

FIG. 6. Enhancement of PGE<sub>2</sub> production by clofazimine in *M. leprae*-infected macrophages. Macrophages were preincubated in the presence of the indicated doses of clofazimine for 4 h, followed by infection with *M. leprae* ( $5 \times 10^6$ /well), and the culture was continued for another 20 h. The amount of PGE<sub>2</sub> in the culture supernatants was measured. The results are representative of three independent cell culture tests.

is known to have a favorable influence on the reversal reaction in human leprosy (2). Browne and Hogenzeil found that clofazimine controlled persistent exacerbations in patients who were corticosteroid dependent for controlling the passing reactions, including ENL, and suggested that the drug may exert a suppressive effect on the development of acute exacerbation in lepromatous leprosy (3). These observations have been followed by those of later workers, and one of the special indications for use of clofazimine is the presence of acute reactions or a chronic recurrent reaction in lepromatous leprosy. These clinical data suggest that the mechanisms underlying the action of the drug in these leprosy patients mainly seem to be anti-inflammatory, although there is no direct evidence. Macrophages are capable of elaborating a series of biochemical products with potent immunomodulatory activities. We have observed enhancement of the production of PGE<sub>2</sub>, when macrophages were pretreated with clofazimine. PGE<sub>2</sub> is released from arachidonic acid by PLA<sub>2</sub>. The enzyme is reported to be stimulated in clofazimine-treated neutrophils (10). With respect to B-cell function, prostaglandins of the E series (PGE) inhibit both B-cell proliferation and the generation of antibody-forming cells, and also, B-cell tolerance is induced by PGE<sub>2</sub> (9, 24). T-cell proliferation is also suppressed by PGE<sub>2</sub> (13). The reaction to leprosy involves antibody (ENL caused by immune complex) and cells (delayed-type hypersensitivity mediated mainly by T cells). The mechanisms underlying the immunomodulatory role of clofazimine are still not clear, but the present study clarifies certain aspects. Apoptosis induced in macrophages might explain the anti-inflammatory activities of clofazimine *in vivo*.

In conclusion, our findings suggest that clofazimine induced apoptosis of macrophages through the activation of caspases. The data indicate that the action of clofazimine in leprosy patients may be at least partially mediated by apoptosis.

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# 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, Binhdin, 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 Quynh Hoa 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  $\mu$ M 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  $\mu$ L) 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  $\mu$ M 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  $1 \times 10^8$ – $1 \times 10^0$  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  $1 \times 10^2$  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)	<i>folP1</i>	GCAGGTTATTGGGGTTTTGA	Present study	312
F2	First PCR (R)	<i>folP1</i>	CCACCAGACACATCGTTGAC	Present study	
F3	Second PCR (F)	<i>folP1</i>	CTTGATCCTGACGATGCTGT	Present study	245
F4	Second PCR (R)	<i>folP1</i>	ACATCGTTGACGATCCGTG	Present study	
F5	Sequencing primer (F)	<i>folP1</i>	ATCCTGACGATGCTGTCCA	Present study	-
F4	Sequencing primer (R)	<i>folP1</i>	ACATCGTTGACGATCCGTG	Present study	-
R1	First PCR (F)	<i>rpoB</i>	CAGACGCTGATCAATATCCGT	Present study	358
R2	First PCR (R)	<i>rpoB</i>	CAGCGGTCAAGTATTTCGATC	Present study	
R3	Second PCR (F)	<i>rpoB</i>	CAATATCCGTCCGGTGGTC	Present study	337
R4	Second PCR (R)	<i>rpoB</i>	GTATTCGATCTCGTCGCTGA	Present study	
R5	Sequencing primer (F)	<i>rpoB</i>	ACGCTGATCAATATCCGTCC	Present study	-
R6	Sequencing primer (R)	<i>rpoB</i>	CGACAA TGAACCGATCAGAC	Present study	-
G1	First PCR (F)	<i>gyrA</i>	ACGCGATGAGTGTGATTGTGG	Present study	336
G2	First PCR (R)	<i>gyrA</i>	TCCAAATAGCAACCTCACC	Present study	
G3	Second PCR (F)	<i>gyrA</i>	GATGGTCTCAAACCGGTACA	Present study	291
G4	Second PCR (R)	<i>gyrA</i>	CCCAAATAGCAACCTCACCA	Present study	
G3	Sequencing primer (F)	<i>gyrA</i>	GATGGTCTCAAACCGGTACA	Present study	-
G4	Sequencing primer (R)	<i>gyrA</i>	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  $1 \times 10^3$ – $1 \times 10^4$  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

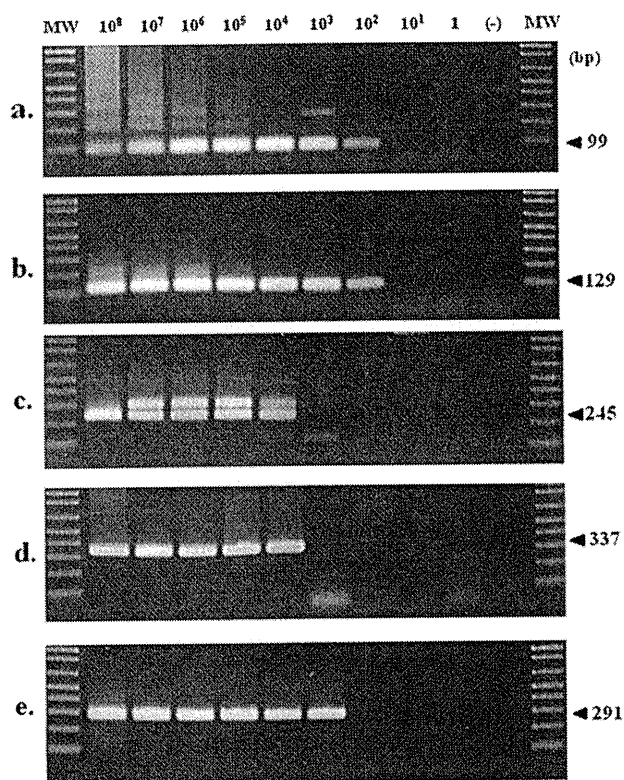
Samples positive by RLEP-nested PCR were applied for mutation experiments on the DRDRs of *folP1*, *rpoB*, and the *gyrA* gene. Nineteen mutations were found in 187 *folP1* samples, but no mutations related to drug resistance were noted in 163 *rpoB* and 147 *gyrA* gene samples. The mutations detected 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 dapson 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 ofloxacin 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

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

Case category	No.	RLEP	<i>folP1</i>	<i>rpoB</i>	<i>gyrA</i>
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%)

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.

#### 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 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 <i>folP1</i>	Mutation on <i>rpoB</i>	Mutation on <i>gyrA</i>
1	DDS	7	5	0	0
2	MDT (24 months)	3	1	1 <sup>a</sup>	0
3	DDS plus MDT (24 months)	2	2	0	0
4	OFX	2	0	0	0
All	...	14	8	1 <sup>a</sup>	0

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

<sup>a</sup> 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)	<i>folP1</i> mutation
A	New	2006 April 3	Abdomen (biopsy)	None <sup>a</sup>
		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 January 17	Arm (smear)	None
		2007 January 30	Earlobe (smear)	55th (CCC → CGC)
		2007 January 30	Arm (smear)	None

<sup>a</sup> 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 dapson-resistant *M. leprae* in patients receiving MDT, suggesting that some of the patients with relapse who were previously treated with dapson monotherapy might have persistent infections with dapson-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 dapson 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 dapson-resistant mutations had histories of treatment with dapson monotherapy. To clarify the relationship between relapse, drug resistance, and dapson monotherapy, it might be necessary to investigate persistence of drug-resistant *M. leprae* through large-scale surveillance.

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## Immunostimulatory Activity of Major Membrane Protein II from *Mycobacterium tuberculosis*<sup>∇‡</sup>

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Previously, we observed that both major membrane protein II of *Mycobacterium leprae* (MMP-ML) and its fusion with *M. bovis* BCG (BCG)-derived heat shock protein 70 (HSP70) (Fusion-ML) are immunogenic and that recombinant BCG secreting either of these proteins effectively inhibits the multiplication of *M. leprae* in mice. Here, we purified *M. tuberculosis*-derived major membrane protein II (MMP-MTB) and its fusion with HSP70 (Fusion-MTB) in a lipopolysaccharide-free condition and evaluated their immunostimulatory abilities. Both MMP-MTB and Fusion-MTB activated monocyte-derived dendritic cells (DC) in terms of phenotype and interleukin-12 (IL-12) production, but Fusion-MTB more efficiently activated them than MMP-MTB did. The IL-12 production was a consequence of the ligation of those recombinant proteins with Toll-like receptor 2. The *M. tuberculosis*-derived and *M. leprae*-derived recombinant proteins activated naïve T cells of both CD4 and CD8 subsets, but *M. tuberculosis*-derived proteins were superior to *M. leprae*-derived proteins and fusion proteins were superior to MMP, regardless of the origin of the protein. Memory-type CD4<sup>+</sup> T cells obtained from BCG-vaccinated healthy individuals seem to be primed with MMP-MTB by the vaccination, and both *M. tuberculosis*-derived recombinant proteins produced perforin-producing CD8<sup>+</sup> T cells from memory-type CD8<sup>+</sup> T cells. Further, infection of DC and macrophages with *M. tuberculosis* H37Ra and H37Rv induced the expression of MMP on their surface. These results indicate that *M. tuberculosis*-derived MMP, as a sole protein or as part of a fusion protein, may be useful for developing new vaccinating agents against tuberculosis.

