾. Specifically, *M. leprae* of the 4-copy strain might have been carried by the people who migrated to Japan through the Korean Peninsula, while the 3-copy strain was spread to the Okinawa Islands by the people who migrated to Japan from Southeast Asia 30,000 years ago. This example demonstrates how VNTRs with small range of polymorphic variation, such as that of the *rpoT* gene, are useful in tracing the global transmission of leprosy.

The geographical distribution of *M. leprae* SNP alleles 1-4 is also suitable for analyzing the global spread of leprosy⁶⁾. An assay using a combination of SNPs and the *rpoT* gene VNTR was applied to determine whether Japanese Brazilian patients newly diagnosed in the mainland of Japan had been infected in Japan or Brazil¹⁴⁾. SNP type 4, detected in two Japanese Brazilian patients, was not found among 46 Japanese patients from the mainland. The isolate with SNP type 3 and the 3-copy polymorphism of the *rpoT* gene, found in 7 isolates from Japanese Brazilian cases, is also uncommon in mainland Japan. Thus it is most likely that the patients were infected in Brazil and developed symptoms of leprosy after they came to Japan. It had been generally assumed that this was the case since the prevalence of leprosy is high in

Brazil, but there was no substantial microbiological evidence to support the idea. The assay combining SNPs and the *mpoT* gene VNTR clearly contributed to the analysis of the global transfer of leprosy incident to the current movement of people.

The distribution of each SNP genotype in Asian countries was also examined in another study¹⁴⁾. Most of the isolates (26 of 29) from Myanmar had SNP type 1. The frequency of this SNP declines in proportion to the distance of the countries from India, as is seen by low proportions in Indonesia, Korea and Japan. Logically, the SNP type 2 isolate could be derived from a type 1 progenitor, followed by evolution to type 3 and type 4 derivatives. All isolates from the southern part of India had SNP type 1⁶⁾. Taken together with data from other studies, it seems likely that leprosy originated in the Indian subcontinent and spread outward to other areas of the world. This idea is compatible with the argument that leprosy originated in the Indian subcontinent and was introduced to Egypt as a result of Persian invasion during the sixth century BC or by the troops of Alexander the Great in the fourth century BC³⁶⁾.

The use of molecular epidemiology through the application of genotyping as a means of tracking the transmission of leprosy began just 4 years ago in 2004, on the basis of a milestone discovery of two VNTRs in the genomic DNA of *M. leprae*. This new field of leprosy research is expected to illuminate obscure mechanisms of leprosy transmission with respect to sources, reservoirs, modes of transmission and the history of leprosy.

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The *Mycobacterium avium* Complex *gftB* Gene Encodes a Glucosyltransferase Required for the Biosynthesis of Serovar 8-Specific Glycopeptidolipid[†]

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Mycobacterium avium complex (MAC) is one of the most common opportunistic pathogens widely distributed in the natural environment. The 28 serovars of MAC are defined by variable oligosaccharide portions of glycopeptidolipids (GPLs) that are abundant on the surface of the cell envelope. These GPLs are also known to contribute to the virulence of MAC. Serovar 8 is one of the dominant serovars isolated from AIDS patients, but the biosynthesis of serovar 8-specific GPL remains unknown. To clarify this, we compared gene clusters involved in the biosynthesis of several serovar-specific GPLs and identified the genomic region predicted to be responsible for GPL biosynthesis in a serovar 8 strain. Sequencing of this region revealed the presence of four open reading frames, three unnamed genes and *gftB*, the function of which has not been elucidated. The simultaneous expression of *gftB* and two downstream genes in a recombinant *Mycobacterium smegmatis* strain genetically modified to produce serovar 1-specific GPL resulted in the appearance of 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl-glucose, which is unique to serovar 8-specific GPL, suggesting that these three genes participate in its biosynthesis. Furthermore, functional analyses of *gftB* indicated that it encodes a glucosyltransferase that transfers a glucose residue via 1→3 linkage to a rhamnose residue of serovar 1-specific GPL, which is critical to the formation of the oligosaccharide portion of serovar 8-specific GPL. Our findings might provide a clue to understanding the biosynthetic regulation that modulates the biological functions of GPLs in MAC.

Mycobacteria are pathogens that cause diseases such as tuberculosis and leprosy. In addition, nontuberculous mycobacteria, which are widely distributed in the natural environment, cause opportunistic pulmonary infections resembling tuberculosis. These mycobacteria are distinguished by a multilayered cell envelope consisting of peptidoglycan, mycolyl arabinogalactan, and surface glycolipids (9, 13). The surface glycolipids are abundant and structurally different, and they may act as a barrier to immune responses (9, 13). Glycopeptidolipids (GPLs) are major glycolipid components present on the surface of several species of nontuberculous mycobacteria (40). All of these GPLs have a conserved core structure that is composed of a fatty acyl tetrapeptide glycosylated with 6-deoxytalose (6-d-Tal) and *O*-methyl-rhamnose (*O*-Me-Rha) and are termed non-serovar-specific GPLs (nsGPLs) (2, 4, 14). On the other hand, the GPLs of *Mycobacterium avium* complex (MAC), nontuberculous mycobacteria consisting principally of two species, *M. avium* and *M. intracellulare*, have various haptenic oligosaccharides linked to the 6-d-Tal residue of nsGPLs, resulting in serovar-specific GPLs (ssGPLs) (2, 4, 40). The oligosaccharide portions of ssGPLs define MAC serovars that are classified

into 28 types. The serovar 1-specific GPL, with Rha linked to the 6-d-Tal residue, is the basic oligosaccharide unit of all ssGPLs (11). The Rha residue of serovar 1-specific GPL is further extended by various glycosylation steps, such as rhamnosylation, fucosylation, and glucosylation (11). These glycosylation steps generate structural diversity in GPLs of MAC (11). However, because of their complexity, most of the biosynthetic pathways for ssGPLs have not been fully determined. We recently showed that the biosynthesis of nsGPLs was regulated by a combination of glycosyltransferases (31). Therefore, each glycosyltransferase might mediate a specific step in the biosynthesis of ssGPLs.

In terms of biological activity, it has been reported that the properties of ssGPLs are notably different from each other and that some of the properties play a role in affecting host responses to MAC infections (3, 5, 21, 27, 37, 38). Moreover, epidemiological studies have shown that serovars 1, 4, and 8 are distributed predominantly in North America and are also frequently isolated from AIDS patients (24, 39, 41). However, in contrast to other ssGPLs, the serovar 8-specific GPL is reported to be able to induce altered immune responses (3, 21). The biosynthetic pathway for serovar 8-specific GPL, particularly its oligosaccharide portion that includes a unique 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl-glucose (Glc) residue (7, 8) that may determine the specificity of serovar 8, remains unknown (Table 1). In this study, we investigated the genomic region assumed to be associated with the biosynthesis of GPL in MAC serovar 8 strain and identified the genes involved in

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TABLE 1. Oligosaccharide structures of serovar 1- and 8-specific GPLs

Serovar	Oligosaccharide	Reference(s)
1	α -L-Rha-(1 \rightarrow 2)-L-6-d-Tal	17
8	4,6-O-(1-carboxyethylidene)-3-O-methyl- β -D-Glc-(1 \rightarrow 3)- α -L-Rha-(1 \rightarrow 2)-L-6-d-Tal	7, 8

the glycosylation pathway leading to the formation of serovar 8-specific GPL.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and DNA manipulation. Table 2 shows the bacterial strains and vectors used in this study. MAC strains were grown in Middlebrook 7H9 broth (Difco) with 0.05% Tween 80 supplemented with 10% Middlebrook ADC enrichment (BBL). Recombinant *M. smegmatis* strains used for GPL production were cultured in Luria-Bertani broth with 0.2% Tween 80. Isolation of DNA and transformation of *M. smegmatis* strains were performed as previously described (32). The genomic regions of MAC strains were amplified by a two-step PCR using TaKaRa *LA Taq* with GC buffer and the following program: denaturation at 98°C for 20 s and annealing-extension at 68°C for an appropriate time depending on the length of the targeted region. *Escherichia coli* strain DH5 α was used for routine manipulation and propagation of plasmid DNA. When necessary, antibiotics were added as follows: kanamycin, 50 μ g/ml for *E. coli* and 25 μ g/ml for *M. smegmatis*; and hygromycin B, 150 μ g/ml for *E. coli* and 75 μ g/ml for *M. smegmatis*. Oligonucleotide primers used in this study are listed in Table 3.

