表 29 Hansen 病の病型分類

菌数による分類	少菌型 (paucibacillary：PB)	多菌型 (multibacillary：MB)	
免疫学的分類 (Ridley-Jopling 分類)	（I 群）TT 型	B 群 BT 型　BB 型　BL 型	LL 型
らい菌に対する細胞性免疫能	良好	低下/なし	
皮膚スメア検査	陰性	陽性	
らい菌	少数/発見しがたい	多数	
皮疹の数	少数	多数	
皮疹の分布	左右非対称性	左右対称性	
皮疹の性状	斑（環状斑）	紅斑（環状斑），丘疹，結節	
皮疹の表面	乾燥性，無毛	光沢，平滑	
皮疹部の知覚障害	高度（触覚，痛覚，温度覚）	軽度/正常	
病理所見	類上皮細胞性肉芽腫	組織球性肉芽腫	
	巨細胞，神経への細胞浸潤	組織球の泡沫状変化	
病理でのらい菌	陰性	陽性	
主たる診断根拠	皮疹部の知覚障害	皮膚スメア検査などでのらい菌の証明	
治療	WHO/MDT/PB 6 か月間 （リファンピシン，DDS）	WHO/MDT/MB 1～3 年間 （リファンピシン，DDS，クロファジミン）	

WHO/MDT：WHO が推奨する多剤による治療法．
DDS：ジアミノジフェニルスルホン．

日本の新患は年間約 5 人であるが，ほとんどが外国人である．

■臨床症状・検査・診断・治療

　らい菌に対する特異的な細胞性免疫能の差によって，病型が分けられる（表 29）．少菌型（PB，TT 型など）は，らい菌に対する免疫能が働き，皮疹は少数で，末梢神経の炎症が強く，皮疹部を中心とした知覚障害を認める．多菌型（MB，LL 型，BL 型など）は，多数の皮疹（図 21）を認めるが，知覚障害は軽度である．

　治療は多剤併用療法（multidrug therapy：MDT）を行う（表 29）．

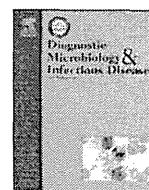　治療薬がなかった時代には末梢神経障害が進み，手足や顔などに変形や後遺症が現れたため，偏見・差別が続き，らい予防法のもと，療養所への隔離政策がとられてきた．

〔石井則久〕

図 21 多菌型（MB，BL 型）の症例
全身に多数の隆起性紅斑局面と環状紅斑を認め，皮疹部は軽度知覚障害がある．

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Mycobacteriology

Evaluation of major membrane protein-I as a serodiagnostic tool of pauci-bacillary leprosy

CrossMark

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ABSTRACT

We have previously shown that the serodiagnosis using major membrane protein-II (MMP-II) is quite efficient in diagnosing leprosy. However, the detection rate of pauci-bacillary (PB) leprosy patients is still low. In this study, we examined the usefulness of major membrane protein-I (MMP-I) from Mycobacterium leprae. The MMP-I–based serodiagnosis did not show significantly high detection rate. However, when the mixture of MMP-I and MMP-II antigens was used, we detected 94.4% of multi-bacillary leprosy and 39.7% of PB patients. There were little correlation between the titers of anti–MMP-I antibodies (Abs) and that of anti–MMP-II Abs in PB patients' sera. Ten out of 46 MMP-II–negative PB leprosy patients were MMP-I positive, so that the detection rate of PB leprosy patient increased from 39.7% to 53.8% by taking either test positive strategy. We concluded that MMP-I can complement the MMP-II–based serodiagnosis of leprosy.

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1. Introduction

Leprosy is chronic infectious disease caused by an infection with *Mycobacterium leprae* (*M. leprae*), and a significant number of new cases are still detected in 2011; for instance, 219,075 new cases were reported (WHO, 2012). Leprosy usually leads to peripheral nerve injury and systemic deformity (Job, 1989; Stoner, 1979), and the development of the deformity might be preventable, if the sufficient chemotherapy is initiated at an early stage of infection. Thus, early detection of leprosy is quite essential. As leprosy is mainly endemic in developing countries, easy and inexpensive diagnosis is strongly desired.

The diagnosis of leprosy is conducted based on microscopic detection of acid-fast bacilli in skin smears or biopsies, along with clinical and histopathological evaluation of suspected lesions. However, these methods have low sensitivity because *M. leprae* bacilli cannot be detected easily (Shepard and McRae, 1968). Although PCR-based molecular methods have been developed (Donoghue et al., 2001; Martinez et al., 2006; Phetsuksiri et al., 2006), it is not practical to perform PCR in resource-poor settings area. In this respect, serodiagnosis is a reasonable method to diagnose leprosy. Phenolic glycolipid-I (PGL-I), which is supposed to be *M. leprae* specific, was discovered in 1981 (Hunter and Brennan, 1981). The PGL-I is currently accepted as the standard target antigen (Ag) for serodiagnosis of leprosy (Meeker et al., 1986; Schuring et al., 2006; Sekar et al., 1993). However, the method using PGL-I may be useful for the detection of multi-bacillary (MB) leprosy but is not sensitive enough for the detection of pauci-bacillary (PB) leprosy at least in some countries (Kai et al., 2008; Soebono and Klatser, 1991). In the previous study, we have focused on major

membrane protein-II (MMP-II) from *M. leprae* (Maeda et al., 2007; Kai et al., 2008). MMP-II is one of the major proteins in the membrane fraction of *M. leprae*, and it induces immune response of host cells during infection (Maeda et al., 2005; Makino et al., 2005). We applied MMP-II as a serodiagnostic tool and found that the MMP-II–based serodiagnosis can increase the detection rate of PB leprosy patient. However, detection rate was still low at 39% (Maeda et al., 2007); thus, it is desirable to improve the sensitivity of the diagnostic tool.

In this study, we focused on major membrane protein-I (MMP-I) from *M. leprae*. MMP-I is 35-kDa major membrane protein expressed in *M. leprae*, which is identified as one of the most dominant Ags of *M. leprae* (Winter et al., 1995).

Although the function of MMP-I is still unknown, MMP-I may induce cell-mediated immune responses (unpublished observation) but has no homology with MMP-II. Therefore, MMP-I could be recognized by the different population of immune cells of leprosy patients and might be worth applying as a serodiagnostic Ag for the improvement of serodiagnosis. We purified recombinant MMP-I Ag using *Mycobacterium smegmatis* and evaluated its usefulness in the detection of both PB and MB leprosy patients.

2. Materials and methods

2.1. Study population

Sera were obtained with informed consent from healthy volunteers and leprosy patients in Japan. Frozen sera samples were used for the study. The samples studied comprised of MB ($n = 72$) and PB ($n = 78$) leprosy patients, either treated or untreated, from the National Sanatorium Oshimaseishoen. Classification of leprosy was performed by using the clinical criteria but was re-classified according to WHO

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recommendations (http://www.who.int/lep/classification/en/index. html) for study purposes. In Japan, children are obligated to get vaccination with *Mycobacterium bovis* bacillus Calmette–Guerin (BCG); therefore, all healthy volunteers (n = 78) are likely to be BCG-vaccinated. Sera from healthy volunteers were used as negative controls in the enzyme-linked immunosorbent assay (ELISA) to determine the cut-off value for the positivity. This study is approved by the ethics committee of the National Institute of Infectious Diseases, Tokyo, Japan.

2.2. Purification of MMP-I and MMP-II

The MMP-I gene (ML0841) was cloned from the genome DNA of *M. leprae*, using primers: 5′-GAGGATCCACGTCGGCTCAGAATGAGTC-3′ and 5′-ATACTAGTTCACTTGTACTCATGGAACT-3′. The amplified gene was expressed in *M. smegmatis* using pMV261 expression vector. The recombinant protein was His6-tagged and purified with Cu^{2+} resin (ABT Agarose Bead Technologies, Tampa, FL, USA). The protein obtained was electrophoresed on sodium dodecyl sulfate–polyacrylamide gels, then the gel was stained with Instant Blue (Expedeon protein solution, San Diego, CA, USA), and a single band of MMP-I protein was observed. The MMP-II gene (ML2038c) was expressed and purified as previously described (Maeda et al., 2007).

2.3. ELISA

The ELISA for the detection of anti-MMP-I antibodies (Abs) was performed as described previously with several modifications (Maeda et al., 2007). Briefly, 96-well plates (Nunc Maxisorp, Thermo Fischer Scientific Inc., Waltham, MA, USA) were coated overnight with MMP-I Ag at a concentration of 1 μg/mL, MMP-II Ag at 2 μg/mL, or the mixture of MMP-I (1 μg/mL) and MMP-II (2 μg/mL) Ags. All Ags were diluted in 0.1 mol/L carbonate buffer (pH 9.5). After blocking

with 10% fetal bovine serum (FBS)-containing phosphate-buffered saline (PBS), the plates were washed 3 times with PBS containing 0.05% Tween 20 (PBS-T). The optimal concentration of both Ags was determined in advance. Human sera diluted 100-fold were added and incubated at room temperature for 2 hours. After washing with PBS-T, biotinylated anti-human IgG Ab (Vector Laboratories, Burlingame, CA, USA) was added at a concentration of 0.5 μg/mL and incubated for 1 hour. Then, the plates were incubated with reagents from a Vecstain ABC kit (Vector Laboratories) for 30 min. These reagents include avidin and biotinylated horse-radish peroxidase, and this enzyme binds to biotinylated anti-human IgG Ab via avidin. After further washing with PBS-T, a substrate solution consisting of 0.2 mg/mL of 2,2′-Azino-bis (3-ethylbenzothiazolone-6-sulfonic acid) and 0.02% H_2O_2 in 0.1 mol/L citrate buffer was added until a blue color developed, and the reaction was stopped by adding 2 N H_2SO_4. Optical density (OD) was measured at 405 nm using a spectrophotometer. Plate-to-plate variations in OD readings were controlled using a common standard serum with an OD value of 0.360. The volume of all solutions used in the 96-well plate was 50 μL/well.

2.4. Statisitical analyses

The data were analyzed using MEDCALC software (MedCalc, Ostend, Belgium). A receiver operating characteristic (ROC) curve was drawn to calculate the cut-off levels using the OD values of MB leprosy patients' sera and healthy controls. The McNemar test was applied to determine the P value. When the number of inconsistent pairs was less than or equal to 25, the calculation of 2-sided P value was done based on the cumulative binomial distribution. The P value of <0.05 was considered to be statistically significant. The κ value was calculated to determine the agreement between the 2 tests.
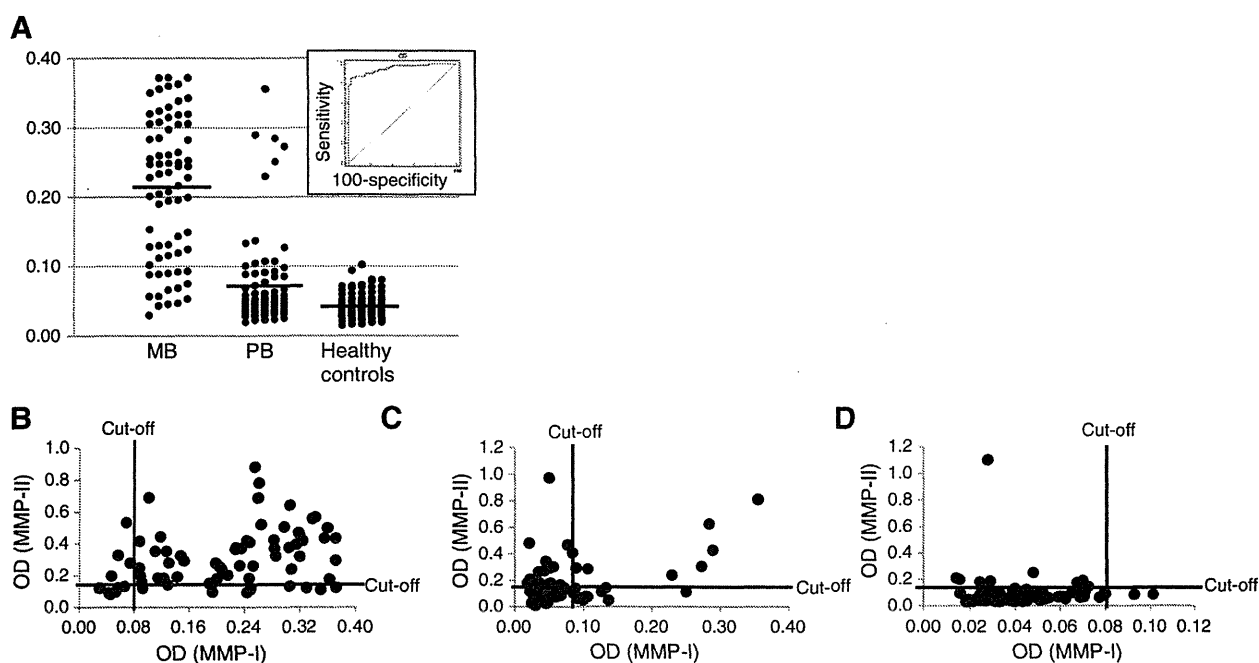
Fig. 1. OD values of each sample were determined by MMP-I-based serodiagnosis and MMP-II-based serodiagnosis. (A) Sample sera from MB leprosy patients (left), PB leprosy patients (middle), and healthy controls (right) were subjected to MMP-I-based ELISA. OD value (wave length: 405 nm) of each sample was plotted. The thick horizontal lines show the average of OD in each group. (Inset) ROC curve analysis of MMP-I-based ELISA. The cut-off value was determined as 0.080, and the area under the ROC curve was 0.952. (B-D) The results of MMP-I-based ELISA and MMP-II-based ELISA were plotted. Sample sera from MB leprosy patients (B), PB leprosy patients (C), and healthy controls (D) were subjected to MMP-I-based or MMP-II-based ELISAs. The x-axis shows the OD value of MMP-I-based ELISA, and the y-axis shows that of MMP-II-based ELISA. Thick lines show the cut-off value of each analysis (MMP-I, 0.080; MMP-II, 0.13).

