based on the detection of bands of 196 bp in size (Figure 2).

## Discussion

Recent advances in molecular biology have raised hopes of producing more effective DNA vaccines as an alternative in preventing diseases in a much more specific and direct manner. Meanwhile, studies on animal models have provided valuable findings on the potentials of the DNA vaccine as a new option in vaccine studies and industry [5]. Prior to this study, MDP1 had been shown to be a potential DNA vaccine adjuvant in BCG, whereby it has a unique ability in blocking DNase activity, and consequently decreasing the amount of DNA necessary for vaccination [20]. Furthermore, studies have showed that MDP1 is an effective adjuvant for DNA vaccine when given separately in different plasmids through intraperitoneal and intramuscular routes of administrations [20]. In this study we showed that chickens immunized at two different sites with plasmids containing H5 and MDP1, respectively, developed higher antibody titer compared to chickens immunized with H5 alone indicating the adjuvant effect of MDP1 on AIV DNA vaccine.

The antibody responses to the H5 and H5 + MDP1 vaccine were measured using both ELISA and HI test. Meanwhile, serum samples obtained from chickens in the groups immunized with PBS, pcDNA3.1 + and pcDNA3.1/MDP1 were negative for antibody titer in both ELISA and HI test. Chickens immunized with H5 + MDP1 vaccines were able to produce detectable AIV H5 antibody 1 week earlier compared to chickens immunized with H5 vaccine alone (Table 3). The mechanisms that associated with this finding are not know where administration of MDP1 facilitate the production of antibody against H5. Furthermore, eight out of nine chickens in the H5 + MDP1 immunized group were able to develop detectable AIV H5 antibody whilst, five out of nine chickens in H5 group were able to show detectable AIV H5 antibody 35 days post immunization.

Based on HI test, the antibody production after immunization was detectable from day 14 and the production had an increasing pattern for two subsequent bleeding sessions (Table 3). The mean antibody production of the group immunized using H5 + MDP1 vaccines was slightly higher compared to the group immunized with H5 vaccine (Table 4). However, the difference was not statistically significant probably due to high standard deviation. Probably, a selection of appropriate expression plasmid construction with optimized codon usages in chickens is essential in improving the expression and regulates the delivery of the DNA vaccine for inducing significant antibody responses [21]. Furthermore, only nine chickens were used in a group in the immunization trials.

Amplification of specific regions from RNA genome was performed using RT-PCR to detect the transcription of the targeted gene in cells. Previously, Ferstl *et al.* (2004) indicated that RT-PCR is an accurate method to study the expression of desired genes in *in vivo* experiments [22].

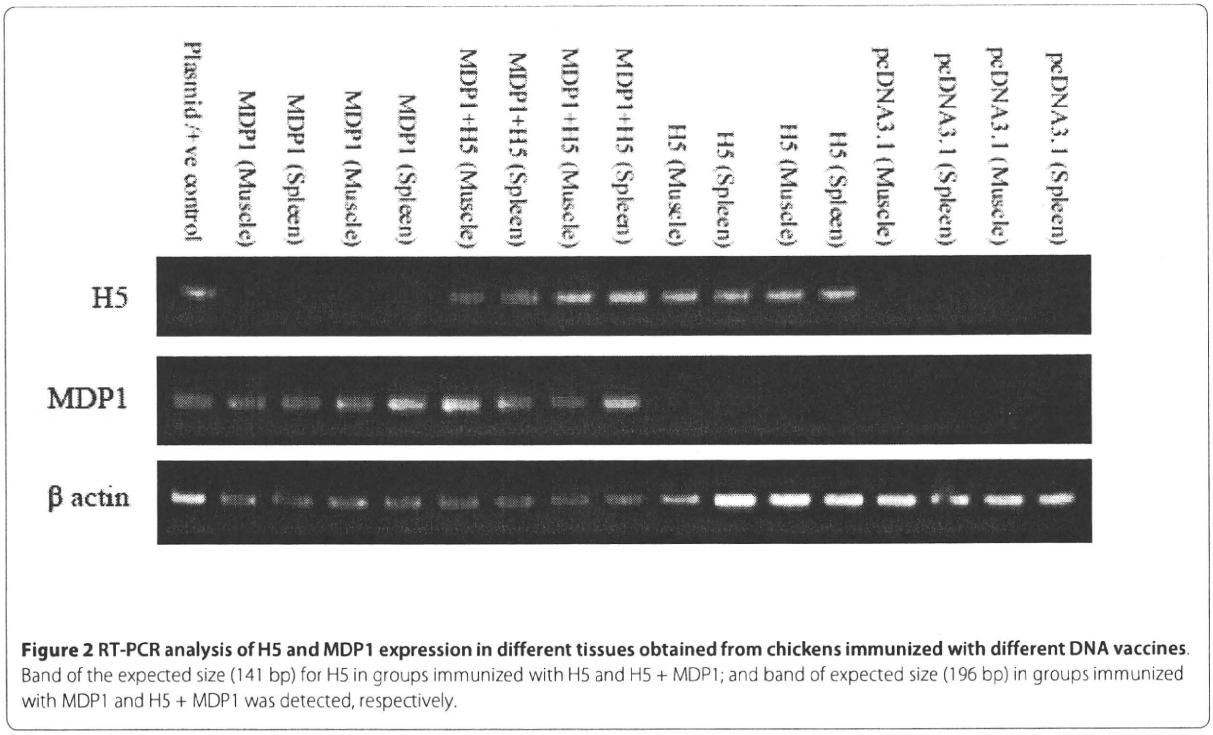


**Figure 2 RT-PCR analysis of H5 and MDP1 expression in different tissues obtained from chickens immunized with different DNA vaccines.** Band of the expected size (141 bp) for H5 in groups immunized with H5 and H5 + MDP1; and band of expected size (196 bp) in groups immunized with MDP1 and H5 + MDP1 was detected, respectively.

The spleen and muscle (immunization site) samples of the chickens immunized with different DNA vaccine constructs were extracted and used as templates for PCR and RT-PCR amplifications. Agarose gel electrophoresis following RT-PCR showed successful expression of H5 mRNA for groups immunized with H5 and H5 + MDP1 vaccines (Figure 2). This finding is consistent with the results of previous studies suggesting the successful delivery and presentation of the target gene to the immune system [14,23-25]. The extracted RNA was analyzed with PCR amplification only in which no band of the expected size was detected (data not shown), indicating that the amplified product from the RT-PCR experiments were from *in vivo* transcription of the target genes.

In this study, the intramuscular immunization was performed using endotoxin-free naked H5 cloned in pcDNA3.1 +, resulted in the production of antibody against the constructed H5 DNA. This result was consistent with a study performed by Le Gall-Recule' and coworkers (2002), who found that AIV H7 cloned into an eukaryotic expression plasmid, pCMV could lead to antibody response, using different administration methods [23]. However, in another study, direct intramuscular immunization using naked plasmid did not produce the same HI titer in all the treatment, probably due to the inaccurate gene delivery system [25]. In this study, a detectable HI titer was successfully produced from the direct immunization of H5 and H5 + MDP1 vaccines in all the treatments (Table 4). Even though the mean HI titer between chicken immunized with H5 vaccine with and without MDP1 was not statistically significant, the HI titers at the different time points during the course of the experiment between the two groups were found to be significantly different and had an increasing pattern. Hence, HI test is more sensitive in detecting H5 antibody in avian compared to ELISA which is consistent with a previous study by Bulbot *et al.* [26].

In this study, the highest HI titer of 13.33 ± 4.13 was observed in chickens immunized with H5 + MDP1 vaccines on day 35 post immunization. Previous studies have shown, post immunization serum HI titre of 32 and above results in protective immunity against H5N1 influenza infection or disease in populations [26,27]. Even though we did not evaluate the constructed vaccines efficacy against viral challenge; but studies showed regardless of low antibody titers following immunization with DNA vaccine, the immunized chickens were protected against lethal challenge probably due to the cellular immune response [27-29].

## Conclusions

Our study demonstrates the potential of MDP1 as a genetic adjuvant for H5 DNA vaccine. However, chickens immunized with H5 + MDP1 vaccines developed the highest HI titer of 16 although antibody titers between chickens immunized with H5 with and without MDP1 were not statistically significant. Our future efforts will concentrate on the analysis of the cellular immune responses following the immunization using constructed H5 + MDP1 DNA vaccine.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

BJ designed and performed the experiments to explore the adjuvancy role of Mycobacterial DNA binding Protein 1 (MDP1) in augmenting H5 DNA vaccine in inducing specific antibody response and wrote the manuscript. ARO supervised the project and edit the manuscript. MHB and NBA co-supervised the experiments. MR participated in animal trial. SM provided the MDP1 gene and monoclonal antibody.

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### Author Details

[1]Institute of Bioscience, University Putra Malaysia, Serdang 43400, Selangor, Malaysia, [2]Faculty of Veterinary Medicine, University Putra Malaysia, Serdang 43400, Selangor, Malaysia, [3]Faculty of Biotechnology and Biomedical Sciences, University Putra Malaysia, Serdang 43400, Selangor, Malaysia and [4]Department of Bacteriology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan

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# Novel Rhamnosyltransferase Involved in Biosynthesis of Serovar 4-Specific Glycopeptidolipid from *Mycobacterium avium* Complex[▽]

Yuji Miyamoto,[1]* Tetsu Mukai,[1] Takashi Naka,[2,3] Nagatoshi Fujiwara,[2] Yumi Maeda,[1] Masanori Kai,[1] Seiko Mizuno,[2,4] Ikuya Yano,[5] and Masahiko Makino[1]

*Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan[1]; Department of Bacteriology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan[2]; MBR Co., Ltd., 7-7-15 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan[3]; Faculty of Human Development, Soai University, 4-4-1 Nanko-naka, Suminoe-ku, Osaka 559-0033, Japan[4]; and Japan BCG Laboratory, 3-1-5 Matsuyama, Kiyose, Tokyo 204-0022, Japan[5]*

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**Glycopeptidolipids (GPLs) are one of the major glycolipid components present on the surface of *Mycobacterium avium* complex (MAC) that belong to opportunistic pathogens distributed in the natural environment. The serovars of MAC, up to around 30 types, are defined by the variable oligosaccharide portions of the GPLs. Epidemiological studies show that serovar 4 is the most prevalent type, and the prognosis of pulmonary disease caused by serovar 4 is significantly worse than that caused by other serovars. However, little is known about the biosynthesis of serovar 4-specific GPL, particularly the formation of the oligosaccharide portion that determines the properties of serovar 4. To investigate the biosynthesis of serovar 4-specific GPL, we focused on one segment that included functionally unknown genes in the GPL biosynthetic gene cluster of a serovar 4 strain. In this segment, a putative hemolytic protein gene, *hlpA*, and its downstream gene were found to be responsible for the formation of the 4-*O*-methyl-rhamnose residue, which is unique to serovar 4-specific GPL. Moreover, functional characterization of the *hlpA* gene revealed that it encodes a rhamnosyltransferase that transfers a rhamnose residue via 1→4 linkage to a fucose residue of serovar 2-specific GPL, which is a key pathway leading to the synthesis of oligosaccharide of serovar 4-specific GPL. These findings may provide clues to understanding the biological role of serovar 4-specific GPL in MAC pathogenicity and may also provide new insights into glycosyltransferase, which generates structural and functional diversity of GPLs.**