Construction of expression vectors. The *rfA* gene was amplified from genomic DNA of *M. avium* strain JATA51-01 using primers RTFA-S and RTFA-A. The PCR products were digested with each restriction enzyme and cloned into the BamHI-PstI site of pMV261 to obtain pMV-*rfA*. To use the site-specific integrating mycobacterial vector more conveniently, we constructed pYM301a containing an AflII site in pYM301. The region encompassing *gftB*, ORF3, and ORF4 was amplified from genomic DNA of MAC serovar 8 strain ATCC 35771 using primers GFTB-S and ORF4-A. In addition, *gftB* was amplified using primers GFTB-S and GFTB-A. The PCR products were digested with each restriction enzyme and cloned into the PstI-EcoRI site of pYM301a to obtain pYM-gtTB-orf3-orf4 and pYM-gtTB (Table 2).

Isolation and purification of GPLs. Harvested bacterial cells were allowed to stand in CHCl₃-CH₃OH (2:1, vol/vol) for several hours at room temperature. After water was added, total-lipid extracts were obtained from the organic phase and evaporated to dryness. Total-lipid extracts were subjected to mild alkaline hydrolysis as previously described (32, 33) to obtain crude GPL extracts. For analytical thin-layer chromatography (TLC), crude GPLs obtained from the same wet weight of harvested bacterial cells were spotted on Silica Gel 60 plates (Merck) using CHCl₃-CH₃OH-H₂O (30:8:1, vol/vol/vol) as the solvent and were visualized by spraying the plates with 10% H₂SO₄ and charring. Purified GPLs were prepared from crude GPLs by preparative TLC on the same plates, and

TABLE 3. Oligonucleotide primers used in this study

Primer	Sequence ^a	Restriction site
RTFA-S	5'-CGGGATCCCATGAAATTTGCTGT GGCAAG-3'	BamHI
RTFA-A	5'-AACTGCAGCTCAGCGACTTCGCT GCGCTTC-3'	PstI
GFTB-S	5'-AACTGCAGAAATGACCGCCACAA CCAGGGC-3'	PstI
GFTB-A	5'-GGAATTCCTCAGGCGCTCAGTGGC TCGTC-3'	EcoRI
ORF4-A	5'-GGAATTCCTAGGGCGCAATTCG ATGAG-3'	EcoRI
GTFB-U4	5'-GGAATTCGGTTCGACTCGACGAAG CCGAC-3'	EcoRI
DRRC-A	5'-GGAATTCGAGCGGGGCGACT CCTGCT-3'	EcoRI

^a Underlining indicates restriction sites.

each GPL was extracted from the corresponding band. Perdeuteriomethylation was carried out as previously described (6, 12, 17).

GC-MS and MALDI-TOF MS analysis. Crude and purified GPLs were hydrolyzed in 2 M trifluoroacetic acid (2 h, 120°C), and the released sugars were reduced with NaBD₄ and then acetylated with pyridine-acetic anhydride (1:1, vol/vol) at room temperature overnight. The resulting alditol acetates were separated and analyzed by gas chromatography-mass spectrometry (GC-MS) with a TRACE DSQ (Thermo Electron) equipped with an SP-2380 column (Supelco) using helium gas. The following program was used: temperature increased from 52 to 172°C at a rate of 40°C/min and then increased from 172 to 250°C at a rate of 3°C/min. To determine the total mass of the purified GPLs, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectra (in the positive mode) were obtained with a QSTAR XL (Applied Biosystems) using a pulse laser with emission at 337 nm. Samples mixed with 2,5-dihydroxybenzoic acid as the matrix were analyzed in the reflectron mode with an accelerating voltage of 20 kV and with operation in positive ion mode.

Nucleotide sequence accession number. The 4.6-kb genomic region amplified from MAC serovar 8 strain ATCC 35771 using primers GFTB-U4 and DRRC-A has been deposited in the DDBJ nucleotide sequence database under accession number AB437139.

RESULTS

Isolation and sequencing of the 4.6-kb genomic region responsible for GPL biosynthesis in MAC serovar 8. Lacking information on the genes responsible for biosynthesis of serovar 8-specific GPL, we compared and analyzed the genomic regions likely to be responsible for GPL biosynthesis in several

TABLE 2. Bacterial strains and vectors used in this study

Strain or vector	Characteristics	Source or reference
Bacteria		
<i>E. coli</i> DH5 α	Cloning host	TaKaRa
<i>M. smegmatis</i> mc ² 155	Expression host	35
<i>M. intracellulare</i> ATCC 35771	MAC serovar 8 strain	29
<i>M. avium</i> JATA51-01	Source of <i>rfA</i>	17
Vectors		
pYM301	Source of pYM301a	30
pYM301a	Site-specific integrating mycobacterial vector carrying an <i>hsp60</i> promoter cassette and AflII site	This study
pMV261	<i>E. coli</i> -Mycobacterium shuttle vector carrying an <i>hsp60</i> promoter cassette	36
pMV- <i>rfA</i>	pMV261 with <i>rfA</i>	This study
pYM-gtTB	pYM301a with <i>gftB</i>	This study
pYM-gtTB-orf3-orf4	pYM301a with <i>gftB</i> , ORF3, and ORF4	This study



FIG. 1. Organization of the 4.6-kb genomic region isolated from MAC serovar 8 strain. Filled triangles indicate the primers used for PCR amplification.

MAC serovars (16, 28). Most of these regions have high homology to each other, while the segment between the *gtfB* and *drrC* genes was found to vary in the strains. Therefore, we assumed that this segment contains genes involved in the formation of the unique Glc residue in serovar 8-specific GPL. To clone the *gtfB-drrC* region by using PCR, we designed various primers containing sequences derived from other MAC strains. By examining combinations of several pairs of primers, a 4.6-kb fragment was amplified from genomic DNA of a MAC serovar 8 strain when primers GTFB-U4 and DRRC-A were used (Fig. 1). Sequencing of this 4.6-kb fragment revealed four complete open reading frames (Fig. 1). The deduced amino acid sequences encoded by ORF1, ORF2, ORF3, and ORF4 were found to be identical to the amino acid sequences of four functionally undefined proteins from *M. avium* strain 104, MAV_3253, MAV_3255, MAV_3256, and MAV_3257, respectively (GenBank accession no. NC_008595.1). *M. avium* strain A5 also possessed a genomic region harboring ORF2, ORF3, and ORF4 (GenBank accession no. AY130970.1). These four open reading frames are predicted to encode the following proteins: ORF1, a putative glycosyltransferase similar to GtfD, which has been identified as a fucosyltransferase involved in the biosynthesis of serovar 2-specific GPL (73% identity) (30); ORF2, a putative glycosyltransferase, designated GtfTB, showing high homology to Rv1516c of *M. tuberculosis* (61% identity) (28); ORF3, a putative polysaccharide pyruvyltransferase similar to MSMEG_4736 and MSMEG_4737 of *M. smegmatis* (61 and 58% identity, respectively) (GenBank accession no. NC_008596.1); and ORF4, a putative *O*-methyltransferase similar to MSMEG_4739 of *M. smegmatis* (55% identity) (GenBank accession no. NC_008596.1).