Table 1

Positivity rates of MMP-I- and MMP-II-based serodiagnosis in various groups of subjects by ELISA.

	MMP-I				MMP-II				McNemar test	Inter-rater agreement
	Tested	Positive	%	95% CI	Tested	Positive	%	95% CI	MMP-I versus MMP-II	κ
MB leprosy	72	62	86.1	75.9–93.1	72	58	80.6	69.5–88.9	P = 0.3877	0.403
PB leprosy	78	20	25.6	16.4–36.8	78	32	41.0	30.0–52.8	P = 0.0518	0.101
Healthy subjects	78	2	2.6	0.3–8.9	78	9	11.5	5.4–20.8	P = 0.0654	−0.044

The data in Fig. 1 were summarized. CI = confidence interval.

3. Results and discussion

3.1. Detection of leprosy patient by the MMP-I-based serodiagnosis

We purified recombinant MMP-I and MMP-II Ags and measured the anti-MMP-I IgG Ab levels in the leprosy patients' sera. We also compared the levels of anti–MMP-I IgG Ab with those of anti–MMP-II IgG Ab. The study population consisted of MB leprosy, PB leprosy patients, and normal healthy BCG-vaccinated individuals from Japan. Some of the patients were already under treatment, so that not all patients were active leprosy patients. The OD value of each sample is plotted in Fig. 1A for MMP-I-based ELISA. The cut-off value was calculated by ROC curve analysis, using the OD values of MB leprosy patients' sera and healthy controls (Fig. 1A, inset). The cut-off value was 0.080 for MMP-I–based ELISA, and the area under the ROC curve was 0.952. The cut-off value for MMP-II–based ELISA was 0.13. From the data thus obtained, we determined the positivity rates of each group and summarized in Table 1. As shown in Table 1, 86.1% of MB (n = 72, 62/72), 25.6% of PB leprosy patients (n = 78, 20/78), and 2.6% of healthy subjects (n = 78, 2/78) had positive anti–MMP-I IgG Ab levels in their sera. We then compared those results with anti–MMP-II IgG Ab levels in their sera. As shown in Table 1, the percent positivities of anti-MMP-II Ab levels were 80.6% in MB leprosy patients (58/72), 41.0% in PB leprosy patients (32/78), and 11.5% in healthy subjects (9/78). The percent positivities of anti-MMP-I Ab in sera of both MB and PB leprosy patients were not significantly high in comparison to anti–MMP-II Ab (P = 0.3877 and P = 0.0518, by McNemar test). To represent as a dotplot, MMP-I OD values were plotted on X axis and that of MMP-II on Y axis in Fig. 1B (MB leprosy), 1C (PB leprosy), and 1D (healthy controls).

3.2. Usefulness of combination of MMP-I and MMP-II for sensitive detection of leprosy

We analyzed the effect of the use of the mixture of MMP-I and MMP-II Ags for the improvement of the detection rate of leprosy.
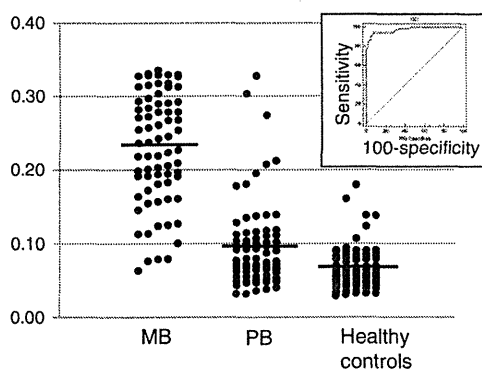
Fig. 2. OD values of each sample were determined by the combinational use of MMP-I and MMP-II Ags for ELISA. Sample sera from MB leprosy patients (left), PB leprosy patients (middle), and healthy controls (right) were subjected to MMP-I- and MMP-II-based ELISA. OD value of each sample was plotted. The thick horizontal lines show the average OD value in each group. The cut-off value was calculated by ROC curve shown in the inset. The cut-off value was determined as 0.094, and the area under the ROC curve was 0.974.

MMP-I Ag and MMP-II Ag were mixed and were used as the Ag for ELISA. All sera used in Table 1 were analyzed. The OD value of each sample is plotted in Fig. 2. The cut-off value was calculated by ROC curve analysis, using the OD value of MB leprosy patients' sera and healthy controls (Fig. 2, inset). The cut-off value was 0.094, and the area under the ROC curve was 0.974. From those data, we determined the positivity rates of each group and summarized in Table 2. The percent positivities against Ag-mixture were 94.4% (68/72) for MB, 39.7% (31/78) in PB leprosy patients, and 9.0% (7/78) for healthy subjects. The detection rate of MB leprosy patients was significantly higher than that using MMP-II Ag alone (P = 0.002, by McNemar test). Since both MMP-I and MMP-II Ags are immunogenic (Maeda et al., 2005, 2007; Winter et al., 1995, and our unpublished observations), it would be speculated that most MB leprosy patients produce IgG Abs against either MMP-I or MMP-II. In contrast, the detection rate of PB leprosy patients remained at the same level as MMP-II–based serodiagnosis.

For improvement in the detection rate of PB leprosy, we focused on the inter-rater agreement between the MMP-I–based and the MMP-II–based serodiagnosis. As shown in Table 1, the κ value was low for PB leprosy (κ value: 0.10). This indicates that there is little correlation between the Ab titers against MMP-I and MMP-II in this population of leprosy patients (Fig. 1C). This speculation directed us to conduct more detailed analysis of the titers of anti-MMP-I and anti–MMP-II Abs in those sample sera. We classified the sample sera into 4 groups, "MMP-I negative and MMP-II negative", "MMP-I negative and MMP-II positive", "MMP-I positive and MMP-II negative", and "MMP-I positive and MMP-II positive" from the results of ELISA obtained in Table 1 (Table 3A). In PB leprosy sera, the proportions of the sera classified into the second group and the third group was higher than expected (28.2% and 12.8%, respectively). Thus, we reclassified the sera into 2 groups, "Consistent" and "Not consistent" (Table 3B). "Consistent" group includes "MMP-I negative and MMP-II negative" and "MMP-I positive and MMP-II positive" groups, those have agreement in the results of ELISA using MMP-I Ag and MMP-II Ag, while "Not consistent" group includes "MMP-I negative and MMP-II positive" and "MMP-I positive and MMP-II negative" sera. Interestingly, only 59.0% of PB leprosy sera showed consistency between the results of anti–MMP-I Ab–based ELISA and anti–MMP-II Ab–based ELISA (Table 3B). The results are in contrary to the results of MB leprosy sera, as 83.3% of sera have consistency (Table 3B). These results suggested the possibility that the MMP-I Ag

Table 2

Positivity rates of MMP-I and MMP-II-mixed serodiagnosis in various groups of subjects by ELISA.

	MMP-I + MMP-II				McNemar test versus MMP-II
	Tested	Positive	%	95% CI	
MB leprosy	72	68	94.4	86.4–98.5	P = 0.002
PB leprosy	78	31	39.7	28.8–51.5	P = 1
Healthy subjects	78	7	9.0	3.7–17.6	P = 0.753

The data in Fig. 2 were summarized.

Table 3
(A) Sample sera were classified by the positivity of anti–MMP-I Ab and anti–MMP-II Ab. The figures in brackets show the rate positivity in each group. (B) Sample sera were reclassified by the consistency of the result of the ELISA. The row "Consistent" shows the number of sera that have been classified in the group "MMP-I negative and MMP-II negative" or "MMP-I positive and MMP-II positive". The row "Not consistent" shows the number of sera that have been classified in the group "MMP-I negative and MMP-II positive" or "MMP-I positive and MMP-II negative".

	MB (n = 72)	PB (n = 78)
(A)		
MMP-I negative and MMP-II negative	6 (7.7%)	36 (46.1%)
MMP-I negative and MMP-II positive	4 (5.6%)	22 (28.2%)
MMP-I positive and MMP-II negative	8 (11.1%)	10 (12.8%)
MMP-I positive and MMP-II positive	54 (75.0%)	10 (12.8%)
(B)		
Consistent	60 (83.3%)	46 (59.0%)
Not consistent	12 (16.6%)	32 (41.0%)

can rescue the people who are diagnosed as negative in MMP-II serodiagnostic test, which may successfully reduce false-negative results. When MMP-II Ag–based serodiagnosis was followed by MMP-I Ag–based analysis, 53.8% of PB leprosy patients (42/78) were Ab positive, and 91.7% of MB leprosy patients (66/72) were Ab positive, although relatively high percentages of healthy subjects (14.1%, 11/78) were Ab positive (Table 4, data of Table 1 were re-analyzed), and the exact reason for this percentage remains to be evaluated. Therefore, both MMP-I Ag and MMP-II Ag may be quite useful diagnostic tools for both MB and PB leprosy.

There is the discrepancy between the results obtained by mixing MMP-I and MMP-II Ags in the same well and those by individual ELISA in the detection rate of PB leprosy. We found that the individual serological tests performed using the Ag mixture are not always successful in detecting all PB leprosy patients in separate ELISA assays. The theoretical calculation indicates that MaxiSorp ELISA plate surface has the capacity to adsorb about 650 ng/cm^2 of globular protein such as IgG; it is likely that both MMP-I and MMP-II Ags can be adsorbed (150 ng/well). The probable explanation for the low detection rate of PB leprosy may be the hydrophobic characteristics of both MMP-I and MMP-II Ags. Such characteristics may interfere with the adsorption of those Ags on ELISA plate. However, as far as we tested other ELISA plates including AGC technoglass Co. Ltd. and the other Ag-coating buffers, we could not improve the detection rate of PB leprosy using mixed Ags. Another way to modify the assay such as using specific Abs to MMP-I and MMP-II Ags for coating the plates and then layering the Ag may also help to detect more Abs, but presently, such specific Abs are not available. On the other hand, some of MB leprosy patients have polyclonally activated B cells, which might lead to produce IgG Abs with high affinity; therefore, the use of mixture of MMP-I and MMP-II Ags could be applicable.