The genus *Mycobacterium* has a unique feature in the cell envelope that contains a multilayered structure consisting of peptidoglycan, mycolyl-arabinogalactan complex, and surface glycolipids (8, 12). It is known that these components play a role in protection from environmental stresses, such as antimicrobial agents and host immune responses (8, 12). Some of them are recognized as pathogenic factors related to mycobacterial diseases, such as tuberculosis and leprosy (8, 12). In case of nontuberculous mycobacteria that are widely distributed in the natural environment as opportunistic pathogens, glycopeptidolipids (GPLs) are abundantly present on the cell envelope as surface glycolipids (34). GPLs have a core structure in which a fatty acyl-tetrapeptide is glycosylated with 6-deoxy-talose (6-d-Tal) and *O*-methyl-rhamnose (*O*-Me-Rha) (2, 5, 13). This structure is common to all types of GPLs, and GPLs with this structure that have not undergone further glycosylation are termed non-serovar-specific GPLs (nsGPLs) (2, 5, 13). Structural diversity generated by further glycosylations, such as rhamnosylation, fucosylation, and glucosylation, is observed for the oligosaccharide portion linked to the 6-d-Tal residue of nsGPLs from *Mycobacterium avium* complex (MAC), a member of the nontuberculous mycobacteria consisting of two species, *M. avium* and *M. intracellulare* (2, 5, 34). Consequently, these nsGPLs with varied oligosaccharides lead to the formation of the serovar-specific GPLs (ssGPLs) that define around 30 types of MAC serovars (10).

The properties of MAC serovars are known to be notably different from each other and also to be closely associated with the pathogenicity of MAC (3, 6, 18, 30, 31, 32). Various epidemiological studies indicate that serovar 4 is the most prevalent type and is also one of the serovars frequently isolated from AIDS patients (1, 20, 33, 36). Additionally, pulmonary MAC disease caused by serovar 4 is shown to exhibit a poorer prognosis than that caused by other serovars (23). With respect to host immune responses to MAC infection, serovar 4-specific GPL is reported to have characteristic features that are in contrast to those of other ssGPLs (21, 30). Structurally, serovar 4-specific GPL contains a unique oligosaccharide in which the oligosaccharide of serovar 2-specific GPL is further glycosylated with 4-*O*-methyl-rhamnose (4-*O*-Me-Rha) residue through a 1→4 linkage (Table 1) (24). Therefore, it is thought that the presence of 4-*O*-Me-Rha and its linkage position are important in exhibiting the specificity of biological activities. The biosynthesis of the oligosaccharide portion in several ssGPLs is currently being clarified (15, 16, 17, 25, 26), while that of serovar 4-specific GPL is still unresolved. In this study, we have focused on the genomic region predicted to be associated with GPL biosynthesis in the serovar 4 strain and explored the key genes responsible

* Corresponding author. Mailing address: Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan. Phone: 81-42-391-8211. Fax: 81-42-394-9092. E-mail: yujim@nih.go.jp.

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TABLE 1. Oligosaccharide structures of serovar 2- and 4-specific GPLs

| Serovar | Oligosaccharide | Reference |
|---|---|---|
| 2 | 2,3-di-*O*-Me-α-L-Fuc-(1→3)-α-L-Rha-(1→2)-L-6-d-Tal | 9 |
| 4 | 4-*O*-Me-α-L-Rha-(1→4)-2-*O*-Me-α-L-Fuc-(1→3)-α-L-Rha-(1→2)-L-6-d-Tal | 24 |

for the formation of 4-*O*-Me-Rha that might determine the specific properties of MAC serovar 4.

## MATERIALS AND METHODS

**Bacterial strains, culture conditions, and DNA manipulation.** Table 2 indicates 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). For GPL production, *Mycobacterium smegmatis* strains were cultured in 2× YT broth (16 g tryptone, 10 g yeast extract, and 5 g NaCl per liter) with 0.2% Tween 80. DNA manipulation of *M. smegmatis* strains was conducted as previously described (27). PCR amplification was done by two-step PCR using TaKaRa LA *Taq* with GC buffer, with 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 DH5α was used for the routine manipulation and propagation of plasmid DNA. Antibiotics were added as required: kanamycin, 50 μg/ml for *E. coli* and 25 μg/ml for *M. smegmatis*; hygromycin B, 150 μg/ml for *E. coli* and 75 μg/ml for *M. smegmatis*. Oligonucleotide primers used in this study are listed in Table 3.

**Construction of expression vectors.** For generation of the serovar 2-specific GPL (GPL-S2)-producing strain, the vector possessing *rtfA*, *mdhtA*, *merA*, and *gtfD* genes was constructed. The *rtfA* gene was amplified from genomic DNA of *M. avium* strain JATA51-01 using primers RTFA-S and RTFA-A. The *mdhtA*, *merA*, and *gtfD* genes were amplified as one operon from the previously constructed vector pMVΔmtfF using primers MDHTA-S2 and GTFD-A2 (26). After construction of pMV261a, in which an AflII site was introduced into pMV261, the above two PCR products were digested with each restriction enzyme and cloned into the BamHI-PstI and PstI-AflII sites of pMV261a to give pMV-rtfA-mdhtA-merA-gtfD (Table 2). The fragments for construction of expression vectors were amplified from genomic DNA of MAC serovar 4 strain (ATCC 35767) using the following primers: HLPA-S and HLPA-A for *hlpA*, HLPA-S and ORF2-A for *hlpA-orf2*, and ORF3-S and ORF5-A for *orf3-orf4-orf5*. These PCR products were digested with each restriction enzyme and cloned into the EcoRI-ClaI, EcoRI-HindIII, and PstI-EcoRI sites of pYM301a to give pYM-hlpA, pYM-hlpA-orf2, and pYM-orf3-orf4-orf5, respectively (Table 2).

**Isolation and purification of GPLs.** To isolate whole-lipid extracts, harvested bacterial cells were mixed with $CHCl_3/CH_3OH$ (2:1 [vol/vol[) for several hours at room temperature. The extracts in organic phase were separated by adding water and evaporated until dry. To remove the lipid components except for GPLs, the whole-lipid extracts were subjected to mild alkaline hydrolysis to prepare the crude GPLs as previously described (27, 28). For analytical thin-layer chromatography (TLC), crude GPLs on silica gel 60 plates (Merck) were developed with $CHCl_3/CH_3OH/H_2O$ (30:8:1 [vol/vol/vol]), followed by spraying with 10% $H_2SO_4$ and charring. Purified GPLs were prepared from crude GPLs by preparative TLC on the same plates and extracted from the bands corresponding to each GPL. To determine the linkage position of sugar moieties, perdeuteriomethylation was performed for purified GPLs as previously described (7, 11, 15).

**GC-MS and MALDI-TOF MS analysis.** Purified and perdeuteriomethylated GPLs were hydrolyzed in 2 M trifluoroacetic acid (2 h, 120°C), and the released sugars were reduced with $NaBD_4$ and then acetylated with pyridine/acetic anhydride (1:1 [vol/vol]) at room temperature overnight. The resulting alditol acetates were analyzed by gas chromatography-mass spectrometry (GC-MS) with a GCMS-QP2010 (Shimadzu) equipped with a SP-2380 column (Supelco) using helium gas. The temperature program was from 52 to 172°C with an increase in temperature of 40°C/min, 172 to 223°C at 3°C/min, and then 223 to 270°C at 40°C/min. To determine the total mass of the purified GPLs, matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectra were acquired with an Ultraflex II (Bruker Daltonics). Samples were dissolved in chloroform-methanol (2:1 [vol/vol]) at a concentration of 1 mg/ml, 1 μl was applied directly to the sample plate, and then 1 μl of 10 mg/ml 2,5-dihydroxybenzoic acid in chloroform/methanol (1:1 [vol/vol]) was added as a matrix. The purified GPL was analyzed in the reflectron mode with an accelerating voltage operating in a positive mode of 20 kV (17).

**Nucleotide sequence accession number.** The 6.8-kb genomic region amplified from the MAC serovar 4 strain (ATCC 35767) by using primers GTFB-S1 and MDHTA-A2 has been deposited in the DDBJ nucleotide sequence database under accession no. AB550236.

## RESULTS

Previously, the A5 strain, one of the MAC serovar 4 strains, was reported to contain a genomic region similar to the GPL biosynthetic gene cluster identified in other serovars (22). However, to date, there are no studies clarifying the biosynthetic pathways involved in the formation of 4-*O*-Me-Rha, which is unique to serovar 4-specific GPL. To explore this glycosylation pathway, we focused on one segment interposed with the *gtfB* and *mdhtA* genes whose organization was shown to vary in strains of other serovars (14, 22). In this study, using another serovar 4 strain, ATCC 35767, whose genomic information is unknown, we designed various primers for PCR amplification of a focused segment based on the sequences from other serovar strains. After the testing of primer pairs, a

TABLE 2. Bacterial strains and vectors used in this study

| Strain or vector | Characteristic(s) | Source or reference |
|---|---|---|
| Bacterial strains | | |
| *E. coli* DH5α | Cloning host | TaKaRa |
| *M. smegmatis* $mc^2155$ | Expression host | 29 |
| *M. intracellulare* ATCC 35767 | MAC serovar 4 strain | 35 |
| *M. avium* JATA51-01 | Source of the *rtfA* gene | 26 |
| Vectors | | |
| pYM301a | Site-specific integrating mycobacterial vector carrying an *hsp60* promoter cassette | 25 |
| pMV261a | *E. coli-Mycobacterium* shuttle vector carrying an *hsp60* promoter cassette with an AflII site | This study |
| pMVΔmtfF | Source of *mdhtA*, *merA*, and *gtfD* genes | 26 |
| pMV-rtfA-mdhtA-merA-gtfD | pMV261a carrying *rtfA*, *mdhtA*, *merA*, and *gtfD* genes | This study |
| pYM-hlpA | pYM301a carrying the *hlpA* gene | This study |
| pYM-hlpA-orf2 | pYM301a carrying the *hlpA* gene and ORF2 | This study |
| pYM-orf3-orf4-orf5 | pYM301a carrying ORF3, ORF4, and ORF5 | This study |

TABLE 3. Oligonucleotide primers used in this study

| Primer | Sequence[a] | Restriction site |
|---|---|---|
| RTFA-S | 5′-CGGGATCCCATGAAATTTGCTGTGGCAAG-3′ | BamHI |
| RTFA-A | 5′-AACTGCAGCTCAGCGACTTCGCTGCGCTTC-3′ | PstI |
| MDHTA-S2 | 5′-GCTCTAGACTGCAGAAAAACCAACTTCTACTGCCTGACCTG-3′ | PstI |
| GTFD-A2 | 5′-GGAATTCTTAAGTCTACGGTTCTGCGCTTCGTTCTTTG-3′ | AflII |
| HLPA-S | 5′-GGAATTCGTGACAACGACGCCACCAGT-3′ | EcoRI |
| HLPA-A | 5′-CCATCGATACTACGCTGCCGCGCTAGGCG-3′ | ClaI |
| ORF2-A | 5′-CCCAAGCTTCTCAGACTCTAACGTACAGTTC-3′ | HindIII |
| ORF3-S | 5′-CACCTGCAGAAATGACCGCCACAACCAGGGC-3′ | PstI |
| ORF5-A | 5′-GCAGAATTCCTACGGCGCCAATTCGATGAG-3′ | EcoRI |
| GTFB-S1 | 5′-GGAACTCCTGCACCTTGGGGCCGT-3′ | |
| MDHTA-A2 | 5′-GGTGCGGGTCAACGTAGAGGTG-3′ | |

[a] Underlining indicates the restriction site.