Identification of the genes required for synthesis of the sugar residue unique to serovar 8-specific GPL. Based on the deduced functions of the genes in the 4.6-kb fragment, we focused on *gtfTB* (ORF2), ORF3, and ORF4 and characterized them by performing expression analyses. Because the serovar 8-specific GPL has a structure in which the Rha residue of serovar 1-specific GPL is further glycosylated (Table 1), it was necessary to prepare a strain producing serovar 1-specific GPL that could be the substrate for the enzymes participating in the biosynthesis of serovar 8-specific GPL. For this, as previously demonstrated, we created a recombinant *M. smegmatis* strain, designated MS-S1, by introducing the plasmid vector pMV-rtfA having the *M. avium* *rtfA* gene, which converts nsGPLs to serovar 1-specific GPL (30). We then introduced the integrative expression vector pYM-gtfTB possessing *gtfTB* into MS-S1 and assessed GPL profiles by performing a TLC analysis (Fig. 2). By comparison with the profile of MS-S1/pYM301a (vector control) (Fig. 2, lane A), two new spots, designated spots GPL-SG-U and -D, were observed in MS-S1/pYM-gtfTB (Fig. 2, lane B), indicating that serovar 1-specific

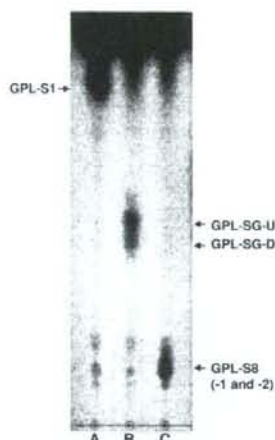


FIG. 2. TLC of crude GPL extracts from recombinant *M. smegmatis* strains MS-S1/pYM301a (A), MS-S1/pYM-gtfTB (B), and MS-S1/pYM-gtfTB-orf3-orf4 (C). GPL extracts were prepared from the total lipid fraction, and this was followed by mild alkaline hydrolysis. Samples were spotted and developed using $\text{CHCl}_3\text{-CH}_3\text{OH-H}_2\text{O}$ (30:8:1, vol/vol/vol).

GPL was converted to structurally different compounds by expression of *gtfTB*. Moreover, when the expression vector pYM-gtfTB-orf3-orf4 containing *gtfTB*, ORF3, and ORF4 was introduced into MS-S1, another new spot, designated GPL-S8, appeared (Fig. 2, lane C), implying that the structure of GPL-SG-U and -D was further modified by the products of ORF3 and ORF4. To confirm that these compounds contain the sugar residues associated with serovar 8-specific GPL, we performed a GC-MS analysis of the monosaccharides released from crude GPL extracts of each recombinant strain and the MAC serovar 8 strain (Fig. 3). The results showed that there was an excess of Glc, together with Rha, 6-d-Tal, 3,4-di-*O*-methyl-Rha, and 2,3,4-tri-*O*-methyl-Rha, in the profile of MS-S1/pYM-gtfTB compared with other profiles, as well as minor Glc peaks presumably derived from traces of trehalose-containing glycolipids (Fig. 3B). This indicates that the *gtfTB* gene mediates the transfer of a Glc residue to serovar 1-specific GPL. In contrast, the profile of MS-S1/pYM-gtfTB-orf3-orf4 revealed the presence of 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl-Glc, which was also detected in the MAC serovar 8 strain (Fig. 3C and D), demonstrating that the three genes are associated with the formation of the unique sugar residue of serovar 8-specific GPL.

Functional characterization of *gtfTB*. Expression analysis showed that serovar 1-specific GPL was converted to new compounds containing Glc when the *gtfTB* gene was expressed (Fig. 2, lane B, and Fig. 3B). Although these results suggested that the product of *gtfTB* participates in the formation of a Glc residue, it is not clear whether *gtfTB* encodes the glycosyltransferase that transfers Glc via 1→3 linkage to the Rha residue of serovar 1-specific GPL, whose linkage was previously detected in serovar 8-specific GPL (7, 8). To elucidate the function of *gtfTB*, we determined the linkage of sugar moieties of GPL-SG-U and -D, which were produced by recombinant strain

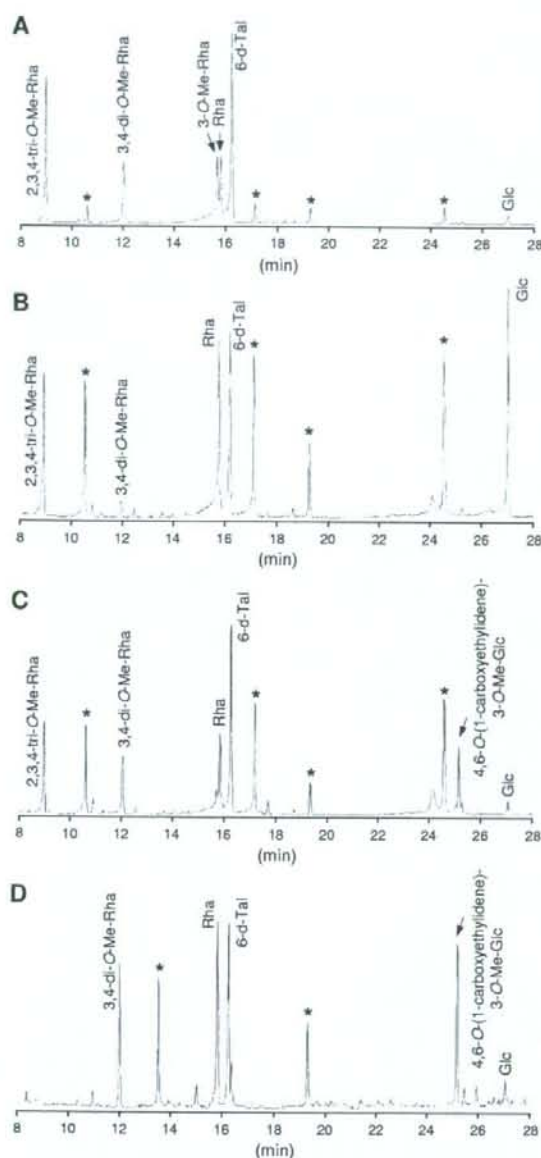


FIG. 3. GC-MS of alditol acetate derivatives from crude GPL extracts of recombinant strains *M. smegmatis* MS-S1/pYM301a (A), MS-S1/pYM-gtfTB (B), and MS-S1/pYM-gtfTB-orf3-orf4 (C) and a MAC serovar 8 strain (D). GPL extracts were prepared from the total-lipid fraction, and this was followed by mild alkaline hydrolysis. Asterisks indicate noncarbohydrates. Me, methyl.

MS-S1/pYM-gtfTB (Fig. 2, lane B). After extraction of the products from the corresponding bands on the TLC plate, purified GPL-SG-U and -D were subjected to perdeuteriomethylation followed by GC-MS. The differences in the TLC profiles of GPL-SG-U and -D might have been due to the

presence or absence of fatty acid methylation, which is often observed in *M. smegmatis* GPLs (23, 31), whereas the GC-MS profiles and fragmentation ions for GPL-SG-U and -D were identical, demonstrating that GPL-SG-U and -D had the same sugar moieties and linkages. Therefore, the profiles of GPL-SG-U shown here are representative of GPL-SG-U and -D. The GC-MS profile of GPL-SG-U contained four peaks corresponding to 6-d-Tal, Rha, Glc, and 2,3,4-tri-*O*-methyl-Rha (data not shown). The characteristic spectra for Glc, Rha, and 6-d-Tal are shown in Fig. 4. The spectrum of Glc had fragment ions at m/z 121, 167, and 168, which represent the presence of deuteriomethyl groups at positions C-2, C-3, and C-4 (Fig. 4A). In contrast, fragment ions at m/z 121, 134, 193, and 240 were detected for Rha, indicating that a deuteriomethyl group was introduced at positions C-2 and C-4 of Rha, in which position C-3 was acetylated (Fig. 4B). In addition, detection of fragment ions at m/z 134, 181, and 193 (Fig. 4C) revealed that there was deuteriomethylation at positions C-3 and C-4 in 6-d-Tal. These results demonstrated that position C-1 of Glc is linked to position C-3 of Rha but not to position C-2 of 6-d-Tal, because it has been determined previously that position C-1 of Rha is linked to position C-2 of 6-d-Tal in the oligosaccharide of serovar 1-specific GPL (17). Accordingly, the oligosaccharide structures of GPL-SG-U and -D were determined to have Glc-(1→3)-Rha-(1→2)-6-d-Tal at D-*allo*-Thr, demonstrating that *gtfTB* encodes the glucosyltransferase that transfers a Glc residue via 1→3 linkage to the Rha residue of serovar 1-specific GPL.

