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].

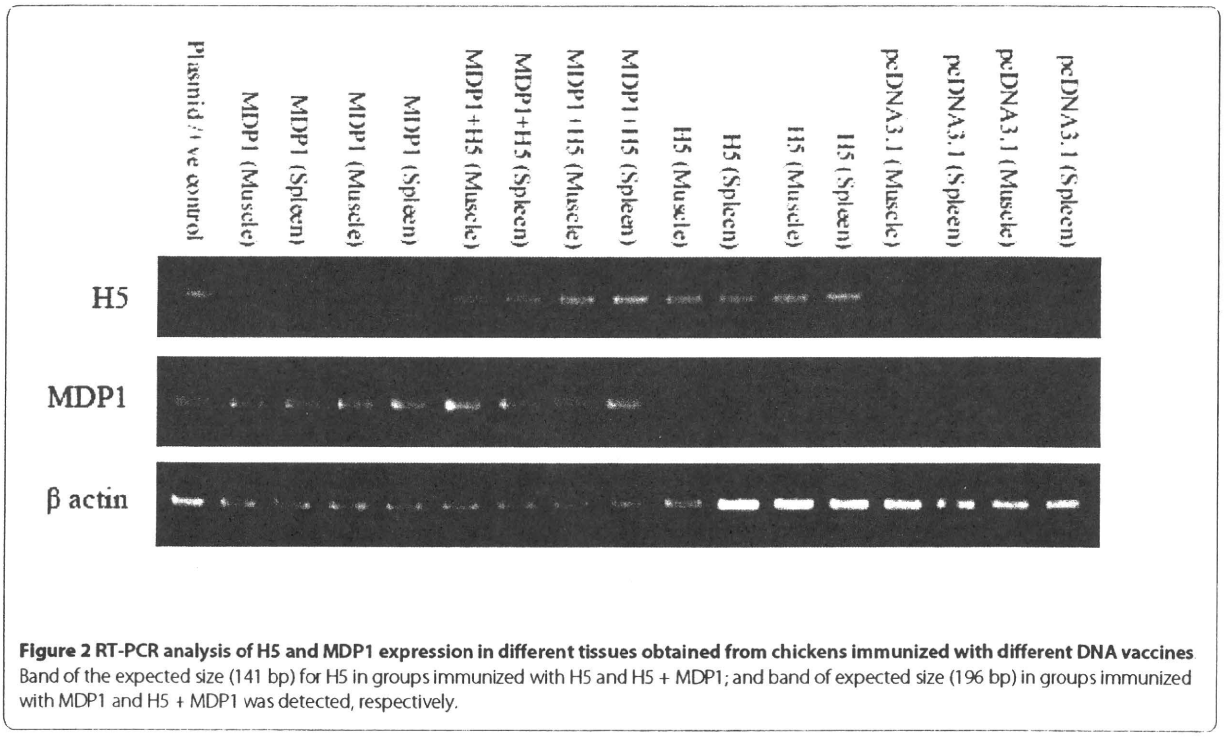

Figure 2 RT-PCR analysis of H5 and MDP1 expression in different tissues obtained from chickens immunized with different DNA vaccines. Band of the expected size (141 bp) for H5 in groups immunized with H5 and H5 + MDP1; and band of expected size (196 bp) in groups immunized with MDP1 and H5 + MDP1 was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

Analysis of Drug-Resistant Strains of *Mycobacterium leprae* in an Endemic Area of Vietnam

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(See brief report by Ramien and Wong, e133–e135.)

Background. Multidrug therapy has effectively reduced the number of leprosy cases in the world. However, the rate of reduction has decelerated over the years, giving early detection of *Mycobacterium leprae* and epidemiological study of relapse renewed relevance in attempts to eliminate the disease.

Methods. A molecular epidemiological survey for drug-resistant *M. leprae* was conducted in the central and highland regions of Vietnam. A total of 423 samples taken from patients, including 83 patients with new cases, 321 patients receiving treatment, and 19 patients with relapse, were studied for detection of *M. leprae* with mutations relating to drug resistance by sequencing the drug resistance determining region of the *folP1*, *rpoB*, and *gyrA* genes, which are responsible for dapsone, rifampicin, and ofloxacin resistance, respectively.

Results. Nineteen mutations were found in the *folP1* gene samples, and no mutations relating to drug resistance were found in either the *rpoB* or *gyrA* genes. Samples from patients with relapse showed *folP1* mutation rates as high as 57%, and the mutation rates in samples from new and recent cases were <10%. Patients with relapse who had histories of treatment with dapsone monotherapy showed high mutation rates (78%), compared with patients with relapse who had previously only received multidrug therapy (33%).

Conclusions. Our study indicated high rates of dapsone resistance in patients with relapse, compared with patients with new and recent cases of leprosy. Moreover, it was observed that many of the patients with relapse who had dapsone-resistant mutations had histories of treatment with dapsone monotherapy.

Leprosy is a chronic infectious disease caused by infection with *Mycobacterium leprae*. The present strategy for leprosy control is based on the multidrug therapy (MDT), recommended by the World Health Organization (WHO) [1], which has successfully reduced the number of leprosy cases in the world. However, transition in the number of registered cases and new cases amounting to ~210,000 and ~250,000, respectively, has almost come to a standstill [2]. Drug-resistant strains were first found in 1964, 1976, and 1997 [3–5]. MDT was designed to prevent the emergence and spread of drug-resistant strains. However, a strain showing resistance to both dapsone and rifampicin was reported in 1993 [6], and at present, there are further reports indicating the emergence of *M. leprae* strains resistant to multiple drugs [5, 7]. At present, the rapid detection and control of such drug-resistant strains is essential in countries approaching leprosy elimination levels, such as Vietnam.