Tuberculosis is a chronic infectious disease caused by intracellular infection with *Mycobacterium tuberculosis* (20). It is estimated that one-third of the global population is latently infected with this inhaled pathogen, which infects primarily macrophages and dendritic cells (DC), and tuberculosis is responsible for more than two million deaths yearly worldwide (11, 34, 36). The emergence of multidrug-resistant strains of *M. tuberculosis* mandates the development of more effective preventive and therapeutic strategies, including the development of improved vaccines (48). Protective immunity against *M. tuberculosis* is conducted chiefly by adaptive cellular immune responses, and gamma interferon (IFN- $\gamma$ )-producing type 1 CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells are key components of this immunity (1, 12, 16). IFN- $\gamma$  produced by activated T cells is believed to be an essential element of the host defense against *M. tuberculosis* (13). Further, the contribution of CD8<sup>+</sup> T cells to protection by lysing infected cells is also important for bacterial killing (7, 19). CD8<sup>+</sup> T cells can kill *M. tuberculosis*-infected host cells via a granule-dependent mechanism involving perforin and granulysin, which has a direct antimicrobial activity (42, 49).

The only approved vaccine currently available against tuberculosis is *M. bovis* bacillus Calmette-Guérin (BCG), an attenuated strain of *M. bovis*. More than four billion doses of BCG have been administered so far, and is established as a safe vaccine (29). BCG appears to be effective at preventing diseases such as tuberculous meningitis and miliary tuberculosis in newborns and toddlers; however, it has no apparent effect on pulmonary tuberculosis in adults (3, 9). The reason why BCG cannot prevent disease development is not fully known, but one of the reasons is based on the fact that BCG has a capacity to block phagosome maturation to inhibit antigen (Ag) processing and presentation to type 1 T cells (14, 32, 38). Indeed, although *M. tuberculosis* directly delivers Ag to the major histocompatibility complex (MHC) class I processing pathway, BCG was less able to activate CD8<sup>+</sup> T cells (35, 41). Further, BCG growing in human macrophages was not recognized by immune CD4<sup>+</sup> T cells, although BCG-infected macrophages continued to express MHC class II molecules (35). These observations indicate the need for the development of a new vaccine against tuberculosis.

Various new vaccine candidates which are based on Ags that are recognized in infected individuals are currently in clinical trials, including early secretory antigenic target 6 (ESAT-6), the Ag85 family, and a polyprotein Ag, designated Mtb72F, derived from *M. tuberculosis* proteins Mtb32 and Mtb59 (1, 2, 17, 18, 37, 39). However, a fully reliable new vaccine has not been established yet.

A situation similar to that of tuberculosis can be found in leprosy, which is caused by infection with *M. leprae*, and the development of a new vaccine capable of inhibiting the multi-

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plication of *M. leprae* is highly desirable. In both tuberculosis and leprosy, the activation of T cells is induced by DC loaded with bacilli or their components, which display various antigenic molecules on their surface, including the immunodominant Ags (15, 30), although there are conflicting results indicating that *M. leprae* inhibits the activation and maturation of DC (33). We are of the opinion that future vaccines, to be successful, must (i) be highly antigenic, (ii) have the capacity to activate both naïve CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells, and (iii) have the ability to be expressed on the surface of mycobacterium-infected Ag-presenting cells (APCs) such as macrophages and DC. Previously, we identified major membrane protein (MMP; gene name, *bfrA* or ML2038) as one of the immunodominant Ags of *M. leprae* (21). *M. leprae*-derived MMP (MMP-ML) ligates Toll-like receptor 2 (TLR2) and consequently activates the NF- $\kappa$ B pathway of host cells (21). DC pulsed with MMP-ML activate memory-type CD4<sup>+</sup> and CD8<sup>+</sup> T cells to produce IFN- $\gamma$  in an Ag-specific fashion (21, 26). Further, MMP-ML is supposed to be recognized *in vivo* by T cells of *M. leprae*-infected individuals, including paucibacillary leprosy patients (26).

Further, when we introduced MMP-ML with the Ag85A secretion signal of *M. tuberculosis* into BCG, the modified BCG, termed BCG-SM, secreted MMP-ML, enhanced the ability of BCG to activate naïve CD4<sup>+</sup>, and further, successfully activated naïve CD8<sup>+</sup> T cells (25). Furthermore, BCG-SM at least partially inhibited the growth of *M. leprae* in C57BL/6 mice subsequently challenged by injection in the footpads (22). These observations indicate that MMP-ML could be a target molecule to be further analyzed as a vaccine candidate, and the fact that BCG-SM can activate both subsets of naïve T cells to produce IFN- $\gamma$  indicates that secretion of MMP-ML, presumably in the phagosome of APCs, is a useful strategy to activate T cells (25). We sought another strategy to further enhance the T cell-stimulating activity of BCG, especially of the ability to activate IFN- $\gamma$ -producing CD8<sup>+</sup> T cells quickly and strongly. To this end, we used heat shock protein 70 (HSP70) as a fusion partner (6, 10, 44, 45). The gene encoding HSP70 of BCG was directly linked with that of MMP and extrachromosomally transformed into BCG (BCG-70 M) (31). BCG-70 M secreted the HSP70-MMP fusion protein (Fusion-ML) and activated not only Ag-specific naïve CD8<sup>+</sup> T cells polyclonally but also naïve CD4<sup>+</sup> T cells strongly (31). Further, the secreted Fusion-ML protein activated DC in terms of phenotype and the production of cytokines such as interleukin-12 (IL-12) (31). Thus, the production and secretion of HSP70 in phagosomes along with MMP-ML, using BCG as a vector, seem to be effective in activating human naïve CD8<sup>+</sup> T cells. These observations led us to speculate that the use of MMP, which is commonly present in pathogenic mycobacteria, or of the HSP70-MMP fusion protein may be useful in inhibiting the multiplication of *M. tuberculosis*. However, the MMP homology between *M. leprae* and *M. tuberculosis* (MMP-MTB; gene name, *bfrA* or Rv1876) is 90.6% at the amino acid level. Therefore, in this study, we purified *M. leprae*- or *M. tuberculosis*-derived MMP and a fusion protein composed of HSP70 and *M. leprae*- or *M. tuberculosis*-derived MMP by using *M. smegmatis* and evaluated their immunostimulatory activities.

## MATERIALS AND METHODS

**Preparation of cells and Ags.** Peripheral blood was obtained from healthy, purified protein derivative-positive individuals after informed consent was obtained. In Japan, BCG vaccination is compulsory for children (0 to 4 years old). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described (23). The viability of T cells obtained from cryopreserved PBMCs was more than 90%, and no functional selection was induced in either monocytes or T cells by the cryopreservation of PBMCs. For the preparation of peripheral monocytes, CD3<sup>+</sup> T cells were removed from either freshly isolated heparinized blood or cryopreserved PBMCs using immunomagnetic beads coated with anti-CD3 monoclonal antibody (MAB; Dynabeads 450; Dynal Biotech, Oslo, Norway). The CD3<sup>-</sup> PBMC fraction was plated on collagen-coated plates, and the non-plastic-adherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (47). Monocyte-derived DC were differentiated as described previously (23, 28). Briefly, monocytes were cultured in the presence of 50 ng of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF; PeproTech EC Ltd., London, England) and 10 ng of rIL-4 (PeproTech) per ml (28). On day 4 of culture, immature DC (purity, 70%) were pulsed with recombinant protein and on day 6 of culture, DC were used for further analyses of surface Ag and for mixed-lymphocyte assays. Macrophages were differentiated as described previously (24, 27). In brief, monocytes were cultured in the presence of 10 ng of rM-CSF (R&D Systems, Inc., Minneapolis, MN) per ml. On day 5 of culture, macrophages were pulsed with recombinant protein and on day 7 of culture, they were used for further analyses of surface Ag and for mixed-lymphocyte assays.

**Preparation of *M. tuberculosis*.** *M. tuberculosis* strains H37Ra and H37Rv, which were originally purchased from the American Tissue Culture Collection, were kindly donated by T. Yamazaki, National Institute of Infectious Diseases. Both H37Ra and H37Rv were cultured *in vitro* using Middlebrook 7H9 broth (BD Biosciences, San Jose, CA) supplemented with 0.05% Tween 80 and 10% albumin-dextrose-catalase (BD Biosciences). The bacilli were grown to log phase and stored at 10<sup>8</sup> CFU/ml at -80°C. Before the infection of DC and macrophages, H37Ra and H37Rv bacteria were counted by the colony assay method.

**Construction of vectors for production of *M. leprae*- or *M. tuberculosis*-derived recombinant MMP and HSP70-MMP fusion protein (Fusion-ML) or Fusion-MTB.** An *Escherichia coli*-*Mycobacterium* shuttle vector, pMV261 (43), was used as a parent vector. To replace the kanamycin resistance gene with a hygromycin resistance cassette, the XbaI-NheI fragment from pYUB854 (5) was cloned into SpeI-NheI-digested plasmids. The resultant vector was a hygromycin-resistant pMV261 vector, pMV261H. The acetamidase promoter was amplified from *M. smegmatis* mc<sup>2</sup>155 genomic DNA. The primers used were F Pace Xba (5'-TTA ATC TAG AGA AGT GAC GCG GTC TCA AGC GTC-3' [underlining indicates an XbaI site]) and R Pace Bam (5'-TTT AGG ATC CGT GGA CTC CCT TTC TCT TA-3' [underlining indicates a BamHI site]). The *Hsp60* promoter region in pMV261H was replaced with the amplified PCR products, and the resulting vector was named p2H Pace.

We inserted an N-terminally His-tagged gene sequence encoding MMP-ML, MMP-MTB, HSP70-MMP-ML fusion (Fusion-ML), or HSP70-MMP-MTB (Fusion-MTB) into the p2H Pace vector using the In-Fusion Advantage PCR Cloning Kit (Clontech Laboratories, Inc.). Briefly, the linearized p2H Pace vector for the In-Fusion reaction was prepared by PCR with F ter (5'-TAG TTA ACT AGC GTA CGA T-3') and R Pace H6 (5'-GTG ATG GTG GTG ATG GTG CAT GTG GAC TCC CTT TCT CTT AT-3'). PCR primers for inserts were designed that share 15 bases of homology with the sequences at the ends of linearized p2H Pace. These primers were used to amplify the insert DNAs for MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB. The resulting PCR products were combined with the linearized vector in the In-Fusion cloning reaction and then transformed into *E. coli*. All clones were verified by sequencing.