Structural assignment of GPL-S8 synthesized by expression of *gtfTB*, ORF3, and ORF4. GC-MS of the crude GPL extract from MS-S1/pYM-gtfTB-orf3-orf4 revealed the presence of 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl-Glc (Fig. 3C). To confirm that this structural component was derived from GPL-S8, we performed GC-MS and MALDI-TOF MS analyses of purified GPL-S8. The results showed that GPL-S8 contained a 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl-Glc residue and two main pseudomolecular ions (m/z 1,565.9 and 1,579.8 [$M + Na$]⁺) (data not shown). Consequently, as shown in Fig. 5, these results were consistent with the proposed structure for GPL-S8-1 and -2 containing 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl-Glc, with differences in pseudomolecular ions due to fatty acid methylation.

DISCUSSION

Structural diversity of the ssGPLs, notably in their sugar residues, defines 28 serovars of MAC. Although these ssGPLs are known to contribute to the virulence of MAC, the mechanisms of their biosynthetic regulation are largely unknown. In this study, we clarified the biosynthetic pathway for serovar 8-specific GPL, specifically the glycosylation step in which a Glc residue is transferred to the Rha residue of serovar 1-specific GPL.

To isolate the genomic region associated with the biosynthesis of serovar 8-specific GPL, we compared the GPL biosynthetic gene clusters in several MAC strains and found significant differences in the *gtfB-drrC* region. The segment flanking the 3' end of the *gtfB-drrC* region includes several genes responsible for the serovar 1-specific GPL whose structure is found in all ssGPLs. On the other hand, it is experimentally

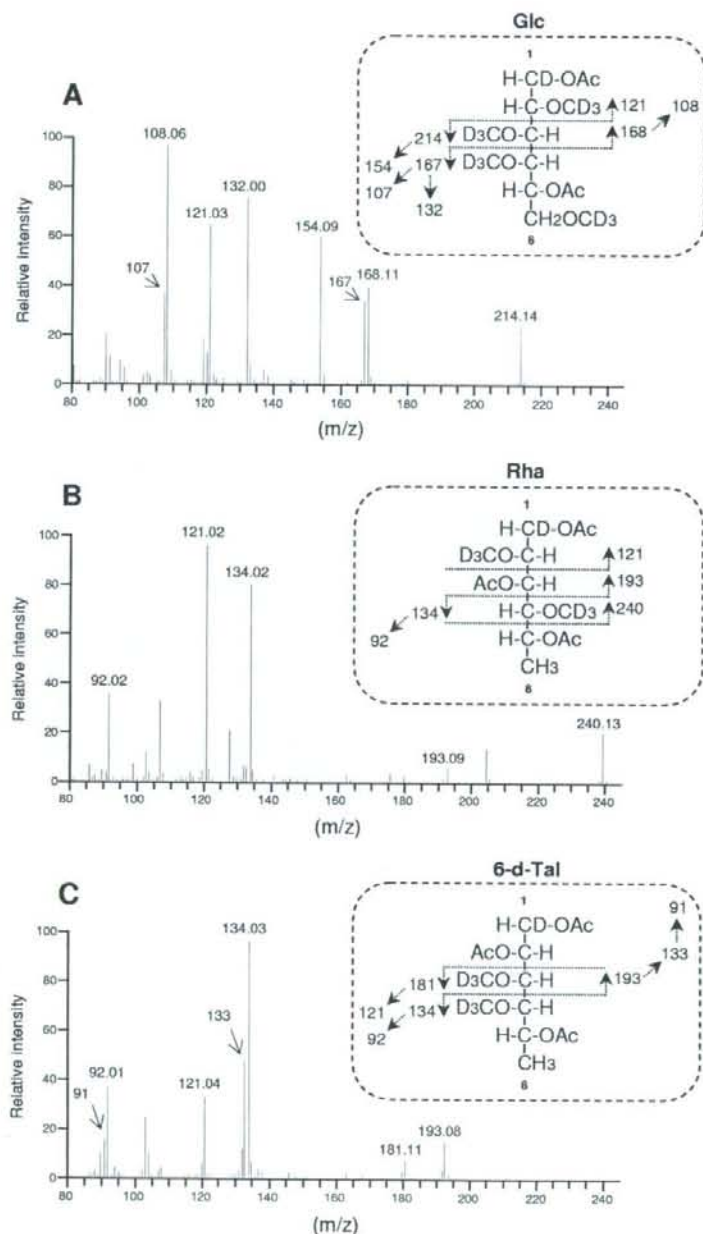


FIG. 4. GC-MS spectra and fragment ion assignments for Glc (A), Rha (B), and 6-d-Tal (C), which were derived from alditol acetates of sugars released from deuteriomethylated GPL-SG-U. Ac, acetate; D, deuterium.

clarified that the *gfb-drrC* regions of serovar 2-, 7-, and 16-specific GPL-producing strains contain the genes involved in the formation of the specific sugar residues that are transferred to the Rha residue of serovar 1-specific GPL (18, 19, 30). Thus,

this region could play an important role in generating the structural diversity of ssGPLs. As shown in this study, the specific functions for formation of sugar moieties of serovar 8-specific GPL were due to the genes present in the *gfb-drrC*

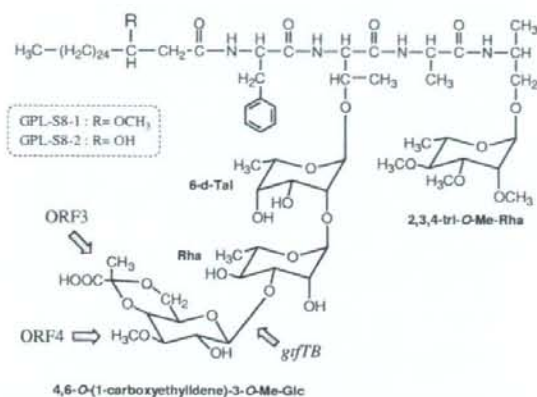


FIG. 5. Proposed structure and biosynthetic genes of GPL-S8 (serovar 8-specific GPL). Me, methyl.

region, suggesting that focusing on this region might provide clues for elucidating the characteristics of other ssGPLs whose biosynthesis is still not known.

It has been reported previously that the *gtfTB* gene in *M. avium* strains 104 and A5 was not likely to be associated with GPL biosynthesis because its ancestral homologue, Rv1516c (61% identity with the *GtfTB* gene), was the gene of *M. tuberculosis*, which produces no GPLs (28). Thus, it was interesting that *gtfTB* encodes a glycosyltransferase that does participate in GPL biosynthesis in which a Glc residue is transferred to serovar 1-specific GPL, yielding the serovar 8-specific GPL. *M. avium* strains 104 and A5 synthesize serovar 1-specific GPL as a final product and intermediate, respectively, while it has been recognized that neither of these strains produces serovar 8-specific GPL in spite of the presence of *gtfTB* in the GPL biosynthetic gene cluster (28). These observations raised the possibility that the transcription of *gtfTB* is inefficient in both strains due to the upstream sequences. Actually, in *M. avium* strain 104, a transposase sequence was observed upstream of *gtfTB*, indicating that this strain might be deficient in glucosylation, and consequently a serovar 1-specific GPL-producing strain is obtained (28). On the other hand, it has been shown that the biosynthetic gene cluster for serovar 7-specific GPL in *M. intracellulare* strain ATCC 35847 contains a putative glycosyltransferase gene which encodes amino acid sequences that are similar to the amino acid sequences encoded by *gtfTB* (59% identity) (18). Structural analysis of sugar moieties in serovar 7-specific GPL indicated that this *GtfTB* homologue may serve as a glycosyltransferase during formation of the terminal amidohexose residue that structurally resembles Glc (18).

