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Use of Protein Antigens for Early Serological Diagnosis of Leprosy[▽]

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Leprosy is a chronic and debilitating human disease caused by infection with the *Mycobacterium leprae* bacillus. Despite the marked reduction in the number of registered worldwide leprosy cases as a result of the widespread use of multidrug therapy, the number of new cases detected each year remains relatively stable. This indicates that *M. leprae* is still being transmitted and that, without earlier diagnosis, *M. leprae* infection will continue to pose a health problem. Current diagnostic techniques, based on the appearance of clinical symptoms or of immunoglobulin M (IgM) antibodies that recognize the bacterial phenolic glycolipid I, are unable to reliably identify early-stage leprosy. In this study we examine the ability of IgG within leprosy patient sera to bind several *M. leprae* protein antigens. As expected, multibacillary leprosy patients provided stronger responses than paucibacillary leprosy patients. We demonstrate that the geographic locations of the patients can influence the antigens they recognize but that ML0405 and ML2331 are recognized by sera from diverse regions (the Philippines, coastal and central Brazil, and Japan). A fusion construct of these two proteins (designated leprosy IDRI diagnostic 1 [LID-1]) retained the diagnostic activity of the component antigens. Upon testing against a panel of prospective sera from individuals who developed leprosy, we determined that LID-1 was capable of diagnosing leprosy 6 to 8 months before the onset of clinical symptoms. A serological diagnostic test capable of identifying and allowing treatment of early-stage leprosy could reduce transmission, prevent functional disabilities and stigmatizing deformities, and facilitate leprosy eradication.

Cases in which *Mycobacterium leprae* infection manifests to cause leprosy present as a bacteriologic, clinical, immunologic, and pathological spectrum ranging from the extremes observed in paucibacillary (PB) and multibacillary (MB) patients (21, 24). PB patients have one or a few skin lesions and a low or absent bacterial index (BI; a measure of the number of acid-fast bacilli in the dermis, expressed on a logarithmic scale) and demonstrate specific cell-mediated immunity against *M. leprae*, but they have low or absent titers of *M. leprae*-specific antibodies and a granulomatous dermatopathology. In marked contrast, MB patients have multiple symmetric skin lesions and a high BI and demonstrate high titers of anti-*M. leprae* antibodies but an absence of specific cell-mediated immunity and a dermatopathology largely devoid of functional lymphocytes (21). Despite the implementation of a WHO-directed eradication program over the last 20 years, the worldwide annual rate of new case detection for leprosy remains stable at approximately 300,000 (17, 18, 26, 27). Earlier and objective diagnosis of leprosy could interrupt transmission and, in the long term, help further reduce the number of new cases and facilitate eradication.

There is no single diagnostic laboratory test for leprosy, and diagnosis remains essentially clinical. Clinical diagnosis of leprosy is dependent upon recognition of disease symptoms and is therefore only possible once the disease has manifested. WHO experts have listed diagnostic criteria as one or more of the following: hypopigmented or reddish skin patches with definite loss of sensation; thickened peripheral nerves; acid-fast bacilli on skin smears/biopsy specimens (WHO Expert Committee on Leprosy, 1998). Pure neuritic leprosy forms, however, present with no skin lesion. Confounding WHO's implementation of a global leprosy eradication strategy is that the number of trained leprologists has diminished. This is inadvertently increasing the likelihood that a clinical diagnosis is delayed or even missed, especially in regions where leprosy has been controlled (1, 13, 16, 25).

The presence of serum immunoglobulin M (IgM) antibody to phenolic glycolipid I (PGL-I) correlates with BI in leprosy patients and has been used to support disease symptoms as a means to categorize leprosy patients. Enzyme-linked immunosorbent assay (ELISA) and rapid lateral flow test formats have been developed for the detection of anti-PGL-I antibody (3, 4, 8, 19, 22, 23, 28). In one study, a lateral flow assay correctly diagnosed 97.4% of MB patients, with a specificity of 86.2% (4). Patients toward the PB end of the leprosy spectrum have low or no BI, however, and the majority of these patients are not identified by PGL-I-based tests (4, 7, 19). In addition, false-positive results in areas of endemicity are relatively high (>10%) (4, 7, 19). Consequently, none of these PGL-I-based tests has been widely implemented in

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field situations. In addition, many studies have demonstrated that MB patients have high titers of *M. leprae*-specific antibodies but PB patients have low or absent titers. For these reasons, the potential for serological diagnosis of low-BI patients, such as PB patients or MB patients who are developing disease, has not been thoroughly pursued.

In a recent small-scale study, we demonstrated that the ML0405 and ML2331 proteins were recognized by sera from MB leprosy patients presenting with high BI (20). In the current study we demonstrate that ML0405 and ML2331 are diagnostically relevant antigens by analyzing a large panel of MB leprosy patient sera from a variety of leprosy-affected regions (the Philippines, central and coastal Brazil, and Japan). We also examine the ability of *M. leprae* protein antigens to diagnose low-BI leprosy (PB patients and early MB patients) and show here the diagnostic potential of ML0405, ML2331, and a newly discovered *M. leprae* antigen, ML1556c. Based on the results, we construct and evaluate a fusion protein comprising ML0405 and ML2331 (designated *l*eprosy *I*DRI *d*iagnostic 1 [LID-1]) and demonstrate that this construct can be used to serologically diagnose leprosy patients among presymptomatic individuals, that is, before a clinical diagnosis is possible. Moreover, ML1556c may be a valuable adduct to LID-1 for the diagnosis of PB leprosy.

MATERIALS AND METHODS

Subjects and samples. Sera were obtained from patients with leprosy (MB and PB) or tuberculosis (TB), healthy household contacts of MB leprosy patients (HHC), and endemic and nonendemic controls (EC and NEC). MB and PB leprosy patient sera used in this study were derived from recently diagnosed, previously untreated individuals who did not have signs of reversal reactions. Leprosy was classified in each case by bacterial, histological, and clinical observations carried out by qualified personnel, with the BI recorded at the time of diagnosis. HHC were defined as adults living in the same house as an MB index case for at least 6 months. TB patients were included to evaluate potential antigen cross-reactivity with other mycobacterial infection. Sera from TB patients were obtained after drawing blood from *Mycobacterium tuberculosis* sputum-positive, human immunodeficiency virus-negative individuals with clinically confirmed pulmonary TB who were undergoing treatment. Normal sera (EC and NEC) were obtained after blood draws from volunteers with no history of leprosy or TB infection. In all cases, drawing of blood was carried out with informed consent (with local institutional review board approval or local ethics committee approval in Brazil, Japan, the Philippines, Seattle, and St. Louis). The composition of each study population is summarized in Table 1.

In Cebu City, leprosy and TB patients were recruited at the Cebu skin clinic and Leonard Wood Memorial Research Center in Cebu City, Cebu (Philippines) from 2003 to 2006. Between 1985 and 1991, sera were collected prospectively from individuals who resided with MB patients (BI > 2) for at least 2 years and were free of leprosy as determined by clinical dermato-neurological examination at the inclusion point of the study. Some of these individuals developed MB leprosy as the study progressed, and these sera have previously been described (11).

In Goiânia, the state capital of Goiás State (western central Brazil), leprosy and TB patients were recruited at the main outpatient clinics of Centro de Referência em Diagnóstico e Terapêutica and Hospital Anuar Auad in 2006. PB leprosy patients were selected from a cohort of leprosy patients with a single skin lesion recruited at Brazilian sites of endemicity from 1999 to 2001, as previously described (9).

In Salvador, the state capital of Bahia State (northeast coastal Brazil), leprosy patients were recruited at Hospital Dom Rodrigo de Menezes in 2006.

In Japan, leprosy patients were recruited at the National Sanatorium Oshimaseishoen, Kagawa.

In St. Louis, sera were collected from U.S.-based individuals at a variety of times following *Mycobacterium bovis* BCG immunization.

All serum specimens were aliquoted and stored at −20°C or −80°C prior to assay.

TABLE 1. Study populations

Site	Sample categorization (total no.)	BI (mean)	Sex ratio[a]	Mean age (yr) (range)
Cebu City, Philippines	MB (17)	2.8	2.4	30 (18–55)
	PB (54)	0.5	0.4	31 (15–45)
	TB (6)		5	45 (35–53)
	EC (8)		1	26 (19–38)
	HHC (10)		0.4	38 (18–60)
Goiânia, Brazil	MB (28)	2.4	1.5	44 (19–81)
	PB (83)	0	0.4	33 (7–76)
	TB (26)		2.7	39 (17–66)
	EC (30)		0.1	20 (19–26)
	HHC (11)		0.5	28 (18–51)
Salvador, Brazil	MB (10)	NA[b]	3.5	35.1 (20–70)
	PB (6)	0	5	31.6 (12–42)
	HHC (11)		0.1	48.5 (25–57)
Kagawa, Japan	MB (30)	NA	NA	60 (48–79)
	PB (30)	0	NA	70 (55–90)
	EC (26)		NA	54 (48–62)

[a] Male/female ratio.
[b] NA, not available.