6.8-kb fragment was amplified with primers GTFB-S1 and MDHTA-A2 (Fig. 1). Nucleotide sequences of the amplified fragments were similar to that of the GPL biosynthetic gene cluster from the A5 strain (94% identity in nucleotide sequences) (GenBank accession no. AY130970.1). This segment contains five complete open reading frame (ORF) genes (Fig. 1): the ORF1 gene, similar to a putative hemolytic protein gene (*hlpA*) previously found in the GPL biosynthetic gene cluster of the serovar 2 strain (69% identity in amino acid sequences) (GenBank accession no. AF125999.1) (14); the ORF2 gene, an undefined gene showing low similarity to some *O*-methyltransferases; and the ORF3, ORF4, and ORF5 genes, with amino acid sequences almost identical to those of three proteins, including GtfTB, which were previously identified as biosynthetic enzymes for serovar 8-specific GPL (GenBank accession no. AB437139.1) (25).

Prior to functional analysis of each ORF, it was necessary to prepare a strain producing the substrate for the enzymes participating in the biosynthesis of serovar 4-specific GPL. Since serovar 4-specific GPL has a structure in which the terminal Fuc residue of serovar 2-specific GPL is further glycosylated with 4-*O*-Me-Rha, we created a recombinant *M. smegmatis* strain (termed MS-S2) by introducing the plasmid vector pMV-rtfA-mdhtA-merA-gtfD possessing *M. avium rtfA*, *mdhtA*, *merA*, and *gtfD* genes, which were previously shown to convert nsGPLs to serovar 2-specific GPL with a terminal Fuc residue (termed GPL-S2) (26). For five ORFs, we first examined the function of the ORF1 (termed *hlpA*) and its downstream ORF2 gene by TLC analysis of recombinant strains, because these have not been functionally defined and it is difficult to predict the role of each gene. In comparison with the profile of the control strain (MS-S2/pYM301a) (Fig. 2, lane A), the new products (GPL-S4) were observed for the strain with the *hlpA* gene introduced (MS-S2/pYM-hlpA) (Fig. 2, lane B). Moreover, when the expression vector covering both *hlpA* and ORF2 was introduced into MS-S2 (MS-S2/pYM-hlpA-orf2), another new product (GPL-S4M) appeared (Fig. 2, lane C). These observations indicated that GPL-S2 was converted to structurally different compounds by the expression of *hlpA* and that the compounds generated by *hlpA* were further modified by ORF2. As for the ORF3, ORF4, and ORF5 genes, which show a high similarity to the biosynthetic genes for serovar 8-specific GPL, we further generated a strain having three ORFs (MS-S2/pYM-orf3-orf4-orf5) and examined the GPL production by TLC analysis (Fig. 2, lane D). The results indicated the appearance of known product GPL-S8, previously shown to have a sugar residue of serovar 8-specific GPL, with no GPL-S4 and GPL-S4M (25), demonstrating that the enzymes encoded by three ORFs might act on the serovar 1-specific GPL which was produced as a precursor of GPL-S2 and subsequently yielded GPL-S8.

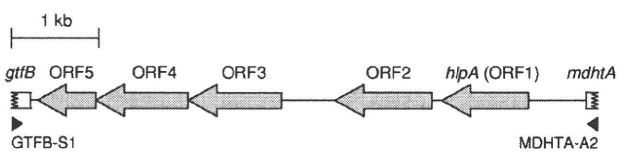


FIG. 1. Organization of the 6.8-kb genomic segment isolated from MAC serovar 4 strain (ATCC 35767). Filled triangles indicate the primers used for PCR amplification.

Because the compounds produced by *hlpA* and ORF2 were structurally unidentified, we performed a GC-MS analysis of the products GPL-S2, GPL-S4, and GPL-S4M, which were purified from recombinant strain MS-S2/pYM301a, MS-S2/pYM-hlpA, and MS-S2/pYM-hlpA-orf2, respectively. Although two spots were seen for each product, this might be due to a different methylation pattern for the fatty acid portion, which is often observed with GPL biosynthesis of *M. smegmatis* and does not affect oligosaccharide structure (19, 25). In GC-MS profiles of GPL-S2 and GPL-S4, the classes of the detected sugar residues, Fuc, 6-d-Tal, Rha, and 2,3,4-tri-*O*-Me-Rha, were found to be identical to each other (Fig. 3A and B). However, it was observed that the intensity of the Rha residue in GPL-S4 was higher than that of the other sugars, while in GPL-S2, the intensity of the Rha residue was lower than that of Fuc, indicating that the proportion of Rha content in GPL-S4 was relatively large compared to that in GPL-S2. These results implied that the *hlpA* gene mediates the transfer of an additional Rha residue to GPL-S2. In contrast, the profiles of GPL-S4M showed the presence of 4-*O*-Me-Rha that is specifically observed for serovar 4-specific GPL (Fig. 3C), demonstrating that ORF2 encodes a rhamnosyl 4-*O*-methyltransferase and that both genes are responsible for the formation of the unique sugar residue of serovar 4-specific GPL. Furthermore, we confirmed the molecular masses of products GPL-S2, GPL-S4, and GPL-S4M by MALDI-TOF MS analysis (Fig. 4). Each product contained two main pseudomolecular ions ($[M + Na]^+$) with 14 mass unit differences, indicating the

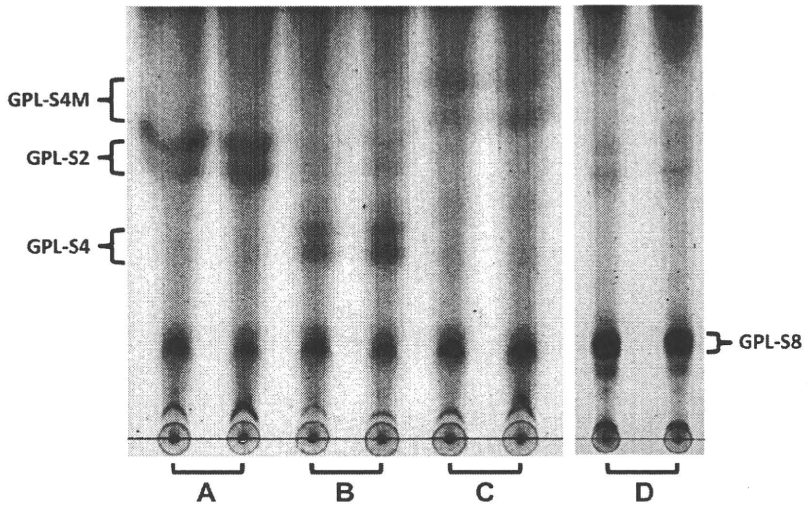


FIG. 2. TLC analysis of crude GPL extracts from recombinant *M. smegmatis* strains MS-S2/pYM301a (A), MS-S2/pYM-hlpA (B), MS-S2/pYM-hlpA-orf2 (C), and MS-S2/pYM-orf3-orf4-orf5 (D). GPL extracts were prepared from the total lipid fraction after a mild alkaline hydrolysis step. Each recombinant strain was tested by two samples derived from independent colonies. Samples were spotted and developed in $CHCl_3$-$CH_3OH$-$H_2O$ (30:8:1 [vol/vol/vol]).

presence or absence of the methyl group in the fatty acid portion as described above. Thus, the results revealed that the mass unit difference between GPL-S2 (*m/z* 1,465.80, 1,479.82) and GPL-S4 (*m/z* 1,611.84, 1,625.85) was 146 and that between GPL-S2 and GPL-S4M (*m/z* 1,625.89, 1,639.90) was 160, demonstrating that the Rha and 4-*O*-Me-Rha residues were further added to the GPL-S2 to yield GPL-S4 and GPL-S4M, respectively.

The results from TLC, GC-MS, and MALDI-TOF MS analyses strongly suggested that *hlpA* and ORF2 are involved in the formation of 4-*O*-Me Rha. However, it is not clear whether the *hlpA* gene product functions as a glycosyltransferase that transfers a Rha via 1→4 linkage to a Fuc residue, which is observed only for serovar 4-specific GPL. To elucidate the function of *hlpA*, we determined the linkage of sugar moieties of GPL-S4 produced by recombinant strain MS-S2/pYM-hlpA (Fig. 2, lane B). The purified GPL-S4 was subjected to perdeuteriomethylation followed by GC-MS and gave four peaks corresponding to Fuc, 6-d-Tal, Rha, and 2,3,4-tri-*O*-Me-Rha (data not shown). The spectra of Rha and 6-d-Tal demonstrated that the linkage position between these two sugar residues is commonly observed in the oligosaccharide of all ssGPLs, and position C-3 of Rha is linked to the next one, which is consistent with the data previously reported (Fig. 5B and C) (25, 26). In addition, the detection of fragment ions at *m/z* 121, 168, and 206 in spectra of Fuc indicated that its positions C-2 and C-3 were deuteriomethylated (Fig. 5D), meaning that position C-1 of Fuc is linked to position C-3 of Rha and position C-4 of Fuc is linked to the next one. These observations were supported by the fact that GPL-S4 was structurally based on the oligosaccharide of serovar 2-specific GPL. The peak of 2,3,4-tri-*O*-Me-Rha was found to include mixed fragment ions (Fig. 5A). A group of fragment ions corresponding to the spectra of 2,3,4-tri-*O*-Me-Rha linked to alaninol of tetrapeptide was observed. The remaining fragment ions at *m/z* 121, 134, 168, and 181 indicate the presence of deuteriomethyl groups at positions C-2, C-3, and C-4 of the other Rha that is linked at the terminus of oligosaccharide in GPL-S4. These results indicate that position C-1 of terminal Rha is linked to position C-4 of Fuc. Accordingly, the oligosaccharide structures of GPL-S4 were determined to have Rha-(1→4)-Fuc-(1→3)-Rha-(1→2)-6-d-Tal at D-*allo*-Thr, demonstrating that *hlpA* encodes a rhamnosyltransferase that transfers a Rha residue via 1→4 linkage to a Fuc residue of serovar 2-specific GPL (Fig. 6).