Previous study has shown that the detection rate of serodiagnosis using MMP-II is similar for leprosy patients in Vietnam to those in Japan. In contrast, the detection rate of PGL-I–based serodiagnosis is significantly lower for Vietnamese patients than Japanese (Kai et al., 2008). The reason why the sensitivity of PGL-I–based serodiagnosis is different between Vietnamese and Japanese is still unclear. It could be

speculated that the major pathogenic and non-pathogenic mycobacterial species found in each region differs and the inhabitants in such region could be influenced by those mycobacteria that would lead to production of region-specific Abs. Therefore, the detection rate of leprosy may differ when MMP-I–based serodiagnosis is applied to leprosy patients of endemic countries.

The sera used in this study were obtained from both treated and untreated leprosy patients. However, because of the lack of records on the treatment history, we could not perform comparative studies between untreated and treated patients. The detailed cohort studies are absolutely required to reveal the utility of MMP-I Ag in primary diagnosis, especially in endemic areas. Also, we need the testing of cross-reactivity of MMP-I and MMP-II Ags with other mycobacterial diseases, especially because homologs of MMP-I are identified in several other mycobacteria and possible infection of those bacteria may have impact on the detection of leprosy. So far, cell-mediated immune responses using MMP-I and MMP-II Ags seem not helpful for the diagnosis of PB leprosy, so that serological tool may be advantageous for the PB diagnosis. Through the establishment of simple tool for the serodiagnosis using both MMP-I and MMP-II Ags, we may be able to achieve easy and inexpensive diagnosis of leprosy of both MB and PB type.

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Table 4
Positivity rates of MMP-I or MMP-II ELISAs in various groups of subjects.

	MMP-I or MMP-II		
	Tested	Positive	%
MB leprosy	72	66	91.7
PB leprosy	78	42	53.8
Healthy subjects	78	11	14.1

Protection against *Mycobacterium leprae* Infection by the ID83/GLA-SE and ID93/GLA-SE Vaccines Developed for Tuberculosis

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Despite the dramatic reduction in the number of leprosy cases worldwide in the 1990s, transmission of the causative agent, *Mycobacterium leprae*, is still occurring, and new cases continue to appear. New strategies are required in the pursuit of leprosy elimination. The cross-application of vaccines in development for tuberculosis may lead to tools applicable to elimination of leprosy. In this report, we demonstrate that the chimeric fusion proteins ID83 and ID93, developed as antigens for tuberculosis (TB) vaccine candidates, elicited gamma interferon (IFN-γ) responses from both TB and paucibacillary (PB) leprosy patients and from healthy household contacts of multibacillary (MB) patients (HHC) but not from nonexposed healthy controls. Immunization of mice with either protein formulated with a Toll-like receptor 4 ligand (TLR4L)-containing adjuvant (glucopyranosyl lipid adjuvant in a stable emulsion [GLA-SE]) stimulated antigen-specific IFN-γ secretion from pluripotent Th1 cells. When immunized mice were experimentally infected with *M. leprae*, both cellular infiltration into the local lymph node and bacterial growth at the site were reduced relative to those of unimmunized mice. Thus, the use of the *Mycobacterium tuberculosis* candidate vaccines ID83/GLA-SE and ID93/GLA-SE may confer cross-protection against *M. leprae* infection. Our data suggest these vaccines could potentially be used as an additional control measure for leprosy.

Prevalence rates for leprosy have sharply declined over the last 20 years, with the major breakthrough being attributed to the provision of free-of-charge treatment to all diagnosed leprosy patients. The stalled decreases in both global prevalence and new case detection rates of leprosy over the last decade indicate that additional measures are likely required. The relative success of leprosy control, however, has prompted the integration of leprosy-specific programs into general health facilities and has also reduced the resources available to research (most notably specialized investigators and funds) (1, 2). During the same period, the World Health Organization (WHO) has declared tuberculosis (TB) a global public health emergency. Indeed, over 2 billion people are now believed to be infected with *Mycobacterium tuberculosis*, and multi- and extremely drug-resistant strains are rapidly emerging (3, 4). Numerous groups are actively engaged in developing replacement or supplementary vaccines as an alternative or additional control strategy for the TB epidemic (5). Defined antigens, delivered as plasmid DNA, vectored DNA, or as recombinant proteins in adjuvant, have proven effective in animal models, and at least nine subunit TB vaccines have entered clinical trials (6–11).

The *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccines represent an important component within TB control programs, since they provide at least partial protection against tuberculosis (12–14) and leprosy (15). In several countries, both TB and leprosy are endemic, and the contribution of mass BCG vaccination is often overlooked as a factor within leprosy control (16). The application of emerging TB vaccines to leprosy control programs could be logistically and economically beneficial as a public health intervention, but the capacity of such vaccine candidates to elicit protective responses against infection with *Mycobacterium leprae* (the causative agent of leprosy) has not been investigated.

As chimeric fusion proteins comprised of three and four *M.*

tuberculosis proteins, the TB vaccine candidate antigens ID83 and ID93 each present *M. tuberculosis* proteins selected from various categories (17). When combined with the synthetic Toll-like receptor 4 ligand (TLR4L) glucopyranosyl lipid adjuvant (GLA) in a stable emulsion (SE), ID93 boosts the effects of BCG, protecting mice and guinea pigs against infection with *M. tuberculosis* (7, 18). The current study was designed to examine the potential of the ID83/GLA-SE and ID93/GLA-SE vaccines to protect against *M. leprae* infection.

MATERIALS AND METHODS

Subjects and samples. Recently diagnosed and previously untreated leprosy patients and controls from an area to which leprosy is endemic (EC) were recruited at Centro de Referencia em Diagnostico e Terapeutica and Hospital Anuar Auad, Goiânia, Goiás State, Brazil. Leprosy patients were categorized as paucibacillary (PB) by clinical, bacilloscopic, and histological observations (bacterial index, skin lesions, nerve involvement, and histopathology) carried out by qualified personnel. Blood was obtained from tuberculosis patients (*M. tuberculosis* sputum-positive, HIV-negative individuals with clinically confirmed pulmonary tuberculosis) who were undergoing treatment. EC were healthy individuals who had never had tuberculosis, had no history of leprosy in the family, and were living in the area to which leprosy is endemic. All had previously been immunized with BCG, and all blood samples were obtained after informed consent and after local ethics committee approval.

TABLE 1 Homology of ID83 and ID93 components between mycobacteria

Vaccine(s)	Component (M. tuberculosis protein)[a]	Homolog (% identity) from:	
		M. leprae[b]	M. bovis
ID83 and ID93	Rv1813	Not found	Mb1843c (100)
	Rv2608	Not found	Mb2640 (99.8)
	Rv3620	ML1055 (57.9 [in 95-aa overlap])	
ID93 only	Rv3619	ML1056 (64.15 [in 92-aa overlap])	

[a] M. tuberculosis H37Rv strain; homologs were searched for using Tuberculist.
[b] M. leprae TN strain. aa, amino acid.

Determining reactivity by 24-h WBA. Whole-blood assays (WBA) were performed with venous undiluted heparinized whole blood (Greiner). Within 2 h of collection, blood was added to each well of a 24-well plate (450 μl/well; Sigma, St. Louis, MO) and incubated with antigens at 37°C, 5% CO_2. For each assay, stimulations were conducted with 10 μg/ml of recombinant protein, 10 μg/ml M. leprae cell sonicate (provided by John Spencer, Colorado State University, Fort Collins, CO, under NIH contract N01 AI-25469), or 1 μg/ml phytohemagglutinin (PHA) (Sigma). After 24 h, plasma was collected and stored at −20°C. Gamma interferon (IFN-γ) content within the plasma was determined by enzyme-linked immunosorbent assay (ELISA), used according to the manufacturer's instructions (QuantiFERON CMI; Cellestis, Carnegie, Australia). The detection limit of the test was 0.05 IU/ml. For data interpretation, we assigned as a positive result a concentration above an arbitrary cutoff point of 0.5 IU/ml.

Mice and immunizations. Wild-type C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were immunized with recombinant protein formulated with saline, SE, or GLA-SE to provide a final protein concentration of 100 μg/ml antigen and 200 μg/ml GLA-SE (19). Mice were immunized up to 3 times by subcutaneous (s.c.) injection of 0.1 ml vaccine at the base of the tail at 2-week intervals. Mice were maintained under specific-pathogen-free conditions, and all procedures were approved by the appropriate institutional animal care and use committees.

Antibody responses. Mouse sera were prepared by collection of retroorbital blood into Microtainer serum collection tubes (VWR International, West Chester, PA), followed by centrifugation at 1,200 rpm for 5 min. Each serum sample was then analyzed by antibody capture ELISA. Briefly, ELISA plates (Nunc, Rochester, NY) were coated with 1 μg/ml recombinant antigen in 0.1 M bicarbonate buffer and blocked with 1% bovine serum albumin (BSA)-phosphate-buffered saline (PBS). Then, in consecutive order and following washes in PBS-Tween 20, serially diluted serum samples, anti-mouse IgG, IgG1, or IgG2c-horseradish peroxidase (HRP) (all from Southern Biotech, Birmingham, AL), and 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) (ABTS)-H_2O_2 (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were added to the plates. Plates were analyzed at 405 nm (EL_x808; Bio-Tek Instruments Inc., Winooski, VT). The endpoint titer was determined as the last dilution to render a positive response, determined as 2 times the mean optical density of the replicates derived from sera from unimmunized mice in the Prism software program (GraphPad Software, La Jolla, CA).

Antigen stimulation and cytokine responses. Single-cell suspensions were prepared by disrupting spleens between sterilized frosted slides. Red blood cells were removed by lysis in 1.66% NH_4Cl solution, and then mononuclear cells were enumerated by ViaCount assay with a PCA system (Guava Technologies, Hayward, CA). Single-cell suspensions were cultured at 2×10^5 cells per well in duplicate in a 96-well plate (Corning Incorporated, Corning, NY) in RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum (FCS) and 50,000 units penicillin-streptomycin (Invitrogen). Cells were cultured in the presence of 10 μg/ml antigen for 96 h, after which culture supernatants were collected and cytokine content was assessed. Cytokine concentrations within culture superna-

tants were determined by ELISA. ELISA kits for determination of mouse IFN-γ, interleukin 5 (IL-5), IL-13 and tumor necrosis factor alpha (TNF-α) were performed according to the manufacturer's instructions (eBioscience, San Diego, CA), and optical density was determined using an ELx808 plate reader (Bio-Tek Instruments Inc., Winooski, VT).

Alternatively, for the elucidation of intracellular cytokine expression, cells were cultured at 37°C for 16 h in the presence of 1 μg/ml phorbol myristate acetate (PMA)-ionomycin (Sigma, St. Louis, MO) and 10 μg/ml recombinant antigen and Golgi Stop (BD Biosciences, San Diego, CA). Cells were fixed and permeabilized in Cytofix/Cytoperm (BD Biosciences, San Diego, CA). To stain, cells were first incubated with the anti-FcγII/IIIR antibody 2.4G2 to block nonspecific binding, before addition of a cocktail of fluorescently conjugated antibodies to identify cytokine-producing antigen-experienced T helper cells (anti-CD4, anti-CD3ε, anti-CD44, anti-IL-2, anti-IFN-γ and anti-TNF [all from eBioscience]). Flow cytometry was performed using an LSR Vantage instrument (BD Biosciences), and the data were analyzed using the FlowJo software program (Treestar, Ashland, OR).

M. leprae-induced inflammation. To assess M. leprae-induced inflammation, live M. leprae bacilli (Thai-53 strain) were purified from the footpads of nu/nu mice at National Hansen's Disease Programs and shipped overnight on ice to the Infectious Disease Research Institute (IDRI) for inoculations (20). Heat-killed M. leprae (HKML) bacteria were obtained by heating bacilli at 70°C for 1 h and then quenching on ice. Mice were inoculated with 1×10^6 bacilli in a volume of 10 μl by intradermal (i.d.) injection into the ear pinnae. Twelve weeks later, single-cell suspensions were prepared from the (auricular) draining lymph nodes (DLN), and cell numbers were determined by ViaCount assay with a PCA system (Guava Technologies).