MDT has been quite successful in Vietnam, and elimination of leprosy (prevalence rate, < 1/10,000 population) was achieved on the national level in 1995 [8]. The prevalence rate per 10,000 population in 2006

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was .07 [8, 9]. However, the majority of patients with leprosy are found in the central and highland regions of Vietnam [10], consisting of 11 provinces, including 4 provinces in the highland region and 7 provinces in the delta region. In 2005, the number of patients with leprosy was 236, spread through 4 provinces of the highland region; the prevalence rate of newly detected cases was 3.5 cases/10,000 population, although the overall prevalence rate was .25 cases/100,000 population on the national level. The rate of newly detected cases in the 7 delta region provinces was 1.38 cases/10,000 population [8, 9]. These cases not only present the danger of being possible infectious sources for leprosy but also harbor the risk of developing into relapse cases. However, little is known regarding the effects of drug-resistant *M. leprae* in patients with leprosy, especially in cases of relapse.

Therefore, in the present study, molecular epidemiological studies on drug-resistant strains were conducted in 11 provinces primarily in the central and highland regions that represent the areas where leprosy is endemic in Vietnam.

MATERIALS AND METHODS

Sensitivity of Polymerase Chain Reaction

The number of bacilli isolated from nude mice footpads was counted using the method described by Shepard et al [11]. Serial 10-fold dilutions of the enumerated *M. leprae* bacilli were used for polymerase chain reaction (PCR) in our study.

Clinical Specimens

Samples (from slit-skin smears or punch biopsies) were taken from patients with leprosy after receipt of informed consent in primarily the central and highland regions of Vietnam (including 11 provinces: Danang, Quangnam, Quangngai, Binhdinh, Phuyen, Khanhhoa, Ninhthuan, Kontum, Gialai, Daknong, and Daklak), and the samples were classified as new (before starting MDT), recent (receiving MDT), and relapse cases. Relapse was defined as development of new skin lesions after completion of MDT and increase in bacterial index by >2 log units in any lesion.

The total of 423 samples included those from 83 patients with new cases, 321 patients with recent cases (receiving treatment), and 19 patients with relapse (collection period: March 2004–August 2009). Among 16 patients with relapse who had positive results of *M. leprae*–specific PCR, 9 cases were determined to be relapse after dapsone monotherapy (7–20 years), 3 as relapse after complete MDT, 2 as second relapse (the first after dapsone monotherapy and the second after MDT), and 2 as relapse after ofloxacin treatment. Samples were obtained from the skin lesions of patients (smear on blade or biopsy soaked in 1 mL of 70% ethanol at room temperature in the field, before being sent to Quyhoa National Leprosy & Dermato-Venereology Hospital laboratory).

DNA Extraction, Nested PCR, and Sequencing

M. leprae templates from both dilutions of *M. leprae* bacilli and slit-skin smears were prepared by treatment with lysis buffer at 60°C overnight, as described elsewhere [12]. Nested PCR amplification of the RLEP regions of *M. leprae* was performed under conditions described elsewhere with minor modifications, using the primers listed in Table 1 [13]. In brief, PCR amplification using special reagents (20 mM Tris-HCl [pH, 7.5], 8 mM magnesium chloride, 7.5 mM DTT, 2.5 mg BSA, 150 μM deoxynucleotides, 1.5 mM magnesium sulphate, and 2.5 units KOD-plus-Ver.2 DNA polymerase [Toyobo]) was performed using sample DNA as templates. Both first and second PCR conditions were as follows; strand separation at 94°C for 4 min, denaturing at 94°C for 40 s, annealing at 55°C for 1 min, and extension at 72°C for 20 s plus 1-s increment per cycle for 25 cycles. Products from the first PCR (0.5 uL) were used as templates in the second PCR. The nested PCR for DRDR was performed using the primer pairs listed in Table 1. Mutations were measured on the *folP1* gene for dapsone [14], the *rpoB* gene for rifampicin, and the *gyrA* gene for ofloxacin [15, 16]. Nested PCR conditions for drug resistance were different from that for RLEP-nested PCR. In brief, PCR amplification using standard reagents (10 mM Tris-HCl [pH, 8.3], 2 mM magnesium chloride, 250 μM dNTPs, and 2.5 units TaKaRa Ex Taq DNA polymerase [Takara shuzo]) was performed using sample genomic DNA as templates. The primer pairs used to amplify the specific drug-resistant genes are shown in Table 1. The reaction condition was 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for 35 cycles.

The amplicons were visualized by agarose gel electrophoresis, and DNA was recovered from the gel using Mini-Elute gel extraction kits (Qiagen). The recovered DNA molecules were sequenced using the ABI Prism BigDye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems) and run on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). The sequence data were analyzed by DNA analysis program Genetyx-MAC, version 15 (GENETYX), and were compared with those in the GenBank database.