## DISCUSSION

It is known that serovar 4 is the most prevalent type, and serovar 4-specific GPL, particularly its oligosaccharide portion, plays a role in exhibiting the specific properties that belong to pathogenic factors. However, to date, the biosynthesis of its oligosaccharide portion has not been clarified. In this study, structural determination of three recombinant products, GPL-S2, GPL-S4, and GPL-S4M, revealed that *hlpA* and its downstream gene (ORF2) in the GPL biosynthetic gene cluster are involved in the formation of 4-*O*-methyl Rha, which is unique to serovar 4-specific GPL (Fig. 6). Previously, it was reported that the GPL biosynthetic gene cluster of MAC serovar 2 strains contained one gene whose amino acid sequences are similar to that of *hlpA* with 69% identity (14). This has been regarded as the gene not associated with GPL biosynthesis, because its amino acid sequences are similar to those of hemolytic proteins distributed in some species of bacteria (4). Thus, as shown in Fig. 6, it was surprising that *hlpA* from serovar 4 was found to encode a rhamnosyltransferase that plays a critical role in the pathway leading from serovar 2-specific GPL to serovar 4-specific GPL. For mycobacterium species, a BLAST analysis of HlpA revealed that its homologues are seen only in MAC serovar 2 and not in other species, including *Mycobacterium tuberculosis*. When we tested the

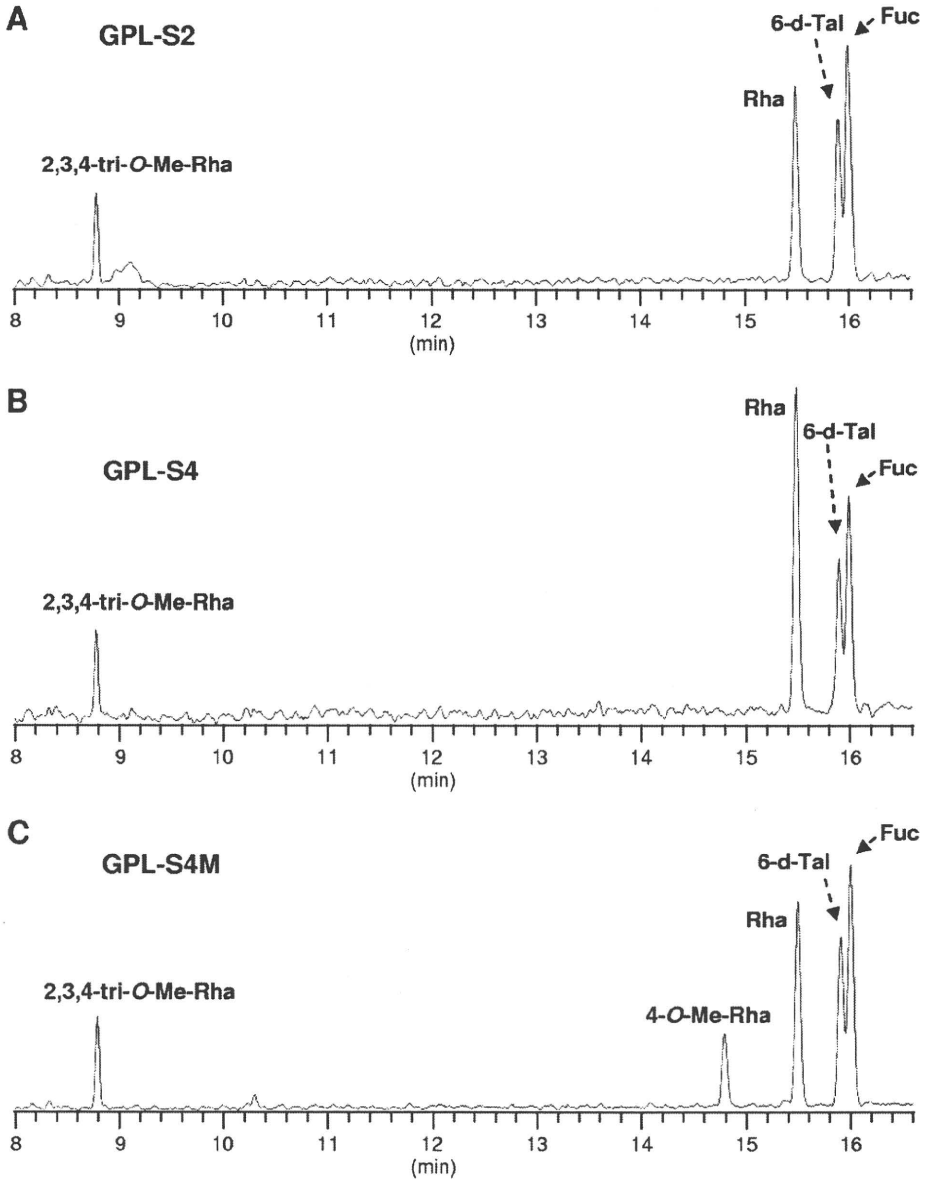


FIG. 3. GC-MS of alditol acetate derivatives from GPL-S2 (A), GPL-S4 (B), and GPL-S4M (C), which were purified from GPL extracts of recombinant *M. smegmatis* strains MS-S2/pYM301a, MS-S2/pYM-hlpA, and MS-S2/pYM-hlpA-orf2, respectively.

function of HlpA from serovar 2, it did not serve as a glycosyltransferase like HlpA from serovar 4 (data not shown). At present, the function of HlpA from serovar 2 is still unclear, because the biosynthesis of the oligosaccharide portion in serovar 2-specific GPL has been fully elucidated (14, 26). The oligosaccharide structure of serovar 2-specific GPL is basic for several ssGPLs, including serovar 4-specific GPL. In the biosynthetic gene cluster of serovar 2-specific GPL, several insertion sequence (IS) elements are observed, raising the possibility that the HlpA from serovar 2 is retained through genomic alterations that induce biosynthetic changes from other ssGPLs to serovar 2-specific GPL. Therefore, HlpA in the serovar 2 strain originally might function as a glycosyltransferase in the biosynthesis of oligosaccharides of other serovars.

Most HlpA homologues are putatively categorized as hemolytic proteins because they are similar to one protein from *Prevotella intermedia*, which is actually proved to have hemolytic activity (4). Since the amino acid sequences of HlpA show 38% identity and 54% similarity to the above protein of *P. intermedia*, we predicted that HlpA also possesses hemolytic activity as an additional function. However, none was detected when *hlpA* was expressed in *M. smegmatis* and *E. coli* by plate assay using a sheep blood agar plate (data not shown). A BLAST analysis of HlpA homologues showed that they also

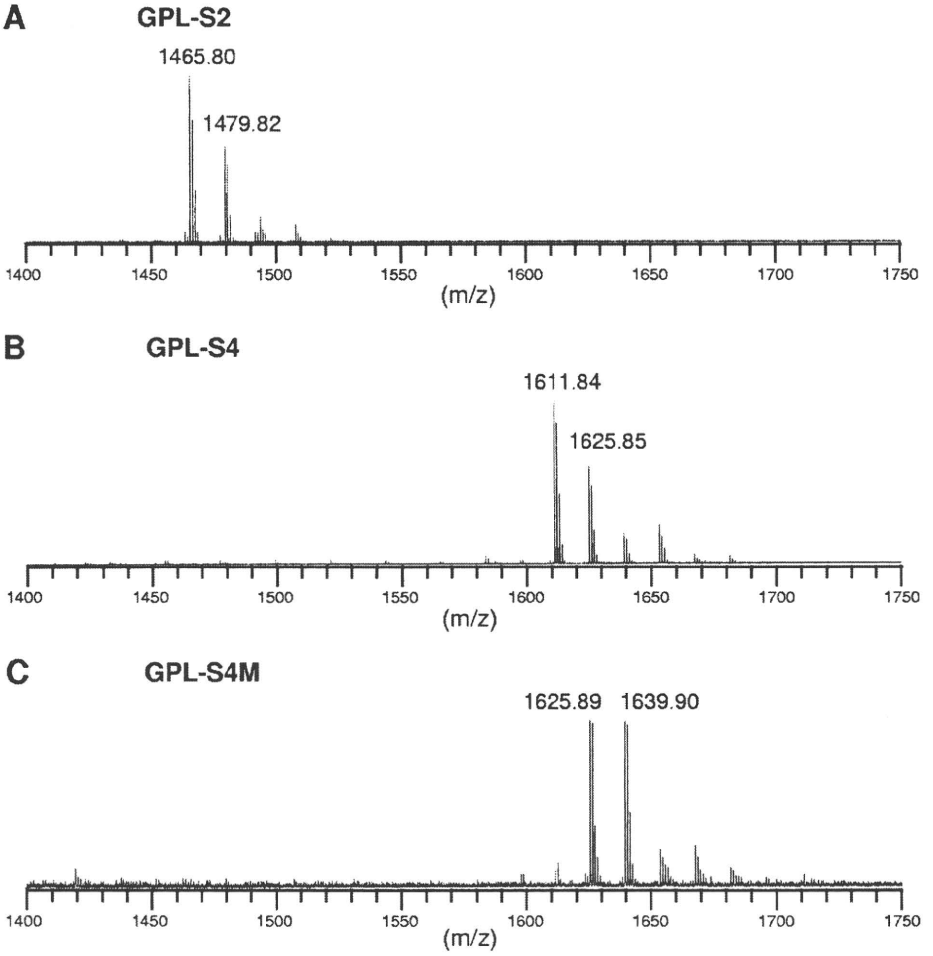


FIG. 4. MALDI-TOF MS of GPL-S2 (A), GPL-S4 (B), and GPL-S4M (C), which were purified from GPL extracts of recombinant *M. smegmatis* strains MS-S2/pYM301a, MS-S2/pYM-hlpA, and MS-S2/pYM-hlpA-orf2, respectively.

contained a partial motif of some glycosyltransferases and methyltransferases. Therefore, it is envisaged that the evolutionary ancestor of HlpA might have lost hemolytic activity in MAC or, conversely, have been altered to retain it in *P. intermedia* in the course of phylogenetic evolution between these bacterial species.