Cloning and purification of target antigens. DNA encoding selected *M. leprae* proteins was PCR amplified from *M. leprae* Thai-53 genomic DNA using *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA). PCR primers were designed to incorporate specific restriction enzyme sites 5′ and 3′ of the gene of interest and excluded in the target gene for directional cloning into the expression vector pET28a (Novagen, Madison, WI). After PCR amplification, purified PCR products were digested, ligated with vector DNA, and used to transform *Escherichia coli*, and individual clones were induced to produce recombinant proteins, as previously described (20). Recombinant proteins were quantified using the bicinchoninic acid protein assay (Pierce, Rockford, IL), and quality was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The characteristics of each *M. leprae* protein evaluated are summarized in Table 2. The ML1556c protein was included because portions of the ML1556 protein were identified in four separate clones during serological expression screening with sera from PB leprosy patients (data not shown) (20). Recognition of the clones was derived from amino acids 58 to 256 of ML1556, which are only 47% identical to the *M. tuberculosis* protein Rv2839 (compared to 82% identity across the entire amino acid sequences of ML1556 and Rv2839).

Determining patient reactivity by ELISA. ELISAs were conducted independently at IDRI, Seattle, WA (Cebu and St. Louis sera); UFG, Goiânia, and UFB, Salvador, Brazil; and NIID, Tokyo, Japan. Polysorp 96-well plates (Nunc, Rochester, NY) were coated with 1 μg/ml recombinant protein or 200 ng/ml of natural disaccharide with octyl linkage (NDO), the synthetically derived B-cell epitope of PGL-I, conjugated to bovine serum albumin (NDO-BSA; kindly supplied by John Spencer, Colorado State University, under NIH contract N01 AI-25469), in bicarbonate buffer overnight at 4°C and blocked for 1 h at room temperature with phosphate-buffered saline–Tween with 1% BSA on a plate shaker. Serum diluted appropriately in 0.1% BSA was added to each well, and plates were incubated at room temperature for 2 h with shaking. Plates were washed with buffer only, and horseradish peroxidase-conjugated IgG or IgM (Rockland Immunochemicals, Gilbertsville, PA), diluted in 0.1% BSA, was added to each well and incubated at room temperature for 1 h with shaking. After washing, plates were developed with peroxidase color substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and the reaction was quenched by the addition of 1 N H_2SO_4. The optical density (OD) of each well was read at 450 nm. Positive responses were defined as an OD of >2× the mean OD of endemic control sera or an OD of >0.1, whichever was higher.

Statistics. *P* values were determined using Student's *t* test.

RESULTS

Recognition of *M. leprae* proteins by Filipino leprosy patient sera. The majority of MB leprosy patients are readily identified

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TABLE 2. Main characteristics of *M. leprae* antigens tested[a]

Gene accession no.	Functional classification[b]	Protein type	Length (bp)	Product size (kDa)	% Identity[c] with:				
					M. tuberculosis H37Rv[e]	*M. bovis* AF2122/97[e]	*M. avium* 104[d]	*M. marinum* ATCC BAA-535[e]	*M. smegmatis* MC2 155[d]
ML0091	II.C.2	28-kDa antigen precursor	711	23.7	53	53	54	54	48
ML0405	V	Conserved hypothetical	765	25.3	62	62	None	NA	None
ML1633	II.C.2	Possible secreted hydrolase	1,608	57.0	25	25	35	81	62
ML2055	IV.A	Probable cell surface protein	864	29.5	72	72	69	73	54
ML2331	II.C.2	Possible secreted protein	771	26.5	80	80	77	80	67
ML2346	VI	Hypothetical	906	33.9	None	None	None	None	None
ML1556	II.A.6	Translation initiation factor	2,775	96.6	84	82		90	

[a] Annotations for gene accession number, functional classification, and protein type are according to the Sanger database.
[b] Functional classifications: II.C.2, surface polysaccharides, lipopolysaccharides, proteins, and antigens; V, conserved hypotheticals; IV.A, virulence; VI, unknowns; II.A.6, protein translation and modification.
[c] BLAST reports were performed in September 2006; tBLASTn was used for comparisons of proteins versus translated DNA. NA, not applicable.
[d] From http://www.tigr.org.
[e] From http://www.sanger.ac.uk/Projects.

by ELISA and lateral flow tests, which assess the capacity of patient IgM to bind *M. leprae* PGL-I or its synthetic analogue (NDO) conjugated to a carrier protein (BSA). In comparison with MB leprosy patients, PB leprosy patients have low or no anti-PGL-I responses and are more difficult to diagnose serologically. We therefore sought to determine whether PB sera recognized protein antigens, expanding our previous analyses and comparing the potential of NDO-BSA, ML0405, and ML2331 to diagnose leprosy, and found that the protein antigens have a similar profile for leprosy diagnosis as that for NDO-BSA; all three test antigens were readily detected by MB patient sera, by some PB patient sera, and by few, if any, EC, HHC, or TB sera (Fig. 1). Thus, similar to NDO-BSA, ML0405 and ML2331 demonstrate good potentials for the diagnosis of leprosy.

Recognition of MB leprosy patient sera with refined ML0405 antigen constructs. To learn more regarding the seroreactivity of ML0405 and enhance recombinant ML0405 expression for purification, we expressed a variety of ML0405 polypeptide fragments and determined whether Filipino MB leprosy patient sera had similar binding capacities to these fragments and to full-length (ML0405FL) protein. All constructs were able to bind MB patient sera (Fig. 2) ($P < 0.01$ for MB versus EC). The reactivity of a truncated form (ML0405Tr) of the protein was equivalent to the reactivity of ML0405FL ($P = 0.885$ for MB patient sera), whereas the reactivity of the protein construct lacking the predicted membrane-spanning region (ML0405Tm) declined slightly (Fig. 2) ($P = 0.047$ and 0.060 for Tm versus FL and Tr forms, respectively, for MB). These data indicate that the majority, if not all, of the B-cell epitopes recognized by antibodies in patient sera are retained and accessible in the truncated form of the protein. Further testing was conducted using either ML0405FL or ML0405Tr.

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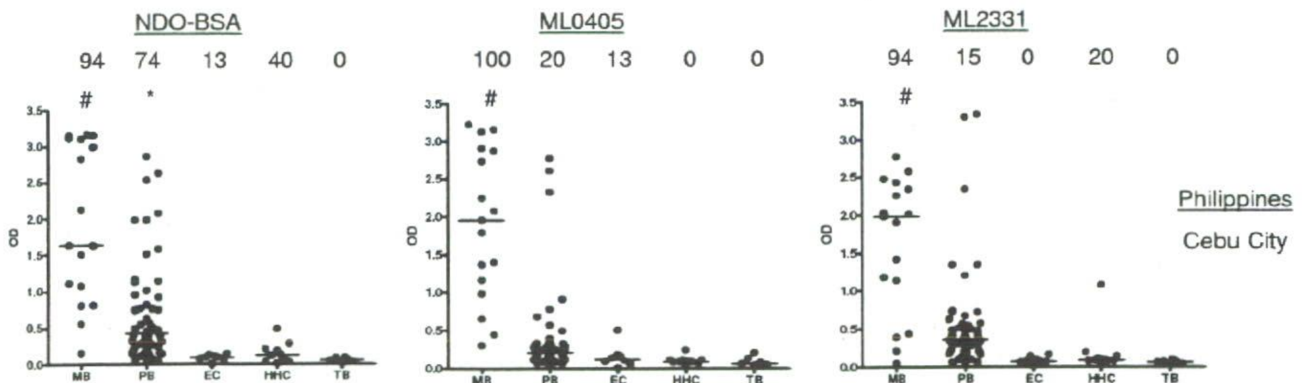

FIG. 1. Sera from Filipino leprosy patients react with recombinant *M. leprae* antigens. Sera from clinically diagnosed MB and PB leprosy patients, EC individuals, and HHC of MB leprosy patients were assessed against NDO-BSA, ML0405, and ML2331. NDO-BSA reactivity was assessed by IgM binding, and protein reactivity was assessed by IgG binding. Sera were from Cebu City, Philippines. Each point represents an individual serum sample, and the median is represented by the line. The number above each data set is the percent positive responses. *, $P < 0.05$; #, $P < 0.001$ versus EC.

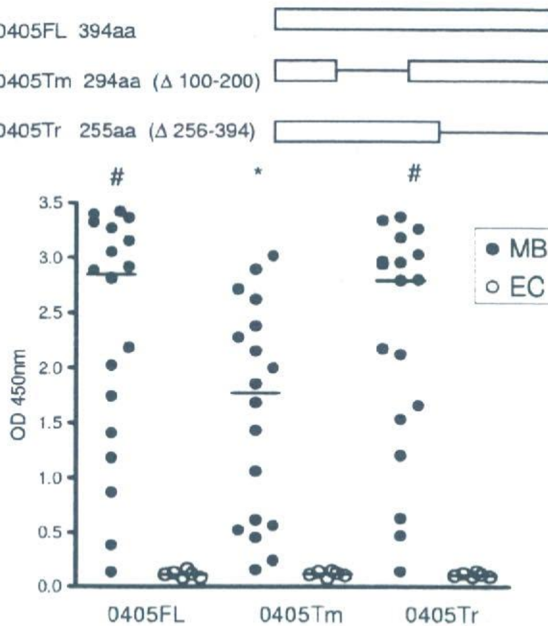

FIG. 2. ML0405 constructs react with MB leprosy patient sera. Different ML0405 constructs were created and expressed as recombinant proteins. The schematic diagram shows the sequence alignment of each of these constructs, with the deleted regions indicated by the line. Each construct was tested for IgG reactivity by ELISA with individual Filipino MB leprosy patient sera (n = 18) or EC sera (n = 6). *, $P < 0.05$; #, $P < 0.001$ versus EC.