RESULTS

PCR Sensitivity

Serial dilutions of the bacilli of 1×10^8–1×10^0 were prepared to determine PCR sensitivities. Genomic DNAs were extracted from the diluents with use of methods described under Materials and Methods [11]. The previously reported RLEP-nested PCR (named RLEP-L) was capable of detecting 1×10^2 bacilli in samples (Figure 1a) [13]. The newly designed RLEP-nested PCR, using K1 and K2 primers for the first PCR and LP1 and LP2 primers for the second PCR (named RLEP-K), is capable of detecting comparable counts of bacilli (Figure 1b), and RLEP-K

Table 1. Sequences of Primers Used in this Study

Name	Usage	Gene	Sequence, 5′→3′	Reference	Size, bp
K1	First PCR (F)	RLEP	CGTGGGTGTGAGGATAGTTGT-	Present study	268
K2	First PCR (R)	RLEP	GATCATCGATGCACTGTTCACT-	Present study	
LP1	First or second PCR (F)	RLEP	TGCATGTCATGGCCTTGAGG-	13	129
LP2	First or second PCR (R)	RLEP	CACCGATACCAGCGGCAGAA	13	
LP3	Second PCR (F)	RLEP	TGAGGTGTCGGCGTGGTC	13	99
LP4	Second PCR(R)	RLEP	CAGAAATGGTGCAAGGGA	13	
F1	Second PCR (F)	*folP1*	GCAGGTTATTGGGGTTTTGA	Present study	312
F2	First PCR(R)	*folP1*	CCACCAGACACATCGTTGAC	Present study	
F3	Second PCR (F)	*folP1*	CTTGATCCTGACGATGCTGT	Present study	245
F4	Second PCR(R)	*folP1*	ACATCGTTGACGATCCGTG	Present study	
F5	Sequencing primer (F)	*folP1*	ATCCTGACGATGCTGTCCA	Present study	–
F4	Sequencing primer (R)	*folP1*	ACATCGTTGACGATCCGTG	Present study	–
R1	First PCR (F)	*rpoB*	CAGACGCTGATCAATATCCGT	Present study	358
R2	First PCR (R)	*rpoB*	CAGCGGTCAAGTATTCGATC	Present study	
R3	Second PCR (F)	*rpoB*	CAATATCCGTCCGGTGGTC	Present study	337
R4	Second PCR (R)	*rpoB*	GTATTCGATCTCGTCGCTGA	Present study	
R5	Sequencing primer (F)	*rpoB*	ACGCTGATCAATATCCGTCC	Present study	–
R6	Sequencing primer (R)	*rpoB*	CGACAA TGAACCGATCAGAC	Present study	–
G1	First PCR (F)	*gyrA*	ACGCGATGAGTGTGATTGTGG	Present study	336
G2	First PCR (R)	*gyrA*	TCCCAAATAGCAACCTCACC	Present study	
G3	Second PCR(F)	*gyrA*	GATGGTCTCAAACCGGTACA	Present study	291
G4	Second PCR (R)	*gyrA*	CCCAAATAGCAACCTCACCA	Present study	
G3	Sequencing primer (F)	*gyrA*	GATGGTCTCAAACCGGTACA	Present study	–
G4	Sequencing primer (R)	*gyrA*	CCCAAATAGCAACCTCACCA	Present study	–

products are visualized more clearly with less smear bands. Therefore, the new RLEP-K system was used for detection in further experimentation with use of clinical samples.

Using DNAs extracted from the serial dilutions of *M. leprae*, we determined the sensitivity of the nested PCR for DRDRs. The limit of amplification by PCR was 1×10^3–1×10^4 bacilli (Figure 1 c–e).

RLEP-nested PCR for Clinical Samples

The PCR methods were applied on 423 clinical samples collected from areas of endemicity in Vietnam. First, we tested RLEP-K for detection of *M. leprae* after extraction of DNA from smear samples. Positive bands were obtained by gel electrophoresis using RLEP-K on 290 samples. The positivity rate was 69%. The patients supplying the 290 samples were divided into 3 categories: new, relapse, and recent cases. Positive rates of RLEP-K by category were 75%, 84%, and 66%, respectively (Table 2).

Mutations in Clinical Samples

Samples positive by RLEP-nested PCR were applied for mutation experiments on the DRDRs of *folP1*, *rpoB*, and the *gyrA* gene. Nineteen mutations were found in 187 *folP1* samples, but no mutations related to drug resistance were noted in 163 *rpoB* and 147 *gyrA* gene samples. The mutations detected on *folP1* were as follows: 6 cases of ACC to ATC in codon 53(threonine to isoleucine), 9 cases of CCC to CGC in codon 55 (proline to arginine), and 4 cases of CCC to CTC (proline to leucine). Two new cases, 8 relapse cases, and 9 recent cases had mutations on *folP1*. Mutation rates in the 3 categories were 6.1%, 57%, and 6.4%, respectively (Table 3).

Some missense mutations, of which the association with drug resistance is unknown, were detected in the *rpoB* gene from clinical samples. The mutations were detected in 7 patients at codons 517, 532, and 556. One patient with relapse showed a mutation from CAG (glutamine) to CAT (histidine) at codon 517. One new patient showed 2 mutations at codon 517 from CAG (glutamine) to CAT (histidine) and at codon 532 from GCG (alanine) to TCG (serine). Sequence electropherograms indicated double peaks of a second nucleotide at codon 556 in 3 patients categorized as having recent cases. One peak was G (identical to that of wild-type), and the other peak was T, which changed the amino acid from glycine (GGC) to valine (GTC; data not shown).

The Relation between Treatment and Drug-Resistant Mutations in Patients with Relapse

Patients with relapse were categorized into 4 groups, by treatment history (Table 4). Group 1 comprised those treated with dapsone monotherapy. Group 2 was treated with MDT for 24 months. Group 3 included patients who had received

