

Postgenomic Approach To Identify Novel *Mycobacterium leprae* Antigens with Potential To Improve Immunodiagnosis of Infection

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Early detection of *Mycobacterium leprae* infection is considered an important component of strategies aiming at reducing transmission of infection, but currently available diagnostic tools often lack sufficient sensitivity and specificity to reach this goal. Recent comparative genomics have revealed the presence of 165 *M. leprae* genes with no homologue in *M. tuberculosis*. We selected 17 of these genes for further study. All 17 genes were found to be expressed at the mRNA level in *M. leprae* from infected mice and from a multibacillary leprosy patient. Additional comparative genomic analyses of all currently available mycobacterial genome databases confirmed 12 candidate genes to be unique to *M. leprae*, whereas 5 genes had homologues in mycobacteria other than *M. tuberculosis*. Evaluation of the immunogenicity of all 17 recombinant proteins in PBMC from 127 Brazilians showed that five antigens (ML0576, ML1989, ML1990, ML2283, and ML2567) induced significant gamma interferon levels in paucibacillary leprosy patients, reactional leprosy patients, and exposed healthy controls but not in most multibacillary leprosy patients, tuberculosis patients, or endemic controls. Importantly, among exposed healthy controls 71% had no detectable immunoglobulin M antibodies to the *M. leprae*-specific PGL-I but responded to one or more *M. leprae* antigen(s). Collectively, the *M. leprae* proteins identified are expressed at the transcriptome level and can efficiently activate T cells of *M. leprae*-exposed individuals. These proteins may provide new tools to develop tests for specific diagnosis of *M. leprae* infection and may enhance our understanding of leprosy and its transmission.

Although the global prevalence of leprosy has dropped dramatically coinciding with the introduction of multiple-drug therapy, new case detection rates have remained stable over the years at approximately 700,000 new cases per year (26). One interpretation of these findings is that transmission of *Mycobacterium leprae* infection is not significantly affected by current leprosy control measures. In addition to delayed or missed diagnosis of infectious leprosy patients, the lack of tests to measure asymptomatic *M. leprae* infection in contacts also hampers more precise assessment of transmission of *M. leprae*. An important goal would therefore be the development of improved diagnostic tools that are able to detect *M. leprae* infection before clinical manifestations arise and to distinguish *M. leprae* infection from infection with other mycobacteria, such as *M. tuberculosis* and environmental mycobacteria (4).

Diagnosis and classification of leprosy in the field is classi-

cally based on clinical assessment and detection of acid-fast bacilli in skin slit smears or biopsies of suspected cases. There is no specific and sensitive test available that can detect asymptomatic *M. leprae* infection or predict progression of infection to clinical disease. A test that is able to detect *M. leprae* infection is based on the detection of immunoglobulin M (IgM) antibodies to phenolic glycolipid-1 (PGL-I), an *M. leprae*-specific cell surface antigen. However, although this test can detect antibodies in most multibacillary leprosy patients, it has limited value in identifying paucibacillary (PB) leprosy patients and their contacts, since these typically develop cellular rather than humoral immunity (reviewed in reference 20).

Alternative tests that measure cellular rather than humoral immunity, such as skin tests, have been developed in various forms since Mitsuda (7, 19). Analogous to the tuberculin skin test, which is commonly used in tuberculosis, these tests have relied on the use of complex and often incompletely defined mixtures of *M. leprae* components or extracts. Such skin tests have limited value due to their inherent high cross-reactivity with other mycobacteria, resulting in low specificity. For leprosy, such cross-reactivity is particularly problematic in coun-

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tries with high incidence rates of tuberculosis, routine BCG vaccination practice, and high levels of exposure to nonpathogenic environmental mycobacteria.

Prior to the completion of the *M. leprae* genome sequence, the search for *M. leprae*-specific antigens that stimulate cellular rather than humoral immunity led to the identification of several antigens and peptides that were able to induce T-cell responses in vitro (12, 16, 17). Unfortunately, for most of these antigens, homologues were later found in other mycobacteria, including *M. tuberculosis*, which limits the diagnostic potential of these antigens for leprosy (6, 21, 22). For example, *M. leprae* antigens encoded by the *M. leprae* genes ML0049 and ML0050 were recognized by T cells from the majority of *M. leprae* responsive leprosy patients (16, 17). However, despite their limited sequence identity with the *M. tuberculosis* homologues Rv 3875 (ESAT6) and Rv 3874 (CFP10) (36 and 40%, respectively), significant cross-reactivity between *M. leprae* and *M. tuberculosis* ESAT6 and CFP10 proteins was found, indicating that the diagnostic potential of ML0049 and ML0050 proteins in leprosy areas of endemicity with a high prevalence of tuberculosis is limited. Based on these observations, we hypothesized that candidate antigens that are unique to *M. leprae* may provide more specific targets to measure cellular immunity. The recent publication of the genome sequences of *M. leprae* (10), *M. tuberculosis* (11, 13), *M. bovis* (15), *M. smegmatis* (<http://www.tigr.org>), and the almost-completed genome sequences of several other mycobacterial species (*M. avium*, *M. marinum*, *M. paratuberculosis*, and *M. ulcerans* [<http://www.sanger.ac.uk/Projects/>, <http://www.tigr.org>, <http://www.ncbi.nlm.nih.gov/genomes/>, and <http://genopole.pasteur.fr/Mulc/BuruList.html>, respectively]) provided an unprecedented opportunity to identify proteins that are unique to *M. leprae*.