Diagnosis of Filipino PB leprosy patients with *M. leprae* proteins. We then went on to more closely investigate the potential of *M. leprae* antigens for diagnosing PB leprosy. Sera from Filipino patients clinically diagnosed with PB leprosy and with a low BI were tested for reactivity with potential diagnostic *M. leprae* antigens (ML0405Tr, ML2331, ML1556c, and NDO-BSA). NDO-BSA was capable of identifying 57% (26 of 46) of these Filipino PB leprosy patients, but a substantial number of samples provided weak positive responses (Fig. 3). ML0405 and ML2331 also reacted with sera from some PB patients (Fig. 3A and B). Most of these Filipino sera that reacted with these proteins also demonstrated strong NDO-BSA responses, however, and so the added benefit of using these antigens for leprosy diagnosis within the Filipino population appeared minimal. In contrast, 4 of 20 sera that were weak positive/negative by NDO-BSA ELISA testing demonstrated strong reactivity to ML1556c (Fig. 3C). This result suggests that ML1556c may be useful as an adjunct to PGL-I testing, or other tests, to improve the sensitivity and clarity of leprosy diagnosis.

To test the specificity of ML1556c as a leprosy diagnostic reagent, we directly compared the reactivities of ML1556c with sera from PB leprosy patients, MB leprosy patients, TB patients, EC, and HHC of MB leprosy patients located in Cebu City, Philippines (Fig. 3D). Positive responses were observed in five of eight additional PB leprosy sera tested, with three of the sera yielding strong responses that could provide a clear diagnosis. Positive responses to ML1556c were also observed in two of seven MB leprosy sera tested in this experiment.

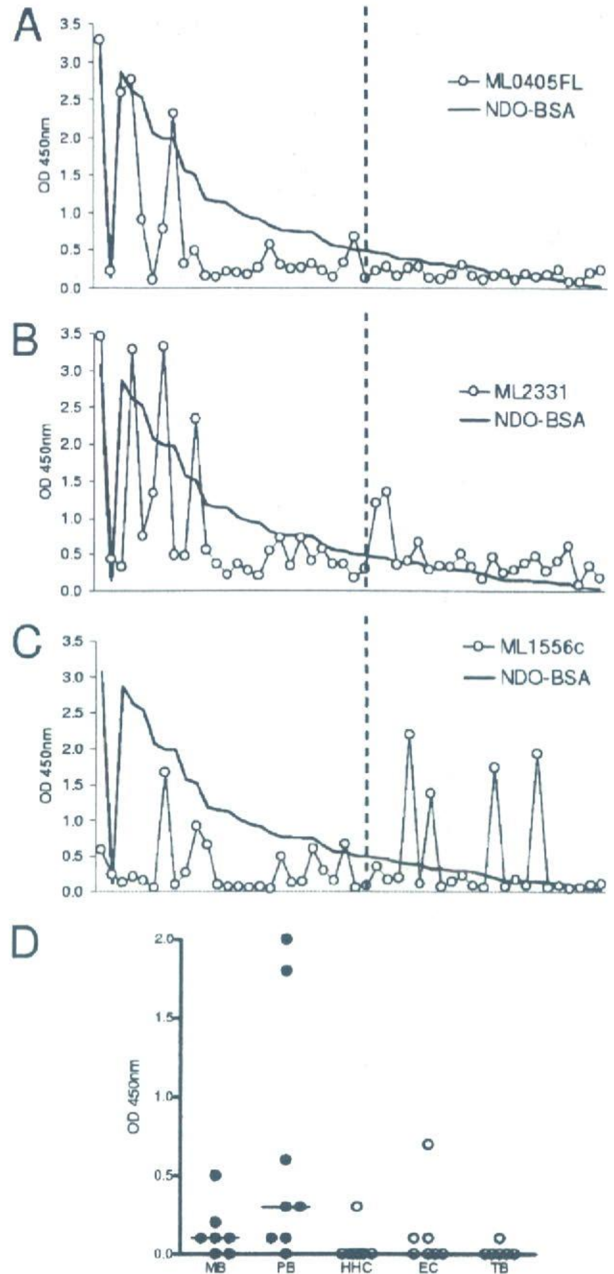

FIG. 3. *M. leprae* proteins react with PB leprosy patient sera. (A to C) Antibody reactivities of sera from a pool of clinically diagnosed MB leprosy patients, from a pool of negative control individuals, and from 46 clinically diagnosed PB leprosy patients were assessed against NDO-BSA and ML0405 (A), ML2331 (B), and ML1556c (C). NDO-BSA reactivity was assessed by IgM binding and, for reference, is shown in each plot. Recombinant protein reactivity was assessed by IgG binding. The first open circle represents the value obtained for pooled MB sera, while the next open circle represents the reactivity of pooled EC sera; individual PB sera are then arranged along the x axis according to their responsiveness versus NDO-BSA. The dashed line indicates the point at which diagnosis by NDO-BSA reactivity becomes unclear. ML1556c reacts with PB leprosy patient sera. (D) IgG reactivities of ML1556c with a small panel of individual sera from EC, leprosy patients (MB and PB), and TB patients were determined by ELISA using samples from Cebu City, Philippines. Each point represents an individual serum sample, and the median is represented by the line.

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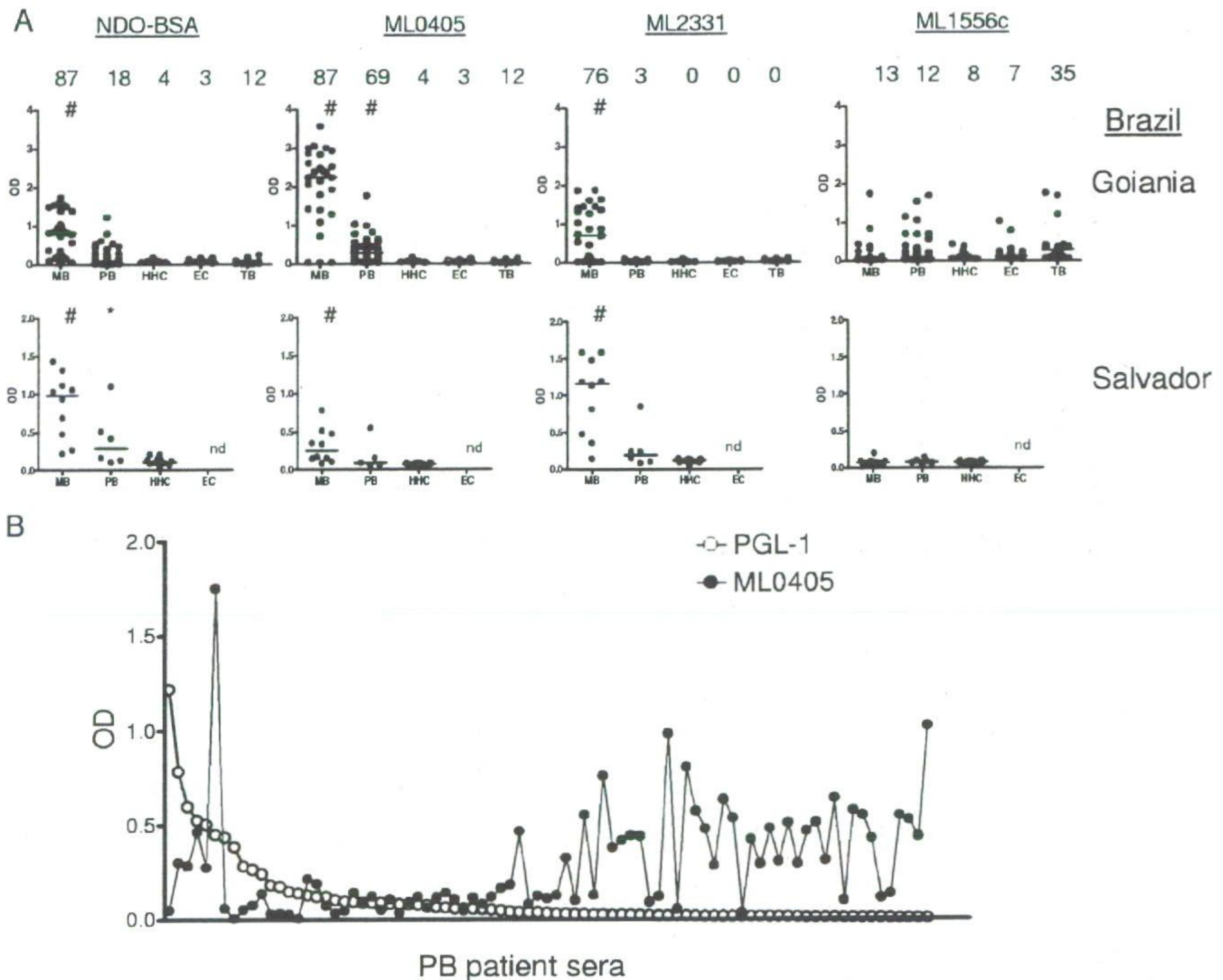

FIG. 4. Sera from Brazilian leprosy patients react with recombinant *M. leprae* antigens. Sera from clinically diagnosed MB and PB leprosy patients, EC individuals, and HHC of MB leprosy patients were assessed against NDO-BSA, ML0405, ML2331, and ML1556c. NDO-BSA reactivity was assessed by IgM binding, and protein reactivity was assessed by IgG binding. Sera were from Goiânia and Salvador (see Table 1). (A) Each point represents an individual serum sample, and the median is represented by the line. The number above each data set is the percent positive responses. *, $P < 0.05$; #, $P < 0.001$ versus EC. (B) To demonstrate complementarity, the individual PB sera from Goiânia are arranged along the x axis according to their responsiveness versus NDO-BSA and overlaid with the response of each serum to ML0405.