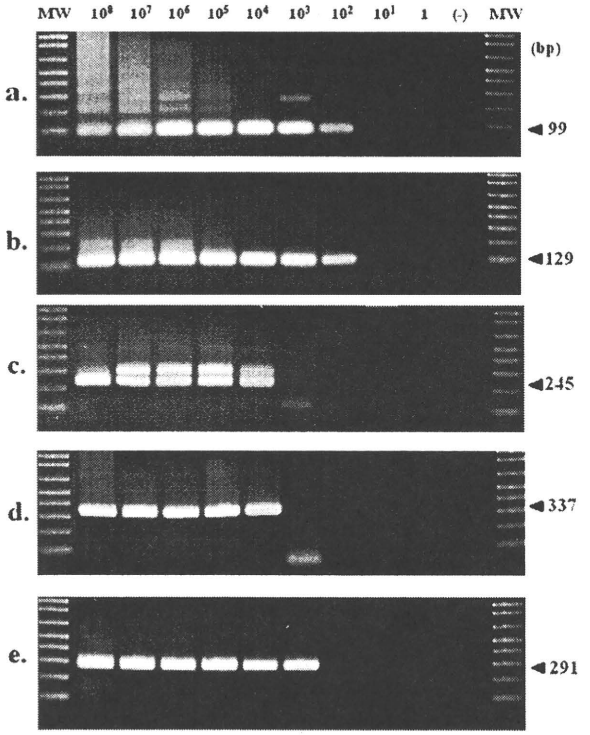

Figure 1. Sensitivity of nested polymerase chain reaction (PCR). The nested PCR products were visualized on 2 % agarose gel. A, RLEP-nested PCR (RLEP-L) using primers, LP1-LP4 (final products size, 99 bp). B, RLEP-nested PCR (RLEP-K) using primers, K1, K2, LP1, and LP2 (final products size, 129bp). C, *folP1*-nested PCR using F1-F4. D, *rpoB*-nested PCR using R1-R4. E, *gyrA*-nested PCR using G1-G4.

a diagnosis of second relapse—once after treatment with dapsone monotherapy and, subsequently, after MDT for 24 months. Group 4 was treated with ofloxacine monotherapy. Eight of the 14 patients with *folP1*-amplified relapse cases (57%) had mutations on the *folP1* gene. Seven (78%) of 9 patients with relapse who were categorized in groups 1 and 3 also had *folP1* mutations. However, 2 patients in group 4 had no mutations on any of the 3 genes.

Monitoring of Mutations in Patients

One hundred seven slit-skin smear samples from 43 patients were taken with consents at different times from each patient for monitoring mutations under treatment. Table 5 shows the difference in mutation results between 5 such patients. The other 38 patients showed no mutation during monitoring. Patients A, B, and C, who had new cases, showed a similar pattern, with no mutation at first testing and mutation in codon 53 on the *folP1* gene during MDT. However, double peaks of T and C in the second base were observed on *folP1* in the 3 patients. Patients D and E, who had relapse cases and finished dapsone monotherapy 20 years earlier, had a mutation on *folP1* in 2005 and no mutation after MDT.

Table 2. Polymerase Chain Reaction Positivity in New, Relapse, and Recent Cases

Case category	No.	RLEP	*folP1*	*rpoB*	*gyrA*
New	83	62 (75%)	33	39	43
Relapse	19	16 (84%)	14	15	13
Recent	321	212 (66%)	140	109	91
Total	423	290 (69%)	187 (64%)	163 (56%)	147 (51%)

DISCUSSION

The most popular PCR method for *M. leprae* detection with high sensitivity and specificity is probably the RLEP-nested PCR method, because the RLEP regions are specific for *M. leprae,* with >28 copies dispersed through the *M. leprae* genome [17]. New primers were designed for the RLEP-nested PCR in our study. This system using the new primers was termed RLEP-K. RLEP-K products appear to be a somewhat sharper and stronger band on agarose gel electrophoresis, compared with that that of previous RLEP-nested PCR (ie, RLEP-L). The RLEP-K detected *M. leprae* in 69% of the Vietnam samples. The remaining 31% of the samples were deduced as being cases either cleared of *M. leprae* by chemotherapy or those having <100 bacilli, which was below the detection limit of RLEP-K. We also designed new primers for amplification and sequencing of DRDR in the drug-resistance related genes *folP1*, *rpoB*, and *gyrA*, which were applied in examining the Vietnam samples. The mutation rates of *folP1* in new and recent cases were 6.1% and 6.4%, respectively. In contrast, the mutation rate in relapse cases was quite high, at 57%. The result indicated a strong correlation between mutation rate and relapse. Two possible reasons were conceived regarding the high positive rate of dapsone resistance in patients with relapse: (1) reinfection by the primary drug-resistant strain (7 of 8 samples indicating relapse were collected in the province in central Vietnam, which had the highest prevalence of leprosy and high rate of relapse (data not shown) and (2) reactivation of dapsone-resistant strains capable of

Table 3. Number of Mutations on *folP1*

Case category	No. of PCR-positive cases	No. of mutations (mutation ratio)	No. of mutation in mutation types
New	33	2 (6.1%)	2 (55th: CCC-CGC)
Relapse	14	8 (57%)	2 (53rd: ACC-ATC)
			3 (55th: CCC-CGC)
			3 (55th: CCC-CTC)
Recent	140	9 (6.4%)	4 (53rd: ACC-ATC)
			4 (55th: CCC-CGC)
			1 (55th: CCC-CTC)

Table 4. Mutations Noted in RLEP-Positive Relapse Cases, by Treatment Group

Group	Past treatment	No.	Mutation on *folP1*	Mutation on *rpoB*	Mutation on *gyrA*
1	DDS	7	5	0	0
2	MDT (24 months)	3	1	1[a]	0
3	DDS plus MDT (24 months)	2	2	0	0
4	OFX	2	0	0	0
All	...	14	8	1[a]	0

Abbreviations: DDS (diaminodiphenylsulfone), dapsone monotherapy; MDT,multidrug treatment; OFX, Ofloxacine monotherapy.

[a] Unknown DR mutation

persisting after chemotherapy, discussed below. Although it is still unclear whether the relapses are caused by reinfection by *M. leprae* or by reactivation of persistent *M. leprae*, close correlation between drug resistance and relapse have been recognized in several studies [18, 19].