Serovar 4 strains, including ATCC 35767, have been recognized as strains producing the serovar 4-specific GPL but not the serovar 8-specific GPL (24, 35). However, as shown in Fig. 1, we found that the GPL biosynthetic gene cluster contains three known genes (the ORF3, ORF4, and ORF5 genes) previously identified as biosynthetic genes responsible for the formation of 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl glucose residue in the oligosaccharide of serovar 8-specific GPL (25). TLC analysis showed that overexpression of three ORFs potentially produces the serovar 8-specific GPL, including the 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl glucose residue (Fig. 2, lane D), demonstrating that in the serovar 4 strain, there is inefficient expression of three genes, which might be caused by genomic alterations affecting their transcription, resulting in the loss of serovar 8-specific GPL. Moreover, HlpA homologues are often found in several species of cyanobacteria but not in other bacterial groups and mycobacterium species, implying the occurrence of a certain kind of "horizontal gene transfer" between these environmental bacteria. Thus, MAC seemed to incorporate foreign genes or realign preexisting genes to modify the oligosaccharide structures of GPLs for their survival in a varied environment. In terms of sugar composition and linkage affecting the properties of ssGPLs, the functional aspects of the 4-*O*-methyl-Rha residue, which influence the interactions with the host cell, are still unclear. In addition, the sugar linkage Rha-(1→4)-Fuc is seen only in serovar 4-specific GPL and not in other ssGPLs, suggesting that it might generate unique properties that differ notably from those generated by other sugar linkages. Also, the rarity of this sugar linkage could be one of the factors that define the specificity of MAC serovar 4, which would be resolved by further studies, including the generation of an *hlpA* knockout mutant. For functional characterization of *hlpA* and ORF2, we have adopted the gene expression experiment

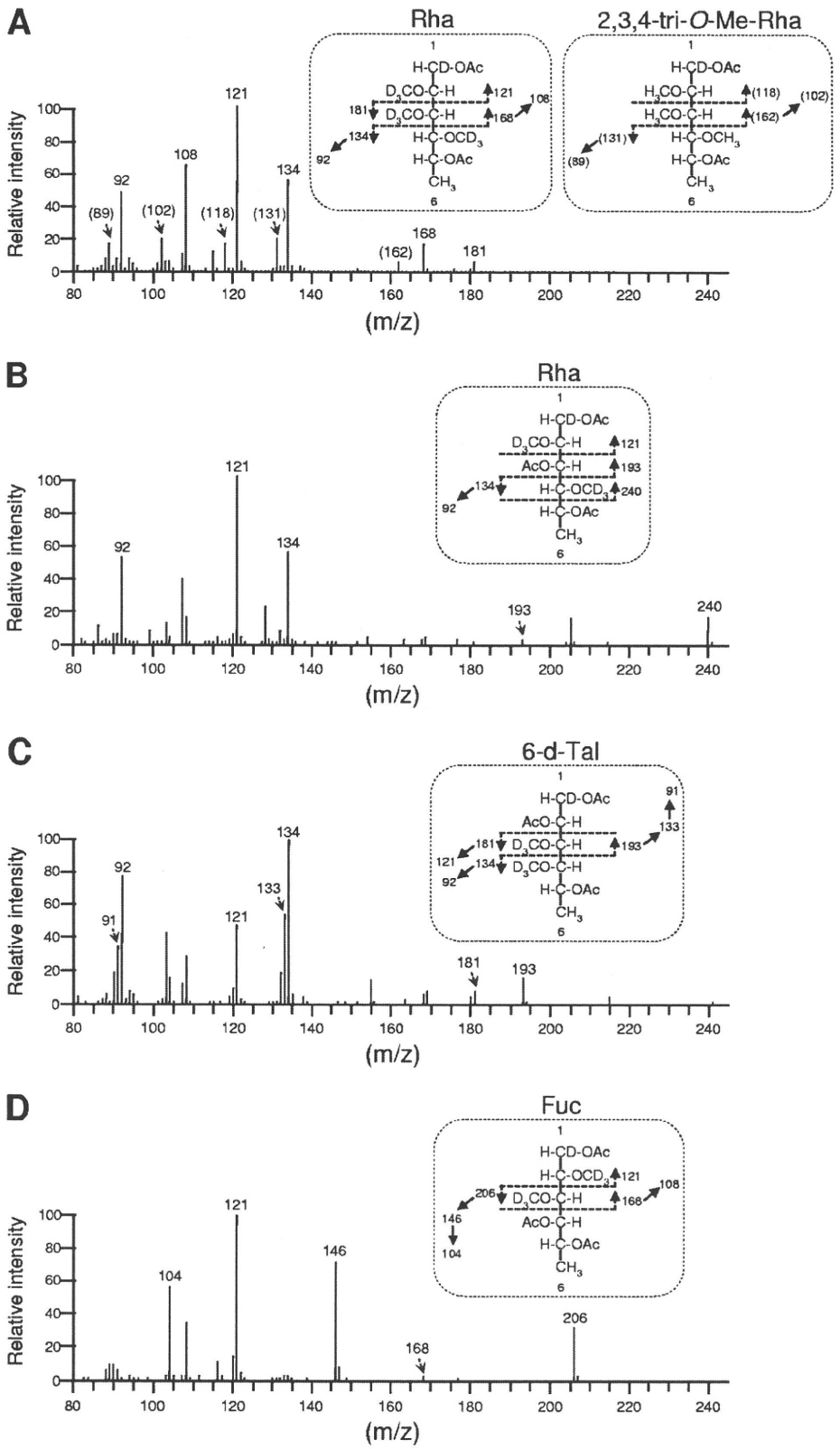


FIG. 5. GC-MS spectra and fragment ion assignments of 2,3,4-tri-*O*-Me-Rha (A), Rha (A and B), 6-*d*-Tal (C), and Fuc (D), which are derived from alditol acetates of sugars released from deuteriomethylated GPL-S4. Ac, acetate; D, deuterium.

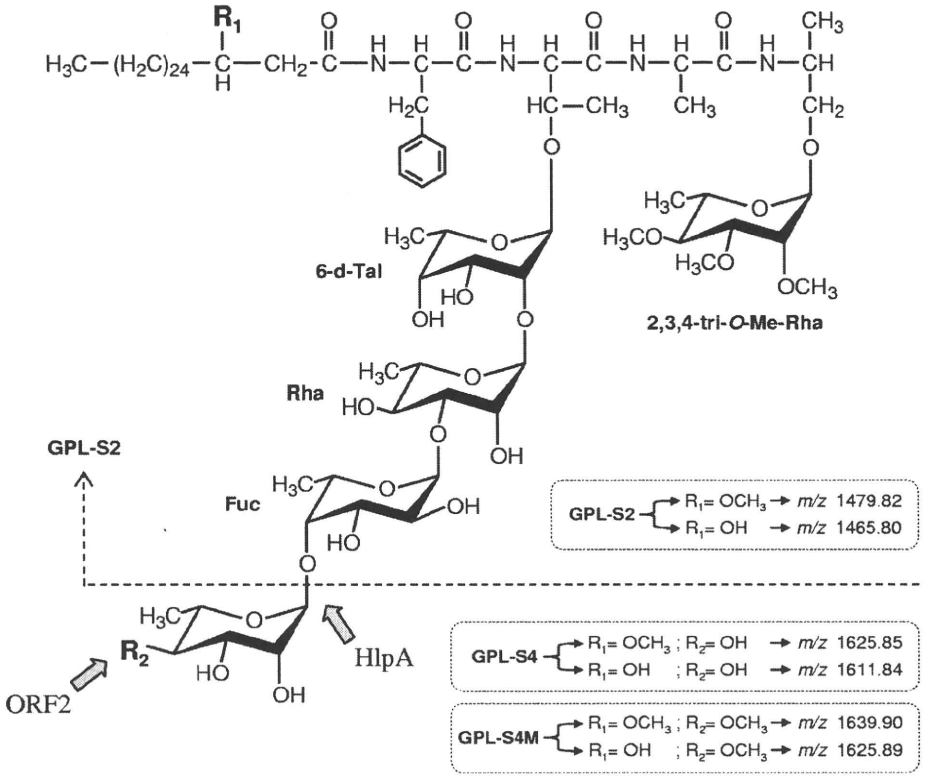


FIG. 6. Proposed structures and biosynthetic pathways for GPL-S2, GPL-S4, and GPL-S4M. Parentheses indicate structural differences between three compounds, which are deduced from MALDI-TOF MS analyses {pseudomolecular ions ($[M + Na]^+$)}.

using the *M. smegmatis* strain. Further enzymatic analyses, such as *in vitro* testing with recombinant proteins, would confirm our results. Taken together, these findings may contribute to understanding the mechanism for generation of structural and functional diversity of GPLs as well as their biological role in MAC.

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# Influence of Advanced Age on *Mycobacterium bovis* BCG Vaccination in Guinea Pigs Aerogenically Infected with *Mycobacterium tuberculosis*[▽]

Shihoko Komine-Aizawa,[1,2]* Toshio Yamazaki,[3] Tsuyoshi Yamazaki,[4] Shin-ichiro Hattori,[1,5] Yuji Miyamoto,[6] Naoki Yamamoto,[1,7] Shinji Haga,[8] Masahiko Sugitani,[9] Mitsuo Honda,[1,2] Satoshi Hayakawa,[1,2] and Saburo Yamamoto[10]

*AIDS Research Center,[1] Division of Biosafety Control and Research,[3] Department of Mycobacteriology, Leprosy Research Center,[6] and Department of Bacteriology,[8] National Institute of Infectious Diseases, Division of Microbiology[2] and Division of Pathology,[9] Department of Pathology and Microbiology, Nihon University School of Medicine, and Japan BCG Laboratory,[10] Tokyo, School of High-Technology for Human Welfare, Tokai University, Kanagawa,[4] and Division of Hematopoiesis, Center for AIDS Research, Kumamoto University, Kumamoto,[5] Japan, and Department of Microbiology, Yong Loo Lin School of Medicine, Singapore[7]*

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***Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is the only tuberculosis (TB) vaccine currently available, but its efficacy against adult pulmonary TB remains controversial. BCG induces specific immune responses to mycobacterial antigens and may elicit protective immunity against TB. TB remains a major public health problem, especially among the elderly, yet the efficacy of BCG in the elderly is unknown. We investigated the ability of BCG vaccination to prevent TB in young (6-week-old), middle-aged (18-month-old), and old (60-month-old) guinea pigs. BCG-Tokyo vaccination reduced the growth of *Mycobacterium tuberculosis* H37Rv in all three groups. By use of an enzyme-linked immunospot (ELISPOT) assay, antigen-specific gamma interferon (IFN-γ)-producing cells were detected in the 60-month-old guinea pigs after a booster vaccination with BCG-Tokyo. Our findings suggest that BCG-Tokyo has a protective effect against tuberculosis infection regardless of age.**

Tuberculosis (TB) remains a major public health problem, especially among elderly people. Patients ≥60 years of age account for ≥50% of new cases in Japan (29). The increasing susceptibility of the elderly to *Mycobacterium tuberculosis* is generally thought to be associated with age-related changes in immune system function, especially losses or delays in antigen-specific $CD4^+$ T-cell function (14). Compromised antigen-specific $CD4^+$ T-cell responses may contribute to increased susceptibility to *M. tuberculosis* infection in mice (27).

*Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is the only TB vaccine currently available. BCG has been used for more than 80 years (41), and vaccination with BCG is the standard for TB prevention in most countries. BCG induces specific immune responses to mycobacterial antigens and may elicit protective immunity against tuberculosis. BCG provides efficient protection against severe and disseminated TB, such as tuberculosis meningitis and miliary tuberculosis, in children (33, 34, 40). Although the long-term efficacy of BCG has been documented (3, 6), with several reports indicating efficient protection against disseminated TB in newborns and children, it appears to have less efficacy against adult pulmonary TB (2). In fact, its efficacy against pulmonary TB in both adults and the elderly is controversial, as is the efficacy of revaccination (5).

In the present study, we examined the efficacy of BCG against TB at different ages in a common guinea pig model (15, 25, 30). We used three age-segregated groups—young (6 weeks old), middle-aged (18 months old), and old (60 months old)—and we measured the number of antigen-specific gamma interferon (IFN-γ)-producing cells as an indicator of the efficacy of the vaccine against TB.