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ML1556c did not react with any of the Filipino TB patient sera tested, was recognized by only one of eight HHC sera, and reacted with only one of six EC sera. Negative results were obtained upon further testing involving another 45 TB sera and 23 NEC sera (data not shown). Taken together, these results generated from sera from the Philippines suggested the utility of ML1556c to improve the diagnosis of PB leprosy.

Identification of leprosy patients in Brazil. We also examined the ability of recombinant *M. leprae* antigens to identify leprosy patients located around Goiânia, Brazil, and Salvador, Brazil. Within the clinically diagnosed leprosy population, PGL-I/NDO-BSA was capable of identifying 87% (33 of 38) of the MB patients (Fig. 4). In agreement with the results obtained by analysis of Filipino leprosy patient sera, ML0405 and ML2331 reacted with large proportions of Brazilian MB patient sera (87% [33 of 38] and 76% [29 of 38], respectively), and ML1556c reacted with only some MB patient sera (13%, 5 of 38) (Fig. 4). In Goiânia, positive responses were also observed against antigens ML0091 (71%, 20 of 28), ML1633 (32%, 9 of 28), ML2055 (75%, 21 of 28), and ML2346 (29%, 8 of 28) (data not shown). The clarity of MB leprosy diagnosis (strength of signal in positive samples versus negative samples) in Goiânia was greater when using ML0405 rather than NDO-BSA, but in Salvador it was greater when using ML2331 rather than NDO-BSA.

We also determined if these antigens were recognized by Brazilian PB patient sera. PGL-I/NDO-BSA was capable of identifying only 20% (18 of 89) of the PB patients, a level not appreciably higher than the proportion of positive responses observed with TB patients (12%, 3 of 26) (Fig. 4A). An IgG

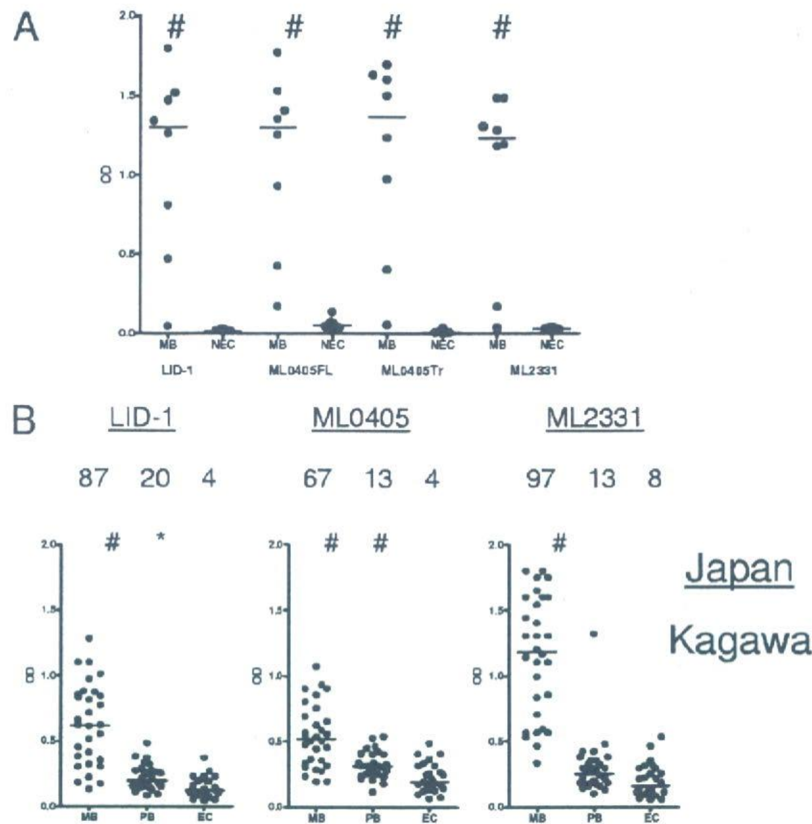

FIG. 5. LID-1 retains reactivity with leprosy patient sera. (A) LID-1 (a fusion construct of ML0405 and ML2331), ML0405FL, ML0405Tr, and ML2331 reactivities were assessed by IgG binding in an ELISA with eight MB leprosy patient serum samples from Salvador and eight NEC serum samples. (B) Sera from clinically diagnosed Japanese MB and PB leprosy patients, and Japanese EC individuals, were assessed for IgG reactivities with LID-1, ML0405, and ML2331. Each point represents an individual serum sample, and the median is represented by the line. The number above each data set is the percent positive responses. $\star$, $P < 0.05$; #, $P < 0.001$ versus EC.

reactivity that permitted serologic diagnosis of an increased number of PB leprosy patients was observed for ML0405 (69%, 61 of 89), but responses to ML2331 were very weak, with very few positives (3%, 3 of 89) (Fig. 4A). The antigens ML0091 (6%, 5 of 83), ML1633 (17%, 14 of 83), ML2055 (13%, 11 of 83), and ML2346 (27%, 22 of 83) were recognized by some PB patient sera, but responses were generally weak (data not shown). Many of the PB patient sera that did not react with PGL-1 had a strong reactivity with ML0405 (Fig. 4B). ML1556c was recognized by only a minor subset of PB leprosy patient sera (12%, 11 of 89) and Brazilian EC individuals (6.7%, 2 of 30), but ML1556c reactivity was detected in a substantial number of Brazilian TB patients (35%, 9 of 26). These data indicate only a minor number of positive results in the Brazilian population if ML1556c is used for leprosy diagnosis, with a further complication of false-positive diagnosis in TB patients. Antigen ML0405, however, did not react with significant numbers of EC sera (3.3%, 1 of 30) or TB sera (12%, 3 of 26) (Fig. 4A). These results indicate that ML0405 can recognize some PB leprosy patients in the Brazilian population and could be used to augment leprosy diagnosis with PGL-1.

Construction of a fusion construct of ML0405-ML2331 (LID-1). Having extended our earlier observation that the single antigens ML0405 and ML2331 have the potential to diagnose leprosy (20), and given the observations that ML0405 appeared better for diagnosis in Goiânia and Cebu City but ML2331 appeared better for diagnosis in Salvador, we constructed a single fusion molecule incorporating both proteins. ML0405Tr was expressed at the C terminus of the molecule and ML2331 in the N terminus. Following recombinant expression, we validated the reactivity of the construct by assaying LID-1 versus a small panel of sera from Salvador that had bound each single component. These sera readily detected LID-1, ML0405FL, ML0405Tr, and ML2331 (Fig. 5A). Importantly, construction of the fusion protein did not introduce false-positive results with NEC sera (Fig. 5A).

We further extended our examination of sera from different geographic locations by assessing sera from Japanese leprosy patients for reactivity with ML0405, ML2331, and LID-1. Positive response were observed with MB patient sera (67% [20 of 30] for ML0405, 97% [29 of 30] for ML2331, and 87% [26 of 30] for LID-1) and PB patient sera (13% [4 of 30] for ML0405, 13% [4 of 30] for ML2331, and 20% [6 of 30] for LID-1), with few responses in EC sera (4% [1 of 26] for ML0405, 8% [2 of 26] for ML2331, and 4% [1 of 26] for LID-1) (Fig. 5B). Taken together, these data indicate that LID-1 is useful as a diagnostic antigen for leprosy.