**Expression and purification of recombinant proteins in *M. smegmatis*.** *M. smegmatis* mc<sup>2</sup>155 was cultured *in vitro* using LB broth supplemented with 0.05% Tyloxapol (Sigma-Aldrich, St. Louis, MO). Expression vectors were introduced into *M. smegmatis* by electroporation (40). Transformants were selected on LB agar (BD Biosciences, San Jose, CA) plates containing 50  $\mu$ g/ml hygromycin. The selected clone was grown in LB broth with 150  $\mu$ g/ml hygromycin. During the logarithmic phase, acetamide was added to the culture medium at a final concentration of 0.2% (8). After an additional 16 h of culture, recombinant *M. smegmatis* was centrifuged and resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 3 M guanidine-HCl) containing proteinase inhibitor and disrupted by sonication. The suspension was centrifuged at 27,000  $\times$  g for 15 min. The supernatant was further filtered through a 0.45- $\mu$ m filter and used as starting material. MMP-ML and MMP-MTB were purified by metal affinity

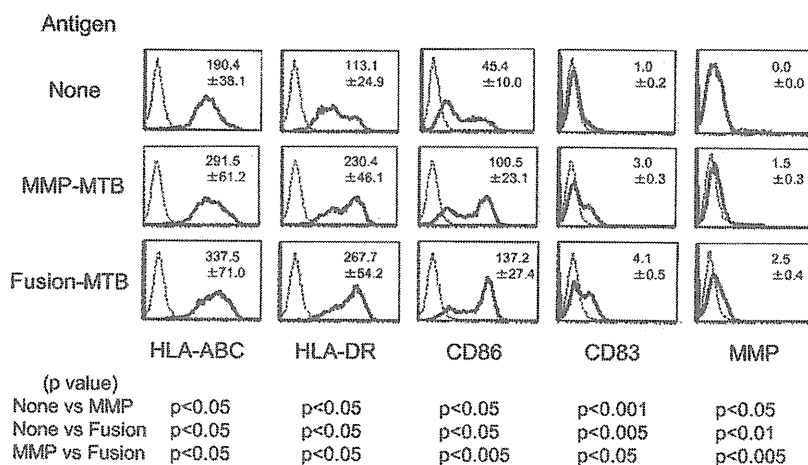


FIG. 1. Expression of APC-associated molecules and MMP on DC by stimulation with recombinant proteins. Immature DC obtained from monocytes in the presence of rGM-CSF and rIL-4 were pulsed with either MMP-MTB or Fusion-MTB at 10  $\mu$ g/ml on day 4 of culture. The DC were gated and analyzed on day 6 after the start of culture. Dotted lines, isotype-matched control IgG or IgM (for MMP); solid lines, the indicated test MAb. Representative results of three separate experiments are shown. The value in the top right corner of each graph is the mean fluorescence intensity of three independent experiments with a control Ig or the test MAb  $\pm$  the standard deviation. Titers were statistically compared using Student's *t* test.

chromatography (TALON Metal Affinity Resins; Clontech Laboratories). Fusion-ML and Fusion-MTB were purified by two purification steps. Passage through a metal affinity column (TALON) was also used in the first step. The eluted crude proteins were applied to a HiLoad Superdex 200 pg column (GE Healthcare, Buckinghamshire, England) for further purification by gel filtration. Three major fractions were detected after the second step; one of them contained the target protein. The purified proteins (MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining (see Fig. S1 in the supplemental material).

**Analysis of cell surface Ag.** The expression of cell surface Ag on DC and lymphocytes was analyzed using a FACScalibur. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma-Aldrich), and  $1 \times 10^6$  live cells were analyzed. For cell surface Ag analysis, fluorescein isothiocyanate (FITC)-conjugated MAbs against HLA-ABC (G46-2.6; BD Biosciences), HLA-DR (L243; BD Biosciences), CD86 (FUN-1; BD Biosciences), and CD83 (HB15a; Immunotech, Marseille, France) were used.

The expression of MMP on Ag-pulsed DC or DC infected with *M. tuberculosis* at an indicated multiplicity of infection (MOI) was determined using the MAb against MMP-ML (M270-13, IgM, kappa), which probably detects MMP complexed with MHC molecules on the surface of DC (26), followed by FITC-conjugated anti-mouse Ig Ab (Tago Immunologicals, Camarillo, CA). The intracellular production of perforin was assessed as follows. Memory-type CD8<sup>+</sup> T cells were stimulated with Ag-pulsed DC for 5 days in the presence of memory-type CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells were surface stained with phycoerythrin-labeled MAb to CD8 and fixed in 2% formaldehyde. Subsequently, the cells were permeabilized using permeabilizing solution (BD Biosciences) and stained with an FITC-conjugated MAb to perforin ( $\delta$ G9; BD Biosciences) or an FITC-labeled isotype control.

**APC functions of DC.** The ability of Ag-pulsed DC and macrophages to stimulate T cells was assessed using an autologous APC-T cell coculture as previously described (15, 28). Purification of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was conducted by using negative-isolation kits (Dynabeads 450; Dynal Biotech) (28). The purity of the CD4<sup>+</sup> and CD8<sup>+</sup> T cells was more than 95% as assessed by FACScalibur. Naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells were produced by further treatment of these T cells with MAb to CD45RO, which was followed by beads coated with MAb to goat anti-mouse IgG (DynaL Biotech). The purity of both subsets of naive T cells was more than 97%. However, there was no contamination of memory-type T cells in the naive T cell preparations. More than 98% of the CD45RA<sup>+</sup> T cells were positive for expression of the CCR7 molecule. Memory-type T cells were similarly produced by the treatment of cells with a MAb to CD45RA Ag. The purified responder cells ( $1 \times 10^5$  per well) were plated in 96-well round-bottom tissue culture plates, and DC or macrophages pulsed with Ag were added to give the indicated APC/T cell ratio. Supernatants of APC-T

cell cocultures were collected on day 4, and the cytokine levels were determined. In some cases, Ag-pulsed DC were treated with MAbs to HLA-ABC (W6/32; mouse IgG2a, kappa), HLA-DR (L243; mouse IgG2a, kappa), CD86 (IT2.2; mouse IgG2b, kappa, BD Biosciences), or MMP (M270-13) or normal mouse IgG or IgM. The treatment of DC with these MAbs did not affect the viability of the DC (not shown). Also, in some cases, Ag-pulsed DC were costimulated with CD40 ligand (CD40L; 1  $\mu$ g/ml; PeproTech). The optimal concentration was determined in advance.

**Measurement of cytokine production.** Levels of the following cytokines were measured: IFN- $\gamma$  produced by CD4<sup>+</sup> and CD8<sup>+</sup> T cells and IL-12p40 produced by DC stimulated for 24 h with Ag. The concentrations of these cytokines were quantified with enzyme-linked immunosorbent assay (ELISA) kits (Opt EIA Human ELISA Set; BD Biosciences). The detection limit of the IFN- $\gamma$  ELISA kit is 3.0 pg/ml.

**Statistical analysis.** Student's *t* test was used to determine statistically significant differences.

## RESULTS

**Activation of DC by *M. tuberculosis*-derived recombinant proteins.** For a recombinant protein to activate T cells, it must have the ability to activate APCs. We assessed the phenotypic change induced in DC by stimulation with MMP-MTB (gene name, Rv1876 or *bfrA*) and a fusion protein composed of BCG-derived HSP70 and MMP-MTB (Fusion-MTB) (Fig. 1). Both recombinant proteins upregulated the surface expression of HLA-ABC, HLA-DR, CD86, and CD83. However, Fusion-MTB more efficiently enhanced the expression of all of these molecules. Further, MMP-MTB- or Fusion-MTB-pulsed DC expressed molecules which react with anti-MMP-ML MAbs. Again, Fusion-MTB was more efficient than MMP-MTB in the induction of expression of the molecules. These results indicated that both recombinant proteins may have the ability to activate DC. To confirm this point, we measured the IL-12p40 production of DC by stimulation with the recombinant proteins (Fig. 2). We comparatively analyzed MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB. All of the recombinant proteins induced the production of IL-12p40, but the levels of IL-12p40 produced by stimulation were as follows: MMP-MTB > MMP-

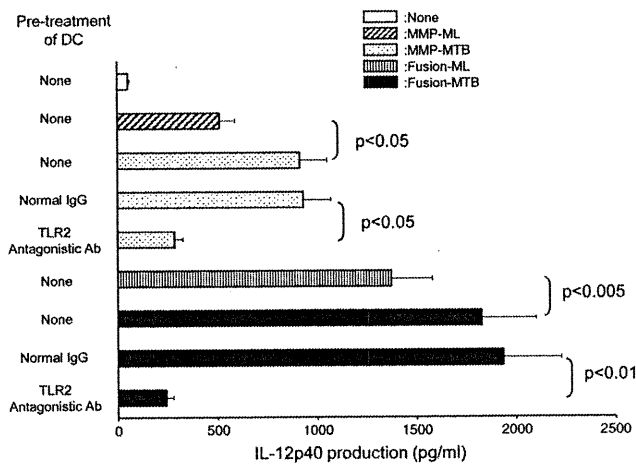


FIG. 2. IL-12p40 production by DC stimulated with recombinant proteins. Monocyte-derived DC from 5 days of culture in the presence of rGM-CSF and rIL-4 were stimulated with the indicated recombinant protein at 10  $\mu$ g/ml for 24 h. In some cases, immature DC were pretreated with normal murine IgG or TLR2-antagonistic Ab (10  $\mu$ g/ml) and subsequently stimulated with recombinant protein for 24 h. The concentration of IL-12p40 was determined by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean  $\pm$  the standard deviation. Titers were statistically compared by Student's *t* test.