Determination of bacterial burden. To assess M. leprae growth, live M. leprae bacilli (Thai-53 strain) were purified from the footpads of nu/nu mice at National Institute of Infectious Diseases. Six C57BL/6 mice per group were s.c. vaccinated with a total of 5 μg/mouse of either the ID83 or ID93 fusion protein or GLA-SE (10 μg/mouse) as a negative control 3 times with an interval of 3 weeks between inoculations and a month later were challenged with 5×10^3 M. leprae bacilli by subcutaneous (s.c.) injection into each footpad. Footpads were harvested 7 months later, and the bacilli were enumerated by direct microscopic counting of acid-fast bacilli according to the method of Shepard and McRae, with a limit of detection of 3,700 bacilli (21). Animal studies were reviewed and approved by the Animal Research Committee of Experimental Animals of the National Institute of Infectious Diseases (Tokyo, Japan) and were conducted according to their guidelines.

Statistics. For human data, the nonparametric Kruskal-Wallis analysis-of-variance test was used to compare the IFN-γ levels among all of the groups, and the Mann Whitney U test was applied for comparison between two groups. The P values for mouse studies were determined using the Student t test. Statistics were generated using the software program MS Excel (Microsoft Corporation, Redmond, WA) or Prism (GraphPad Software, Inc., La Jolla, CA). Statistical significance was considered when the P values were <0.05.
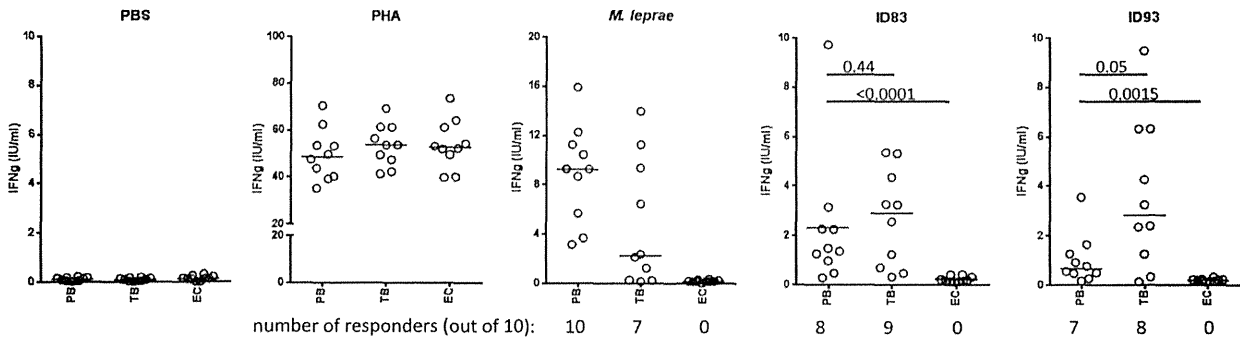
- 41 -

number of responders (out of 10):   10   7   0   8   9   0   7   8   0

FIG 1 The ID83 and ID93 fusion proteins are recognized by PB leprosy patients. Whole blood from the PB, TB, and EC groups was cultured for 24 h in the presence of antigen, and IFN-γ content in the plasma was measured by ELISA. Data from each individual is represented by a point, and the black bar indicates the median IFN-γ value. *P* values are indicated.

RESULTS

Recognition of ID83 and ID93 by leprosy patients. Replication and dissemination of *M. leprae* is limited in PB leprosy patients, suggesting that their potent cellular immune response is associated with limited or localized disease. Antigens that are recognized by immune cells of PB patients may therefore be the key to identifying an effective subunit vaccine against leprosy. We assessed the abilities of two TB vaccine candidate antigens we have developed, ID83 (a fusion of three *M. tuberculosis* proteins, Rv1813, Rv2608, and Rv3620) and ID93 (a fusion of four *M. tuberculosis* proteins, Rv1813, Rv2608, Rv3619, and Rv3620) (Table 1), to stimulate patient-specific IFN-γ recall responses in WBA. While none of the EC samples exhibited IFN-γ levels above 0.5 IU/ml, blood from TB patients reacted strongly to these proteins and secreted significant levels of IFN-γ (Fig. 1). IFN-γ levels were ob-

served above this threshold in 8 of 10 TB patient samples stimulated with either protein. Although not as potently recognized as by blood from TB patients, ID83 and ID93 were also well recognized by PB leprosy patient blood (Fig. 1). IFN-γ levels above 0.5 IU/ml were observed in 8 of 10 PB patient samples stimulated with ID83 and 6 of 10 samples stimulated with ID93. Taken together, these data indicate that the TB vaccine candidate antigens ID83 and ID93 are also applicable to leprosy.

Impact of vaccine formulation on immune response. To examine this vaccine potential, mice were immunized with either ID83/SE or ID83/GLA-SE. Immunization with either formulation induced an antigen-specific IgG response (Fig. 2). However, although antigen-specific IgG1 responses were similar, GLA-SE induced a significantly greater anti-ID83 IgG2c response than SE (Fig. 2). These data indicate that immunization with the GLA-SE,

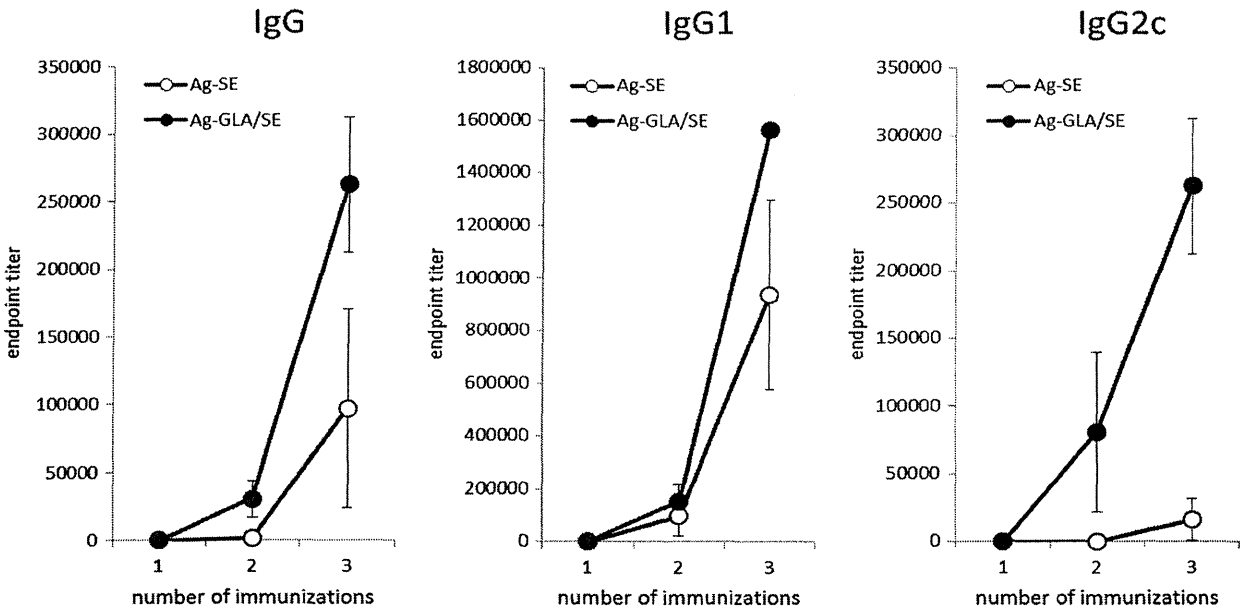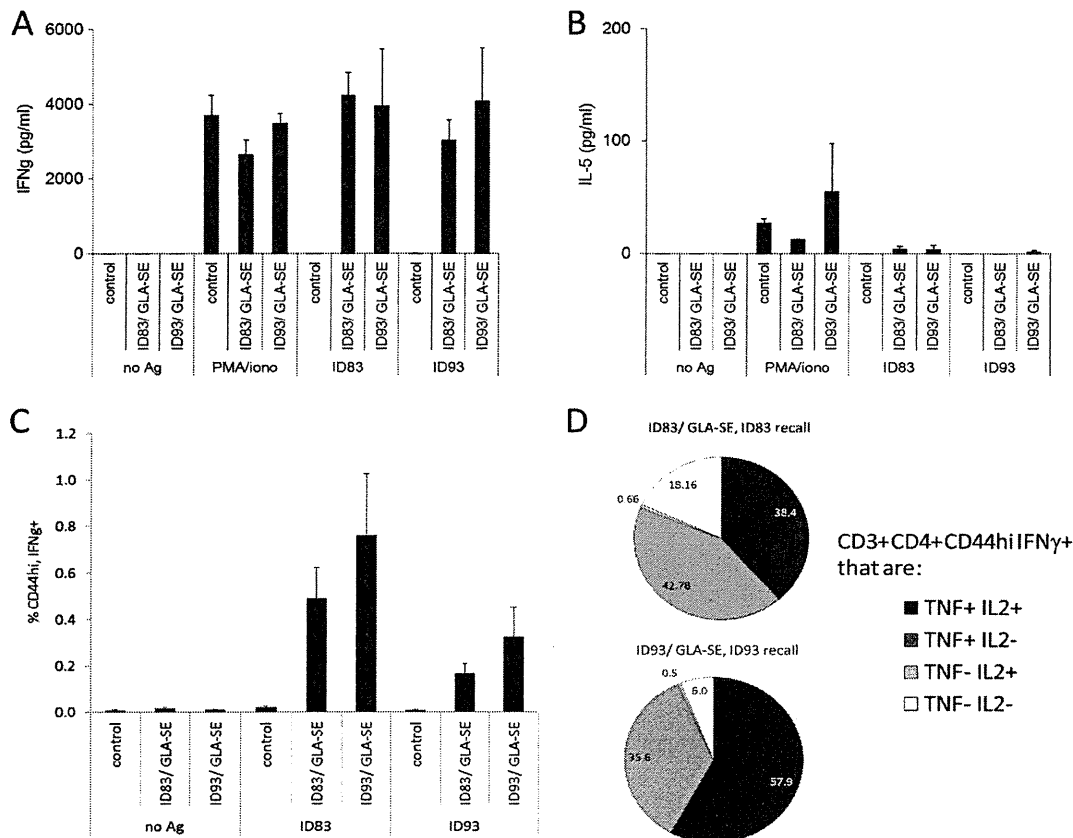**FIG 2** Immunization with ID83/GLA-SE but not ID83/SE promotes strong antigen-specific IgG2c responses. Mice were injected s.c. with ID83/SE and ID83/GLA-SE at biweekly intervals for a total of 3 immunizations. Serum was collected before each immunization and 1 month after the final immunization. Antigen-specific serum IgG, IgG1, and IgG2c endpoint titers were determined by antibody-capture ELISA.

- 42 -

FIG 3 Immunization with either ID83/GLA-SE or ID93/GLA-SE stimulates pluripotent Th1 antigen (Ag)-specific responses. Mice were injected s.c. with ID83/GLA-SE and ID93/GLA-SE at biweekly intervals for a total of 3 immunizations. Single-cell suspensions of spleen cells were prepared 1 month after the final immunization and cultured with 10 μg/ml protein. Culture supernatants were collected after 4 days, and IFN-γ (A) or IL-5 (B) content was determined by ELISA. Results are shown as means and SE; $n = 3$ per group. iono, ionomycin. Alternatively, cells were cultured with antigen and BD Golgi Stop for 16 h and then fixed and stained to determine the percentage (C) of CD3$^+$ CD4$^+$ CD44^{hi} IFN-γ$^+$ cells by flow cytometry. Results are shown as means and SE; $n = 3$ per group. The phenotype of each CD3$^+$ CD4$^+$ CD44^{hi} IFN-γ$^+$ cell was further delineated by costaining for IL-2 and/or TNF. In panel D, results are shown as percent CD3$^+$ CD4$^+$ CD44^{hi} IFN-γ$^+$ cells exhibiting each phenotype. Data are representative of at least 3 independent experiments.

but not the SE, formulation promotes a Th1-like response that could provide cross-protection against *M. leprae* infection.