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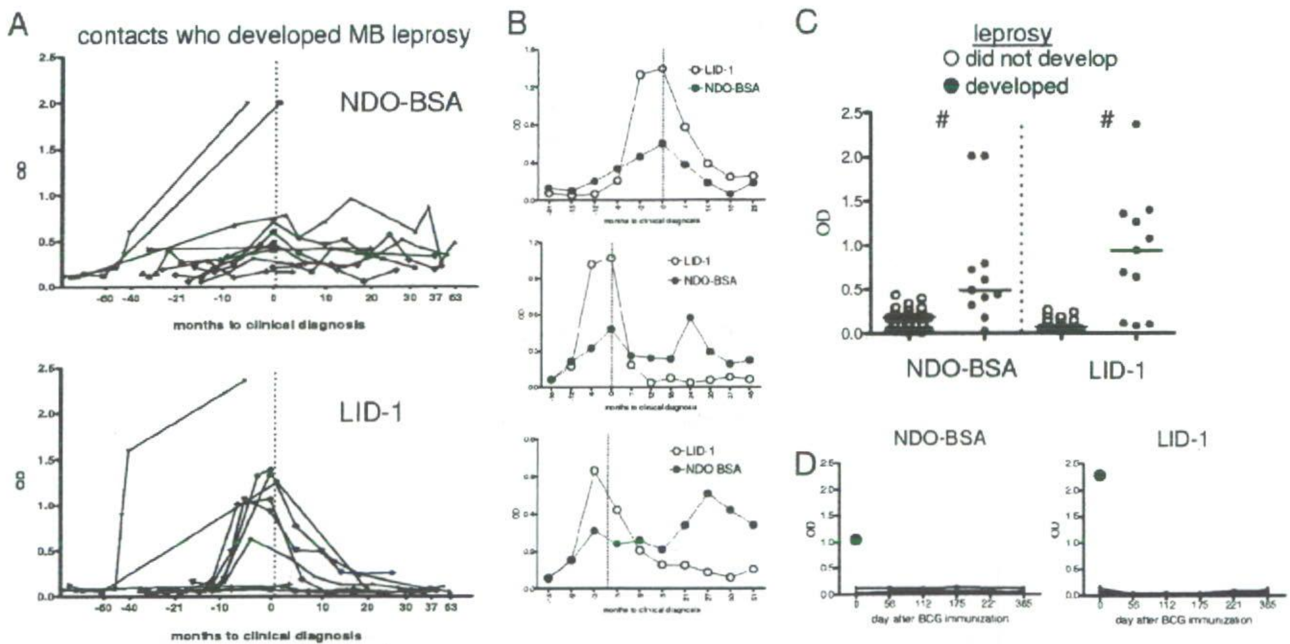

FIG. 6. LID-1 reactivity can diagnose leprosy before clinical symptoms. (A) LID-1 and NDO-BSA reactivities within sera from a prospective study conducted in Cebu City, Philippines, were assessed by either IgG or IgM binding in an ELISA. Sera were collected at a variety of times prior to the clinical diagnosis of MB leprosy in 11 patients and at a variety of times after the commencement of treatment. (B) Representative plots for individual patients are shown. (C) Sera were collected from 57 household contacts that did not develop clinical leprosy and were compared with single serum samples from each individual contact that developed leprosy (serum samples were collected within 3 months of clinical diagnosis). #, $P < 0.001$. (D) LID-1 and NDO-BSA reactivities within sera from a prospective study using 10 U.S.-based individuals who were immunized with BCG were assessed. Sera were collected at regular intervals following BCG immunization. The solid circle at day zero designates the reactivity of a leprosy patient serum sample that was included as a positive control.

LID-1 reactivity can diagnose leprosy before clinical symptoms. Having demonstrated that the LID-1 fusion molecule retained the ability to diagnose leprosy patients but lacked responses to EC sera, we obtained sera from a prospective study conducted in Cebu City, Philippines, between 1985 and 1991 (11). In that study, household contacts of leprosy patients were monitored over a prolonged period of time, and some developed clinical MB leprosy. In sera from the individuals who developed MB leprosy, as previously reported, anti-PGL-I levels increased before leprosy was diagnosed by clinical exam (Fig. 6A). Our data also indicate that anti-LID-1 antibody levels began to increase markedly as soon as 1 year prior to clinical diagnosis (Fig. 6A). For many of the patients (7 of 11, 64%) the increase in the anti-LID-1 IgG response was strikingly more obvious than the increase in the anti-PGL-I IgM response (Fig. 6B). Those patients that developed clinical leprosy had anti-PGL-1 antibody levels not dissimilar to many individuals who did not develop leprosy (Fig. 6C). The difference in anti-LID-1 antibody levels was much clearer, with a much larger differentiation between the positive responses of patients who developed leprosy compared with the extremely low levels of anti-LID-1 antibody in individuals who did not develop leprosy (Fig. 6C). Taken together, these data indicate that LID-1 is capable of providing an early serological diagnosis of leprosy.

LID-1 does not react with sera from individuals recently exposed to BCG. To examine in detail if leprosy diagnosis could be complicated by exposure to or infection with other mycobacteria, we also examined sera collected longitudinally from 10 U.S.-based individuals who were immunized with BCG. None of these BCG-immunized individuals developed positive serological responses against LID-1 or NDO-BSA (Fig. 6D). These data indicate that LID-1 can provide a clear diagnosis of *M. leprae* infection prior to the onset of signs that permit clinical leprosy diagnosis and that LID-1-based diagnostic tests could be used to expedite leprosy treatment.

DISCUSSION

Current diagnosis of leprosy is based on the appearance of clinical signs, and it is well established that the earlier a patient is identified the better their response to treatment. In addition, MB leprosy patient household contacts have a higher risk of developing clinical leprosy than contacts of PB leprosy patients (10, 12). This has been attributed to increased shedding and spreading of viable bacteria by MB leprosy patients (2). Accurate and early detection of *M. leprae*-infected individuals will open the possibility of earlier treatment that could both prevent disability and significantly reduce leprosy transmission.

We have evaluated the serological responses to a variety of *M. leprae* protein antigens in an attempt to discover antigens that can improve diagnosis of leprosy by detecting patients with a low BI (PB leprosy patients or early MB leprosy patients). We demonstrated that (i) ML0405 and ML2331 can be used to diagnose MB leprosy patients independently of geographic location; (ii) ML1556c can recognize some PB patients (al-

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though it is recognized by some TB sera as well); (iii) ML0405 and ML2331 can be used for diagnosis of some PB patients; (iv) a fusion construct of ML0405 and ML2331 (LID-1) retains diagnostic capability; and (v) LID-1 can provide a clear leprosy diagnosis before the onset of clinical symptoms. These findings will improve both leprosy diagnosis and patient care.

One approach for the early detection of *M. leprae* infection is through serological diagnosis. We have conducted screening to identify *M. leprae* antigens that have not previously been described, and we then evaluated the diagnostic potential of these antigens with leprosy patient sera. In this study, the diagnostic potential of select antigens was assessed in clinically disparate leprosy patient groups, ranging from MB patients who presented with large bacterial burdens and large skin lesions to PB patients who presented with low or absent bacterial burdens and a few, small skin lesions. As expected, MB leprosy patients were easier to identify by serological assays and typically yielded higher responses than PB patients. Unexpectedly, close examination of patients with a low BI from the Philippines indicated that some patients exhibited strong responses against the ML1556c protein. The responses of Filipino PB patients to ML1556c were often greater than those of MB patients. These results suggested the utility of this protein either as an adjunct to antigens that could identify MB patients to provide a cross-spectrum leprosy diagnosis or as a stand-alone protein for PB leprosy diagnosis. An objective and differential diagnosis of MB or PB leprosy could lead to better treatment of patients by guiding the multidrug therapy regimen provided to them.

We also analyzed the diagnostic potential of each antigen within geographically disparate groups of patients, from the Philippines and two sites in Brazil. In the Brazilian (Goiânia) PB leprosy patient group, ML1556c provided only a few positive responses; this dampened the enthusiasm for ML1556c to be a widely used diagnostic or prognostic leprosy antigen. Of interest, many PB leprosy patients in Brazil (both Goiânia and Salvador) could be diagnosed by ML0405 reactivity, and several PB patients (Salvador) could be diagnosed with ML2331 reactivity. It is unclear if the differences in the responses of patients from different geographic locations are related to differences in *M. leprae* strains or to regional variations in host genetics. These possibilities might be addressed by analysis of patient sera on fragments of ML1556c or by a survey of anti-ML1556c antibody on lysates of different *M. leprae* strains. Regardless, the observed differences indicate the importance of examining antigen-specific responses in several regions when considering their ability to diagnose leprosy globally.

Given that the ML0405Tr and ML2331 proteins could provide diagnosis of leprosy, we made a fusion protein (LID-1) of these individual components. After ensuring the fusion protein retained reactivity against leprosy sera from Salvador, Brazil, we tested the antigens against sera from Japan. As with results obtained using sera from Brazil, Japanese MB leprosy patient sera reacted as strongly with the fusion LID-1 as with the ML0405 and ML2331 components. In addition, some Japanese PB leprosy patient serum antibodies recognized these antigens.

Studies have argued that the presence of anti-PGL-I antibodies is an indicator of leprosy development, but this has been debated (5, 6, 14, 15). Many contacts of leprosy patients have anti-PGL-I antibodies but do not develop disease, limiting the capacity of PGL-I-based assays to predict disease development. Indeed, PGL-I-based tests are typically marketed as a support reagent to confirm clinical diagnosis and aid leprosy classification but are not recommended for use as a stand-alone for diagnosis (19). The differential in responses of sera from contacts that developed leprosy compared with contacts that did not develop leprosy was much greater for LID-1 than PGL-1. We demonstrated that LID-1 is capable of providing an early serological diagnosis of MB leprosy. A clear and early diagnosis was achieved in 7 of 11 contacts of leprosy patients who themselves went on to develop clinical leprosy. For the small panel of sera tested, the time benefit of a LID-1-based diagnosis over a clinical-based diagnosis was 6 to 8 months. Thus, screening for LID-1-reactive antibodies, either in the general population or within more focused at-risk populations, could significantly expedite treatment of leprosy patients and, also, affect transmission rates by reducing the number of individuals who develop large bacterial burdens. As another benefit, antibody levels against LID-1 dropped following the implementation of drug treatment in these individuals, and thus the reduction and disappearance of antibodies against LID-1 may be a useful measure of multidrug therapy efficacy.