ML, Fusion-MTB > Fusion-ML, and Fusion-MTB > MMP-MTB. In order to reveal the mechanisms leading to the activation of DC by MMP-MTB or Fusion-MTB, we pretreated immature DC with TLR2-antagonistic Ab and subsequently stimulated the treated DC with recombinant proteins, since both MMP-ML and Fusion-ML are reported to activate the NF- $\kappa$ B pathway through ligation with TLR2 (21, 31). While pretreatment of DC with normal murine IgG did not affect the production of IL-12 by stimulation with recombinant proteins, the pretreatment with TLR2-antagonistic Ab significantly inhibited the cytokine production caused by MMP-MTB and Fusion-MTB (Fig. 2). Also, we tested the effect of TLR4-antagonistic Ab on IL-12 production; however, the pretreatment of DC with TLR4-antagonistic Ab did not inhibit cytokine production (not shown).

**Activation of T cells by recombinant protein.** The enhanced activation of DC by *M. tuberculosis*-derived MMP and fusion proteins may enable autologous T cell activation. The T cell-activating ability of the recombinant proteins was assessed chiefly by using DC as APCs. Memory-type CD4<sup>+</sup> T cells were purified from healthy, BCG-vaccinated individuals. All of the recombinant proteins activated the CD4<sup>+</sup> T cells with a small dose (~2.0  $\mu$ g/ml) of Ags and a small dose of recombinant protein-pulsed DC (T cell/DC ratio, 80:1) (Fig. 3A). However, MMP-MTB and Fusion-MTB more efficiently activated the T cells than *M. leprae*-derived proteins did, and Fusion-MTB induced a significantly larger amount of IFN- $\gamma$  than MMP-MTB did. When we used a higher concentration of recombinant proteins, such as 5 or 10  $\mu$ g/ml, and used a higher number of DC, such as a T cell/DC ratio of 10:1, as a stimulator, similar statistically significant differences were observed (not shown). Further, only Fusion-MTB successfully activated memory-type

CD4<sup>+</sup> T cells through macrophages, although a large dose of Ag and a high number of Ag-pulsed macrophages were required (T cell/macrophage ratio, 5:1) (Fig. 3A). The CD4<sup>+</sup> T cell-stimulating activity of MMP-MTB and Fusion-MTB was confirmed by using CD45RO-negative naive CD4<sup>+</sup> T cells. All of the recombinant proteins, including MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB, activated naive CD4<sup>+</sup> T cells, and Fusion-MTB was the most effective (Fig. 3B). Compared to memory CD4<sup>+</sup> T cells, naive CD4<sup>+</sup> T cells required a larger dose of Ag (~10  $\mu$ g/ml) and a higher number of Ag-pulsed DC (T cell/DC ratio, 10:1) to be activated. To address the mechanisms leading to the activation of naive CD4<sup>+</sup> T cells by Fusion-MTB, Fusion-MTB-pulsed DC were treated with MAbs against HLA-DR, CD86, and MMP-ML molecules and subsequently used to stimulate naive CD4<sup>+</sup> T cells (Fig. 3C). IFN- $\gamma$  production by these naive CD4<sup>+</sup> T cells was significantly inhibited by the surface treatment of the DC with the MAbs, and similarly, IL-2 production by naive CD4<sup>+</sup> T cells was inhibited (not shown). The ability of MMP-MTB and Fusion-MTB to activate memory-type CD8<sup>+</sup> T cells was then assessed (Fig. 4A). Although, in contrast to memory-type CD4<sup>+</sup> T cells, a large dose of recombinant proteins was required, both *M. tuberculosis*-derived recombinant proteins induced significant production of IFN- $\gamma$  from memory-type CD8<sup>+</sup> T cells. Further, the additional treatment of Ag-pulsed DC with CD40L upregulated the production of IFN- $\gamma$  by CD8<sup>+</sup> T cells. In both cases, i.e., without and with CD40L treatment, Fusion-MTB induced significantly greater IFN- $\gamma$  production than MMP-MTB did. In order to confirm the CD8<sup>+</sup> T cell-stimulating abilities of both MMP-MTB and Fusion-MTB, naive CD8<sup>+</sup> T cells were also examined as responders. In this case, purified proteins from *M. leprae* were used as a control (Fig. 4B). Both MMP-MTB and Fusion-MTB activated naive CD8<sup>+</sup> T cells to produce IFN- $\gamma$ ; however, the concentration of IFN- $\gamma$  released from naive CD8<sup>+</sup> T cells was low and a cytokine concentration of less than 35 pg/ml was produced, and the concentration of IFN- $\gamma$  produced from naive CD8<sup>+</sup> T cells by stimulation with Fusion-MTB was significantly lower than that from memory CD8<sup>+</sup> T cells ( $P < 0.005$ ). The naive CD8<sup>+</sup> T cell-stimulating activities of the recombinant proteins were as follows: MMP-MTB > MMP-ML, Fusion-MTB > Fusion-ML, and Fusion-MTB > MMP-MTB. The IFN- $\gamma$  production by naive CD8<sup>+</sup> T cells was enhanced by the additional treatment of Ag-pulsed DC with CD40L, and the highest production of IFN- $\gamma$  was achieved by Fusion-MTB; in this case, Fusion-MTB could induce an IFN- $\gamma$  concentration of more than 100 pg/ml. To elucidate the mechanisms of the activation of naive CD8<sup>+</sup> T cells by Fusion-MTB, Fusion-MTB pulsed DC were treated with MAbs to HLA-ABC and CD86 and subsequently used as a stimulator (Fig. 4C). IFN- $\gamma$  production by naive CD8<sup>+</sup> T cells was significantly inhibited by the treatment of the DC. One of the aims of CD8<sup>+</sup> T cell activation in terms of the host defense against *M. tuberculosis* is to produce cytotoxic CD8<sup>+</sup> T cells. To measure the production of cytotoxic CD8<sup>+</sup> T cells, we assessed the intracellular production of perforin in CD8<sup>+</sup> T cells which were stimulated with MMP-MTB or Fusion-MTB in the presence of CD4<sup>+</sup> T cells (Fig. 4D). Both recombinant proteins produced perforin-producing CD8<sup>+</sup> T cells, and Fusion-MTB seemed to produce them more efficiently.

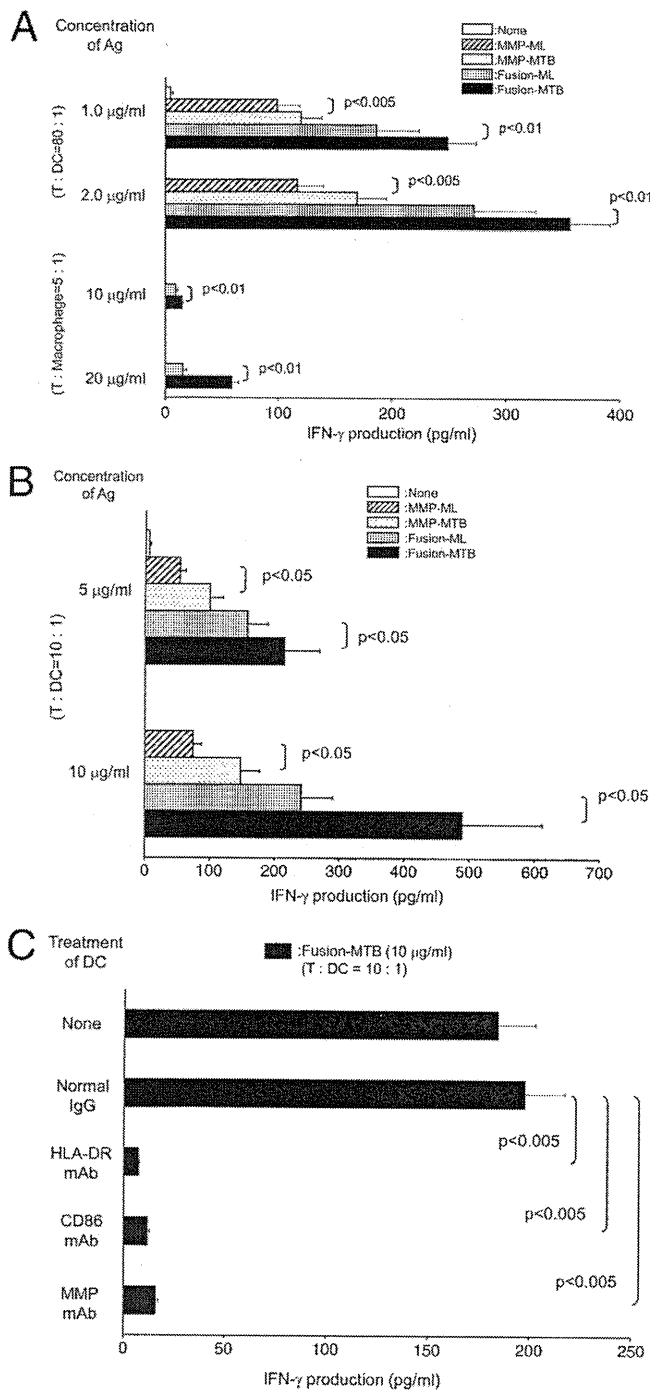


FIG. 3. (A) IFN- $\gamma$  production by memory-type CD4<sup>+</sup> T cells stimulated with recombinant proteins. Monocyte-derived DC or macrophages were pulsed with the indicated recombinant protein at the indicated concentration and used to stimulate memory-type CD4<sup>+</sup> T cells in a 4-day culture. Responder CD4<sup>+</sup> T cells ( $1 \times 10^5$ ) were stimulated with the indicated dose of Ag-pulsed DC or macrophages. (B) IFN- $\gamma$  production by naive CD4<sup>+</sup> T cells by stimulation with recombinant protein. Monocyte-derived DC were pulsed with the indicated recombinant protein at 5 or 10  $\mu\text{g/ml}$  and used to stimulate naive CD4<sup>+</sup> T cells in a 4-day culture. Responder CD4<sup>+</sup> T cells ( $1 \times 10^5$ ) were stimulated with the Ag-pulsed DC at a T cell/DC ratio of 10:1. (C) Inhibition of naive CD4<sup>+</sup> T cell activation by treatment of Ag-pulsed DC with MAb. Monocyte-derived DC were pulsed with Fusion-MTB at 10  $\mu\text{g/ml}$  and subsequently treated at 10  $\mu\text{g/ml}$  with

**Expression of MMP on APCs infected with *M. tuberculosis*.** The molecule used as a vaccinating agent should be expressed on APCs infected with the pathogen. To reveal the expression of MMP on the surface of APCs, DC and macrophages were infected with *M. tuberculosis* H37Ra and H37Rv and analyzed by flow cytometry (Fig. 5). Both DC and macrophages expressed MMP molecules on their surface after infection with H37Ra and H37Rv. Expression levels seemed to be dependent on the dose of *M. tuberculosis* used for infection (not shown).