The proportion of samples showing mutation on the *folP1* gene related to dapsone resistance was 10.2% (19 of 187) in samples from the central and highland regions of Vietnam (Table 3). Comparison with previous reports from South Korea (19.2%) indicates lower rates of relapse in these regions of Vietnam [20].

No mutation was found in the DRDR regions of *rpoB* in all samples. Mutation frequencies of the *rpoB* gene are also very low in other reports. Regarding other areas in Southeast Asia, no cases of rifampicin resistance have been detected in the Philippines, 1 (1.9%) of 54 cases in Myanmar, and 4 (3.3%) of 121 cases in Indonesia. However, in Japan, where the prevalence of leprosy is very low, the reported rate of rifampicin resistance is very high, at 29.5% (26 of 88 cases) [21]. The long-term use of drugs outside the standard MDT regimen in Japanese leprosy cases might have been instrumental in promoting this rifampicin resistance.

As such, no mutations have been found in the DRDR of the *M. leprae rpoB* gene derived from patients with leprosy, including relapse cases in Vietnam. A possible explanation for this could be the success of leprosy control in Vietnam and efficacy of properly administered MDT in which rifampicin—with its bactericidal properties—was effective in suppressing the occurrence of drug-resistant bacilli. In contrast, dapsone (not bactericidal in itself, although capable of suppressing growth), which had previously been used as monotherapy, may have enabled bacteria surviving in the patient receiving treatment to develop mutations, giving them resistance against the drug. Although occurrence of drug-resistant *M. leprae* was kept very low after application of MDT, 7 of 9 samples with drug-resistant mutations had previously been treated by dapsone monotherapy (Table 4). Jing et al [22] reported that patients with multibacillary leprosy who were retreated with MDT after dapsone monotherapy may have lower risk of early relapse while continuing to carry the risk of late relapse. Our observations suggest the possibility that efficacy of MDT may be hampered in some patients by the presence of surviving dapsone-resistant *M. leprae* in their bodies, which could develop into late relapse. Similar observations have been reported, suspecting involvement of the effects of dapsone monotherapy in patients with relapse [23].

There was no mutation in the major sites for drug resistance on the *rpoB* gene. However, we observed mutations at 3 positions, codons 517, 532, and 556, which have not been associated with rifampicin resistance. These mutations in the *rpoB* gene are a finding calling for further clarification.

Table 5. Monitoring of 5 Patients with Multibacillary Leprosy for *folP1* Mutation

Patient	Case category	Date of sample obtainment	Sample site (method of obtainment)	*folP1* mutation
A	New	2006 April 3	Abdomen (biopsy)	None[a]
		2007 January 30	Earlobe (smear)	53rd (ACC→ATC/ACC)
		2007 January 30	Abdomen (smear)	53rd (ACC→ATC/ACC)
B	New	2005 May 31	Earlobe (smear)	None
		2006 March 24	Skin (smear)	None
		2007 November 2	Skin (smear)	53rd (ACC→ATC/ACC)
C	New	2006 July 20	Skin (smear)	None
		2007 January 30	Skin (smear)	53rd (ACC→ATC/ACC)
		2007 January 30	Skin (smear)	53rd (ACC→ATC/ACC)
D	Relapse	2005 November	Earlobe (smear)	55th (CCC→CGC)
		2007 January	Skin (smear)	None
E	Relapse	2007 Jannuary 17	Arm (smear)	None
		2007 January 30	Earlobe (smear)	55th (CCC→CGC)
		2007 January 30	Arm (smear)	None

[a] ACC ATC/ACC indicates double peaks in second base at codon 53.

To reveal the possible relation between treatment and gene mutation, some patients with leprosy were monitored for gene mutations in light of drug treatments. The results showed incidence of dapsone-resistant *M. leprae* in patients receiving MDT, suggesting that some of the patients with relapse who were previously treated with dapsone monotherapy might have persistent infections with dapsone-resistant *M. leprae*. Furthermore, samples derived from different sites of lesions in the same patient sometimes showed different results (Table 5). The results suggest that we need to know the relation between the situation of patients with leprosy and drug resistance.

Overall, our study indicated a high ratio of dapsone resistance in patients with relapse, compared with the other patients with leprosy. In contrast, an unexpected outcome of our study was that we were unable to find mutations on the *rpoB* gene in patients with relapse. Moreover, it was shown that many of the patients with relapse who had dapsone-resistant mutations had histories of treatment with dapsone monotherapy. To clarify the relationship between relapse, drug resistance, and dapsone monotherapy, it might be necessary to investigate persistence of drug-resistant *M. leprae* through large-scale surveillance.

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

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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

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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^{2}155	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

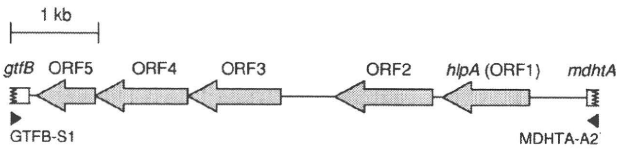

FIG. 1. Organization of the 6.8-kb genomic segment isolated from MAC serovar 4 strain (ATCC 35767). Filled triangles indicate the primers used for PCR amplification.

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.