Elicitation of pluripotent Th1 responses by TB vaccines. To further characterize this vaccine potential, mice were immunized with either ID83/GLA-SE or ID93/GLA-SE. Spleen cells from immunized mice responded to antigen stimulation by secreting large amounts of IFN-γ but very little IL-5, indicating the generation of a strong Th1 response (Fig. 3A and B). Furthermore, many of the antigen-specific IFN-γ-secreting cells also secreted both IL-2 and TNF, and the vast majority secreted at least one of these additional cytokines (Fig. 3C).These data indicate that immunization with either ID83/GLA-SE or ID93/GLA-SE promotes a Th1-like response that could be protective against *M. leprae* infection.

M. leprae-induced inflammation is reduced in mice immunized with TB vaccines. We previously demonstrated that *M. leprae* infection of the ear causes local inflammation that can be interrupted by drug treatment (22, 23). We hypothesized that appropriate vaccination with ID83 and ID93 would limit development of local inflammation. Mice were immunized by s.c. injection at the base of the tail and then infected in the ears with *M.*

leprae. In agreement with our hypothesis, 12 weeks after infection, fewer cells were recovered from DLN of ID83/GLA-SE- or ID93/GLA-SE-immunized mice than from DLN of mock immunized mice (Fig. 4A). The DLN cell numbers of infected, immunized mice were similar to those of HKML-inoculated, infected mice (Fig. 4A). Taken together, these data indicate that the local inflammation observed following *M. leprae* ear infection can be limited by immunization with the candidate TB vaccines.

Immunization with TB vaccines reduces *M. leprae* burden. To investigate if the vaccines could limit growth of *M. leprae*, immunized mice were infected with *M. leprae* in the footpad and bacilli numbers were assessed 7 months later. Immunization with either vaccine decreased bacterial numbers compared with those of mice injected with adjuvant alone (Fig. 4B). Taken together, our experimental data indicate that defined subunit vaccines intended for TB could also be useful for leprosy.

DISCUSSION

Proteins that elicit IFN-γ responses from PB leprosy patients and healthy household contacts of multibacillary (MB) patients

- 43 -
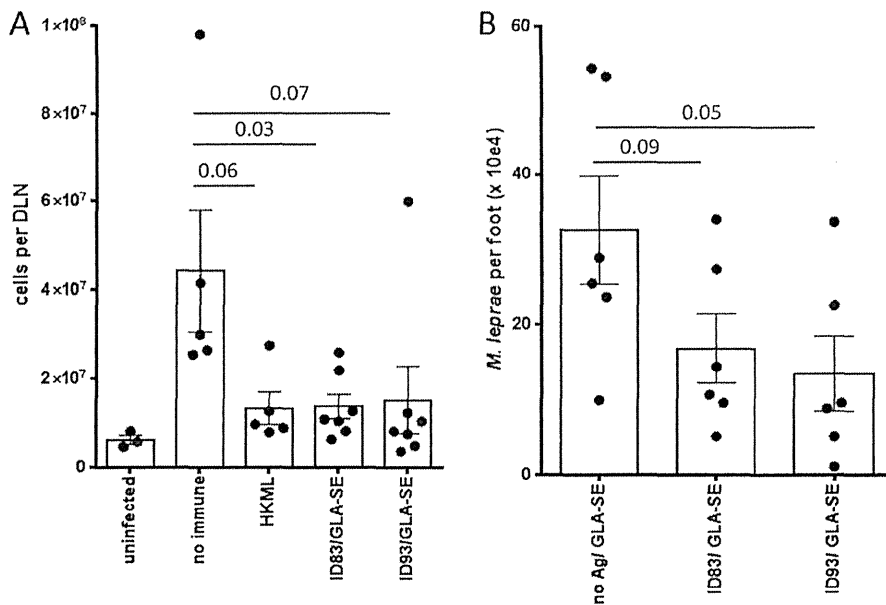
FIG 4 Immunization with TB vaccines reduces lymphadenopathy induced by *M. leprae* infection and reduces *M. leprae* burden. Mice were injected s.c. with ID83/GLA-SE and ID93/GLA-SE at biweekly intervals, for a total of 3 immunizations. In panel A, 1 month after the last immunization, mice were infected with 1×10^6 *M. leprae* bacilli in each ear, and DLN cell numbers were determined 12 weeks later. Results are shown as means and SE ($n = 5$ per group), and data are representative of three independent experiments. Student's t test was used to calculate P values between each group. In panel B, 1 month after the last immunization, mice were infected with 5×10^3 *M. leprae* bacilli in each foot, and bacillus numbers were determined 7 months later. The Mann-Whitney test was used to calculate P values between each group, and results are shown as individual points for each animal, group mean, and SE ($n = 6$ per group).

(HHC) are potential targets of the immune response that naturally limits *M. leprae* infection. Utilizing such proteins within subunit vaccines thus has the potential to disrupt disease development and bacterial dissemination. In this report, we demonstrate that the chimeric fusion proteins ID83 and ID93, each developed as antigen candidates within TB vaccines, are recognized by blood from PB leprosy patients. When either antigen was formulated with a TLR4L-containing adjuvant (GLA-SE), immunization stimulated strong, pluripotent Th1 responses and inhibited *M. leprae*-induced inflammation and bacterial growth in mice. Thus, the use of the *M. tuberculosis* candidate vaccines ID83/GLA-SE and ID93/GLA-SE may confer cross-protection against *M. leprae* infection and could potentially be used as a control measure for leprosy.

While many groups are attempting to develop vaccines for other neglected tropical diseases, difficulties inherent in leprosy research (e.g., our inability to culture *M. leprae in vitro* and the very long duration of experimental infections) have severely restricted the investigation and advancement of leprosy vaccines. There is an enormous effort, however, to develop vaccines against *M. tuberculosis*, and at least nine subunit vaccines have entered clinical trials (11). Taking advantage of TB research efforts could provide an efficient way to codevelop a vaccine for both leprosy and TB. As chimeric fusion proteins comprised of three and four *M. tuberculosis* proteins, ID83 and ID93 each present *M. tuberculosis* proteins selected from various categories (the PE/PPE family of proteins and the EsX family of virulence factors, associated with latent growth of *M. tuberculosis* and expressed during hypoxia, respectively). In this study, our original intent was to examine the T cell responsiveness of Brazilian TB patients to the ID83 and

ID93 vaccine antigen candidates, using leprosy patients as a known mycobacterium-infected control group to determine specificity of the responses. Indeed, TB patients did respond strongly through the secretion of IFN-γ. *In silico* predictions revealed extremely low homology between the amino acid sequences of the individual proteins contained in ID83 and ID93 and the published *M. leprae* genome (24) (Table 1). It was therefore surprising that PB leprosy patients responded well to antigen stimulation. The response of leprosy patients was most likely due to *M. leprae* infection and, because EC did not respond, not due to previous BCG immunization or other factors, such as possible latent *M. tuberculosis* infection or exposure to other environmental mycobacteria. These data provide validation for the potential use of these vaccine candidates for the prevention of leprosy. It has previously been demonstrated that even though the identities between *M. tuberculosis* and *M. leprae* ESAT-6 and CFP-10 (36% and 40%, respectively) were very low and the heterologous proteins were not cross-reactive in terms of serum antibody responses in leprosy patients, there was a strong cross-reactive response at the T cell level in both TB and leprosy patients (25). Thus, the cell-mediated responses for low-homology proteins appear to be more promiscuous and are likely contained within similar T cell epitopes within the heterologous proteins.

When *M. leprae* infection manifests disease, leprosy can present across a diverse bacteriologic, clinical, immunologic, and pathological spectrum. The hallmark neuropathy associated with leprosy arises not only from the direct infection of peripheral nerves by *M. leprae*, a unique trait among bacteria, but also from the inflammatory and immunologic responses to the infection. Indeed, immune-inflammatory episodes known as leprosy reac-

- 44 -

tions are the main cause of irreversible nerve damage and can be severe enough to require hospitalization. Thus, the promotion of strong immune responses could theoretically precipitate immune pathology, and it is therefore of paramount importance to ensure the safety of a vaccine for leprosy. ID83 subunit vaccines containing synthetic TLR4 or TLR9 agonists generated a Th1 immune response and protected mice against challenge with *M. tuberculosis* (19). Experimental infection of mice with *M. leprae* does not precipitate the nerve damage that is a common feature in leprosy patients (21), but it is noteworthy that vaccination with the TB vaccines reduced the *M. leprae*-induced lymphadenopathy. This observation implies that the vaccines, at least at the DLN level, do not elicit strong infection-site inflammation that causes immune pathology. Further exploration in armadillos that do develop neuropathy following *M. leprae* infection appears prudent (26–28).

Currently the Leprosy Control Program in Brazil recommends BCG vaccination for all intradomiciliary contacts of both MB and PB leprosy that already have a BCG scar or the ones that haven't been previously vaccinated. In this regard, a new TB vaccine that could also protect against leprosy could also provide additional protection for exposed contacts of leprosy patients. The majority of research directed toward a vaccine for leprosy dates back a few decades and has focused on the use of related whole mycobacteria or purification of protein fractions from *M. leprae*. Purified and/or recombinant 10-kDa, 25-kDa, and 65-kDa proteins provided protection when administered with Freund's adjuvant (29). Modern vaccine standards, regulations, and safety concerns suggest that more refined products should be developed. The development, production, and advancement of vaccines through the necessary regulatory processes is not trivial or inexpensive, however, and it is therefore noteworthy that at least nine subunit TB vaccines have already passed such scrutiny and have entered clinical trials (11). Our strongest indication that any of these TB vaccines could also contribute toward leprosy control is the demonstration of reduced *M. leprae* burdens in ID83- and ID93-immunized mice following experimental infection.

In summary, despite the positive impact that the widespread provision of multidrug therapy has had on the global prevalence of leprosy, there are indications that further effort and additional strategies are required in the push for elimination. It is our strong opinion that this effort should include an effective vaccine and that perhaps the smoothest path toward this would be the use of TB vaccines that also protect against leprosy. Our data suggest that new TB vaccine initiatives that are advancing the ID83- and ID93-based vaccine candidates could also be highly beneficial for the sustained control of leprosy.

ACKNOWLEDGMENTS

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- 46 -

Intra-subspecies sequence variability of the MACPPE12 gene in Mycobacterium avium subsp. hominissuis

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ABSTRACT

The PE (Pro-Glu) and PPE (Pro-Pro-Glu) multigene families are unique to mycobacteria, and are highly expanded in the pathogenic members of this genus. We determined the intra-subspecies genetic variability of the MACPPE12 gene, which is a specific PPE gene in Mycobacterium avium subsp. hominissuis (MAH), using 334 MAH isolates obtained from different isolation sources (222 human isolates, 145 Japanese and 77 Korean; 37 bathroom isolates; and 75 pig isolates). In total, 31 single-nucleotide polymorphisms (SNPs), which consisted of 16 synonymous SNPs and 15 nonsynonymous SNPs, were determined through comparison with the MACPPE12 gene sequence of MAH strain 104 as a reference. As the result, the 334 MAH isolates were classified into 19 and 13 different sequevars at the nucleic acid level (NA types) and amino acid level (AA types), respectively. Among the 13 AA types, only one type, the AA02 type, presented various NA types (7 different types) with synonymous SNPs, whereas all other AA types had a one-to-one correspondence with the NA types. This finding suggests that AA02 is a longer discernible lineage than the other AA types. Therefore, AA02 was classified as an ancestral type of the MACPPE12 gene, whereas the other AA types were classified as modern types. The ubiquitous presence of AA02 in all of the isolation sources and all different sequevars classified by the hsp65 genotype further supports this classification. In contrast to the ancestral type, the modern types showed remarkable differences in distribution between human isolates and pig isolates, and between Japanese isolates and Korean isolates. Divergence of the MACPPE12 gene may thus be a good indicator to characterize MAH strains in certain areas and/or hosts.

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1. Introduction

Mycobacterial infections caused by strains of the Mycobacterium avium complex (MAC) are becoming increasingly prevalent in animals and humans (Falkinham, 2010; Turenne et al., 2006; Winthrop, 2010). In particular, Mycobacterium avium subsp. hominissuis (MAH) is a frequent agent of human and pig mycobacteriosis (Mijs et al., 2002). Although MAH is typically considered to be an opportunistic bacterium for immunocompromised persons, it also frequently occurs in immunocompetent individuals and generally

manifests as a slowly progressive, often debilitating lung disease. Recently, middle-aged and elderly females without any predisposing conditions have been suggested to bear the brunt of this disease (Inagaki et al., 2009). Therefore, it has been speculated that MAH-associated mycobacteriosis is caused not only by host characteristics but also by bacterial factors (Ichikawa et al., 2009).