## DISCUSSION

*In vivo* studies using various knockout mice indicate that adaptive immunities play an important role in inhibiting the multiplication of *M. tuberculosis* and that the activation of both CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells is an essential element of the control of *M. tuberculosis* infection (1, 12, 16). While CD4<sup>+</sup> T cells chiefly act in the initial phase of infection, CD8<sup>+</sup> T cells either producing IFN- $\gamma$  or having cytotoxic killing activity contribute to the chronic or stationary phase of infection (7, 19, 46). Thus, the antigenic molecules which are used as an essential component of a vaccine should have the ability to activate not only naive CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells but also APCs, including DC. So far, we have found MMP to be one of the immunodominant Ags of *M. leprae* (21) and found evidence that MMP-ML activated DC through ligation with TLR2, which resulted in the activation of the NF- $\kappa$ B pathway of host cells, and that DC pulsed with MMP-ML stimulated both CD4<sup>+</sup> and CD8<sup>+</sup> T cells to produce IFN- $\gamma$  in an Ag-specific manner (21, 26). Further, MMP-ML is supposed to be recognized *in vivo* by both T cell subsets of *M. leprae*-infected individuals, including paucibacillary leprosy patients (26).

In addition, HSP70, one of the heat shock proteins, plays various roles in the upregulation of the ability of APCs to stimulate T cells (6, 10, 44, 45). Further, HSPs of both mammalian host cell and bacterial origins are reported to have chaperon activity (6, 44) and can effectively prime a cytolytic response (10, 45). In fact, we previously reported that HSP70 effectively induced the cross-priming of CD8<sup>+</sup> T cells through the cytosolic pathway when secreted from recombinant BCG in the phagosome of DC as part of a fusion protein (31). Also, others have reported that HSP65 activated naive CD8<sup>+</sup> T cells and a DNA vaccine containing the *hsp65* gene inhibited the development of tuberculosis that is induced by the multiplication of subsequently challenged *M. tuberculosis* (50). Furthermore, vaccination of mice with recombinant BCG that secreted either MMP-ML or Fusion-ML, in which BCG was used as a vehicle, efficiently inhibited the multiplication of subsequently challenged *M. leprae*, although the fusion protein was more efficient in both activating naive T cells and inhibiting *M. leprae* multiplication (22, 25, 31).

MAB to HLA-DR, CD86, MMP, or normal murine IgG or IgM. These DC were used to stimulate naive CD4<sup>+</sup> T cells ( $1 \times 10^5$ ) at a T cell/DC ratio of 10:1. IFN- $\gamma$  produced from T cells was measured by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as means  $\pm$  standard deviations. Titers were statistically compared by Student's *t* test.

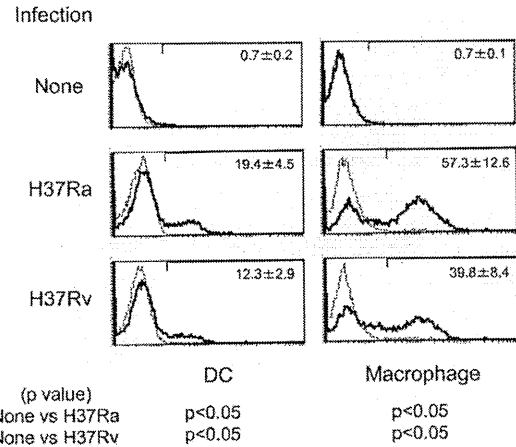
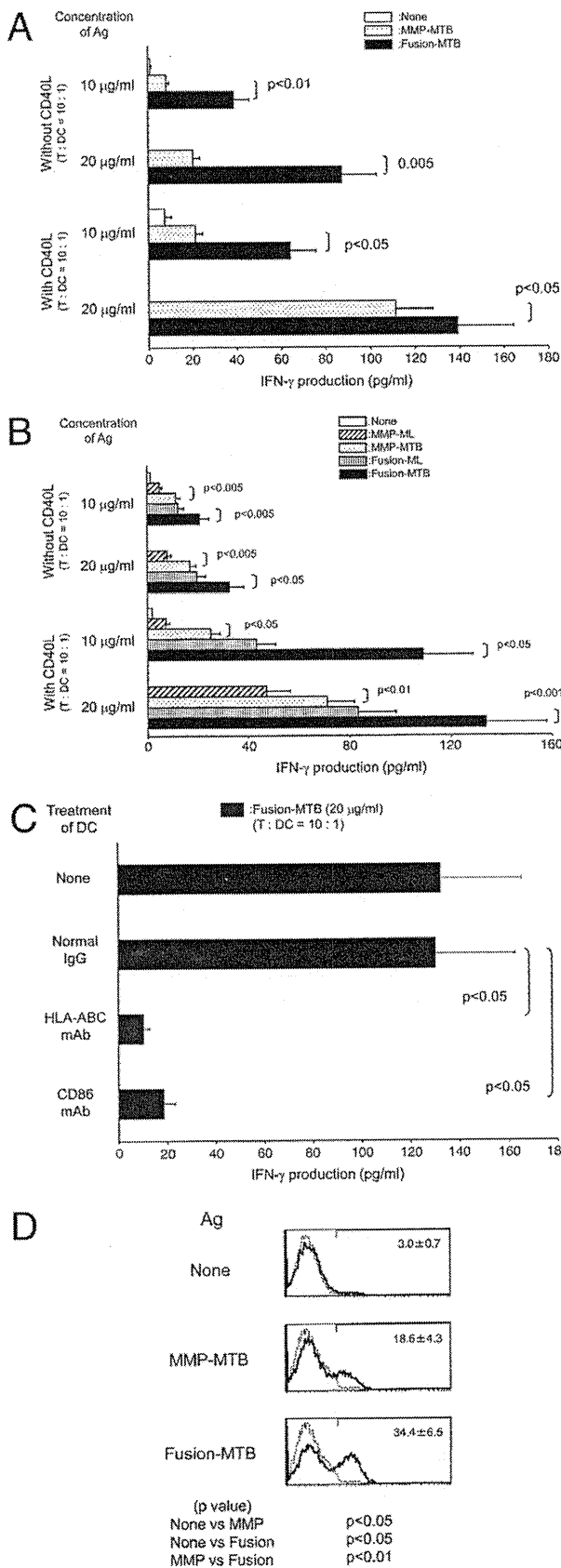


FIG. 5. Expression of MMP on DC and macrophage infected with *M. tuberculosis*. Monocyte-derived DC or macrophages were infected with either H37Ra or H37Rv at an MOI of 1.0 and cultured for another 2 days in the presence of rGM-CSF plus rIL-4 or rM-CSF, respectively. The DC and macrophages were gated and analyzed on day 5 after the start of culture. Dotted lines, control IgM; solid lines, MMP MAb. Results representative of three separate experiments are shown. The values are the mean percentages of major membrane protein II-positive DC or macrophages in three independent experiments and the standard deviations. Titers were statistically compared using Student's *t* test.

MMP is commonly expressed in both pathological mycobacteria and BCG, so that it may be that MMP-MTB plays a substantial role in inhibiting the replication of *M. tuberculosis*; however, the homology of MMP between *M. leprae* (ML2038)

FIG. 4. (A) IFN-γ production by memory-type CD8<sup>+</sup> T cells by stimulation with recombinant protein. Monocyte-derived DC were pulsed with MMP-MTB or Fusion-MTB at 10 or 20 µg/ml, costimulated with or without CD40L (1.0 µg/ml), and used to stimulate memory-type CD8<sup>+</sup> T cells in a 4-day culture. Responder CD8<sup>+</sup> T cells (1 × 10<sup>5</sup>) were stimulated with the Ag-pulsed DC at a T cell/DC ratio of 10:1. (B) IFN-γ production by naive CD8<sup>+</sup> T cells stimulated with recombinant proteins. Monocyte-derived DC were pulsed with the indicated recombinant protein at 10 or 20 µg/ml, further costimulated with or without CD40L (1.0 µg/ml), and used to stimulate naive CD8<sup>+</sup> T cells in a 4-day culture. Responder CD8<sup>+</sup> T cells (1 × 10<sup>5</sup>) were stimulated with the Ag-pulsed DC at a T cell/DC ratio of 10:1. (C) Inhibition of naive CD8<sup>+</sup> T cell activation by treatment of Fusion-MTB-pulsed DC with MAb. Monocyte-derived DC were pulsed with MMP-MTB at 20 µg/ml, costimulated with CD40L (1.0 µg/ml), and subsequently treated at 10 µg/ml with MAb to HLA-ABC, CD86, or normal murine IgG. These DC were used to stimulate naive CD8<sup>+</sup> T cells (1 × 10<sup>5</sup>) at a T cell/DC ratio of 10:1. IFN-γ produced by T cells was measured by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as means ± standard deviations. Titers were statistically compared by Student's *t* test. (D) Intracellular production of perforin by CD8<sup>+</sup> T cells. Monocyte-derived DC were pulsed at 10 µg/ml with either MMP-MTB or Fusion-MTB and cultured with unseparated memory-type T cells (T cell/DC ratio, 40:1) for 5 days. The stimulated CD8<sup>+</sup> T cells were gated and analyzed for perforin production. Values are the mean percentages of the CD8<sup>+</sup> T cell population that were perforin positive in three independent experiments and the standard deviations. Titers were statistically compared using Student's *t* test. A representative of three separate experiments is shown.

and *M. tuberculosis* (Rv1876) is only 90.6% at the amino acid level. Therefore, we assessed the immunostimulatory activity of *M. tuberculosis*-derived MMP and its fusion with BCG-derived HSP70 by using MMP-ML and Fusion-ML as controls.