The deduced amino acid sequences encoded by ORF3 and ORF4 showed that these genes putatively encode polysaccharide pyruvyltransferase and *O*-methyltransferase, respectively. Expression of ORF3 and ORF4 together with *gtfTB* led to structural alterations in which Glc was modified with both 4,6-*O*-(1-carboxyethylidene) and 3-*O*-methyl groups. Based on these observations, it is strongly suggested that ORF3 is associated with the formation of the 4,6-*O*-(1-carboxyethylidene) group that is synonymous with the cyclic pyruvate ketal and that ORF4 is associated with the 3-*O*-methylation of the Glc

residue (Fig. 5). In mycobacteria, homologues of ORF3 and ORF4 were found only in *M. smegmatis*, as MSMEG_4736 (for ORF3), MSMEG_4737 (for ORF3), and MSMEG_4739 (for ORF4). *M. smegmatis* also produces glycolipids containing 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl-Glc as a sugar moiety (25, 34), which suggests that both homologues participate in the synthesis of these glycolipids. Sugar residues with a 4,6-*O*-(1-carboxyethylidene) group substitution have been found in carbohydrates such as extracellular polysaccharide and N-linked glycan, which are produced by some bacteria and yeasts (1, 15, 20, 22, 26). It has been shown that an increase in 4,6-*O*-(1-carboxyethylidene)-containing sugar residues leads to enhanced viscosity of extracellular polysaccharide from *Xanthomonas* sp., which alters the cell surface properties related to cellular attachment and protection from environmental stress (10). Accordingly, in terms of the properties of serovar 8-specific GPL, the presence of the 4,6-*O*-(1-carboxyethylidene) group might influence the pathogenicity of MAC serovar 8.

With regard to the antibody reactivity, it is unclear whether serovar 8-specific antibodies react with GPL-S8 because there are minor structural differences in the methylated positions of fatty acids and the terminal Rha residue linked to the tetrapeptide between GPL-S8 and serovar 8-specific GPL of MAC. Evaluation of the antibody response to GPL-S8 using serovar 8-specific antibodies would facilitate understanding the immunoreactivity mediated by ssGPLs.

In this study, we proved that *gtfTB* and adjacent genes in the GPL biosynthetic gene cluster in MAC serovar 8 strain are responsible for the formation of a unique glucose residue in serovar 8-specific GPL (Fig. 5). In particular, *gtfTB* encodes the glycosyltransferase that plays a critical role in the pathway leading from serovar 1-specific GPL to serovar 8-specific GPL. Through further study, including generation of *gtfTB* knockout mutants of MAC serovar 8 strains, results relevant to the biosynthesis of serovar 8-specific GPL might help clarify the biological function of ssGPLs and their role in the host-pathogen relationships of MAC.

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RESEARCH ARTICLE

GM-CSF-mediated T-cell activation by macrophages infected with recombinant BCG that secretes major membrane protein-II of *Mycobacterium leprae*

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Introduction

Leprosy is a chronic infectious disease induced by parasitic infection with *Mycobacterium leprae* (Stoner, 1979). Despite the marked reduction in the number of both registered leprosy cases and new cases, a significant number of new cases (254 525 for the year 2007) are still detected each year (World Health Organization, 2008). The emergence of multidrug-resistant *M. leprae* (Kai *et al.*, 2004), although still few in number, and the complexity of leprosy reactions are distressing (Moschella, 2004). These observations indicate the urgent need to develop an efficacious vaccine against leprosy. *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) has been known to provide partial protection against the development of leprosy (Ponnighaus *et al.*, 1992). However, meta-analyses conducted by Setia *et al.* (2006) demonstrated an overall protective effect of only 26% against leprosy. There seem to be several reasons why BCG is not as effective as previously predicted. One of them may be

Abstract

The potential of *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) needs to be augmented to efficiently activate CD4⁺ T cells through macrophages. *Mycobacterium leprae*-derived recombinant major membrane protein (MMP)-II induced GM-CSF production from macrophages. A recombinant BCG-SM that secretes MMP-II more efficiently produced GM-CSF and activated interferon (IFN)- γ -producing CD4⁺ T cells than did vector control BCG when infected with macrophages. The T-cell activation by BCG-SM was dependent on the GM-CSF production by macrophages. Interleukin (IL)-10 production by macrophages stimulated with *M. leprae* was inhibited in a GM-CSF-dependent manner when the precursor monocytes were infected with BCG-SM. BCG inducing GM-CSF production was effective in macrophage-mediated T-cell activation partially through IL-10 inhibition.

that the human immune cells most susceptible to BCG infection are macrophages (Grode *et al.*, 2005). On entry into macrophages, mycobacteria inhibit phagosome–lysosome fusion, which results in a less efficient stimulation of interferon (IFN)- γ -producing type 1 CD4⁺ T cells (Ridley & Jopling, 1966; Frehel & Rastogi, 1987). Further, BCG as well as pathogenic mycobacteria can induce the production of an abundant amount of interleukin (IL)-10 from macrophages (Yamamura *et al.*, 1991), which inhibits activation of CD4⁺ T cells (Jonuleit *et al.*, 2001; Granelli-Piperno *et al.*, 2004). Moreover, it has been demonstrated in a murine study that BCG primarily infects macrophages *in vivo*, and the active proliferation of T cells *in vivo* needs the enrolment of dendritic cells (DC). Further, DC are known to be the most professional antigen-presenting cells (APC) in terms of T-cell activation. Thus, the transfer to DC of antigens produced by the processing of intracellular BCG or of proteins secreted from the mycobacteria in macrophages, seems to be important (Winau *et al.*, 2006).

We previously identified major membrane protein (MMP)-II (gene name, *bfrA* or ML2038), which is originally identified as bacterioferritin (Pessolani *et al.*, 1994) and localized in the cell membrane, as one of the dominant antigen of *M. leprae* (Maeda *et al.*, 2005; Makino *et al.*, 2005). Recombinant (r) MMP-II-pulsed DC activate naïve CD4⁺ T cells to produce IFN- γ in an antigen-specific manner, and also stimulate T cells from not only paucibacillary leprosy, a representative clinical leprosy at one pole of the clinical spectrum, but also multibacillary leprosy, a representative leprosy at the opposite pole (Makino *et al.*, 2005). The activation of type 1 CD4⁺ T cells is closely associated with the inhibition of the spread of *M. leprae* *in vivo* as observed in paucibacillary leprosy (Sieling *et al.*, 1999). In this respect, it was interesting to find that T cells from some paucibacillary leprosy patients seemed to be primed with MMP-II antigen *in vivo* (Makino *et al.*, 2005). Therefore, MMP-II was considered to be an immunodominant antigen of *M. leprae*. We constructed an rBCG strain (BCG-SM) that secretes MMP-II of *M. leprae* (Makino *et al.*, 2006). BCG-SM-infected DC stimulated quite efficiently both human naïve CD4⁺ T cells and naïve CD8⁺ T cells *in vitro*, and MMP-II-specific memory T cells were produced in mice inoculated with BCG-SM (Makino *et al.*, 2006).

Macrophages are heterogeneous in various aspects (Randolph *et al.*, 1999), and their differentiation is largely influenced by the cytokine milieu (Nakata *et al.*, 1991; Akagawa, 2002). Previously, we analysed the characteristics of two distinct macrophage subsets: rGM-CSF-mediated macrophages (GM-M ϕ) and rM-CSF-mediated macrophages (M-M ϕ) (Makino *et al.*, 2007). Both macrophages were equally susceptible to mycobacterial infection *in vitro*, but M-M ϕ infected with *M. leprae* did not activate CD4⁺ T cells even after activation using both CD40 ligand and exogenous IFN- γ . Likewise, *Mycobacterium tuberculosis*-infected M-M ϕ failed to stimulate T cells (Verreck *et al.*, 2004). Further, a large amount of IL-10 was produced from M-M ϕ on stimulation with mycobacteria. Therefore, the fact that mycobacteria are highly susceptible to phagocytosis by M-M ϕ and poorly stimulate T cells through M-M ϕ , may be closely associated with the affinity of mycobacteria to macrophages, the induction of a latent infection and, in some cases, the development of disease. Likewise, M-M ϕ is one of the major target immune cells of BCG infection. However, to control the subsequently invading pathogenic mycobacteria, such as *M. leprae*, by producing memory T cells, modified BCG including the newly developed recombinant BCG-SM is required to be able to fully stimulate T cells even if M-M ϕ are the initial target host cells.