We are currently evaluating additional antigens, diagnostic formats, and different geographic sources of patient sera with the objective of early and simple identification of leprosy patients regardless of incidence locality.

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The frequency of drug resistance mutations in *Mycobacterium leprae* isolates in untreated and relapsed leprosy patients from Myanmar, Indonesia and the Philippines

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Summary

Introduction The magnitude of drug resistance in *Mycobacterium leprae* to dapsone, rifampicin, and ofloxacin was studied in three Southeast Asian countries with a high prevalence of leprosy.

Methods *M. leprae* from the skin of leprosy patients was collected in North Maluku and North Sulawesi in Indonesia, Yangon in Myănmar, and Cebu in the Philippines. Mutations in the drug resistance determining regions in the *folP1*, *rpoB*, and *gyrA* genes, which have been proven to confer resistance, were analysed. In addition, samples from 51 newly diagnosed cases and 13 patients with leprosy relapse in Cebu were submitted for susceptibility testing in the mouse footpad.

Results Of 252 isolates obtained from new cases, 3% were dapsone resistant and 2% were rifampicin resistant. In samples taken from patients with relapsed leprosy ($n = 53$), significantly more resistance mutations were detected: 15% had dapsone resistance mutations, and 8% had rifampicin resistance mutations. Two patients

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with relapsed leprosy had mutations for both dapsone and rifampicin resistance. No mutations conferring quinolone resistance were detected. No mutations were detected in the *folP1* gene of *M. leprae* isolates with a low degree of resistance to dapsone.

Discussion Detection of drug-resistant cases by mutation detection in the drug resistance determining region of the genome is a practical method for monitoring resistance. A comparison of the results obtained in this study with previous data obtained prior to the use of multidrug therapy (MDT), does not indicate clearly whether the magnitude of drug resistance has changed. Larger studies of resistance mutations in *M. leprae* isolated from patients with relapsed leprosy are needed to confirm our results.

Conclusion We recommend monitoring the magnitude of drug resistance globally, by testing *M. leprae* DNA from relapse cases and a representative sample of new cases.

Introduction

Multidrug therapy (MDT) was introduced for leprosy control to minimise the development of drug resistance in *Mycobacterium leprae*.[1] Implementation of MDT in leprosy control markedly decreased the global prevalence of the disease during the last two decades, as expected,[2] but isolates with resistance to one or more antibiotics have been detected in many areas.[3–9] Comprehensive data on the magnitude of drug resistance is crucial to evaluate the efficacy of MDT and to maintain the effectiveness of the current leprosy control strategy; the mouse footpad method for drug susceptibility testing is not, however, applicable for large-scale surveillance of the global level of resistance, because it is cumbersome, time-consuming, and available in only a few laboratories in the world. Also, although knowledge of the drug susceptibility of the causative organism of individual patients initiating treatment may be beneficial, the footpad method is impractical for this purpose. Resistance to the anti-leprosy drugs, dapsone, rifampicin and ofloxacin, evolves by amino acid substitution at the binding sites of these drugs. The elucidation of mechanisms for resistance enables us to examine susceptibility to these drugs by a DNA-based assay of PCR-direct DNA sequencing.[4–16]

In the present study, the frequency of *M. leprae* mutations in the drug resistance determining region (DRDR) in the *folP1*, *rpoB*, and *gyrA* genes, which have been proven to confer resistance to dapsone, rifampicin, and ofloxacin, respectively, were examined. With this methodology, a large number of isolates were tested to obtain useful data for exact analysis of drug resistance levels, and the frequency of drug resistance was determined by pertinent DNA sequencing of *M. leprae* isolates from new and relapse cases in three Southeast Asian countries, namely, Indonesia, Myanmar, and the Philippines.

Materials and Methods

M. leprae from the skin of leprosy patients was collected in North Maluku and North Sulawesi in Indonesia (2000–2005), Yangon in Myanmar (2003–2005), and Cebu in the Philippines (2001–2006). The samples were obtained from patients before starting MDT (new cases), from patients treated with MDT for up to 4 months (recent cases), and from patients with relapse (defined as patients who developed new skin lesions after the completion of MDT and whose BI had increased by more than 2 log units at any site[17]).

Bacterial specimens were obtained from the skin lesions of patients by the standard slit skin smear method commonly utilised for assessment of the bacterial index (BI), using a disposable scalpel blade.[18] The material remaining on the blade after doing the smear was used for the study, the blade being soaked in 1 ml of 70% ethanol and kept in a separate vial at room temperature until analysis.

Additionally, *M. leprae* from skin biopsies from 64 patients, including 51 newly diagnosed and 13 relapse cases in Cebu, was submitted for susceptibility testing in the mouse footpad.[19] Groups of mice were infected in both hind footpads with 5000 *M. leprae* and fed continuously a diet containing either no drug, dapsone 0·01%, dapsone 0·001%, dapsone 0·0001%, or clofazimine 0·001%, while other mice received rifampicin 10–20 mg/kg/5 times weekly by gastric gavage. Six months after footpad inoculation *M. leprae* was enumerated from those footpads. Drug resistance was deemed to be present when the number of *M. leprae* exceeded 100,000 viable bacilli in drug treated mice. Sequences in the DRDR of each gene were analysed, in DNA recovered from bacilli which grew in the footpads of mice treated with dapsone.

For the analysis of drug resistance by mutation detection, the blades were sent to Japan in separate, labeled tubes and the bacilli-containing tissues were removed from the tip of the blade using a sterile toothpick. One toothpick was used for each blade to avoid cross-contamination. DNA templates were prepared using a previously described method.[20] Mutations in the *folP1*, *rpoB*, and *gyrA* genes were analysed by PCR-direct DNA sequencing. DNA fragments containing codons known to be associated with resistance for dapsone, rifampicin, and ofloxacin were amplified by nested PCR. Nested PCR was carried out using a G mixture of the FailSafe PCR System (EPICENTRE, Madison, WI, USA) in a 25 μl volume.

Primers were designed according to the sequence of *folP1* (accession No. AL583917, Gene ML0224), *rpoB* (accession No. AL583923, Gene ML1891), and *gyrA* (accession No. AL583917, Gene ML0006) of *M. leprae*. The sequences of the primers are listed in Table 1.

DNA fragments corresponding to the whole *folP1* gene of isolates found to be dapsone resistant to a low degree, were sequenced as described by Kai *et al.*[15] The PCR programme consisted of one hold cycle of 2 min at 94 °C linked to a three-step cycle of 30 s at 94 °C, and 30 s at 56 °C, and 30 s at 72 °C for 30 cycles followed by a final hold cycle of 5 min at 72 °C. PCR fragments were purified and sequenced according to the same protocol as previously described.[20]

Table 1. Sequences of oligonucleotide primers for *M. leprae*

		Primer	Sequence (5′-3′)
folP1 gene	Outer primers	folP1-F1	CTTGATCCTGACGATGCTGT
		folP1-R1	CCACCAGACACATCGTTGAC
	Inner primers	folP1-F2	GATCCTGACGATGCTGTCCAG
		folP1-R2	ACATCGTTGACGATCCGTG
rpoB gene	Outer primers	rpoB-F1	ACGCTGATCAATTATCCGTCC
		rpoB-R1	GTATTCGATCTCGTCGCTGA
	Inner primers	rpoB-F2	CTGATCAATATCCGTCCGGT
		rpoB-R2	CGACAATGAACCGATCAGAC
gyrA gene	Outer primers	gyrA-F1	ATGACTGATATCACGCTGCCA
		gyrA-R1	ATAACGCATCGCTGCCGGTGG
	Inner primers	gyrA-F2	GATGGTCTCAAACCGGTACATC
		gyrA-R2	ACCCGGCGAATTGAAATTG

Isolates with mutations at codons 53 and 55 in the *folP1* gene, at codons 407, 410, 420, 425, 427, in the *rpoB* gene, and at codon 91 in the *gyrA* gene were defined as resistant to dapsone, rifampicin, and ofloxacin, respectively. These mutations have been confirmed to confer resistance to the drug, dapsone,[5,6,8,9,15,16] rifampicin,[4–8,10,13] or quinolone[4–6,8] by the mouse footpad susceptibility test and by mutation detection in the DRDR for each drug. Frequencies of resistance were compared by the Fisher's exact test.

The study was approved by the institutional ethics committee of the National Institute of Infectious Diseases, Japan, and the three local institutional review boards. Informed consent was obtained prior to the collection of bacterial samples.