As expected, MMP derived from *M. tuberculosis* activated DC in terms of phenotypic change and cytokine production, and the cytokine production was associated with the ability of MMP-MTB to ligate with TLR2. MMP-MTB-pulsed DC activated both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. In this respect, only a very small amount of MMP was required to induce vigorous activation of CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells, obtained from BCG-vaccinated healthy donors. These results may indicate that some subsets of CD4<sup>+</sup> T cells are primed with MMP by vaccination with BCG, whose MMP is 100% homologous to that of *M. tuberculosis*, as in the case of leprosy patients whose T cells were primed by *M. leprae* infection. However, in contrast to leprosy patients, only CD4<sup>+</sup> T cells are primed with MMP by BCG vaccination, which may be linked with the fact that the parent BCG less efficiently activates naïve CD8<sup>+</sup> T cells. Activation of T cells usually depends on APCs expressing Ags, so that successful production of MMP-reactive memory-type T cells could be achieved by administration of MMP since MMP could be expressed on the surface of DC after infection with *M. tuberculosis* H37Ra and H37Rv. This speculation might be supported by our preliminary experiments in which administration of MMP-MTB to C57BL/6 mice produced memory-type splenic T cells reactive to MMP-MTB *in vitro*, which produced IFN- $\gamma$  because of this stimulation.

Fourteen amino acids of *M. leprae* MMP differ from those of *M. tuberculosis* MMP, and substitutions of amino acids between these mycobacteria are known to occur randomly. However, a MAb which recognizes the epitope expressed on DC pulsed with *M. leprae*-derived MMP could also detect a peptide expressed on the surface of DC pulsed with *M. tuberculosis*-derived MMP or infected with *M. tuberculosis*. The MAb against MMP-ML inhibited the activation of naïve CD4<sup>+</sup> T cells by stimulation with MMP-MTB-pulsed DC. These observations indicated that the regions common to the MMPs of *M. leprae* and *M. tuberculosis* were chiefly used as antigenic epitopes of CD4<sup>+</sup> T cells. However, the T cell activation by *M. tuberculosis*-derived MMP and Fusion-MTB is significantly stronger than that by *M. leprae*-derived proteins. The exact mechanism leading to the difference between the T cell-stimulating activities of the MMPs derived from these two pathological mycobacterial strains remains to be elucidated, but one possibility is that some parts of *M. tuberculosis*-derived MMP other than common regions have APC-immunomodulating activities that are associated with T cell activation. In fact, *M. tuberculosis*-derived MMP more efficiently activated DC than MMP-ML did, in terms of IL-12 production. However, both MMP-ML and MMP-MTB ligate TLR2; thus, MMP-MTB may have other unknown mechanisms that can induce the activation of DC more strongly. In this respect, we assessed the IL-1 $\beta$ -producing ability of MMP, but there was no apparent difference between the MMPs obtained from *M. tuberculosis* and *M. leprae* (not shown). It has been reported that the replacement of one amino acid of the T cell epitope of the antigenic determinant of Ag85B of *M. tuberculosis* strongly affects its T cell-stimulating activity, i.e., the ability to induce IFN- $\gamma$  production (4). Therefore, a similar change may have

occurred in the MMP system, although it has not been clearly defined.

When we compared the immunostimulating activities of MMP-MTB and Fusion-MTB in terms of the activation of APC and T cells, the latter showed higher activity in the activation of both DC and CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The exact mechanism of the high immunostimulating activity of the fusion protein is not fully known, but it may be associated with previous reports indicating that HSPs play a varied role in enhancing the ability of APCs to stimulate T cells (6, 10, 44, 45). In fact, the fusion protein induced the expression of higher levels of APC-associated molecules on DC than MMP did. Further, Fusion-MTB may be useful to produce cytotoxic CD8<sup>+</sup> T cells because the fusion protein efficiently produced perforin-producing CD8<sup>+</sup> T cells, although both MMP-MTB and Fusion-MTB produced cytotoxic CD8<sup>+</sup> T cells. Moreover, the fusion protein upregulated the expression of CD40 on DC (not shown) and treatment of Fusion-MTB-pulsed DC with CD40L induced the production of a larger dose of IFN- $\gamma$  from both naïve CD4<sup>+</sup> T cells (not shown) and naïve CD8<sup>+</sup> T cells (Fig. 4B). These results indicate that the use of HSP70 as part of a fusion protein may make APCs susceptible to various conditioning molecules, including CD40L. This observation is in the line with the fact that only Fusion-MTB-pulsed monocyte-derived macrophages successfully activated CD4<sup>+</sup> T cells, probably MMP primed, when conditioned with CD40L.

Taken together, the data present here suggest that MMP, alone or as part of fusion protein, is highly immunogenic and may be useful for developing new vaccine against tuberculosis, at least in combination with BCG, ESAT-6, or other molecules.

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## Mutation Analysis of the *Mycobacterium leprae* *folP1* Gene and Dapsone Resistance<sup>∇</sup>

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Diaminodiphenylsulfone (dapsone) has long been used as a first-line drug worldwide for the treatment of leprosy. Diagnosis for dapsone resistance of *Mycobacterium leprae* by DNA tests would be of great clinical value, but the relationship between the nucleotide substitutions and susceptibility to dapsone must be clarified before use. In this study, we constructed recombinant strains of cultivable *Mycobacterium smegmatis* carrying the *M. leprae folP1* gene with or without a point mutation, disrupting their own *folP* gene on the chromosome. Dapsone susceptibilities of the recombinant bacteria were measured to examine influence of the mutations. Dapsone MICs for most of the strains with mutations at codon 53 or 55 of *M. leprae folP1* were 2 to 16 times as high as the MIC for the strain with the wild-type *folP1* sequence, but mutations that changed Thr to Ser at codon 53 showed somewhat lower MIC values than the wild-type sequence. Strains with mutations at codon 48 or 54 showed levels of susceptibility to dapsone comparable to the susceptibility of the strain with the wild-type sequence. This study confirmed that point mutations at codon 53 or 55 of the *M. leprae folP1* gene result in dapsone resistance.

The massive use of dapsone for treatment of leprosy led to the isolation of resistant strains of *Mycobacterium leprae* as early as 1964 (11), only a few years after discovery of the drug. Dapsone is structurally related to the sulfonamides. The mechanism of dapsone resistance in *M. leprae* is thought to be associated with dihydropteroate synthase (DHPS) in a manner similar to the mechanism of resistance to sulfonamides developed in other bacteria. The sulfonamides are structural analogs of *p*-aminobenzoate (PABA) and act as antimetabolites by competing with PABA for the active site of DHPS (4). DHPS catalyzes the reaction between dihydropteridine pyrophosphate and PABA as a part of the biosynthetic pathway leading to tetrahydrofolate (5, 12), which acts as a cofactor in the biosynthesis of purines, pyrimidines, and amino acids. Resistance to the sulfonamides has been shown to be mediated by mutations of the chromosomal *folP* gene encoding DHPS (7, 14, 15). Point mutations in the *folP1* gene have been identified in dapsone-resistant strains of *M. leprae* (9, 10, 16). Because *M. leprae* cannot be cultivated on any artificial medium and requires 13 days to double in experimentally infected mice, DNA diagnosis to detect dapsone-resistant bacteria would be highly useful. However, not all nucleotide substitutions in the *folP1* gene give rise to drug resistance. Therefore, the relationship between drug susceptibility and each nucleotide substitution observed in clinical isolates requires clarification. Dapsone-resistant *M. leprae* isolates have shown mutation at codon 53 or 55 in the *folP1* gene (6, 10, 16). Mutation at codon 48 has also been detected in our clinical specimens (unpublished data). Williams et al. have analyzed two types of mutations at codons 53 and 55 of the *M. leprae folP1* gene using a *folP*-deficient

*Escherichia coli* (16). However, their analysis is as yet insufficient for direct application as molecular diagnosis for dapsone resistance.

In this study, site-directed mutagenesis techniques were used to alter the wild-type *M. leprae folP1* gene at codons shown to be mutated in clinical isolates for testing the effects of these mutations on dapsone susceptibility in a *folP*-disrupted *Mycobacterium smegmatis* host.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5 $\alpha$  cells were grown in Luria-Bertani (LB) medium. *M. smegmatis* mc<sup>2</sup>155 and its transformants were grown in Middlebrook 7H9 medium (Difco, Detroit, MI) supplemented with 0.5% bovine serum albumin (fraction V), 0.2% glucose, 0.085% NaCl, 0.2% glycerol, and 0.1% Tween 80.

**Site-directed mutagenesis.** The wild-type *M. leprae folP1* gene was amplified by PCR from *M. leprae* Thai-53 and cloned into pMV261. Site-directed mutagenesis was performed using PCR with KOD DNA polymerase (Toyobo, Osaka, Japan) and the primers listed in Table 2. PCR products were purified and phosphorylated with T4 kinase and ATP and then ligated to become circular. The ligation mixture was used to transform *E. coli* DH5 $\alpha$ , and kanamycin-resistant colonies were isolated. Plasmids were extracted from the transformants, and the mutated sequences were confirmed by sequencing. Mutations introduced in the *M. leprae folP1* gene are shown in Fig. 1A.