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

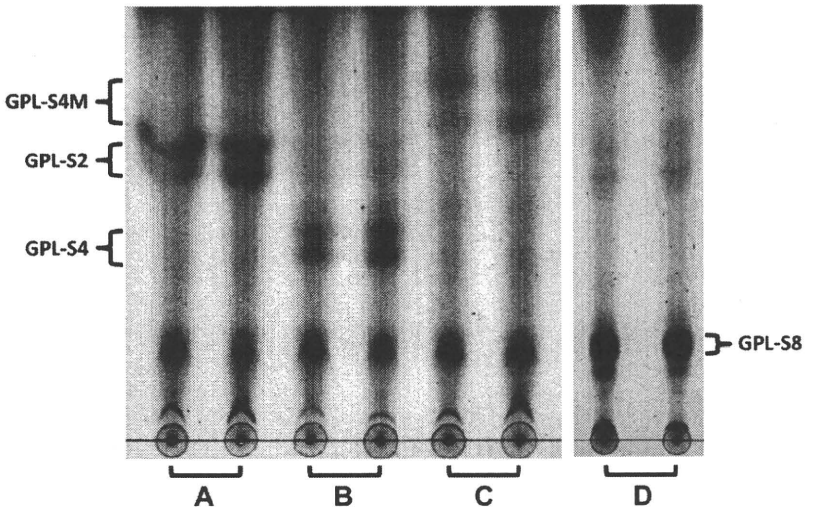

FIG. 2. TLC analysis of crude GPL extracts from recombinant *M. smegmatis* strains MS-S2/pYM301a (A), MS-S2/pYM-hlpA (B), MS-S2/pYM-hlpA-orf2 (C), and MS-S2/pYM-orf3-orf4-orf5 (D). GPL extracts were prepared from the total lipid fraction after a mild alkaline hydrolysis step. Each recombinant strain 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

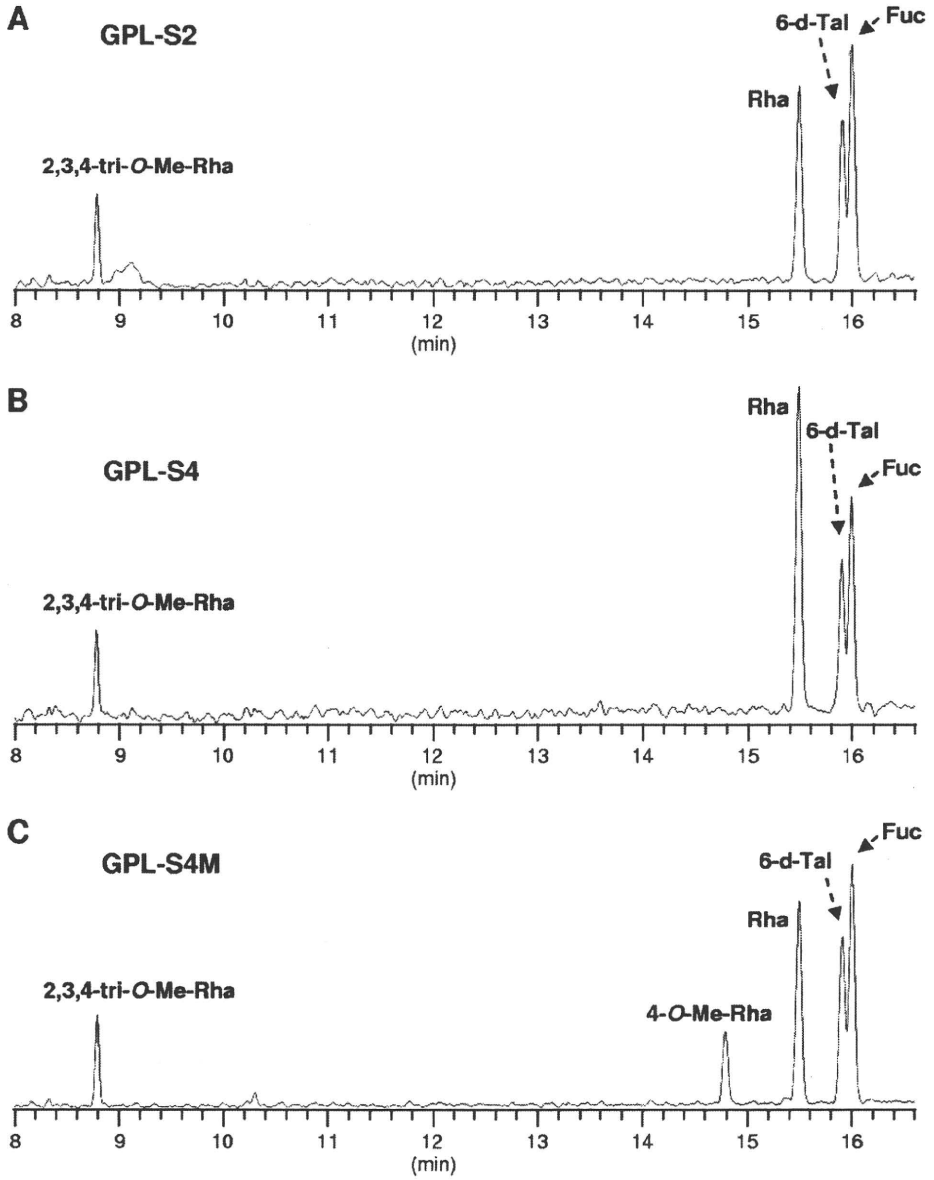

FIG. 3. GC-MS of alditol acetate derivatives from GPL-S2 (A), GPL-S4 (B), and GPL-S4M (C), which were purified from GPL extracts of recombinant *M. smegmatis* strains MS-S2/pYM301a, MS-S2/pYM-hlpA, and MS-S2/pYM-hlpA-orf2, respectively.