We therefore selected several *M. leprae* candidate antigens that have no homologue in *M. tuberculosis* or other known mycobacteria and studied these as T-cell stimulatory antigens, as determined by gamma interferon (IFN- γ) production in peripheral blood mononuclear cells (PBMC) obtained from leprosy patients, household contacts, tuberculosis patients, and healthy individuals from Brazil. The identification of proteins or peptides unique to *M. leprae* may provide a next generation of tools that may help to distinguish *M. leprae* infection from infection with *M. tuberculosis* or environmental mycobacteria or vaccination with BCG.

MATERIALS AND METHODS

Purification and reverse transcription of *M. leprae* RNA. Two *M. leprae* isolates from geographically distinct regions of the world were used for the present study. *M. leprae* Thai-53 was originally isolated from a skin lesion of an untreated multibacillary (MB) leprosy patient in Thailand in 1982. The other *M. leprae* isolate (NHDP98) was obtained from a lesion of an untreated, Mexican-born, MB leprosy patient in 1990. *M. leprae* was purified from hind footpads of BALBc athymic nude mice (Hsd:Athymic Nude; Harlan, Indianapolis, IN) at the Laboratory Research Branch of the National Hansen's Disease Programs, Baton Rouge, LA, as described previously (24). A punch biopsy from a skin lesion of a MB leprosy patient from the United States, previously obtained for diagnostic testing and stored frozen at -70°C in O.C.T. cryoprotective medium (Tissue Tek, Columbus, OH), was thawed at room temperature and removed from the cryopreservative medium. The tissue was washed three times in 1 ml of sterile phosphate-buffered saline (PBS; Sigma, St. Louis, MO) and minced finely. *M. leprae* RNA was extracted from purified bacteria or from minced biopsy tissue by using a previously described, single-tube homogenization-RNA extraction protocol (25). cDNA was made from 10 μl of *M. leprae* RNA by using random

hexamer primers (25), the Advantage cDNA Polymerase Mix, and Advantage RT-for-PCR kit in a final volume of 50 μl according to manufacturer's recommendations (BD Biosciences, Clontech, Palo Alto, CA). Controls for DNA contamination consisted of total *M. leprae* RNA incubated with the reverse transcription reagents excluding reverse transcriptase. Specificity controls consisted of cDNA made from BALB/c mouse spleen total RNA (BD Biosciences, Clontech) and human PBMC-derived RNA.

PCR amplification and DNA sequencing. DNA was purified from 2×10^{10} mouse footpad-derived *M. leprae* Thai-53 (25). DNA concentration was obtained by spectrophotometric means, and total DNA was stored at -80°C . PCR primers and amplification protocols were designed for 19 *M. leprae* genes by acquiring gene sequences from the *M. leprae* genome database (http://www.Sanger.ac.uk/Projects/M_leprae) and by using Omega 2.0 Primer Design software (Oxford Molecular, Ltd.). PCR assays were initially characterized by using 1 ng of *M. leprae* Thai-53 DNA and mouse and human cDNA as specificity controls. PCR fragments were separated by gel electrophoresis (25). The PCR products were purified by using QIAquick PCR purification kit spin columns (QIAGEN, Carlsbad, CA) and the DNA sequence of each PCR fragment was obtained by using automated DNA sequencing (GeneLab; SVM, LSU, Baton Rouge, LA). Initially, cDNA from *M. leprae* isolates was analyzed for the presence of a 507-bp fragment of the *M. leprae* gap transcript by using 30 cycles of PCR. cDNA templates from all *M. leprae* preparations were adjusted to yield equivalent amounts of cDNA for subsequent PCR analysis based on these amplification results. mRNA expression analysis was then performed for all genes using cDNA from each *M. leprae* preparation and 40 cycles of each PCR assay.

Production and testing of *M. leprae*-specific recombinant antigens. Nineteen *M. leprae* candidate genes (see Results for selection) were amplified by PCR from genomic DNA of *M. leprae* (generously provided by the late M. J. Colston) and cloned by using the Gateway technology platform (Invitrogen, Carlsbad, CA) with pDEST17 expression vector containing an N-terminal histidine tag (Invitrogen). Sequencing was performed on selected clones to confirm identity of all cloned DNA fragments. Recombinant proteins were overexpressed in *Escherichia coli* BL21(DE3) and purified as described to remove possibly present endotoxin (14). Each purified *M. leprae* protein was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Coomassie brilliant blue staining and Western blotting with an anti-His antibody (Invitrogen) to confirm size and purity. Endotoxin contents were below 50 IU of recombinant protein/mg as tested by using a Limulus Amebocyte Lysate assay (Cambrex, East Rutherford, NJ). All proteins were tested to exclude antigen nonspecific T-cell stimulation by using PBMC-based IFN- γ release assays of different healthy, *M. leprae*-unexposed, and BCG-negative donors.