Results

Biopsies and slit skin smears were analysed from 121 new or recent cases and 10 relapse cases from Indonesia, 54 new or recent cases and 24 relapse cases from Myanmar, and 77 new or recent cases and 19 relapse cases from Cebu. All newly detected cases were treated with WHO MDT.[21] Almost all patients in Indonesia who relapsed were retreated with same WHO regimen. The MB patients who relapsed in Cebu, were retreated with monthly doses of rifampicin 600 mg, ofloxacin 400 mg and minocycline 100 mg for a total of 12 doses. In Myanmar, when susceptibility test results were known after relapse, patients with susceptible bacilli were treated with same WHO regimen. If dapsone resistance was found, patients were treated with clofazimine 300 mg monthly, clofazimine 50 mg daily, and rifampicin 600 mg monthly for one year. In general, these patients have responded well to the alternative treatment, although final follow-up details are not yet available.

The frequency of drug resistance to the three antibiotics studied varied between countries, and between new and relapse cases (Table 2).

In Indonesia, dapsone resistance mutations was found in 1/121 (1%) new and recent cases and 1/10 (10%) relapse cases; in Myanmar, in 4/54 (7%) new and recent cases and 2/24 (8%) relapse cases; and in the Philippines in 2/77 (3%) new cases and 5/19 (26%) relapse cases. In Indonesia, 4/121 (3%) of *M. leprae* isolates from new and recent cases were found to have rifampicin resistant mutations, while 2/10 (20%) relapse cases were found to have rifampicin resistant mutations. In Myanmar, 1/54 (2%) *M. leprae* isolates from new and recent cases were found to have rifampicin resistance mutations, while isolates from 2/24 (8%) relapse cases had rifampicin resistance mutations. In the Philippines, 0/77 (0%) *M. leprae* from new and recent cases had rifampicin resistance mutations and 0/19 (0%) relapse cases had

Table 2. Prevalence of drug resistance in *M. leprae* isolates from Asian countries

New or recent case			Relapse case		
Dapsone	Rifampicin	Ofloxacin	Dapsone	Rifampicin	Ofloxacin
Indonesia (North Maluku and North Sulawesi)					
1/121 (0·8%)	4/121 (3·3%)	ND	1/10 (10%)	2/10 (20%)	ND
Myanmar (Yangon)					
4/54 (7·2%)	1/54 (1·8%)	0/54	2/24 (8·3%)	2/24 (8·3%)	0/24
Philippines (Cebu)					
2/77 (2·6%)	0/77	0/77	5/19 (26%)	0/19	0/19

rifampicin resistance mutations. The frequency of resistance mutations for both dapsone and rifampicin was consistently higher in patients with leprosy relapse than in new cases, in each of the areas studied. In fact, the frequency of both dapsone and rifampicin resistance mutations was significantly higher in the full cohort of relapse cases than in new and recent cases, $P < 0{\cdot}001$ and $P < 0{\cdot}05$ respectively. Ofloxacin resistance was not evaluated in patients in Indonesia, and was found in no new cases (131) or patients with relapse (43) in Myanmar or the Philippines.

Dapsone resistance mutations in isolates in new or recent cases were detected in all three areas. Four isolates with rifampicin resistance mutations were detected in Indonesia and one in Myanmar, among new or recent cases. An isolate with dapsone resistance mutations was found in an Indonesian patient treated for 2 months. Of four patients with dapsone resistance mutations in Myanmar, three were collected before the start of MDT, and one was from a patient treated for 2 months. Two isolates with dapsone resistance mutations were obtained from patients in Cebu before starting MDT. Of four new or recent cases in Indonesia with rifampicin resistance mutations, one sample was obtained before the start of MDT, two were from patients treated for 2 months, and one was from a patient treated for 4 months with MDT. One isolate from a newly diagnosed case in Myanmar had rifampicin resistance mutations. One isolate in Myanmar and another in Indonesia, both from patients with relapse, had both dapsone and rifampicin resistance mutations. No Multidrug resistance was detected other than these two cases, in all three areas.

The mutations detected were as follows. Mutations in the *folP1* gene included one case of ACC to GTC (Thr → Val) and one case ACC to AGA (Thr → Arg) at codon 53; seven cases of CCC to CTC (Pro → Leu), two cases to TCC (Pro → Ser), two cases to CGC (Pro → Arg), and two cases to CGT (Pro → Arg) at codon 55. Mutations in the *rpoB* gene included one case of GAT to TAT (Asp → Tyr) at codon 410, one case of CAC to GAC (His → Asp) at codon 420; six cases of TCG to TTG (Ser → Leu) and one case of TCG to ATG (Ser → Met) at codon 425. The high frequency of the mutation TCG to TTG at codon 425 is the same result as previously observed in other areas.[10,12] No mutation was demonstrated in the *gyrA* gene of isolates from any area.

Of 64 isolates tested by the mouse footpad method, one isolate had dapsone resistance mutations to a high degree (HD), two had dapsone resistance mutations to an intermediate degree (ID), and 5 had dapsone resistance mutations to a low degree (LD) (Table 3).

Table 3. The results of susceptibility testing for dapsone by the mouse footpad method and sequencing of the *folP1* gene in *M. leprae*

	Mutation			Mouse footpad method results			
Isolate	DHPS substitution	*folP1* mutation	Degree of resistance	0·0001%	0·001%	0·01%	Controls
01Mat02	Thr53Val	ACC53GTC	High	5/5	5/5	5/5	5/5
NCR	Thr53Arg	ACC53AGA	Intermediate	5/5	6/6	0/6	7/7
02Mat47	Pro55Leu	CCC55CTC	Intermediate	5/6	4/4	0/5	6/6
01Mat01	None	None	Low	5/5	0/3	0/6	3/6
01Mat03	None	None	Low	5/8	0/7	0/7	8/8
02Mat25	None	None	Low	4/5	0/5	0/5	5/5
EER	None	None	Low	5/7	0/5	0/8	11/11
MMR	None	None	Low	3/5	0/6	0/6	6/6

The mutation ACC to GTC at codon 53 in the *folP1* gene was detected in the HD isolate, while mutations ACC to AGA at codon 53, and CCC to CTC at codon 55 were detected in the ID isolates. No mutation was demonstrated at either codon in the *folP1* gene of the five isolates with LD in Cebu.

Discussion

The proportion of isolates with dapsone resistance mutations among new and recent cases was 0·8%, 7·2% and 2·6% in Indonesia, Myanmar and the Philippines, respectively. These frequencies of primary dapsone resistance, though of some concern in Myanmar, are generally low, as previously found in San Francisco[22] and the Philippines,[23] and are far lower than the almost 1/3 of cases found in Louisiana,[24] Ethiopia[25] and later in the Philippines,[26] the latter groups being almost entirely LD resistance without known mutation of the *folP1* gene. While the number of patients with leprosy relapse assessed for dapsone resistance in this study was small, fully 8/53 (15%) were found to harbour dapsone-resistant genes. Though this frequency is high, except for the two relapse cases with both dapsone and rifampicin resistance, the reinstitution of WHO MDT, containing the only bactericidal agent in that regimen, rifampicin, as well as clofazimine, currently recommended by the WHO for leprosy relapse following MDT,[21] would likely prove effective. It is unclear whether or not the frequency of dapsone resistance has declined since the wide implementation of MDT, since prior to that time the majority of patients with isolates with dapsone resistance mutations harboured LD strains in many areas[24–26] for which there is no identifiable mutation in the *folP1* gene.

Dapsone resistance in *M. leprae* is known to be the result of specific mutations in codons 53 and 55 within the *folP1* gene coding dihydropteorate synthase (DHPS).[5,6,8,9,15,16] Cambau *et al.* showed that of 10 HD or ID isolates with dapsone resistance mutations, 9 isolates harboured mutations at codon 53 or 55, while one ID isolate showed no mutation in the *folP1* gene.[9] Of 6 LD dapsone resistant isolates, five isolates showed no mutation and one showed a mutation at codon 53.[9] No mutation was detected in 22 susceptible isolates.[9] In other studies, all 15 HD isolates with dapsone resistance mutations showed mutations at codon 53 or 55, while 7 susceptible strains showed no mutation in the *folP1* gene.[5,6,8,15,16] Five LD isolates from the Philippines in our study harboured no mutation in the *folP1* gene. These were all resistant in the mouse foot pad to 0·0001% dapsone in the diet, but not to higher levels. Therefore, almost all isolates identified as dapsone resistance by mutation detection are resistant to dapsone to a high or intermediate degree. However almost no low degree isolates with dapsone resistance mutations could be detected as resistant by mutation detection. Though Shepard[27] found that *M. leprae* obtained from untreated leprosy patients in an earlier era were consistently inhibited by 0·0001% dapsone in diet, Rees[28] found a few were not inhibited at that level. The finding that isolates in the mouse with resistance to dapsone at a concentration of 0·0001% is not associated with a mutation in the *folP1* gene suggests perhaps that resistance to that level of dapsone is found at the far extreme of the dapsone-sensitive *M. leprae* distribution. This concept is important as the vast majority of previously identified dapsone resistance, both primary and secondary, was found resistant only to this level and not higher ones. In any event, it is considered that such cases have no clinical significance, since administration of 0·0001 g DDS per 100 g mouse diet is of the same order as that observed in humans receiving 1 mg DDS daily[29] and the usual dosage of DDS in MDT is 100 mg daily.

As all isolates with HD or ID isolates with dapsone resistance mutations exhibited mutations at codon 53 or 55, the PCR direct sequencing method will detect all clinically significant dapsone resistant cases and the method is feasible for detecting isolates with dapsone resistance mutations.