**Disruption of the *folP* gene on the *M. smegmatis* chromosome.** *M. smegmatis* mc<sup>2</sup>155 cells were transformed with plasmids carrying the *M. leprae folP1* with or without a point mutation. Recombinants were selected on LB medium containing kanamycin. Allelic exchange mutants were constructed by the temperature-sensitive mycobacteriophage method (3). Using the *M. smegmatis* mc<sup>2</sup>155 genome sequence (accession number CP000480), the upstream and downstream flanking DNA sequences were used to generate a deletion mutation in the *folP* gene (MSMEG\_6103). In order to disrupt the *folP* gene, DNA segments from 736 bp upstream through 286 bp downstream of the initiation codon of *M. smegmatis folP* and from 198 bp upstream through 832 bp downstream of the termination codon were cloned directionally into the cosmid vector pYUB854, which contains a *res-hyg-res* cassette and a *cos* sequence for lambda phage assembly. Plasmids thus produced were digested with *PacI* and ligated to the PH101 genomic DNA excised from the phasmid pHA87 by *PacI* digestion. The ligated DNA was packaged using GigaPackIII Gold Packaging Extract (Stratagene, La Jolla, CA), and the resultant mixture was used for transduction of *E. coli* STBL2 (Life Technologies, Carlsbad, CA) to yield cosmid DNA. After *E.*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<b>Strains</b>		
<i>E. coli</i>		
DH5 $\alpha$	Cloning host	
STBL2	Cloning host	
C600 $\Delta$ <i>folP</i> ::Km <sup>r</sup>	<i>folP</i> mutant	7
<i>M. smegmatis</i> mc <sup>2</sup> 155		
<b>Plasmids</b>		
pYUB854	Cosmid vector	3
phAE87	Phasmid vector carrying full length DNA of mycobacteriophage PH101	3
pMV261	<i>E. coli</i> -mycobacteria shuttle plasmid vector (multicopy in mycobacteria)	13
pNN301 <sup>a</sup>	pMV361-type integrative vector (single copy in mycobacteria)	13; this study

<sup>a</sup> pNN301 has an *int-attP* fragment of mycobacteriophage L5 instead of *oriM*.

*coli* was transduced and the transductants were plated on hygromycin-containing medium, phasmid DNA was prepared from the pooled antibiotic-resistant transductants and electroporated into *M. smegmatis* mc<sup>2</sup>155. Bacterial cells were incubated at 30°C to produce the recombinant phage. The *M. smegmatis* transformant carrying the *M. leprae folP1* gene was infected by the produced temperature-sensitive phage at 37°C for allelic exchange, and kanamycin- and hygromycin-resistant colonies were isolated. Two colonies for each point mutation were subjected to subsequent tests.

**Dapsone susceptibility testing.** The MIC values for *M. smegmatis* recombinant clones were determined by culture on Middlebrook 7H10 agar plates containing 2-fold serial dilutions of dapsone (0.25 to 64  $\mu$ g/ml). The MIC value for each strain was defined as the lowest concentration of dapsone needed to inhibit bacterial growth.

RESULTS

**Construction of recombinant *M. smegmatis* strains.** We prepared plasmids with point mutations in the *M. leprae folP1* gene. Each plasmid has 1 of 21 single point mutations at codon 48, 53, 54, or 55 (Fig. 1A). The first or second nucleotide at each codon was replaced by another nucleotide to change the amino acid residue. Mutated sequences were confirmed by sequencing. Plasmids carrying the *M. leprae folP1* with or without a point mutation were individually introduced into *M. smegmatis*. The *M. smegmatis* transformants were subjected to allelic exchange to disrupt the *folP* gene on their own chromosome (Fig. 1B). PCR analysis confirmed that the *folP* sequences in the recombinant strains were replaced by hygromycin resistance gene sequences (Fig. 2). Isolation of a *folP*-disrupted *M. smegmatis* strain carrying the *M. leprae folP1* with mutation 48-4 (mutation 4 at codon 48) was unsuccessful. All the strains except for the strains with mutation 48-5 or 53-4 showed comparable growth rates. The strains with mutation 48-5 or 53-4 grew a little more slowly than the strain with the wild-type sequence. These two mutations may reduce DHPS activity.

**Dapsone susceptibility.** Dapsone susceptibilities of the recombinant *M. smegmatis* strains were tested. As shown in Fig. 3, the MIC of dapsone for recombinant *M. smegmatis* carrying the wild-type *M. leprae folP1* gene was 0.5  $\mu$ g/ml. MIC values for most of the strains with mutations at codon 53 or 55 were 2 to 16 times as high as the MIC for the strain with the wild-type sequence. Interestingly, two strains with alterations

TABLE 2. Primers used in this study

Primer	Sequence <sup>a</sup>	Application
MLFPWTF	GCGAA <u>TT</u> CGTGAGTTTGGCGCCAGTGCA	Cloning of <i>M. leprae folP1</i> , forward
MLFPWTR	GCA <u>AGC</u> TTTCAGCCATCACATCTAACCT	Cloning of <i>M. leprae folP1</i> , reverse
MSFPUF	GC <u>AGG</u> CCTGTATCCTCATCCCGGACAGC	<i>folP</i> disruption, upstream forward
MSFPUR	GCTCTAGATGGTGTTCGATGCTGATCGTG	<i>folP</i> disruption, upstream reverse
MSFPDF	GCAGATCTCGAAACGTTTCTCTCGGTAC	<i>folP</i> disruption, downstream forward
MSFPDR	GCACTAGTACTGGTCGATCTCCGACAGC	<i>folP</i> disruption, downstream reverse
MSFPF	TCACCGAGTACGGCATGAGC	Detection of <i>folP</i> disruption, forward
MSFPR	TAGAGCGCATGGATCAGCAG	Detection of <i>folP</i> disruption, reverse
MLFPR1	CGATTTCGCCACCGACGTCGAC	Introduction of point mutations for codons 53, 54, and 55
MLFPR2	GTCGACAATCGCCGCGCCTT	Introduction of point mutations for codon 48
MLFP48-1	ATCGGTGGCGAAATCGACCCG	Introduction of point mutation 48-1
MLFP48-2	CTCGGTGGCGAAATCGACCCG	Introduction of point mutation 48-2
MLFP48-3	TTCGGTGGCGAAATCGACCCG	Introduction of point mutation 48-3
MLFP48-4	GACGGTGGCGAAATCGACCCG	Introduction of point mutation 48-4
MLFP48-5	GCCGGTGGCGAAATCGACCCG	Introduction of point mutation 48-5
MLFP48-6	GGCGGTGGCGAAATCGACCCG	Introduction of point mutation 48-6
MLFP53-1	GCCGGGCCCGGTGCCATTAG	Introduction of point mutation 53-1
MLFP53-2	ATCCGGCCCCGGTGCCATTAG	Introduction of point mutation 53-2
MLFP53-3	TCCCGGCCCGGTGCCATTAG	Introduction of point mutation 53-3
MLFP53-4	CCCCGGCCCCGGTGCCATTAG	Introduction of point mutation 53-4
MLFP53-5	AACCGGCCCGGTGCCATTAG	Introduction of point mutation 53-5
MLFP53-6	AGCCGGCCCCGGTGCCATTAG	Introduction of point mutation 53-6
MLFP54-1	ACCAGGCCCGGTGCCATTAG	Introduction of point mutation 54-1
MLFP54-2	ACCCGGCCCCGGTGCCATTAG	Introduction of point mutation 54-2
MLFP54-3	ACCTGGCCCCGGTGCCATTAG	Introduction of point mutation 54-3
MLFP55-1	ACCCGGACCGGTGCCATTAG	Introduction of point mutation 55-1
MLFP55-2	ACCCGGGCCCGGTGCCATTAG	Introduction of point mutation 55-2
MLFP55-3	ACCCGGTCCGGTGCCATTAG	Introduction of point mutation 55-3
MLFP55-4	ACCCGGCACGGTGCCATTAG	Introduction of point mutation 55-4
MLFP55-5	ACCCGGCGCGGTGCCATTAG	Introduction of point mutation 55-5
MLFP55-6	ACCCGGCTCGGTGCCATTAG	Introduction of point mutation 55-6