## MATERIALS AND METHODS

**Animals.** Female pathogen-free outbred Hartley guinea pigs were purchased from Japan SLC (Shizuoka, Japan). The guinea pigs were divided into the three groups described above and were housed in accordance with the guidelines for animal experimentation of the Japanese Association for Laboratory Animal Science (1987) and in full compliance with the Law for the Humane Treatment and Management of Animals (Japan). The guinea pigs were fed and maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (NIID), Japan. Once approved by an institutional committee for animal experiments, these studies were conducted at the Animal Facility of Toyama Campus, NIID, Japan, in accordance with the requirements specifically stated in the Laboratory Biosafety Manual of the World Health Organization.

**BCG vaccination.** The guinea pigs were vaccinated with $5 \times 10^5$ CFU of BCG (strain Tokyo 172) injected subcutaneously into the left or right inguinal region. The vaccination schedules were as follows. The old guinea pigs were vaccinated with BCG, maintained for 60 months, and then revaccinated with BCG 6 weeks before *M. tuberculosis* infection (group 1; *n*, 2). The middle-aged guinea pigs were vaccinated either 18 months or 6 weeks before the infection (groups 2 and

* Corresponding author. Mailing address: Division of Microbiology, Department of Pathology and Microbiology, Nihon University School of Medicine, 30-1 Oyaguchi-Kamicho, Itabash-ku, Tokyo, Japan. Phone: 81-3-3972-8111. Fax: 81-3-3972-9560. E-mail: ashiho@med.nihon-u.ac.jp.

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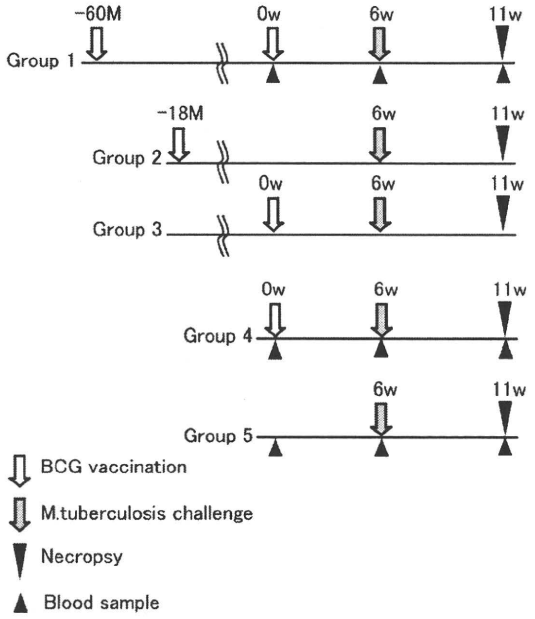


FIG. 1. Experimental design. Guinea pigs in the old group were vaccinated with BCG at the age of 6 weeks and were then maintained for 60 months before revaccination with BCG 6 weeks before *M. tuberculosis* infection (group 1). Guinea pigs in the middle-aged groups either were vaccinated with BCG at the age of 6 weeks and were then maintained for 18 months before infection (group 2) or were not vaccinated with BCG until 6 weeks before infection (group 3). Guinea pigs in the young groups either were vaccinated 6 weeks before infection (group 4) or were not vaccinated (group 5).

3, respectively; *n*, 3). The young animals either were vaccinated 6 weeks before the infection (group 4; *n*, 3) or were not vaccinated (group 5; *n*, 4) (Fig. 1).

**Aerosol challenge with *M. tuberculosis* H37Rv.** Virulent *M. tuberculosis* H37Rv (NIHJ1633) was grown in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with albumin dextrose catalase (ADC) enrichment and 0.05% Tween 80 for 14 to 21 days at 37°C. The bacilli were subjected to gentle sonication in order to obtain a single-cell suspension and were frozen at −80°C until use. Thawed aliquots were diluted in phosphate-buffered saline (PBS) containing 0.05% Tween 20 to the desired inoculum concentration. BCG-vaccinated and unvaccinated guinea pigs were exposed to 2.5 ml of *M. tuberculosis* H37Rv at $5 \times 10^4$ CFU/ml by using an inhalation exposure system, model 4212 (Glas-Col, Terre Haute, IN). Then BCG-vaccinated and unvaccinated guinea pigs were infected with approximately 10 CFU of virulent *M. tuberculosis* H37Rv via the respiratory route. The animals were housed under biosafety level 3 conditions in a manner consistent with the international animal care and use guidelines of the National Institute of Infectious Diseases of Japan.

**DTH skin test.** To investigate delayed-type hypersensitivity (DTH) skin reactions, 0.2 μg of tuberculin purified protein derivative (PPD) was injected intradermally into BCG-vaccinated and unvaccinated guinea pigs, and the skin reactions were measured after 24 h.

**Microbial enumeration.** At 5 weeks postchallenge, specimens from the lungs, tracheal lymph node, and spleen from each (BCG-vaccinated or unvaccinated) aerosol-challenged guinea pig were removed aseptically and were homogenized separately in 1 ml of sterile saline using a Stomacher-80T instrument (Organo, Tokyo, Japan). Appropriate dilutions were inoculated onto 1% Ogawa medium (Kyokuto, Tokyo, Japan) and were incubated at 37°C for 3 weeks. The number of *M. tuberculosis* H37Rv colonies on the medium was counted and expressed as the mean $\log_{10}$ CFU per tissue.

**Histopathology.** The dissected lung samples from each guinea pig were fixed with 10% neutral-buffered formalin and were embedded in paraffin wax. The sections from these tissues were 4 μm thick and were stained with hematoxylin and eosin (H&E) or with Ziehl-Neelsen stain for acid-fast bacilli.

**Preparation of cells.** Mononuclear cells were isolated from the peripheral blood of the guinea pigs. Approximately 10 ml of blood was harvested from the animals by cardiac puncture at 0, 6, and 11 weeks after BCG vaccination. Before blood collection, the animals were anesthetized with ketamine (44 mg/kg). Peripheral blood mononuclear cells (PBMCs) were prepared with Lymphosepar (IBL Co., Ltd., Gunma, Japan) and were then adjusted to $1 \times 10^6$/ml in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum). Cell viability was determined by a trypan blue dye exclusion test. Single-cell suspensions were cultured with or without PPD (10 μg/ml) at 37°C in a humidified 5% $CO_2$ environment for 40 h. Phytohemagglutinin (PHA) (1 μg/ml) was used as a positive control to stimulate whole T cells.

**IFN-γ ELISPOT assay.** IFN-γ-secreting cells were assessed by an enzyme-linked immunospot (ELISPOT) assay that was modified and improved to detect guinea pig IFN-γ-producing cells. Due to the low cross-reactivity of murine, rat, and guinea pig IFN-γ, we obtained a novel rabbit polyclonal anti-guinea pig IFN-γ antibody and developed a guinea pig IFN-γ ELISPOT assay system. Briefly, based on the predicted amino acid sequence of guinea pig IFN-γ described previously(16), we synthesized peptides (GG-1, GG-2, GG-3, GG-4, and GG-5). The immunogen (100 mg of peptide) was injected subcutaneously into rabbits, and 7 weeks after immunization, the rabbits were bled from the ear artery (50 to 100 ml). Antibodies were purified from antisera by affinity chromatography with immobilized synthetic peptides. Immunoblot analysis showed that only antisera against GG-2 and GG-5 reacted with a protein band of about 20 kDa, which is the putative molecular mass of guinea pig IFN-γ. Furthermore, the binding affinities of the purified antibodies for recombinant guinea pig IFN-γ were assessed. Recombinant guinea pig IFN-γ was prepared using the baculovirus system. Guinea pig IFN-γ cDNA was transfected into a baculovirus genome (Abv baculovirus; Katakura Industries Co. Ltd., Tokyo, Japan) with the pYNG transfer vector. Recombinant guinea pig IFN-γ was purified from the culture supernatant by an immunoaffinity column, and its bioactivity was measured based on the inhibition of the cytopathic effect of encephalomyocarditis virus (EMCV) on 104C1 guinea pig fibroblasts. We modified a previously described IFN-γ ELISPOT assay protocol (18) to detect guinea pig IFN-γ-producing cells. A polyclonal rabbit antibody to a guinea pig IFN-γ peptide was allowed to adhere overnight at 4°C to a 96-well nitrocellulose plate (MultiScreen-HA; Millipore, Billerica, MA) at a concentration of 5 μg/ml. The plate was washed with PBS–0.05% Tween 20 (PBST) and was then blocked with PBS supplemented with 1% bovine serum albumin (BSA) for 2 h at room temperature. Guinea pig PBMCs were transferred to the antibody-coated 96-well nitrocellulose plate in triplicate at an input cell number of $1 \times 10^5$ per well and were then incubated for 5 h at 37°C in a humidified 5% $CO_2$ environment. After 5 h of culturing, the plate was washed with PBST to remove cells and was then incubated with a biotinylated rabbit anti-IFN-γ secondary antibody at a concentration of 5 μg/ml for 2 h at room temperature. After a wash with PBST, the plate was treated with streptavidin-alkaline phosphatase and the substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP)-nitroblue tetrazolium (NBT) (ELISPOT blue color module; R&D Systems, Minneapolis, MN). Spot-forming cells (SFCs) were quantified using the KS ELISPOT compact system (Carl Zeiss Japan, Tokyo, Japan).

**Statistical analysis.** The data were analyzed using the Tukey-Kramer test and Pearson's correlation coefficient test using Statcel 2 software. Differences between treatments were determined by the least-squares significant-difference multiple-comparison method. A probability level of 5% ($P$, $<0.05$) was considered statistically significant.

## RESULTS

**DTH skin responses of guinea pigs to PPD.** DTH was assessed on the skin of guinea pigs 6 or 11 weeks after BCG inoculation both before and after challenge with *M. tuberculosis*. At week 6, significant DTH responses to PPD were detected in all of the guinea pigs vaccinated with BCG, while no response to PPD was detected in unvaccinated guinea pigs. The mean diameters of the indurations were as follows: 17.0 ± 4.2 mm (group 1), 20.0 ± 0.6 mm (group 2), 18.0 ± 1.7 mm (group 3), 15.3 ± 2.1 mm (group 4), and 5.5 ± 1.3 mm (group 5). No significant difference was observed among the groups vaccinated with BCG. DTH responses were detected in all groups at 5 weeks after the challenge with *M. tuberculosis* (Fig. 2).

**BCG-induced PPD-specific T-cell responses.** We examined the stimulation by PPD of IFN-γ production by the PBMCs of

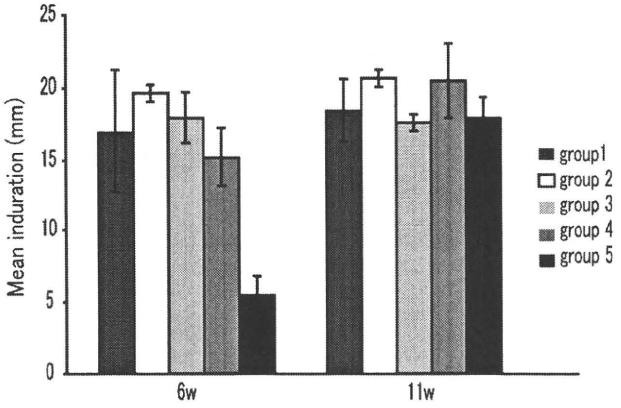


FIG. 2. PPD skin reactions of the guinea pigs. DTH was assessed on the basis of the skin reactions of guinea pigs 6 or 11 weeks (6w or 11w) after BCG inoculation both before and after challenge with *M. tuberculosis*. Error bars represent standard deviations. No significant difference among the groups vaccinated with BCG was observed.