In this report, we examined the T-cell-stimulating ability of BCG-SM-infected M-M ϕ , and further assessed the influence of BCG-SM on the IL-10-producing activity of M ϕ upon a challenge with *M. leprae*.

Materials and methods

Preparation of cells and bacteria

Peripheral blood was obtained from healthy purified protein derivative (PPD)-positive individuals with informed consent. In Japan, most healthy individuals are PPD-positive due to a compulsory BCG vaccination for children (0–4 years old). Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use as previously described (Makino & Baba, 1997). For the preparation of peripheral monocytes, CD3⁺ T cells were removed from freshly isolated heparinized blood or from cryopreserved PBMC using immunomagnetic beads coated with anti-CD3 monoclonal antibody (mAb) (Dynabeads 450, Dynal, Oslo, Norway). The CD3⁻ PBMC fraction was plated on collagen-coated plates and nonadherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (Makino & Baba, 1997). Macrophages were generated by culturing monocytes in the presence of 20% foetal calf serum and either rM-CSF (R and D Systems, Abingdon, UK) (M-M ϕ) or rGM-CSF (PeproTech EC Ltd, London, UK) (GM-M ϕ) (Makino *et al.*, 2007). Both GM-M ϕ and M-M ϕ were pulsed with rBCGs on day 3 or 5 of culture, and were used as a stimulator of T cells on day 5 or 7 (Makino *et al.*, 2007). A recombinant BCG that secretes *M. leprae*-derived MMP-II was constructed as described previously (Makino *et al.*, 2006). In brief, a shuttle vector, pMV-261, having a kanamycin resistance gene and origins of replication for *Escherichia coli* and mycobacteria was used to construct pMV-SM (Secreting MMP-II) having the MMP-II cDNA fragment. The BCG substrain Pasteur was cultured *in vitro* using Middlebrook 7H9 broth (BD Biosciences Pharmingen, San Jose, CA) supplemented with 0.05% Tween 80 and 10% albumin–dextrose–catalase (BD). Expression vectors were introduced into BCG by electroporation (Snapper *et al.*, 1988). Transformants were selected on Middlebrook 7H10 agar (BD) plates supplemented with 10% OADC (BD) and 25 $\mu\text{g mL}^{-1}$ kanamycin. Mycobacteria were subsequently grown in Middlebrook 7H9 broth containing 25 $\mu\text{g mL}^{-1}$ of kanamycin. BCG containing pMV-SM as an extrachromosomal plasmid is referred to as BCG-SM, and BCG containing pMV-261 is referred to as BCG-pMV (vector control BCG). *Mycobacterium leprae* (Thai-53) was isolated from the footpads of BALB/c-*nu/nu* mice (McDermott-Lancaster *et al.*, 1987). The isolated bacteria were counted using Shepard's method (McDermott-Lancaster *et al.*, 1987). The multiplicity of infection (MOI) was determined based on the assumption that macrophages were equally susceptible to infection with BCG or *M. leprae* (Hashimoto *et al.*, 2002). A recombinant MMP-II protein was produced as reported previously (Maeda *et al.*, 2005). Briefly,

the MMP-II gene (ML2038) was inserted into the expression plasmid pET28 (Novagen, Madison, WI) and transformed into *E. coli* strain ER2566 (New England BioLabs, Ipswich, MA). The expressed protein was eluted using Whole Gel Eluter (Bio-Rad Laboratories, Hercules, CA). As a control for *M. leprae* antigen, we have purified hsp18 (ML1795) in *E. coli* using the PET expression system. The cytosolic fraction of the parental BCG was obtained as described previously (Maeda *et al.*, 2003).

Antigen-presenting function of rBCG-infected macrophages

The ability of rBCG-infected macrophages to stimulate T cells was assessed using an autologous macrophage-T cell coculture system as previously described (Wakamatsu *et al.*, 1999; Hashimoto *et al.*, 2002). The responder CD4⁺ T cells were purified from freshly thawed PBMC using a CD4-negative isolation kit (Dynabeads 450) (Wakamatsu *et al.*, 1999). The purity of CD4⁺ T cells was > 95% as assessed by FACS. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates and macrophages were added to give the indicated macrophage:CD4⁺ T-cell ratio. Supernatants of macrophage-T cell cocultures were collected on day 4. To identify molecules restricting T-cell activation, the following purified mAbs were used: anti-HLA-DR Ab (L243) and anti-CD86 Ab [IT2.2, Becton Dickinson (BD), San Jose, CA]. The concentration of IFN- γ produced by CD4⁺ T cells was quantified using an enzyme assay kit, OptEIA Human enzyme-linked immunosorbent assay (ELISA) Set (BD). In some cases, M-M ϕ were pulsed with BCG-SM in the presence of $10 \mu\text{g mL}^{-1}$ of either normal rat IgG or neutralizing mAb to GM-CSF (rat IgG2a) (BD).

Production of IL-10 and GM-CSF by macrophages

The ability of M-M ϕ to produce IL-10 on stimulation with *M. leprae* was assessed. The monocytes were pretreated with the indicated dose of rBCG and subsequently made to differentiate into M-M ϕ by culturing for 5 days in the presence of rBCG and M-CSF. These macrophages were stimulated with *M. leprae* at the indicated MOI for 24 h. In some cases, monocytes were infected with BCG-SM in the presence of $10 \mu\text{g mL}^{-1}$ of neutralizing mAb to GM-CSF. Also, the ability of M-M ϕ to produce GM-CSF on stimulation with rBCG for 24 h was assessed. The concentration of these cytokines was quantified using OptEIA Human ELISA Set (BD).

Statistical analysis

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

Results

Effect of rBCG-infected macrophages on T-cell-stimulating activity

We analysed the T-cell-stimulating activity of rBCG-infected GM-M ϕ and M-M ϕ (Fig. 1). GM-M ϕ infected with either BCG-SM or BCG-pMV significantly stimulated CD4⁺

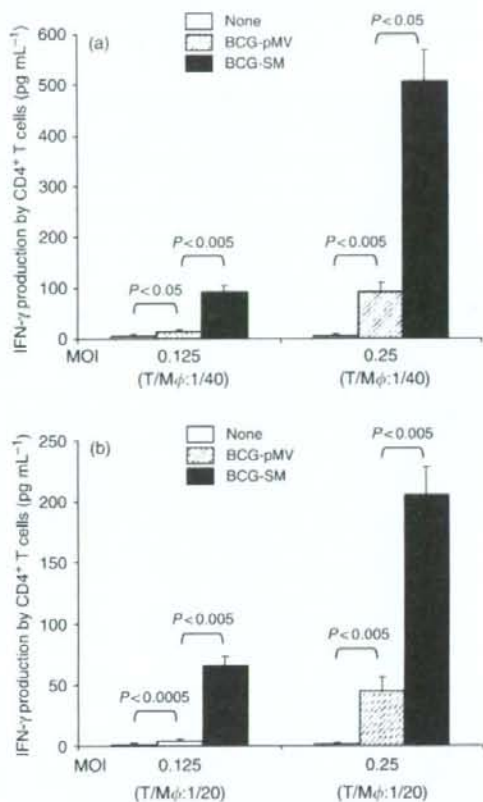


Fig. 1. Production of IFN- γ by CD4⁺ T cells. (a) GM-M ϕ , differentiated by 3 days of culture with rGM-CSF from monocytes, were infected with BCG-pMV (vector control BCG) or BCG-SM (rBCG that secretes MMP-II) at the indicated MOI, and cultured for another 2 days. These GM-M ϕ were used as a stimulator of CD4⁺ T cells (1×10^5 cells per well) at a T cell : GM-M ϕ ratio of 40 : 1 in a 4-day culture. (b) M-M ϕ , differentiated by 5 days of culture with rM-CSF from monocytes, were infected with BCG-pMV or BCG-SM at the indicated MOI, and cultured for another 2 days. M-M ϕ were then used as a stimulator of CD4⁺ T cells (1×10^5 cells per well) at a T cell : M-M ϕ ratio of 20 : 1 in a 4-day culture. A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean \pm SD. Titres were statistically compared using Student's *t*-test.