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

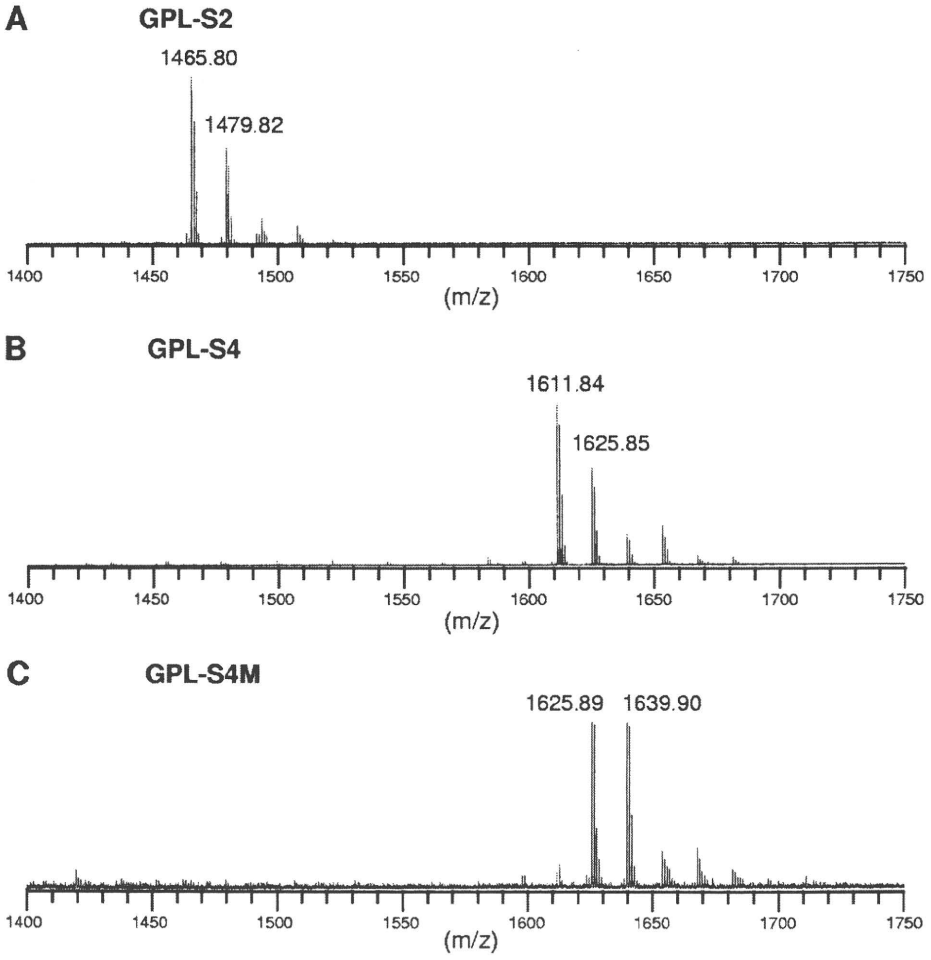

FIG. 4. MALDI-TOF MS of GPL-S2 (A), GPL-S4 (B), and GPL-S4M (C), which were purified from GPL extracts of recombinant *M. smegmatis* strains MS-S2/pYM301a, MS-S2/pYM-hlpA, and MS-S2/pYM-hlpA-orf2, respectively.