Although two patients with dapsone resistance mutations among new or recent cases were treated for 2 months with MDT, they can be classified as primary dapsone resistant cases, since the multiplication of the bacilli is very slow. Resistant strains could not replace susceptible strains in the patient within such a short time.

A striking finding of the study is the detection of isolates with rifampicin resistance mutations amongst patients newly or recently detected and a greater frequency amongst relapse cases. Though in the areas studied the rate of primary rifampicin resistance, 2%, is reasonably low, the rate in patients with leprosy relapse, 8%, is of concern, as well as the two relapse cases who were resistant to both dapsone and rifampicin. In these two instances, retreatment with WHO MDT[21] would need to be prolonged for the improvement of condition since MDT for these cases is monotherapy with clofazimine. Though the number of cases with leprosy relapse in this study is small and as previously mentioned, rifampicin is the key and sole bactericidal component of MDT, perhaps reconsideration of an alternative treatment for those who relapse after MDT is in order; this might reasonably include minocycline[30] and moxifloxacin.[31] Larger studies of rifampicin resistance mutations in relapse cases are needed to ascertain if our current results are generally representative.

Rifampicin resistance is conferred by mutations in the beta subunit of RNA polymerase coded by the *rpoB* gene. Mutations at codon 407, 410, 420, 425 and insertions between 408 and 409 have been confirmed as associated with rifampicin resistance.[4–8,10,11,13,14] Mutations at codons 401,[7] 416,[32] and 427[5,7] have also been found but it has not been revealed clearly whether these mutations confer rifampicin resistance in *M. leprae*. Although mutations at 401 were detected in the *rpoB* gene of rifampicin resistance isolates,[7] it is not proven whether this mutation is associated with rifampicin resistance or not, since the mutation occurred simultaneously with a mutation at codon 420 which is known to be associated with rifampicin resistance. Mutations at 416 were also detected but no confirmatory data from the mouse foot pad susceptibility tests were available,[32] although it is known that this mutation confers rifampicin resistance in *Mycobacterium tuberculosis*.[31] A mutation at codon 427 was detected in one clinical isolate[5] and one rifampicin resistant isolate.[7] The former case was not confirmed by the mouse footpad method and the latter one was detected concordantly with a mutation at 425, although the mutation at this position is known to be associated with rifampicin resistance in *Mycobacterium tuberculosis*.[33] Clarification of the association between these mutations and rifampicin resistance by the mouse footpad method is highly recommended. In studies so far reported on the association of rifampicin resistance with mutations in the *rpoB* gene, only one rifampicin resistant isolate showed no mutation.[5] Taking these results into consideration, detecting rifampicin resistant cases by mutation detection in the *rpoB* gene is a practical method for monitoring resistance to rifampicin.

The prevalence of rifampicin resistance mutations in cases with leprosy relapse was higher than the incidence in new cases, so this also must be monitored carefully. A previous study showed that among a total of 404 multibacillary patients who had been treated with various rifampicin containing regimens, 22 (5·4%) were resistant to rifampicin.[34] Although a small sample size, the prevalence of rifampicin resistance mutations in Indonesia and Myanmar is higher than that found previously, before MDT was implemented. Two possible

reasons for a high prevalence of drug resistance are poor compliance, both with self-administration of drugs and premature discontinuation of therapy,[35] and prior monotherapy with rifampicin, either for leprosy or as part of the standard chemotherapy for tuberculosis which would also expose leprosy patients to rifampicin monotherapy.

Of the two patients with leprosy relapse with doubly resistant mutations (dapsone and rifampicin), the one from Myanmar had previously received monotherapy with dapsone (1973–1977), followed by monotherapy with rifampicin (1982–1986).[36] Of the five patients with isolates with dapsone resistance mutations in the Philippines, three had received prior dapsone, one as monotherapy, and two others either as the sole agent or in one instance combined with clofazimine and in another combined with clofazimine and rifampicin. Though the other relapse patients in the Philippines, as well as those from Indonesia and Myanmar, were treated with WHO MDT, it is unclear whether patients had adhered to the regimen and completed therapy.

Five isolates with ofloxacin resistance mutations have been reported,[4–8] all isolates having the mutation GCA to GTA (Ala → Val) at codon 91 (numbering system as used for *M. leprae*) in *gyrA* gene. A strain with the mutation GGC to TGC (Gly → Cys) at codon 89 was reported previously,[5] but resistance to ofloxacin was not confirmed by the mouse footpad method. Two other amino acid changes, Ser at 91, and Asp at 94 (numbering system as used for *M. tuberculosis*), in the *gyrA* gene of *M. tuberculosis* are associated with quinolone resistance.[37] It seems mutations at the codons 89, 92, and 95 in the *gyrA* gene of *M. leprae* also cause quinolone resistance. No mutation at these codons, 89, 91, 92, and 95, was detected in the samples tested. Thus the level of quinolone resistance in the areas investigated is still very low.

The study indicated the existence of primary and secondary resistance to dapsone and rifampicin in countries where many leprosy cases are still detected. A comparison of the results obtained in this study with previous data obtained prior to the use of MDT, does not indicate clearly whether the magnitude of drug resistance has changed.[23,26,33] We consider this study as a first effort to assess the magnitude of drug resistance in the MDT era. In order to preserve the efficacy of MDT and prevent the spread of drug resistant bacilli, carefully designed global studies are recommended, as suggested previously.[38] Monitoring the susceptibility of isolates from each case of leprosy relapse allows optimal treatment to be chosen, by avoiding ineffective drugs and choosing effective compounds. The longitudinal surveillance of levels of drug resistance in new cases in some areas with a high prevalence of leprosy might contribute to predicting the spread of drug resistant strains. The application of the susceptibility test by mutation detection should be attempted, especially in cases where treatment failure seems a possibility.

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Molecular Analysis of RANKL-Independent Cell Fusion of Osteoclast-Like Cells Induced by TNF-α, Lipopolysaccharide, or Peptidoglycan

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Abstract Focusing on the final step of osteoclastogenesis, we studied cell fusion from tartrate-resistant acid phosphatase (TRAP)-positive mononuclear cells into multinuclear cells. TRAP-positive mononuclear cells before generation of multinuclear cells by cell fusion were differentiated from RAW264.7 cells by treatment with receptor activator of nuclear factor kappa B ligand (RANKL), and then the cells were treated with lipopolysaccharide (LPS), followed by culturing for further 12 h. LPS-induced cell fusion even in the absence of RANKL. Similarly, tumor necrosis factor (TNF)-α and peptidoglycan (PGN) induced cell fusion, but M-CSF did not. The cell fusion induced by RANKL, TNF-α, and LPS was specifically blocked by osteoprotegerin (OPG), anti-TNF-α antibody, and polymyxin B, respectively. LPS- and PGN-induced cell fusion was partly inhibited by anti-TNF-α antibody but not by OPG. When TRAP-positive mononuclear cells fused to yield multinuclear cells, phosphorylation of Akt, Src, extracellular signal-regulated kinase (ERK), p38MAPK (p38), and c-Jun NH2-terminal kinase (JNK) was observed. The specific chemical inhibitors LY294002 (PI3K), PP2 (Src), U0126 (MAPK-ERK kinase (MEK)/ERK), and SP600125 (JNK) effectively suppressed cell fusion, although SB203580 (p38) did not. mRNA of nuclear factor of activated T-cells c1 (NFATc1) and dendritic cell-specific transmembrane protein (DC-STAMP) during the cell fusion was quantified, however, there was no obvious difference among the TRAP-positive mononuclear cells treated with or without M-CSF, RANKL, TNF-α, LPS, or PGN. Collectively, RANKL, TNF-α, LPS, and PGN induced cell fusion of osteoclasts through their own receptors. Subsequent activation of signaling pathways involving PI3K, Src, ERK, and JNK molecules was required for the cell fusion. Although DC-STAMP is considered to be a requisite for cell fusion of osteoclasts, cell fusion-inducing factors other than DC-STAMP might be necessary for the cell fusion. J. Cell. Biochem. 101: 122–134, 2007. © 2006 Wiley-Liss, Inc.

Key words: TNF-α; lipopolysaccharide; peptidoglycan; cell fusion; osteoclasts

Abbreviations used: Akt, PKB, protein kinase B; c-Fos, cellular homolog of v-fos; c-Src, cellular homolog of v-src; DC-STAMP, dendritic cell-specific transmembrane protein; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; HRP; horse radish peroxidase; IL-1β, interluekine-1β; IL-6, interluekine-6; JNK, c-Jun NH2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAPK-ERK kinase; MyD88, myeloid differentiation factor 88; NFATc1, nuclear factor of activated T-cells c1; NF-κB, nuclear factor kappa B; OPG, osteoprotegrin ligand; P-, phosphorylated; PBS, phosphate-buffered saline; PGN, peptidoglycan; p38, p38MAPK; p65, 65 kD subunit of NF-κB (RelA); RANKL, receptor activator of nuclear factor kappa B ligand; SDS, sodium dodecyl sulfate; TBST, 150 mM NaCl and 0.1% Tween-20 in 25 mM Tris/HCl, pH 7.6; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor; TRAP, tartrate-resistant acid phosphatase.

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