T cells. However, a larger amount of IFN- γ was produced by the T cells when GM-M ϕ were infected with BCG-SM (Fig. 1a), although BCG vaccination did not prime for MMP-II-specific T-cell response (not shown). We then analysed the T-cell-stimulating activity of BCG-infected M-M ϕ (Fig. 1b). Again, M-M ϕ infected with BCG-SM induced a higher amount of IFN- γ production by T cells than did BCG-pMV-infected M-M ϕ , although the IFN- γ production was less efficient than that induced by rBCG-infected GM-M ϕ even though higher doses of BCG-infected M-M ϕ were used as a stimulator.

Factors associated with the induction of the T-cell-stimulating activity of M-M ϕ

To define the factors associated with the CD4⁺ T-cell activation by BCG-SM-infected M-M ϕ , we phenotypically analysed M-M ϕ infected with either BCG-pMV or BCG-SM. There was no significant difference between BCG-pMV-infected M-M ϕ and BCG-SM-infected M-M ϕ in the expression of HLA-DR, CD86 or CD40 molecules (not shown). The cytokines produced by M-M ϕ stimulated with rBCGs, including GM-CSF and IL-23, were examined. Both rBCGs induced GM-CSF production, but BCG-SM did so more efficiently than BCG-pMV (Fig. 2). However, IL-23 was not produced by M-M ϕ on stimulation with either BCG-pMV or BCG-SM. We also assessed whether rMMP-II protein can induce GM-CSF production in macrophages. Whereas *M. leprae*-derived cytosolic protein (not shown), other mycobacterial proteins such as BCG-derived cytosolic protein (5–10 $\mu\text{g mL}^{-1}$), control recombinant *M. leprae* antigen (hsp18), and lipopolysaccharide (amount present with rMMP-II protein) did not stimulate M ϕ , MMP-II induced GM-CSF production in a concentration-dependent manner (Fig. 3). rMMP-II also efficiently induced the production of other cytokines including tumour necrosis factor (TNF) α and IL-12p40 from M ϕ (not shown).

We examined the influence of surface antigens on M-M ϕ . The T-cell-stimulating activity of BCG-SM-infected M-M ϕ was significantly inhibited when the infected M-M ϕ were pretreated with the mAb to HLA-DR or CD86 antigens, whereas the control IgG did not affect IFN- γ production by T cells (Fig. 4a). However, IFN- γ production was partially inhibited when BCG-SM-infected M-M ϕ were treated with the mAb to MMP-II (not shown). Next, we examined the effect on T-cell activation of GM-CSF produced by M-M ϕ stimulated with BCG-SM (Fig. 4b). When M-M ϕ were infected with BCG-SM in the presence of the neutralizing mAb to GM-CSF, IFN- γ production by CD4⁺ T cells was significantly inhibited. The T-cell-stimulating activity of BCG-SM-infected M-M ϕ was not affected by normal rat IgG.

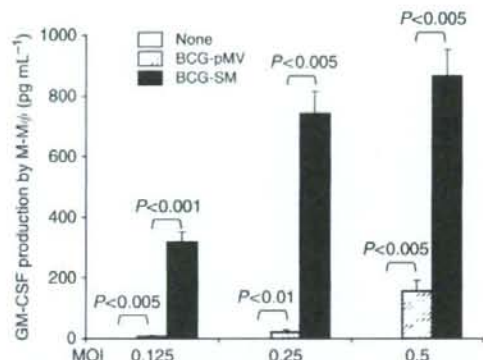


Fig. 2. Production of GM-CSF by M-M ϕ . M-M ϕ differentiated by 5 days of culture with rM-CSF from monocytes, were stimulated with BCG-pMV or BCG-SM for 24 h at the indicated MOI. A representative experiment based three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean \pm SD. Titres were statistically compared using Student's *t*-test.

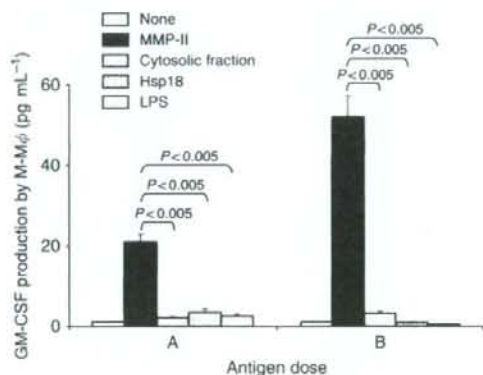


Fig. 3. GM-CSF production by M-M ϕ . M-M ϕ obtained after 5 days of culture with rM-CSF were stimulated for 24 h with rMMP-II, the BCG-derived cytosolic fraction or *Mycobacterium leprae*-derived hsp18 antigen [dose of antigen in (a) 5 $\mu\text{g mL}^{-1}$ and that in (b) 10 $\mu\text{g mL}^{-1}$]. Lipopolysaccharide, assumed to be present with rMMP-II protein (660 ng mg^{-1} MMP-II protein), was used as a negative control (lipopolysaccharide in (a) 3.3 ng mL^{-1} and that in (b) 6.6 ng mL^{-1}). A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean \pm SD. Titres were statistically compared using Student's *t*-test.

Effect of infection of monocytes with BCG on IL-10 production by M-M ϕ

Macrophages are one of the cells most sensitive to *M. leprae* infection and M-M ϕ produce abundant IL-10 when infected with the bacteria (Makino et al., 2007). As precursor

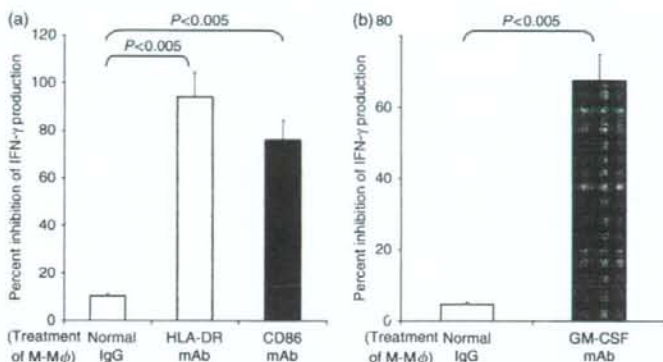


Fig. 4. (a) Inhibition of IFN- γ production by CD4⁺ T cells by pretreatment of BCG-SM-infected M-M ϕ with mAb to HLA-DR or CD86. M-M ϕ differentiated from monocytes using rM-CSF were infected with BCG-SM at an MOI of 0.25 on day 5 of culture and cultured for another 2 days. The BCG-SM-infected M-M ϕ were treated with mAb to HLA-DR or CD86, or isotype-matched control IgG (10 μ g mL⁻¹), and used as a stimulator of CD4⁺ T cells, at a T cell : M-M ϕ ratio of 20 : 1 and cultured for another 4 days. The optimal concentration of mAb was determined in advance. Non-pretreated BCG-SM-infected M-M ϕ induced the production of 220.8 pg mL⁻¹ of IFN- γ by CD4⁺ T cells. This titre was taken as 0% inhibition. (b) Inhibition of IFN- γ production by CD4⁺ T cells by neutralizing GM-CSF produced from BCG-SM-infected M-M ϕ . M-M ϕ , differentiated from monocytes by culturing for 5 days with rM-CSF, were infected with BCG-SM (MOI 0.25) in the presence of neutralizing mAb to GM-CSF or isotype-matched control IgG (10 μ g mL⁻¹). These M-M ϕ were used as a stimulator of CD4⁺ T cells as in (a). The optimal concentration of mAb was determined in advance. M-M ϕ infected with BCG-SM in the absence of any Ab induced the production of 168.3 pg mL⁻¹ of IFN- γ by CD4⁺ T cells. This titre was taken as 0% inhibition. A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean \pm SD. Titres were statistically compared using Student's t-test.