The PE (Pro-Glu) and PPE (Pro-Pro-Glu) multigene families are unique to mycobacteria and are suspected to be involved in immunostimulation and virulence (Gey van Pittius et al., 2006; Mackenzie et al., 2009; Sampson, 2011). The PE and PPE gene families are highly expanded in the pathogenic species of this genus (Gey van Pittius et al., 2006). Recently, Mackenzie et al., 2009 identified 12 PE and 49 PPE orthologs in the major groups of the MAC; Mycobac-

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terium avium subsp. paratuberculosis, M. avium subsp. hominissuis, Mycobacterium avium subsp. avium, and Mycobacterium intracellulare. A genomic comparison among them identified the subspecies-specific PE/PPE genes and the missing PE/PPE genes from one subspecies but present in at least two members of the MAC (Mackenzie et al., 2009). The former are likely to emerge and/or be acquired after divergence into the certain subspecies, whereas the latter are likely to correspond to earlier deletions in the certain subspecies. Two PPE genes, i.e., Mav 0790c and Mav 2006, which are now denoted as MACPPE4 and MACPPE12, respectively, under the newly proposed uniform PE and PPE locus names for all members of the MAC, were specific for MAH strain 104. The corresponding gene products could be used to identify immune responses against this M. avium subspecies, and misinterpretations caused by cross-reactivity in current diagnostics for Johne's disease would thus be avoided (Mackenzie et al., 2009).

In this study, we first confirmed that MACPPE12 is ubiquitous in this subspecies, whereas MACPPE4 is not widely distributed in strains other than MAH strain 104. To determine the intra-subspecies genetic variations of the MACPPE12 gene, we sequenced the full length of the gene (1341 bp) using 334 isolates that were obtained from different sources, i.e., 222 human isolates (145 Japanese and 77 Korean), 37 bathroom isolates, and 75 pig isolates. We also determined whether the genetic variation was associated with the isolation source.

2. Material and methods

2.1. Bacterial isolates

We used crude DNA extracted from 334 isolates for this study. Of these isolates, 257 overlapped with 258 isolates that were previously identified as MAH by hsp65 sequencing analyses and used to analyze genetic diversity (Iwamoto et al., 2012). One isolate from our previous study bank was excluded because of a lack of volume. We newly added 77 isolates from 77 human patients that were obtained from 7 different cities in Korea between 2010 and 2011. They were originally identified as M. avium through sequencing of the 16S rRNA gene (Devulder et al., 2005) at the Korean Institute of Tuberculosis. The hsp65 sequencing analyses that were performed in this study confirmed that all of the isolates belonged to MAH. To compare the genetic diversity of the 77 MAH isolates with the previously obtained data for the other 257 isolates, the same genetic markers used in the previous study (Iwamoto et al., 2012), i.e., the 3′ portion of the hsp65 gene sequence, presence of ISMav6, and genotypes of the 19-locus variable number of tandem repeat (VNTR) sequence, were analyzed for the 77 isolates. The datasets used in this study consisted solely of sequence data and no personal data were disclosed at any point.

2.2. PCR and sequencing of MACPPE12

The MACPPE12 gene, the locus name of which in MAH strain 104 (accession number in GenBank, NC_008595) is Mav_2006, was amplified using the primer sets MAV2006F (5′-TGC GTG GTA ACA AAA GCA AC) and MAV2006R (5′-CTT GCT GCG TAA TGC GAT AA). The PCR reaction consisted of 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. PCR was performed using Ex Taq Hot Start Version (TaKaRa Bio Inc., Shiga, Japan) with GC buffer I (TaKaRa Bio Inc., Shiga, Japan), and the PCR products were subjected to sequence analysis using an AB3500 genetic analyzer system (Applied Biosystems, Foster City, CA). The same primers used for PCR were also used for the sequencing of forward and reverse fragments. In addition, the interim primers MAV2006F634

(AAC GCG CTG CAG AAT CTC) and MAV2006R824 (TCC GTC ATC TTG TGT TCA GC) were used for the sequencing of forward and reverse fragments, respectively. Detailed information regarding VNTR genotypes, hsp65 code types, presence of ISMav6, and MAC-PPE12 sequevars of the 334 isolates in this study are summarized in Supplemental Table 1 (Table S1).

2.3. Phylogenetic analysis

The split-network phylogeny of the complete MACPPE12 gene sequence (1341 bp) was computed by NeighborNet analysis in SplitTree Version 4.8 (Huson and Kloepper, 2005). Recombination events in the MACPPE12 gene within the 334-isolate set were evaluated using DnaSP 4.10 (Rozas et al., 2003).

2.4. Nucleotide accession numbers

Sequences of the complete MACPPE12 gene representing each sequevar recognized in this study (NA types 2 to 19) were deposited in GenBank under accession Nos. AB820302 to AB820319.

3. Results

3.1. Presence of MACPPE4 and MACPPE12 in MAH

In a preliminary study, we first evaluated the ubiquitous presence of two previously reported MAH-specific MACPPE genes, i.e., MACPPE4 and MACPPE12, in MAH by using 16 randomly selected MAH isolates obtained from humans ($n = 6$), bathroom samples ($n = 2$), and pigs ($n = 8$). We attempted to detect the MACPPE4 gene using two PCR primer sets, one targeting the outside regions of MACPPE4, which can amplify the whole MACPPE4 gene with its flanking region, and the other targeting the inside sequences of MACPPE4, which can amplify partial regions of the gene. These primer sets produced expected sizes of PCR products from MAH strain 104 but the amplicons were not obtained from 16 other strains (data not shown). We therefore assumed that the MACPPE4 gene is not universally present in this subspecies, MAH. On the other hand, MACPPE12 was detected from all of the 16 isolates using the primer set targeting the outside regions of the gene, which can amplify whole MACPPE12 gene with its flanking region. Our expanding analysis for all of 334 samples could detect MACPPE12 from all of them. Therefore, it is highly likely that MACPPE12 is a ubiquitous gene in MAH.

3.2. Sequence variation of the MACPPE12 gene

First, we assured our sample set consisted of reasonably high heterogeneous isolates for the evaluation of the genetic variability and distribution of the MACPPE12 gene in MAH by 19-locus VNTR analysis. Actually, we retrieved the data from our previous study (Iwamoto et al., 2012) for 257 isolates and added newly analyzed data for 77 Korean isolates. The data demonstrated a reasonably high degree of genetic diversity in this sample set (Table 1 and Table S1). In brief, 99 genotypes in 145 Japanese isolates, 49 genotypes in 77 Korean isolates, 27 genotypes in 37 bathroom isolates, and 38 genotypes in 75 pig isolates.

The sequence analysis of the full length of the MACPPE12 gene for 334 MAH isolates identified in total 31 SNPs, which formed 19 different MACPPE12 sequevars at the nucleic acid (NA) level (NA type) through comparison with the MACPPE12 gene sequence of MAH strain 104 as a reference (Table 1). Of the 31 SNP positions, 15 positions were nonsynonymous SNPs (nsSNPs) that caused amino acid substitutions. This relatively high ratio of nsSNPs resulted in the formation of 13 different sequevars at the amino acid (AA)

Table 1
SNPs among the *M. avium* subsp. *hominissuis* in comparison with prototype strain *M. avium* subsp. *hominissuis* strain 104 (MAH strain 104).

Amino acid type	Nucleic acid type	Nucleotide at the base pair position of strain MAH 104[a]																																				Total number of:[b]			Genotypes
		4*	66	88*	272*	468	558	571*	605*	715*	733	744	752*	765	822	829*	831	834	866*	867	868*	870	877*	924	978	1000	1005	1150*	1161	1163*	1192*	1330*		nsSNP	sSNP	nsSNP Isolates (n=334)	
AA01	NA01	C	A	A	T	T	G	C	C	C	U	C	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	G	G	U	C	A	0	1	31	12
AA02	NA02	C	C	A	T	T	G	C	C	U	C	U	A	G	C	A	T	G	C	C	G	G	C	G	U	U	G	G	G	O	C	C	2	1	130	86
AA02	NA03	C	A	A	U	T	U	C	C	C	C	U	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	G	G	U	C	C	1	1	8	6
AA02	NA04	C	G	A	A	T	G	C	C	C	C	U	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	G	G	U	C	C	1	1	3	3
AA02	NA05	C	A	A	A	T	G	C	C	C	C	U	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	G	G	U	C	C	1	1	2	2
AA02	NA06	C	C	A	A	T	G	C	C	C	C	U	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	G	G	U	C	C	1	1	1	1
AA02	NA07	C	A	A	A	U	G	C	C	C	C	U	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	G	G	U	C	C	1	1	1	1
AA02	NA08	C	A	A	A	T	G	T	T	C	C	U	A	A	C	A	T	G	C	C	G	G	T	G	U	U	G	G	G	U	C	C	1	1	1	1
AA03	NA09	C	A	G	A	T	G	C	C	C	C	U	A	G	A	U	T	G	C	C	A	G	T	G	U	U	G	G	G	U	C	C	4	1	89	44
AA04	NA10	T	A	A	A	U	G	C	C	C	C	U	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	G	G	U	C	C	2	1	7	6
AA05	NA11	C	G	A	A	T	G	C	C	C	C	U	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	A	G	U	C	C	3	1	1	1
AA06	NA12	C	A	A	A	T	G	C	C	C	C	U	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	G	A	U	C	C	2	1	19	14
AA07	NA13	C	A	A	A	U	G	C	C	C	C	U	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	A	G	U	C	C	2	1	19	9
AA08	NA14	C	G	A	A	T	G	C	C	C	C	U	A	G	U	A	T	G	C	C	G	G	T	G	U	U	G	G	G	U	C	C	4	1	10	5
AA09	NA15	C	A	A	A	T	U	C	C	C	C	U	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	G	G	U	C	C	4	1	3	3
AA10	NA16	C	A	A	A	T	G	C	C	C	C	U	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	G	G	O	C	C	5	1	3	1
AA11	NA17	C	A	A	A	T	U	C	C	C	C	U	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	G	G	U	C	C	3	1	1	1
AA12	NA18	C	A	A	A	T	U	C	C	C	C	U	A	G	C	A	T	G	C	C	G	G	T	G	U	U	G	G	G	U	A	C	3	1	1	1
AA13	NA19	A	G	A	A	C	C	C	C	C	C	A	C	A	A	A	A	A	A	A	A	A	A	A	A	U	A	A	A	A	C	A	4	1	6	3

[a] Asterisks denote nonsynonymous position. AA01 and NA01 is the sequevars for MAH strain 104.
[b] sSNP, synonimous SNP; nsSNP, nonsynonymous SNP; genotypes were defined by 19-VNTR.

level (AA types) (Table 1). Among the 13 AA types, only AA02 showed variation in the nucleic acid sequence, i.e., it matched seven different NA types (NA02 to 08) that possessed various synonymous SNPs (sSNPs) (Table 1). All other AA types were associated in a one-to-one correspondence with NA types. These results suggest that AA02 has a longer history than the other AA types in MAH. We, therefore, classified AA02 as the ancestral type of MACPPE 12, whereas the other AA types were classified as modern types. When we used DnaSP, the minimum number of recombination events for the MACPPE12 gene in the sample set was estimated to be 3. The unrooted phylogeny for the gene sequences determined by SplitsTree4 demonstrated complex web-like topology (Fig. 1).