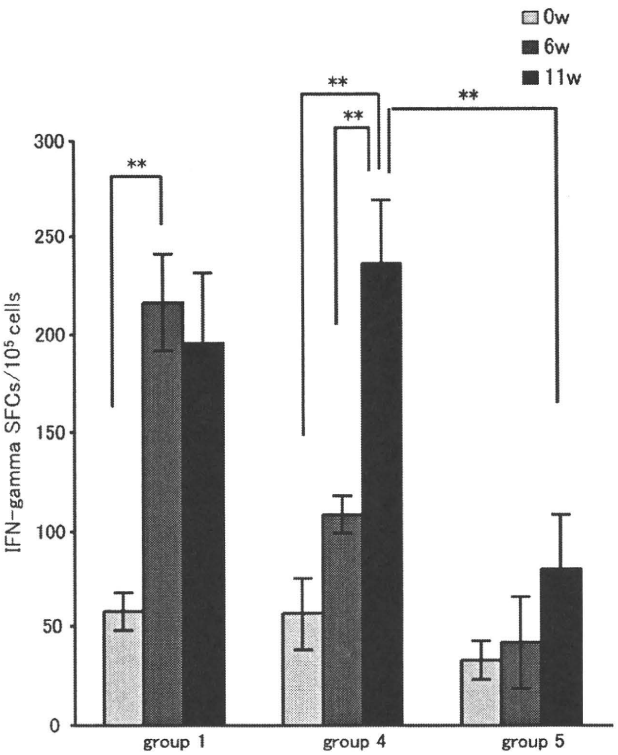


FIG. 3. BCG-induced PPD-specific T-cell responses. To investigate T-cell functions specific for PPD in groups 1, 4, and 5, an IFN-γ ELISPOT assay was performed at weeks zero, 6, and 11 after BCG vaccination. Error bars represent standard deviations. Asterisks indicate that the mean numbers of IFN-γ SFCs were significantly different. **, $P < 0.01$, as determined by analysis of variance (ANOVA) followed by a posthoc Tukey-Kramer test. There was no difference in IFN-γ SFCs between the old and young guinea pigs vaccinated with BCG.

the guinea pigs. To investigate T-cell functions specific for PPD, an IFN-γ ELISPOT assay was performed for groups 1, 4, and 5. The old guinea pigs of group 1 were inoculated with BCG 60 months before the infection and at week zero, and the young guinea pigs of group 4 were inoculated with BCG at week zero. Another group of young guinea pigs, group 5, was not inoculated. IFN-γ production by PBMCs was examined in each group at weeks zero, 6, and 11. At week 6 after the BCG vaccination, significant and specific IFN-γ responses to PPD were detected in groups 1 and 4 (Fig. 3). The mean numbers of SFCs among the PBMCs from the animals in groups 1 and 4 were 216.25 ± 24.50 and 108.75 ± 9.57, respectively. No significant IFN-γ production was detected in group 5. PPD-specific IFN-γ-secreting cells were more frequent among the PBMCs from group 1 than among those from group 4. Five weeks after the challenge with *M. tuberculosis* (at week 11 after the BCG vaccination), a significant increase in IFN-γ production by PBMCs following stimulation with PPD was observed in groups 1 and 4, although there was no difference between the groups in the mean frequency of cells responding specifically to PPD. The number of SFCs was also higher in group 5 after the challenge with *M. tuberculosis*. However, the number of SFCs was significantly lower than those in the BCG-vaccinated groups ($P, < 0.01$). At week zero of BCG vaccination, no increase in IFN-γ production was detected by the ELISPOT assay in group 1 in spite of the early BCG vaccination; groups 4 and 5 also showed no increase.

**Effect of BCG vaccination on bacterial growth in young, middle-aged, and old guinea pigs challenged with *M. tuberculosis* H37Rv.** To determine the impact of BCG vaccination on bacterial growth in young, middle-aged, and old guinea pigs, bacterial replication in the lungs (Fig. 4A), tracheal lymph nodes (Fig. 4B), and spleen (Fig. 4C) was examined for each group. In all cases, those animals vaccinated with BCG showed less bacterial growth in the lungs and tracheal lymph nodes than unvaccinated animals. In the spleen, no bacterial replication was detected except in groups 2 and 5. In group 2, which received BCG vaccination 18 months before the *M. tuberculosis* challenge, the effect of BCG may have been attenuated. Group 1, which was revaccinated with BCG before the challenge, showed significantly less bacterial growth in the lungs than the unvaccinated guinea pigs ($P, <0.05$). In the tracheal lymph nodes, bacterial growth was also reduced, but the difference was not significant. We also found a significant negative correlation between the number of IFN-γ SFCs and the residual number of bacteria in the lungs (expressed in $\log_{10}$ CFU) 5 weeks after *M. tuberculosis* challenge ($r$, −0.6696; $P$, 0.04852) in groups 1, 4, and 5 (Fig. 5). This finding suggests that PPD-specific T-cell responses induced by BCG are crucial for the host defense against *M. tuberculosis* infection. Thus, BCG appears to have a protective effect in guinea pigs at all ages.

**Histopathology.** Figure 6 shows histopathological images of the lungs of young unvaccinated guinea pigs (Fig. 6a and b) and BCG-vaccinated guinea pigs (Fig. 6c and d) 5 weeks after *M. tuberculosis* challenge. Figure 6c shows a lung from a young BCG-vaccinated guinea pig (group 4), and Fig. 6d shows a lung from an old BCG-revaccinated guinea pig (group 1). In the lungs from unvaccinated guinea pigs, large granuloma nodules with central necrosis were predominant and consisted of epithelioid cells. Acid-fast bacilli were detected in the granulomas by Ziehl-Neelsen staining (Fig. 6b). Although granuloma nodules were also observed in the lungs of vaccinated guinea pigs

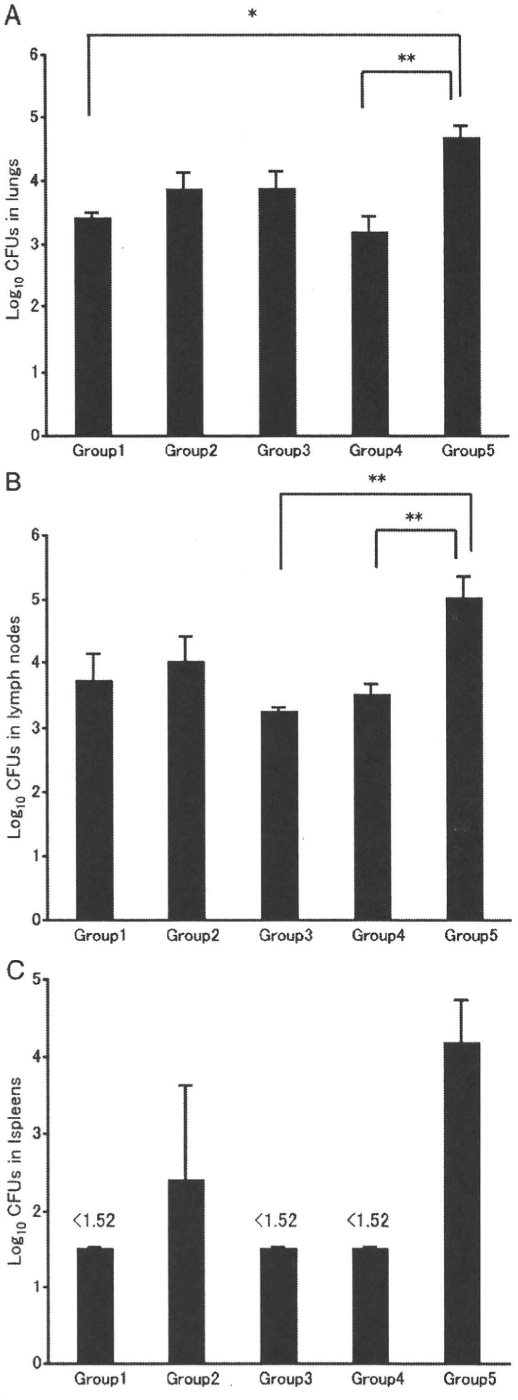


FIG. 4. Effects of BCG vaccination on bacterial growth in young, middle-aged, and old guinea pigs challenged with *M. tuberculosis* H37Rv. To determine the impact of BCG vaccination on bacterial growth, bacterial replication in lung (A), tracheal lymph node (B), and spleen (C) specimens from each guinea pig was examined. The minimum detectable level of bacilli in the tissue homogenate was 1.52 $\log_{10}$ CFU. Error bars represent standard deviations. Asterisks indicate that the mean numbers of *M. tuberculosis* CFU in an organ were significantly different. *, $P < 0.05$, and **, $P < 0.01$, as determined by analysis of variance (ANOVA) followed by a posthoc Tukey-Kramer test.

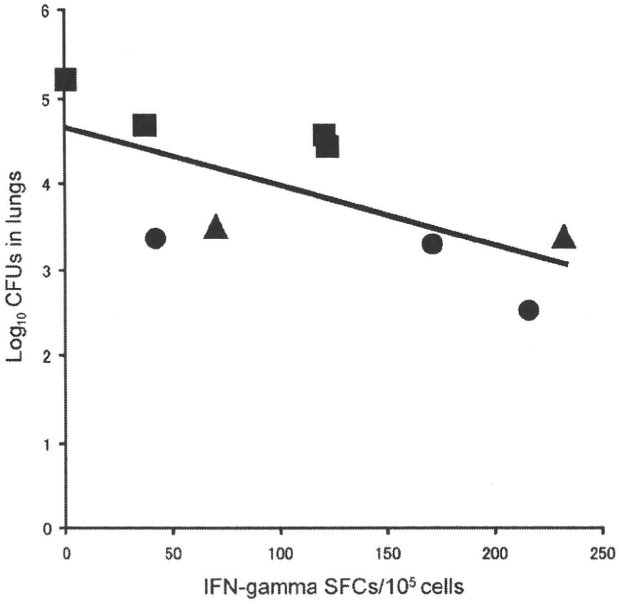


FIG. 5. Correlation between IFN-γ production and bacterial growth in the lungs. There was a statistically significant negative correlation between the number of IFN-γ SFCs detected 5 weeks after *M. tuberculosis* challenge and bacterial growth in the lungs ($r$, −0.6696; $P$, 0.04852 as determined by Pearson's correlation coefficient test). Circles, group 4 (young, vaccinated); squares, group 5 (young, unvaccinated); triangles: group 1 (old, revaccinated).

(Fig. 6c and d), vaccination with BCG reduced granuloma nodule formation in the lungs of both young and old guinea pigs, and no acid-fast bacilli were detected in the granulomas by Ziehl-Neelsen staining (data not shown).