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

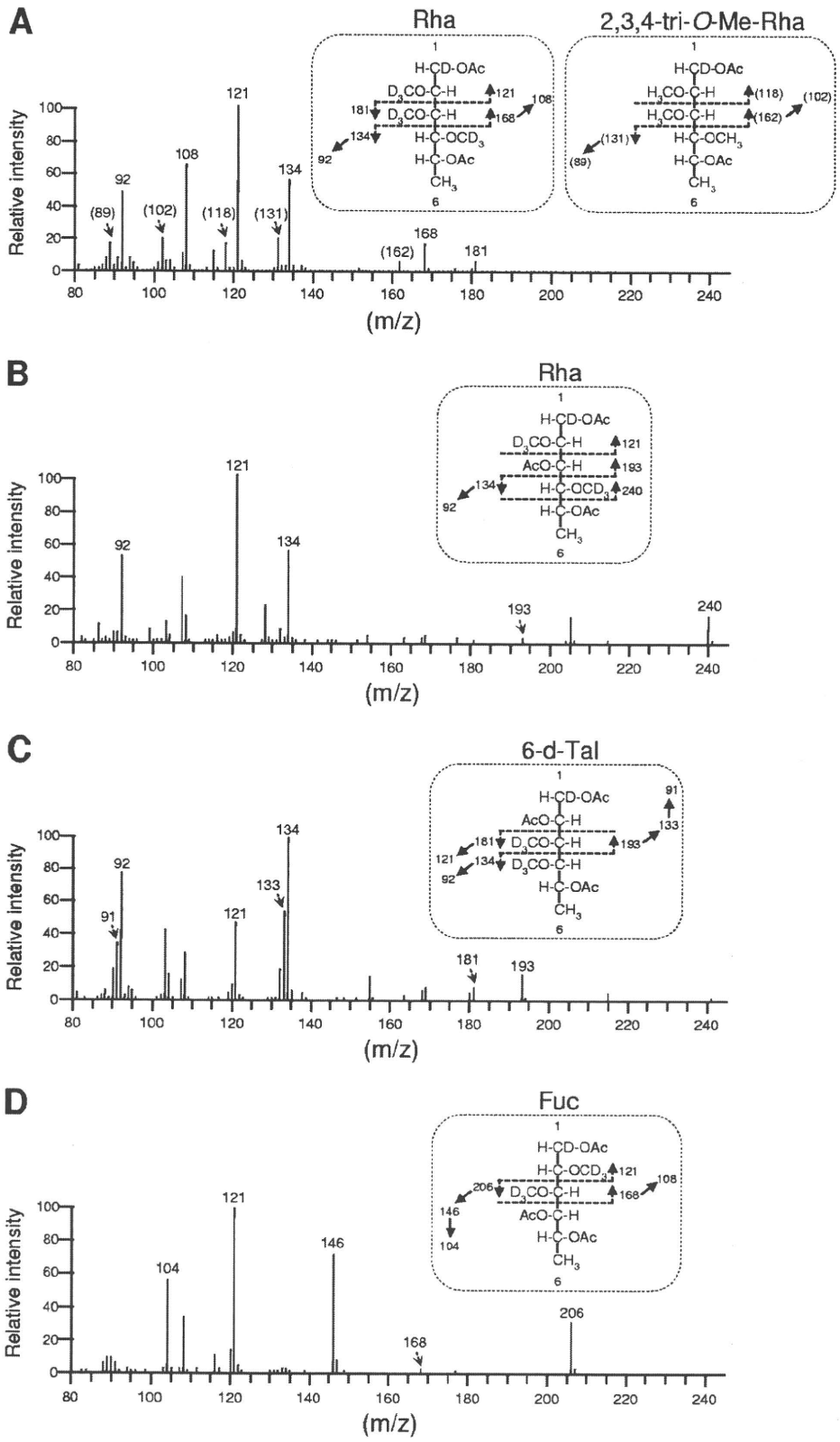

FIG. 5. GC-MS spectra and fragment ion assignments of 2,3,4-tri-*O*-Me-Rha (A), Rha (A and B), 6-*d*-Tal (C), and Fuc (D), which are derived from alditol acetates of sugars released from deuteriomethylated GPL-S4. Ac, acetate; D, deuterium.

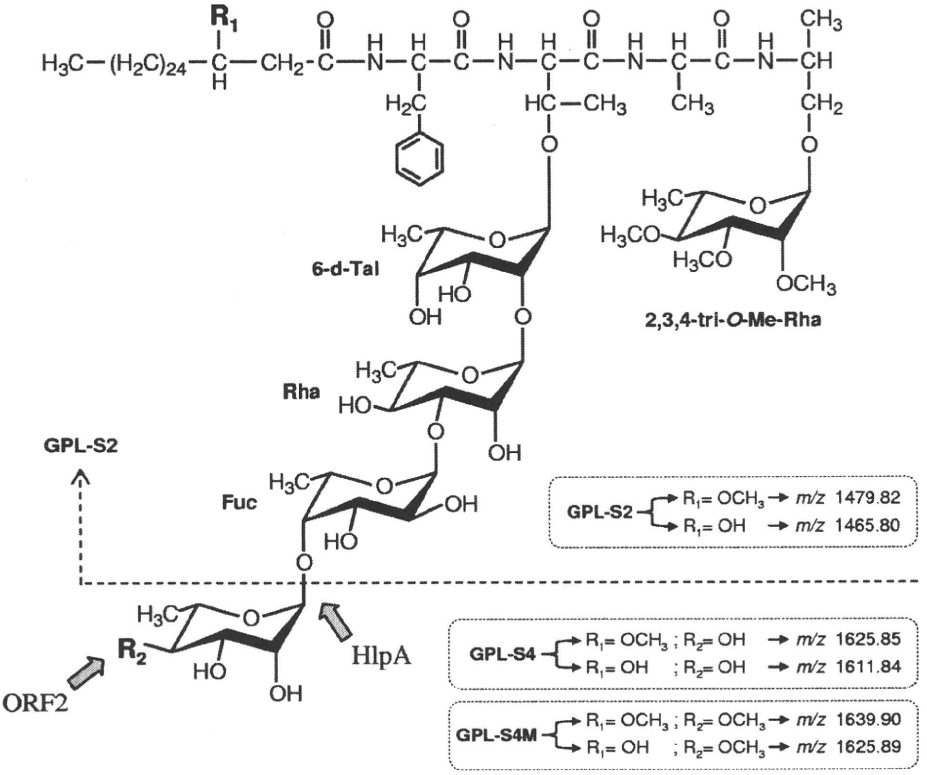

FIG. 6. Proposed structures and biosynthetic pathways for GPL-S2, GPL-S4, and GPL-S4M. Parentheses indicate structural differences between three compounds, which are deduced from MALDI-TOF MS analyses {pseudomolecular ions ($[M + Na]^+$)}.

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*[▽]

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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 5×10^5 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

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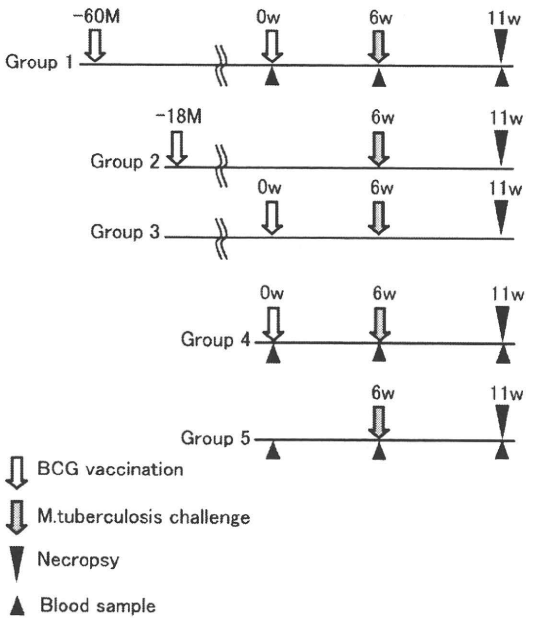

FIG. 1. Experimental design. Guinea pigs in the old group were vaccinated with BCG at the age of 6 weeks and were then maintained for 60 months before revaccination with BCG 6 weeks before *M. tuberculosis* infection (group 1). Guinea pigs in the middle-aged groups either were vaccinated with BCG at 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 5×10^4 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 1×10^6/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 1×10^5 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