3.3. Comparison of the divergence of MACPPE12 in different isolation sources

The ancestral AA type of MACPPE12, AA02, was observed in all of the different isolation sources in this study (Table 2). In contrast, the modern types varied according to the isolation source. AA01 and AA07-09 were mostly present in pigs; AA03, in Japanese humans and bathroom samples; and AA13, in Korean humans. The topological positions of these AA types in the unrooted tree, except for that of AA01, were distinct from the position of the ancestral type (AA02) (Fig. 1).When we compared variation of MACPPE12 AA types with that of the *hsp65* gene sequevar, which consists of only sSNPs in this sample set, the AA02 type was observed in all 11 *hsp65* code types (Table 2). This would be natural due to the ancestral feature of AA02. Apart from the ancestral type, most of the modern types were distributed across more than two *hsp65* code types and not in a single lineage. This data strongly suggested that these two genes diverged independently, not in parallel to each other, during the history of MAH. It would be noteworthy that 57 pig isolates with *hsp65* code type 1, which was characterized as predominant code type of pig isolates in our previous study (Iwamoto et al., 2012), were subclassified into 5 AA types: AA01 (n = 26), AA02 (n = 4), AA07 (n = 12), AA08 (n = 11), and AA09 (n = 4) (Table 2, and Table S1).

A further finding in this study is the commonality and differences between Japanese and Korean human clinical isolates. A high prevalence of IS*Mav6*, which was reported in the genetic characterization of MAH in Japan (Ichikawa et al., 2009), was also observed in Korean isolates (42/77, 54.5%) as was reported by (Niimi et al., 2012). The characteristics of Korean isolates was demonstrated by their high prevalence of *hsp65* sequevar code 16 (26/77, 33.8%), which is a rare sequevar in other countries (Ichikawa et al., 2009; Iwamoto et al., 2012; Turenne et al., 2006). The majority of Korean isolates were ancestral type (AA02), whereas Japanese isolates predominated both AA02 and AA03. Moreover, other modern AA types showed different distributions between samples from these two countries.

4. Discussion

The precise functions of the PE and PPE families are still unknown except for a certain small number of members (Karboul et al., 2008; Mishra et al., 2008; Sassetti and Rubin, 2003), but these families are highly suspected to play key roles in the interaction between pathogens and their host (habitat) (Brennan et al., 2001; Sampson, 2011). A recent comparative study (Mackenzie et al., 2009) of the whole genomes of different MAC organisms revealed that two PPE paralogs, MACPPE4 and MACPPE12, were specifically found in MAH strain 104. Therefore, it would be a reasonable assumption that the two PPE genes reflect bacteriological characteristics of MAH in comparison with other MAC species.

Table 2
Characterization of MACPPE12 gene sequevars according to hsp65 gene sequence and source of the 334 isolates.

| MAC PPE 12 | | hsp65 Code Type | | | | | | | | | | | Source | | | |
AA type	NA type	C1	C2	C3	C7	C9	C15	C16	C17	N1	N2	N3	Human (Japan)	Human (Korea)	Bath-room	Pig
AA01	NA01	26	4	1	–	–	–	–	–	–	–	–	3	–	–	28
AA02	NA02-08	9	68	2	4	3	21	32	2	1	3	1	72	57	12	5
AA03	NA09	1	21	–	–	1	59	2	5	–	–	–	57	10	22	–
AA04	NA10	–	3	–	–	–	3	–	1	–	–	–	5	–	2	–
AA05	NA11	–	–	–	–	–	1	–	–	–	–	–	1	–	–	–
AA06	NA12	–	–	–	–	–	–	–	1	–	–	–	1	–	–	–
AA07	NA13	13	5	–	–	–	–	–	1	–	–	–	2	–	–	17
AA08	NA14	11	5	–	–	1	1	1	–	–	–	–	4	–	–	15
AA09	NA15	4	2	–	–	–	–	–	1	3	–	–	–	–	–	10
AA10	NA16	–	1	–	–	–	2	–	–	–	–	–	–	2	1	–
AA11	NA17	–	–	–	–	–	–	1	–	–	–	–	–	1	–	–
AA12	NA18	–	–	–	–	–	–	–	–	–	1	–	–	1	–	–
AA13	NA19	–	–	6	–	–	–	–	–	–	–	–	–	6	–	–
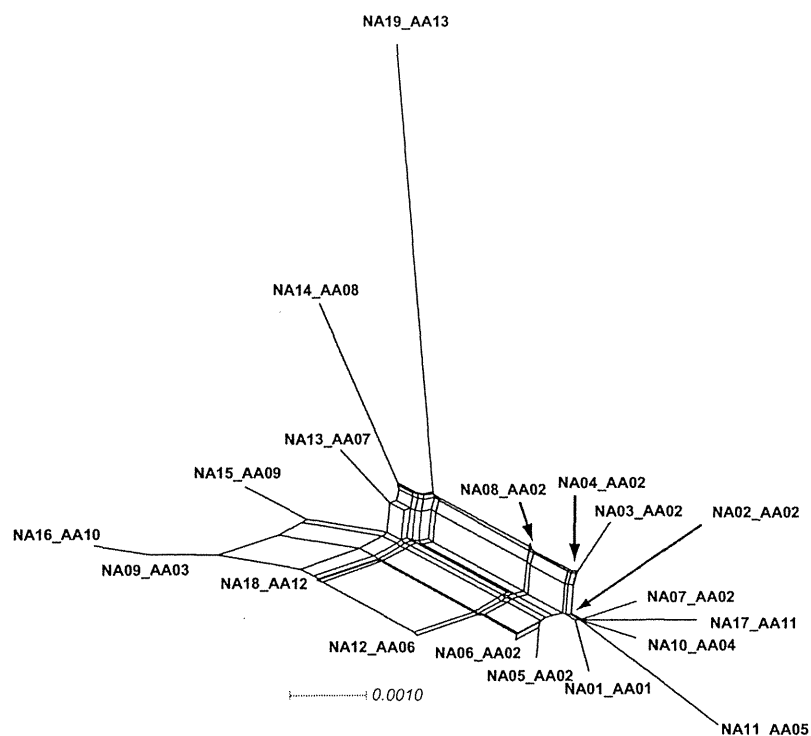
Fig. 1. Phylogenetic representation of each AA and NA type determined in this study and generated in SplitsTree4.

In this study, we evaluated the ubiquitous presence of these two PPE genes in MAH, and their genetic variability and association with the isolation sources and different genetic markers.

Our preliminary study using 16 MAH isolates suggested that MACPPE4 is not ubiquitous in MAH, although it is not certain that if this PPE gene is specific only for MAH strain104 or limited in subgroups of MAH. On the other hand, MACPPE12 was present in all of the 334 MAH isolates. Since our 334 isolates were a set of high heterogeneous isolates, the ubiquitous presence of the PPE gene in this sample set strongly supports the idea that this gene was present in the most recent common ancestor of MAH and universally retained in the subspecies. Because of the absence of the MACPPE12 gene in other members of the MAC and the ubiquitous presence in MAH isolates, MACPPE12 can be considered as a relatively new gene but ubiquitous in MAH.

By using the large number of isolates obtained from different sources, we demonstrated the variability of MACPPE12, which includes 19 different NA types and 13 AA types (Table 1). The web-like topology of the unrooted phylogeny (Fig. 1) and the estimated minimum number of recombination events suggest that genetic recombination plays a role in the divergence of this gene, although its mechanism is unknown. On the basis of the distribution of SNPs, isolation sources, and hsp65 code types in the different MACPPE12 gene AA types, we classified the PPE gene into two groups; one is an ancestral type (AA02) and the other is a modern type. MAH is generally characterized as its ubiquitous host distribution and heterogeneous grouping (Turenne et al., 2008, 2007). However, when we look closely at the correlation between AA types and isolation sources, the distribution of the modern AA types well reflect their isolation sources, whereas the ancestral AA type (AA02) was observed in all of the different isolation sources. Thus, it can be hypothesized that emergence of the modern AA types somehow reflects an on-going evolution of MAH toward specialization (narrower range for host specificity and higher fitness to its habitat)

from the generalism that is characterized by the ancestral AA type. Further investigations in combination with phylogenetic analysis would provide clear evidence for this hypothesis, i.e., evidence to judge if the emergence of modern AA types relies on convergent evolution, which has the advantage of adaptation to the hosts.

The ancestral AA type, AA02, was observed in all of the different *hsp65* code types (Table 2). This implies that AA02 had existed in the MAH (or its immediate ancestor) prior to the occurrence of the divergence of the *hsp65* gene in MAH. Although no NA type variations were found in any of the modern AA types (one-to-one correspondence between NA types and AA types), most of them were distributed in more than two *hsp65* sequevars (Table 2). The absence of sSNPs suggests that insufficient time has elapsed since the emergence of these variants to fix them in the genome. Therefore, it is unlikely that the modern AA types were present prior to the occurrence of the divergence of the *hsp65* gene in MAH. These two genes, MACPPE12 and *hsp65*, would diverge independently; thus the combination of these two genes can provide further discrimination of sub-groups. Indeed, 57 pig isolates with *hsp65* code type 1, which was characterized as predominant code type of pig isolates in our previous study (Iwamoto et al., 2012), were subclassified into 5 AA types; AA01 (*n* = 26), AA02 (*n* = 4), AA07 (*n* = 12), AA08 (*n* = 11), and AA09 (*n* = 4) (Table 2, and Table S1). This subclassification might be a useful approach when we compare the clinical significance at the strain levels.

In conclusion, the present study highlighted the variability of the MACPPE12 gene, which is absent in subspecies other than MAH. The MACPPE12 variants were classified into two groups: ancestral type (AA02) and modern types. AA02 reflects the general concept of MAH, i.e., ubiquitous host distributions and heterogeneity. The distribution of the modern types correlated with their major habitats (hosts). The divergence of the MACPPE12 gene and its distribution may be a good indicator to characterize MAH strains prevalent in certain areas. Further studies using global sample sets may shed light on the variation and distribution of the MACPPE12 gene at a global level.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.08.010.

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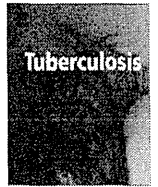
MOLECULAR ASPECTS

Molecular characterization of *Mycobacterium tuberculosis* isolates from elephants of Nepal

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ARTICLE INFO	SUMMARY
	Mycobacterium tuberculosis was cultured from the lung tissues of 3 captive elephants in Nepal that died with extensive lung lesions. Spoligotyping, TbD1 detection and multi-locus variable number of tandem repeat analysis (MLVA) results suggested 3 isolates belonged to a specific lineage of Indo-Oceanic clade, EAI5 SIT 138. One of the elephant isolates had a new synonymous single nucleotide polymorphism (SNP) T231C in the *gyrA* sequence, and the same SNP was also found in human isolates in Nepal. MLVA results and transfer history of the elephants suggested that 2 of them might be infected with *M. tuberculosis* from the same source. These findings indicated the source of *M. tuberculosis* infection of those elephants were local residents, presumably their handlers. Further investigation including detailed genotyping of elephant and human isolates is needed to clarify the infection route and eventually prevent the transmission of tuberculosis to susceptible hosts. © 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Tuberculosis (TB) in elephants is an emerging disease primarily caused by *Mycobacterium tuberculosis*. Although infection with *Mycobacterium bovis* and non-tuberculous mycobacteria (NTM) species has been documented [1–5], the majority of reported cases in captive elephants have been caused by *M. tuberculosis*. Many elephants infected with TB do not manifest clinical signs; however,

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some may have chronic weight loss, anorexia, and weakness. Exercise tolerance may be seen in working elephants. In some cases, the elephants may show symptoms only in the terminal stage of disease or are diagnosed- postmortem [1,2]. Postmortem lesions typically include granulomatous nodules in the lungs and bronchial lymph nodes sometimes with caseous foci. In the advanced stage of the disease, extensive caseocalcareous and cavitating lesions may be observed throughout the entire lung with enlarged bronchial and thoracic lymph nodes [1].

Nepal has a population of more than 200 captive elephants that are used for patrolling the protected areas, in eco-tourism and for wildlife research projects [6]. TB was first identified in the Nepalese captive elephant population in 2002. The government of Nepal has endorsed the Nepal Elephant Tuberculosis Control and Management Action Plan (2011–2015) that detail guidelines for the management of TB including the diagnosis and treatment of TB in elephants of Nepal [7]. Nepal is a country with a high burden of TB in humans [8]. Since captive elephants are in close contact with humans, it is likely that elephants contracted TB from humans at some point in time as TB has not been reported in wild elephants except for one case in an ex-captive African elephant [9]. Exposure

to infected elephants has resulted in transmission of TB to humans as evidenced by tuberculin skin test conversions [10–12] or active disease [13]. To clarify the transmission route, an epidemiological study including precise typing of isolated bacteria is needed. However, to date, few genotyping studies have been done on TB isolates from elephants [14,15]. In the current study, we performed genotyping on three *M. tuberculosis* isolates obtained from 3 captive elephants and compared them with human isolates in Nepal.