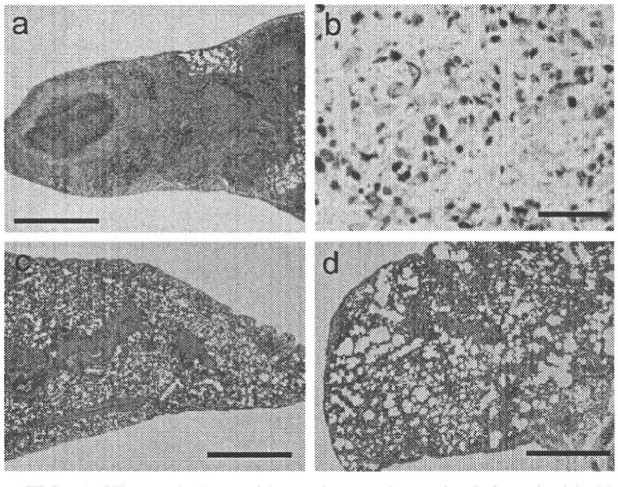


FIG. 6. Histopathology of lungs from guinea pigs infected with *M. tuberculosis* H37Rv. Shown are histopathological observations in the lungs of young unvaccinated (a and b), young BCG-vaccinated (c), and old BCG-revaccinated (d) guinea pigs 5 weeks after *M. tuberculosis* challenge. Bars, 1 mm (a, c, and d) and 50 μm (b).

## DISCUSSION

BCG is the only TB vaccine currently available, and it has been used since 1921. It is inexpensive and safe, with few complications reported in infants. BCG provides efficient protection against severe and disseminated TB, such as tuberculosis meningitis and miliary TB, in children (33, 34, 40). However, its efficacy at preventing pulmonary TB in adults is controversial. Aronson et al. reported that BCG vaccination had long-term efficacy for American Indians and Alaskan natives (3), suggesting that a single dose offered protection for 50 to 60 years. The long-term efficacy estimates from clinical trials, observational case-control studies, and contact studies range from 0 to 80% (7), although the efficacy for the elderly is unknown. In the present study, we demonstrated that revaccination of elderly guinea pigs with BCG-Tokyo reduced bacterial replication in the lungs, alveolar lymph nodes, and spleen. In addition, in 60-month-old guinea pigs, PPD-specific IFN-γ responses were observed after the BCG-Tokyo booster vaccination. These findings suggest that BCG-Tokyo has a protective effect at all ages.

However, the efficacy of BCG revaccination is a matter of international debate (5). Several studies have shown that BCG revaccination had no protective efficacy against TB (19, 28, 32). Fjällbrant et al. reported that both primary vaccination and revaccination of tuberculin skin test-negative young adults caused a significant increase in the T-helper type 1 (Th1) immune response (12), a result consistent with the present findings in the old guinea pig model. This result suggests that BCG revaccination has a protective effect against TB. However, other factors that determine the efficacy of BCG revaccination, including age, duration of vaccination, and the influence of environmental mycobacteria, must be considered.

Cell-mediated immune responses play an essential role in the control of *M. tuberculosis* infection and TB. In particular, $CD4^+$ and $CD8^+$ T-cell subsets are considered to play important roles in the production of cytokines such as IFN-γ and tumor necrosis factor alpha (TNF-α). These cytokines are involved in inflammatory processes, including macrophage activation, control of *M. tuberculosis* replication, and granuloma formation (1, 10, 13). Using guinea pig models, Jeevan et al. suggested that BCG vaccination induces upregulation of IFN-γ and TNF-α after *M. tuberculosis* challenge (16). In the present study, we investigated IFN-γ responses by using an ELISPOT assay. To the best of our knowledge, this is the first study that used a guinea pig model together with an antigen-specific ELISPOT assay to show that BCG induces PPD-stimulated IFN-γ responses. The secretion of PPD-specific IFN-γ was observed in both young and old BCG-vaccinated guinea pigs. The number of PPD-specific IFN-γ-secreting cells was greater in 60-month-old vaccinated guinea pigs (group 1) than in young vaccinated guinea pigs (group 4). Because this was the second BCG vaccination for group 1, a booster effect may have occurred. Bacterial growth in the lungs, lymph nodes, and spleen was higher in unvaccinated guinea pigs (group 5) than in the vaccinated groups. The number of PPD-specific IFN-γ-producing PBMCs in the unvaccinated guinea pigs was significantly lower than that in the vaccinated guinea pigs. The results of our *M. tuberculosis* aerosol infection experiment suggest that the number of PPD-specific IFN-γ-producing PBMCs correlates with the level of protection against *M. tuberculosis*. While TNF-α is another important cytokine that protects against *M. tuberculosis*, BCG vaccination appears to modulate the potentially harmful effects of TNF-α and to reduce *M. tuberculosis* replication (26, 42). Recent studies have shown that general immune responses are important for resistance to *M. tuberculosis*. Interleukin-12 (IL-12) is required for dendritic cell migration (22), maintenance of pulmonary Th1 cells (11), and macrophage activation and subsequent production of IFN-γ. IL-27 has both proinflammatory and anti-inflammatory properties. IL-12 and/or *M. tuberculosis*-induced IL-27 gene expression in human macrophages may regulate macrophage function during *M. tuberculosis* infection (31). In addition, the importance of Th17 responses, including IL-17 and IL-23, in the pathophysiology of *M. tuberculosis* infection has been reported recently (4, 20, 21). *M. tuberculosis*-specific Th1 (IL-12 and IFN-γ) and Th17 responses play roles in the increased expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1), while IL-23 induces IFN-γ and supports the IL-17 response in the lungs. McMurray and colleagues, using a laser capture microdissection (LCM) technique, reported cytokine mRNA responses *in situ* in the pulmonary granulomas of nonvaccinated and BCG-vaccinated guinea pigs (23, 24). TNF-α mRNA was dominant in primary lesions microdissected from nonvaccinated guinea pigs at both 3 and 6 weeks postinfection, while the cytokine profiles of granulomas from BCG-vaccinated guinea pigs shifted from type 1 cytokine mRNA (IFN-γ and IL-12p40) at 3 weeks to a predominantly anti-inflammatory profile dominated by transforming growth factor-β (TGF-β) at 6 weeks (23, 24). These results suggest that BCG vaccination modulates cytokine responses in the lungs to promote antimycobacterial functions while controlling the potentially damaging inflammatory response.

A DTH skin test for PPD has been employed in the diagnosis of TB. While the test is highly sensitive for PPD, its specificity in the diagnosis of TB infection is controversial, because after BCG vaccination, a DTH response is detected. In the present study, DTH responses to PPD were detected in all of the guinea pigs vaccinated with BCG, and no significant difference was observed among the age groups. However, IFN-γ production by PBMCs was significantly different between the groups, and the number of PPD-specific IFN-γ-producing PBMCs correlated with the degree of protection against *M. tuberculosis*. These results suggest that ELISPOT assays that detect TB-specific immune responses may be the most accurate means of monitoring immunity against TB.

In Japan, individuals 65 years old or older represented 22.1% of the population in 2008, and this age group is expected to grow to one-third of the population by 2035 (8). This trend is seen in other countries as well. Currently, in Japan, more than 50% of the new cases of TB occur in patients ≥60 years old (29). The increasing susceptibility of the elderly to *M. tuberculosis* is generally thought to be associated with immune senescence, the most significant change being the loss or delayed production of antigen-specific $CD4^+$ T cells (14). In mice, an inadequate antigen-specific $CD4^+$ T-cell response is thought to contribute to increased susceptibility to *M. tuberculosis* infection (27). However, the mouse model has revealed that old mice express early resistance to pulmonary tuberculo-

sis infection (9, 39). $CD8^+$ T cells contribute to TB resistance via IL-12p70-dependent production of IFN-γ (35–38). However, this innate immune response is antigen independent, and the early resistance cannot be sustained. The bacterial load in the lungs of old mice increases about 90 days after infection (9), and the lungs of old mice are eventually more susceptible to bacterial growth (39). In the present study, antigen-specific IFN-γ production was observed after BCG revaccination of 60-month-old guinea pigs. In humans, the elderly have more preexisting diseases, such as diabetes mellitus (DM) and hypertension, some of which may be associated with an increased risk of TB (17). Clearly, further evaluation of BCG in the elderly is necessary.

In conclusion, we found that vaccination of elderly guinea pigs with BCG-Tokyo reduces bacterial replication in the lungs, alveolar lymph nodes, and spleens of infected animals. In addition, PPD-specific IFN-γ responses were observed after the second BCG-Tokyo vaccination. These findings suggest that BCG-Tokyo has a protective effect at all ages.

## ACKNOWLEDGMENTS

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# Immunogenicity of latency-associated antigens of *Mycobacterium tuberculosis* in DNA-vaccinated mice

Yasuhiro Yamamura[a], Kunio Tsujimura[a], Shintaro Seto[a], Masato Uchijima[a], Hironao Hozumi[a,b], Toshi Nagata[c], Yukio Koide[d]*

[a]*Department of Infectious Diseases, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Higashi-ku, Hamamatsu 431-3192, Japan*
[b]*Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Higashi-ku, Hamamatsu 431-3192, Japan*
[c]*Department of Health Science, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Higashi-ku, Hamamatsu 431-3192, Japan*
[d]*Executive Director for Education and International Exchange, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Higashi-ku, Hamamatsu 431-3192, Japan*

**Abstract**

*Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB), persists within infected macrophages for long periods of time in a metabolically inactive but reversible state known as dormancy. Since the majority of adult pulmonary TB is caused by the reactivation of persistent *Mtb*, novel vaccines to protect against disease reactivation and novel biomarkers to provide the basis of new diagnosis of latent infection are urgently needed. To meet this demand, we assessed the immunogenicity of 32 latency-associated proteins in DNA-vaccinated mice. By using a Helios gene gun, BALB/c and C57BL/6 mice were vaccinated with plasmids carrying DNAs for latency-associated antigens encoded by the DosR regulon. Of the 32 antigens tested, 12 induced antigen-specific T-cell responses in vaccinated BALB/c mice and 9 induced responses in C57BL/6 mice. Five antigens (Rv1998c, Rv2031c, Rv2032, Rv2623, and Rv3132c) induced T-cell responses in both mice strains. In addition, at least 12 and 3 antigens induced antigen-specific antibody production in vaccinated BALB/c and C57BL/6 mice, respectively. Of these, 3 antigens (Rv2029c, Rv2626c, and Rv3132c) induced strong antibody production in both mice strains. These results might be applicable for the future development of a novel vaccine and biomarkers for latent TB infection, although further analyses in human blood samples are necessary.



*Key words*: DNA vaccine; *Mycobacterium tuberculosis*; DosR regulon; Latent infection; Dormancy

## 1. Introduction

Tuberculosis (TB) has plagued humans for thousands of years, and more than 2 billion people (equal to approximately one-third of the world's population) are currently infected with the causative agent *Mycobacterium*

* Corresponding author. Tel.: +81-53-435-2335; fax: +81-53-435-2335.
*E-mail address*: koidelb@hama-med.ac.jp

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