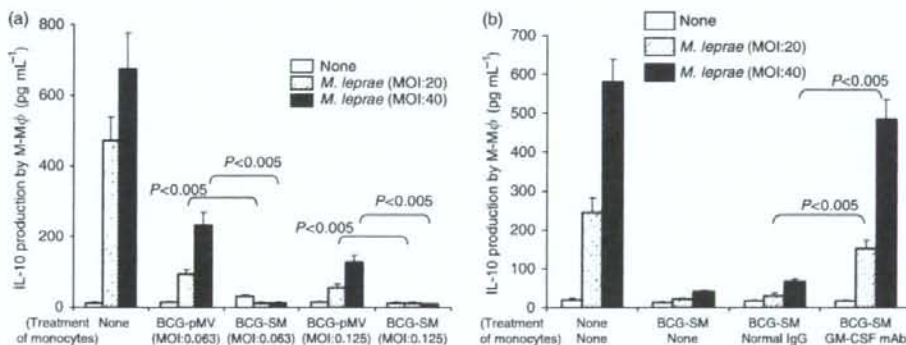


Fig. 5. (a) Production of IL-10 by M-M ϕ . Monocytes were infected with the indicated dose of BCG-pMV or BCG-SM, and subsequently differentiated into M-M ϕ by culturing for 5 days in the presence of M-CSF and rBCGs. These rBCG-preinfected M-M ϕ were stimulated with *Mycobacterium leprae* at the indicated MOI for 24 h. (b) Recovery of IL-10 production by M-M ϕ . Monocytes were infected with BCG-SM (MOI 0.063) in the presence of neutralizing GM-CSF mAb or isotype-matched control IgG and were subsequently differentiated into M-M ϕ by culturing for 5 days. These BCG-SM-preinfected M-M ϕ were stimulated with *M. leprae* at the indicated MOI for 24 h. The optimal concentration of mAb was determined in advance. A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean \pm SD. Titres were statistically compared using Student's t-test.

monocytes also produced GM-CSF on stimulation with BCG in a BCG-SM-predominant fashion (not shown), we examined the effect of infection with rBCGs in monocytes on IL-10 production by M-M ϕ challenged with *M. leprae* (Fig. 5). M-M ϕ differentiated from monocytes untreated with any bacteria produced > 400 pg mL⁻¹ of IL-10 on

stimulation with *M. leprae*; however, the production of cytokine by M-M ϕ pretreated with rBCGs was significantly inhibited (Fig. 5a). The inhibition was more significant when BCG-SM was used as a stimulator of monocytes, and IL-10 production by M-M ϕ was almost completely inhibited. The inhibition was dependent on the dose of BCGs

used for pretreatment. In addition, pretreatment of monocytes with BCG-SM inhibited the IL-10 production induced even by lipopolysaccharide (not shown).

Furthermore, M-M ϕ differentiated from monocytes infected with BCG-SM in the presence of normal IgG did not produce IL-10 on stimulation with *M. leprae* (Fig. 5b). However, a significant level of IL-10 was produced when monocytes were infected with BCG-SM in the presence of the neutralizing mAb to GM-CSF. These results indicate that endogenously produced GM-CSF can inhibit IL-10 production.

Discussion

The host defence against intracellular parasitic pathogens such as *M. leprae* is closely associated with the activation of IFN- γ -producing type 1 T cells (Hashimoto *et al.*, 2002). In fact, in patients with paucibacillary leprosy, the activation of CD4⁺ T cells results in inhibition of the intracellular multiplication and intercellular spread of *M. leprae* (Sieling *et al.*, 1999). The T-cell activation largely depends on the extent of the activation of APC, in which DC play an extremely important role, as DC are the most powerful T-cell stimulators among the APC (Hashimoto *et al.*, 2002). However, if T cells are not efficiently activated due to poor participation of DC, *M. leprae* may be predominantly retained in macrophages. In fact, multibacillary leprosy patients retain numerous *M. leprae* in their macrophages which, in some cases, allow the multiplication and intercellular spread of the bacteria (Ridley & Jopling, 1966).

The tissue resident macrophages, represented by GM-M ϕ and M-M ϕ , are heterogeneous in terms of function (Nakata *et al.*, 1991; Randolph *et al.*, 1999; Akagawa, 2002), despite being similarly susceptible to mycobacterial infection (Makino *et al.*, 2007). GM-M ϕ infected with *M. tuberculosis* or *M. leprae* significantly stimulated CD4⁺ T cells, whereas M-M ϕ failed to stimulate CD4⁺ T cells (Verreck *et al.*, 2004; Makino *et al.*, 2007). In this study, we found that, similar to those pathogenic mycobacteria, vector control BCG (BCG-pMV)-infected GM-M ϕ significantly stimulated CD4⁺ T cells, whereas the BCG-pMV-infected M-M ϕ were less efficient in stimulating these cells. These results indicate the possibility that parental BCG may long reside in M-M ϕ and stimulate T cells inadequately, like the *M. leprae*-infected resident macrophages in multibacillary leprosy. In contrast to BCG-pMV, rBCG that secretes MMP-II (BCG-SM) has the ability to enlist not only GM-M ϕ , but also M-M ϕ , for T-cell activation. Further, the production of IFN- γ by CD4⁺ T cells stimulated with BCG-SM-infected M-M ϕ was significantly inhibited by pretreatment of the M-M ϕ with the mAb to HLA-DR or CD86 antigens. In addition, the pretreatment of M-M ϕ infected with both BCG-SM and BCG-pMV effectively inhibited CD4⁺ T-cell activation (not

shown). Therefore, the BCG-SM-infected M-M ϕ seemed to stimulate CD4⁺ T cells in an antigen-specific manner. Furthermore, there was a striking difference between BCG-pMV and BCG-SM in the induction of GM-CSF production. Not only from M-M ϕ , but also from GM-M ϕ , BCG-SM more efficiently induced GM-CSF production than BCG-pMV, and, further, rMMP-II protein, though less efficient, induced significant GM-CSF production. Previously, we reported that rMMP-II is highly immunogenic and induces production of various cytokines, including IL-12 and TNF- α , from APCs such as macrophages and DC (Maeda *et al.*, 2005). These findings indicate that the enhanced production of GM-CSF on stimulation by BCG-SM was at least partially associated with the secretion of MMP-II from BCG-SM.

As the activation of T cells by BCG-SM-infected M-M ϕ was largely inhibited when endogenously produced GM-CSF was neutralized by the mAb to GM-CSF, the endogenously produced GM-CSF may be closely associated with the enhanced T-cell activation by BCG-SM. Although we could not identify the most relevant antigen for T-cell activation, GM-CSF may change the activation status of macrophages or may at least partially transform the BCG-SM-infected M-M ϕ to GM-M ϕ (Makino *et al.*, 2007). Therefore, BCG-SM seems to be a unique rBCG capable of producing GM-CSF and utilizing M-M ϕ for T-cell stimulation.

Another important characteristic of mycobacteria which contributes to the inhibition of T-cell activation is the abundant production of IL-10 by M-M ϕ (Jonuleit *et al.*, 2001; Mochida-Nishimura *et al.*, 2001; Granelli-Piperno *et al.*, 2004). The major purpose of a vaccination is the production of memory T cells which can rapidly respond to subsequently invading pathogenic mycobacteria. However, IL-10 inhibits the re-activation of memory T cells *in vitro*. We found that the ability of BCG-SM to induce production of GM-CSF is useful to inhibit IL-10 production. Monocytes were quite sensitive in the production of GM-CSF, and both BCG-pMV and BCG-SM induced cytokine production by monocytes, although BCG-SM predominated at lower doses (not shown). Thus, even BCG-pMV inhibited IL-10 production at higher doses. However, macrophages differentiated from monocytes which were infected with a small dose of BCG-SM completely inhibited IL-10 production upon subsequent challenge with *M. leprae*, and the inhibitory activity was at least partially cancelled out by the neutralization of endogenously produced GM-CSF. Further, heat-killed BCG-SM, which does not secrete MMP-II (Makino *et al.*, 2006), did not inhibit IL-10 production. These observations indicate that macrophages treated with GM-CSF endogenously diminished the ability to produce IL-10 upon stimulation with *M. leprae*. Previously, we observed that addition of GM-CSF exogenously blocked the ability to produce IL-10 (Makino *et al.*, 2007), which agrees with the