2. Materials and methods

2.1. Study isolates

2.1.1. Elephant isolates

M. tuberculosis isolates from 3 elephants were included in the study. All 3 elephants were owned by the Government of Nepal and kept in 2 protected areas. Elephants A and C were located at Chitwan National Park (CNP), and Elephant B was located at Koshi Tappu Wildlife Reserve (KTWR) (Figure 1). These elephants were used to patrol the protected areas for wildlife management and conservation purposes. The elephants were housed in open-air, roofed stables adjacent to other elephants. The elephants at each facility foraged and worked together for most time of the day, often coming in contact with domestic and wild animals such as rhinos and various deer species. Each captive elephant is taken care by 3 handlers and these handlers spend a long-time together with their elephants.

Elephant A was an adult female about 65 years old. She was brought to CNP from Motipur area of Sarlahi district near to the Indian border (Figure 1) when she was about 34 years. She was suspected to be suffering from TB and was in permanent segregation for almost 2 years before she died. Several trunk wash cultures collected from her failed to yield a positive isolate. Her body condition deteriorated significantly in the last 6 months before she collapsed and died in August 2009.

Elephant B was a female aged approximately 60 years old. She was brought to KTWR from a town Sitamarhi northern India (Figure 1) when she was about 30 years old. This town is located near to Sarlahi, a district where the Elephant A was previously kept.

She had never been tested for TB before she died in September 2009. For the last 2–3 months before she collapsed, she did not sleep well and lost weight resulting in poor body condition.

Elephant C was a male elephant aged approximately 31 years old. He was born in KTWR and was together with Elephant B for 4 years before he was transferred to CNP at the age of 7. He lost weight and began coughing 6 months before he collapsed in September 2012.

2.2. Human isolates

M. tuberculosis isolates from 7 patients in Nepal having the same spoligotypes with the elephant isolates were selected for this study. All of them were picked up from the isolates banked at German Nepal Tuberculosis Project (GENETUP), Nepal, which were collected from 2007 to 2010. One person was from Chitwan near CNP, 4 were from Kathmandu, 1 from Butwal and 1 from Birgunj (Figure 1). One person each from Birgunj and Hetauda had migrated to Kathmandu. DNA was extracted and the genetic analyses were performed in these isolates as described elsewhere [16].

2.3. Necropsy

All 3 postmortem examinations were carried out at the sites where each elephant collapsed. All personnel involved in the procedure used personal protective equipment including N-95 masks. The abdomen was opened first, and the gastro-intestinal tract and other visceral organs including liver and spleen were observed. The thoracic cavity was approached through the diaphragm per recommendations [17] and the caudal lobe of the lung was observed. Because suspected TB lesions were seen, the thoracic cavity was not further exposed due to the risk of spreading the organism in the environment. Representative lung lesions were collected in sterile screw-top tubes for laboratory analysis.

2.4. Culture

The lung tissue samples were processed according to guidelines of European Society for Mycobacteriology [18]. In brief, the lung
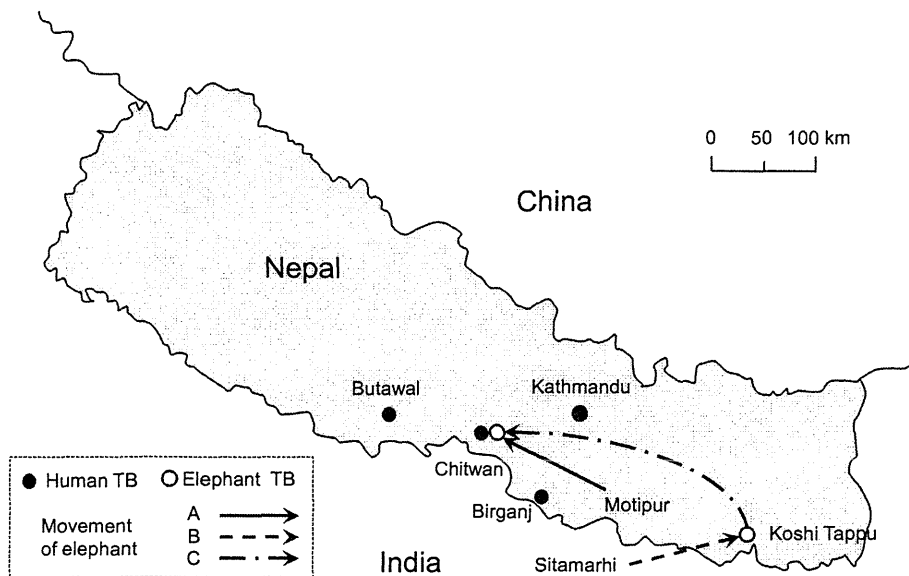

Figure 1. Movement of elephants and the distribution of elephant and human TB isolates in Nepal. Chitwan and Koshi Tappu are locations of the protected areas where the elephants were kept. Elephant A was stationed at a small town, Motipur, in Southern Nepal near to the Indian border before she was transferred to Chitwan. Elephant B was previously kept in an Indian town, Sitamarhi, near to the Nepalese border and transferred to Koshi Tappu. And elephant C was kept at Koshi Tappu and transferred to Chitwan.

tissue was aseptically cut into small pieces using a surgical blade, mixed with 4% sulfuric acid, and incubated in a sterile falcon tube for 20 min at room temperature. Then the sample was neutralized with 4% sodium hydroxide using bromo-thymol blue indicator and centrifuged at 3000 g for 20 min. The supernatant was discarded and then sample was washed once with sterile distilled water, followed by centrifugation at 3000 g for 20 min. The supernatant was discarded and the inoculation was done from the deposit into L-J media. The tubes were examined for growth weekly for 8 weeks.

2.5. DNA extraction

The DNA extraction was done for molecular studies using the GenoType® DNA isolation kit (Hain Lifescience GMBH, Nehren, Germany) from the colony that grew on the culture media. The colonies on the culture media were scraped and suspended in 300 µL of molecular biology grade water in a sterile Twist Top 1.7 ml conical vial and heated for 20 min at 95 °C in water bath. Then the sample was incubated for 15 min in an ultrasonic bath for cellular disruption, followed by centrifugation at 13,000 g for 5 min. Finally, the supernatant was taken containing the bacterial DNA.

2.6. Drug susceptibility test

Drug susceptibility test was performed on the mycobacterial isolates from all the elephants by the proportional method on L-J solid media with critical concentration of 0.2 µg/mL of isoniazid, 40 µg/mL of rifampin, 2 µg/mL of ethambutol and 4 µg/mL of streptomycin on all 3 isolates.

2.7. Genetic analyses

Bacterial species was identified by a multiplex PCR targeting cfp32, RD9 and RD12 [19] and was confirmed by a gyrB sequence analysis [20]. The spoligotype was determined as previously described [21]. Briefly, the direct-repeat (DR) region was amplified with a primer pair and the PCR products were hybridized to a set of 43 oligonucleotide probes corresponding to each spacer, which were covalently bound to the membrane. The spoligo-international type (SIT) was determined by comparing spoligotypes with the international spoligotyping database (SpolDB4) [22]. DR region rearrangement was confirmed by a PCR and sequencing with following primers, IS-LiP-TB3': CAACGCCAGAGACCAGCCGCCGGCTGAG, spacer37R: GACTGTGGACGAGTTCGCGCTC and DR region-R: TCACCGTCAACGCCGCCATCATGCTC. TbD1 detection was carried out by PCR as previously described [20]. Multi-locus variable number of tandem repeat analysis (MLVA) [23] was performed as described [24] with following 18 chosen loci, which showed higher variability among EAI isolates; VNTR424, ETR-C, MIRU4, MIRU40, MIRU10, VNTR1955, QUB11a, QUB11b, ETR-A, VNTR2401, ETR-B, MIEU26, MIRU31, QUB3232, QUB3336, VNTR3690, QUB26 and MIRU39. A dendogram was drawn by UPGMA with BioNumerics ver. 6.0. Genetic regions thought to be associating with drug resistance, i.e., partial rpoB, katG, inhA promoter region, gyrA and rrs sequences, were sequenced and analyzed as described [16,24]. Sequences that had mutations were compared with the public database using NCBI blast search system (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Seven human derived isolates having the same spoligotype were also subjected to the same analyses.

3. Results

The necropsy results of Elephant-A showed that she had liquefied caeseous lesions in lungs. The post-mortem findings of Elephant-B showed that the right lung had tuberculous - like lesions. Similarly, the necropsy findings of Elephant-C showed that the left lung at its dorso-posterior section had abscesses containing white pus. Upon excision, the mediastinal lymph node contained yellowish caseated material.

3.1. Culture

There was growth of M. tuberculosis complex from the representative lung lesion samples from elephants A, B and C.

3.2. Drug susceptibility testing

The isolates from the elephants A, B and C were susceptible to isoniazid, rifampin, ethambutol and streptomycin.

3.3. Species determination and genetic analyses

Bacterial species was determined as M. tuberculosis by a multiplex PCR and was confirmed by gyrB sequencing [19,20]. In gyrB sequence, all the elephant isolates had a single nucleotide polymorphism (SNP) from G to C at the position 990 that leads an amino acid substitution of Met 330 Ile. This mutation was revealed as lineage specific in strains belonging to EAI or Indo-Oceanic lineage [22,25] by NCBI blast search. Elephant C isolate (Elp-C) had a spoligotype belonging to the Indo-Oceanic lineage (EAI5, SIT138) while the other 2 had different new spoligotypes that were not found in the SpolDB4 database [22]. Elephant A isolate (Elp-A) showed only 2 spacers, spacer 38 and 39, positive. In elephant B isolate (Elp-B), the spacer 1 to 28 and 35 to 39 were positive and the pattern is 1 spacer, spacer 33, differed from spoligotype SIT 138 belonging to EAI5 clade (Table 1). Both of the DR region rearrangements, which were the cause of the spoligotype alteration, were confirmed by sequencings. In Elp-A, IS6110 was inserted at the position of spacer 37, and in Elp-B, the spacer 33 was deleted presumably by a homologous recombination (Figure 2) [26]. In TbD1 detection PCR, all 3 samples were positive and determined as ancestral type of M. tuberculosis [20]. The gyrA sequence of Elp-A had a synonymous SNP from T to C at the position of 231, while Elp-B and C had a wild type sequence. This gyrA SNP was not found in the public database, however, the same SNP was detected in two human samples, having spoligotype SIT138, collected in Nepal [16] (Table 1, Figure 3). Other drug resistance determination region sequences, rpoB, katG, inhA promoter region and rrs, were wild type in all the samples. In MLVA, Elp-B and Elp-C made a cluster with 1 locus difference. Elp-A formed a cluster with human isolates having the same gyrA SNP, T231C (Figure 3).

4. Discussion

M. tuberculosis infections in 3 Asian elephants with extensive TB lesions in the lungs are described. The clinical signs shown by these 3 elephants varied although the body condition of all elephants was deteriorating. All 3 elephants had similar lesions in the lungs during necropsy. As in humans, TB in elephants appears to primarily affect the lungs [27].

The diagnosis of TB by culture is considered the gold standard; however, it has very poor sensitivity, especially for ante-mortem diagnosis in elephants [28–30]. A study in Thailand reported that M. tuberculosis was isolated from only 2 out of 60 trunk wash samples from 3 elephants with positive postmortem culture isolations [15]. In another study, only 58% of elephants with confirmed TB infection at necropsy had positive isolations from trunk wash samples [28]. All of the trunk wash samples of Elephant A were negative on culture in the current study.

Our findings demonstrated that these 3 elephants were infected with M. tuberculosis. For the first time, M. tuberculosis was isolated