<sup>a</sup> Restriction sites are underlined

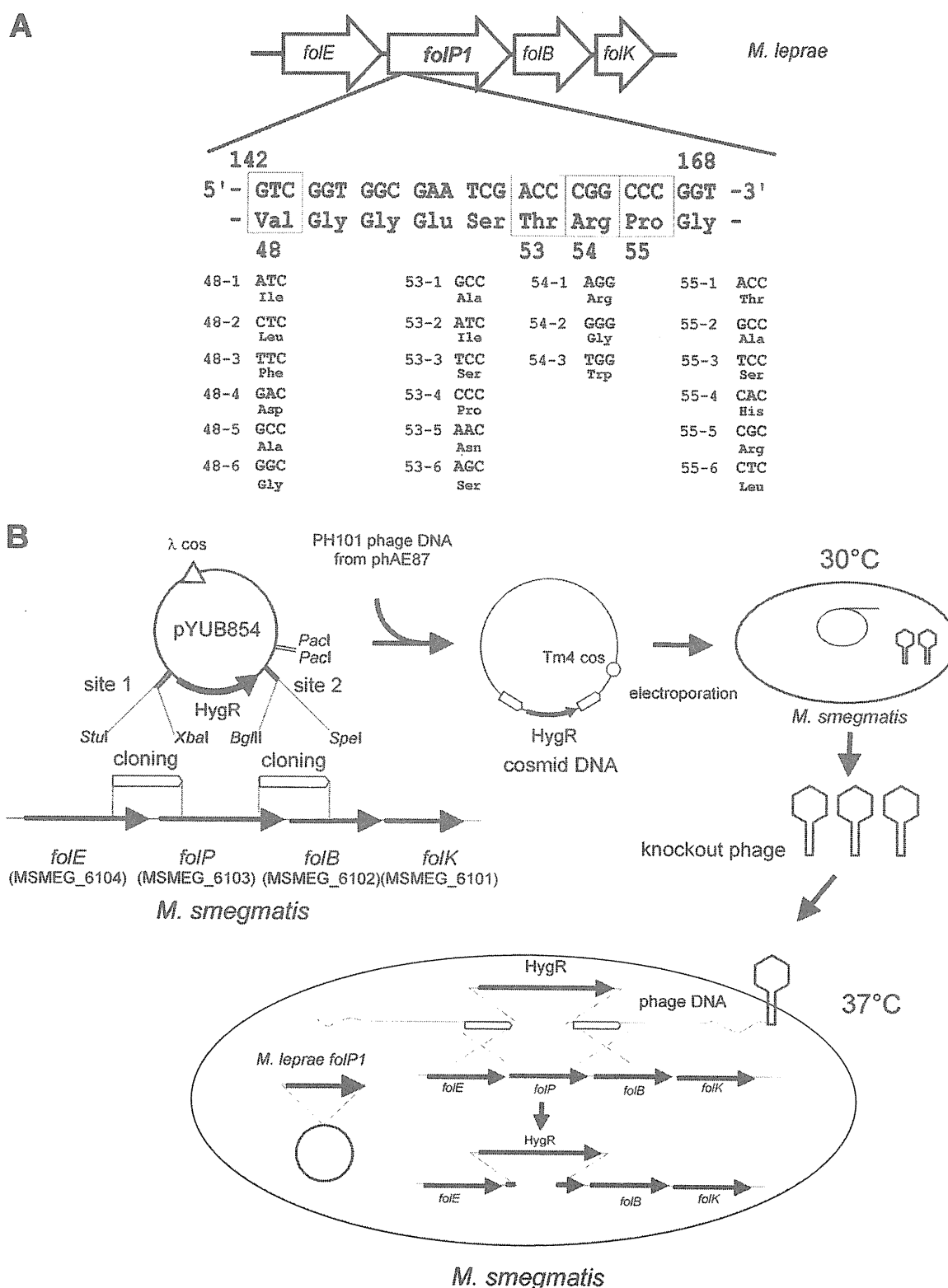


FIG. 1. Construction of recombinant *M. smegmatis* strains for dapsones susceptibility testing. (A) Point mutations introduced in the *M. leprae* *folP1* gene. Single nucleotide substitutions introduced in the *M. leprae* *folP1* at codons 48, 53, 54, and 55 are shown. Deduced amino acid residues are shown below the triplets. (B) Construction of *M. smegmatis* recombinants by allelic exchange.

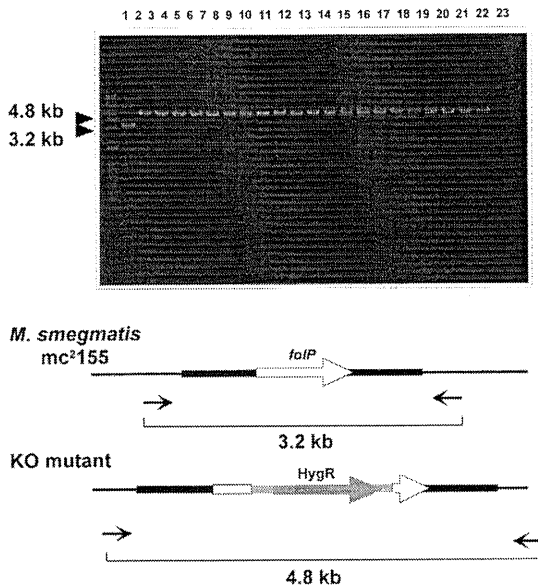


FIG. 2. PCR analysis to confirm the disruption of *folP*. Black arrows represent primers MSFPF and MSFPR for the PCR amplification. Lane 1, *M. smegmatis* mc<sup>2</sup>155; lanes 2 to 22, *M. smegmatis* strains carrying the *M. leprae folP1* without mutation and *folP1* with mutations 48-1, 48-2, 48-3, 48-5, 48-6, 53-1, 53-2, 53-3, 53-4, 53-5, 53-6, 54-1, 54-2, 54-3, 55-1, 55-2, 55-3, 55-4, 55-5, and 55-6, respectively; lane 23, negative control. KO, knockout.

in amino acids from threonine to serine (T53S) encoded by different nucleotide sequences (53-3 and 53-6) were more susceptible to dapsonе than strains with the wild-type *folP1* sequence. MIC values for strains with mutations at codon 48 or 54 were comparable to MICs for strains with the wild-type sequence. MIC values of dapsonе for the recombinant *M. smegmatis* strains are listed in Table 3. Using a multicopy plasmid may affect the expression levels of the *M. leprae folP1* and MIC values. Therefore, we tested all the mutations using

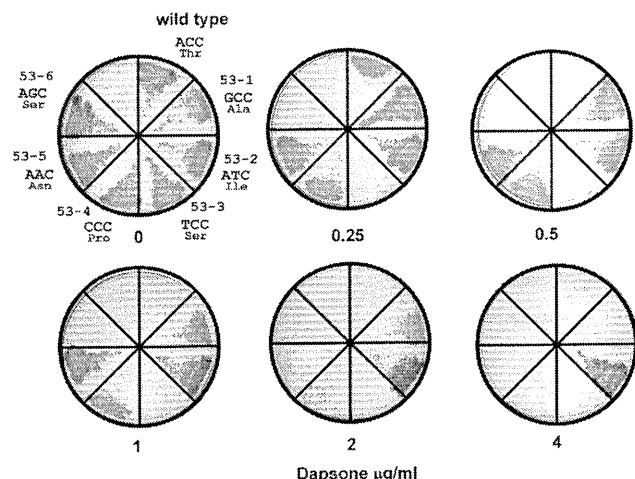


FIG. 3. Dapsonе susceptibility of recombinant *M. smegmatis*. Results for *M. smegmatis* strains with point mutations at codon 53 of the *M. leprae folP1* are shown. Dapsonе concentration is depicted below each plate.

TABLE 3. Dapsonе susceptibility of the recombinant *M. smegmatis* strains

Strain or mutation	Dapsonе MIC (µg/ml)	Reference of footpad test
Wild type	0.5	
48-1 (Val → Ile)	0.5	
48-2 (Val → Leu)	0.5	
48-3 (Val → Phe)	1.0	
48-4 (Val → Asp)	— <sup>a</sup>	
48-5 (Val → Ala)	1.0	
48-6 (Va → Gly)	1.0	
53-1 (Thr → Ala)	4.0	6
53-2 (Thr → Ile)	8.0	6, 10, 16
53-3 (Thr → Ser)	0.25	
53-4 (Thr → Pro)	2.0	
53-5 (Thr → Asn)	2.0	
53-6 (Thr → Ser)	0.25	
54-1 (Arg → Arg)	0.5	
54-2 (Arg → Gly)	1.0	
54-3 (Arg → Trp)	0.5	
55-1 (Pro → Thr)	1.0	
55-2 (Pro → Ala)	2.0	
55-3 (Pro → Ser)	2.0	
55-4 (Pro → His)	2.0	
55-5 (Pro → Arg)	8.0	6, 16
55-6 (Pro → Leu)	4.0	6, 10

<sup>a</sup> Isolation of an *folP*-disrupted *M. smegmatis* strain carrying the *M. leprae folP1* with mutation 48-4 was unsuccessful.

pNN301, a single-copy integrative vector, instead of pMV261 and obtained MIC values identical to those obtained with pMV261, suggesting that the expression levels did not influence the MIC values.

DISCUSSION

We first attempted using *E. coli* C600 Δ*folP*::Km<sup>r</sup> transformants to determine the MIC of dapsonе, but susceptibility of the recombinant *E. coli* strains to dapsonе was not stable even in Mueller-Hinton medium. Subsequently, we tried to isolate a *folP*-deficient *M. smegmatis* strain by allelic exchange, given the closer association of *M. smegmatis* to *M. leprae* than *E. coli*. The selection held great promise as total-sequence comparison of *M. leprae* DHPS with *M. smegmatis* DHPS indicated 83% identity, whereas the identity between *M. leprae* DHPS and *E. coli* DHPS is only 41%, indicating the higher potential of *M. smegmatis* as a host for measuring MIC values of dapsonе for *M. leprae* DHPS. However, isolation of *folP*-deficient *M. smegmatis* was unsuccessful. In *E. coli*, DHPS is not essential for bacterial growth when the cells are cultured with thymidine (7), but DHPS activity may be essential for the growth of *M. smegmatis* as it could not be replaced by any of the supplemented culture media tested. Hence, we then attempted to disrupt the *folP* gene on the *M. smegmatis* chromosome after introducing the *M. leprae folP1* gene into the cell to compensate for DHPS activity.

Comparison of the DHPS structures of *E. coli*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis* has suggested that Ser53 and Pro55 of the *M. tuberculosis* DHPS, which correspond to Thr53 and Pro55 in *M. leprae*, may be the major sites of interaction with PABA, dapsonе, and sulfonamides (1, 2, 8). In the present study, all mutations that cause amino acid sub-

stitutions at codon 55 resulted in dapsone resistance. Mutations at codon 53 also gave rise to dapsone resistance except for the T53S substitution, which resulted in less resistance to dapsone than the wild-type sequence (Fig. 3). The results for mutation 53-1, 53-2, 55-5, and 55-6 are consistent with the mouse footpad dapsone susceptibility testing of the *M. leprae* clinical isolates (6, 10, 16). Mutations at codon 48 or 54 showed comparable levels of susceptibility to dapsone as the wild-type sequence using dapsone susceptibility testing, but the MIC values for mutations 48-3, 48-5, 48-6, and 54-2 were slightly higher than the MIC for the wild-type sequence. Mutation 48-5 for V48A, which has been detected in our clinical samples (unpublished data), might give rise to low-level resistance to dapsone in *M. leprae*. This level of resistance should be very carefully examined by comparison with the results of footpad testing and clinical data. These data will help the molecular diagnosis of dapsone-resistant *M. leprae* with the goal of avoiding the wrong choice of drugs for chemotherapy.

Although these results should always be initially confirmed by clinical susceptibility testing as well, we believe that the method established in this study should have great utility in further attempts to determine the mutations responsible for giving rise to the dapsone resistance of *M. leprae*. The advantage of this method lies in the ability to functionally replace an essential gene of fast-growing mycobacteria with the *M. leprae* counterpart. The method may also be applicable to analysis of the rifampin resistance and quinolone resistance of *M. leprae*.

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