Study subjects. Brazilian leprosy patients were recruited from the Leprosy Out-Patient Unit, Leprosy Laboratory (Oswaldo Cruz Institute, Rio de Janeiro, Brazil). Among these were 15 PB leprosy patients, 19 MB leprosy patients, 13 MB patients with leprosy reactions (Rx), and 21 healthy household contacts of MB leprosy patients (HHC). Leprosy patients were diagnosed and classified based on clinical, bacteriological, and sometimes histopathological findings. MB patients were treated with rifampin, dapson, and clofazimine, and PB patients were treated with rifampin and dapson. All MB patients were skin slit smear positive, whereas all PB patients were skin slit smear negative. The 43 endemic healthy controls were blood donor volunteers attending the Blood Bank of the Santa Casa da Misericórdia, Rio de Janeiro, Brazil, and consisted of 30 *M. leprae* T-cell nonresponders and 13 *M. leprae* T-cell responders, as determined by their T-cell response (IFN- γ) to whole *M. leprae* bacterial extracts (see below). All controls were examined for clinical signs of leprosy and the presence of a BCG vaccination scar. An additional 16 Brazilian tuberculosis patients were recruited from the Ambulatory Service, District Hospital Raphael de Paula e Souza, Rio de Janeiro, Brazil. All patients tested in the present study were human immunodeficiency virus type 1 seronegative. Written informed consent was obtained from all individuals before participating in the study. Ethical approval of the study protocol was obtained through the Institutional Review Boards. Details of all studied groups are summarized in Table 1.

Cell culture and stimulation. Venous blood was obtained from study participants in heparinized tubes and PBMC isolated by Ficoll density centrifugation. PBMC (2×10^6 cells/ml) were plated in duplicate or triplicate cultures in 96-well flat-bottom plates (Costar Corp., Cambridge, Mass.) in 200 μl of Adoptive Immunotherapy medium (AIM-V; Gibco/well supplemented with 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM L-glutamine (Invitrogen). Recombinant protein or irradiated armadillo-derived *M. leprae* sonicate (Colorado State University, Fort Collins) were added at final concentrations of 10 $\mu\text{g}/\text{ml}$. After 5 days of culture at 37°C at 5% CO_2 and 70% relative humidity, 75- μl supernatants were removed from each well, pooled, and frozen in aliquots at -20°C . IFN- γ levels were determined by enzyme-linked immunosorbent assay

TABLE 1. Brazilian study subjects^a

Parameter	MB	MB in Rx	PB	HHC	ML+HC	ML-HC	TB
No. of individuals per group	19	13	15	21	13	30	16
Clinical leprosy diagnosis	+	+	+	-	-	-	-
% T-cell response to <i>M. leprae</i> (%) ^b	0	92	100	62	100	0	31
% Detected with anti-PGL-I IgM	100	69	13	14	0	0	19
% Detected using <i>M. leprae</i> antigen ^c	0	85	93	62	100	7	50

^a MB, multibacillary; PB, paucibacillary; MB in Rx, leprosy patients with reaction; HHC, healthy household control; ML+HC, healthy endemic controls showing significant T-cell responses to *M. leprae* extracts; ML-HC, showing no T-cell responses to *M. leprae* extracts; TB, tuberculosis patients.

^b T-cell responses to *M. leprae* extracts were considered positive if IFN- γ production levels in response to one or more antigens were >200 pg/ml.

^c T-cell responses to five individual *M. leprae* antigens (ML0576, ML2283, ML2567, ML1989, and ML1990) were analyzed. Individuals were considered positive if they recognized one or more antigens (>200 pg/ml).

(ELISA; R&D Systems, Inc., Minneapolis, MN). The cutoff value to define positive responses was set beforehand at 200 pg/ml. The level of sensitivity was 20 pg/ml. Values for unstimulated cell cultures (usually <50 pg/ml) were subtracted from the values found for stimulated cultures in all analyses. As a positive control, phytohemagglutinin (1%) and purified protein derivative (tuberculin, RT49; Statens Serum Institute, Copenhagen, Denmark) of *M. tuberculosis* (5 μ g/ml) were used. Due to the sometimes-limited numbers of PBMC that could be isolated, not all 19 *M. leprae* antigens could be tested for all individuals.

Anti-PGL-I IgM antibodies. Detection of IgM antibody directed against phenolic glycolipid I (PGL-I) of *M. leprae* was analyzed by ELISA as described previously (9). Briefly, blood was allowed to clot for 30 min, and serum was harvested by centrifugation. Sera were kept frozen at -20°C until use. The antigen used in ELISA was NT-P-BSA, and cutoff values for positive responses were set beforehand at an optical density at 450 nm (OD₄₅₀) of 0.200 (9).

Statistical analysis. Differences in IFN- γ levels between groups of patients, controls, and antigens were analyzed with the SPSS software package by using the Mann-Whitney U test. *P* values were corrected for multiple comparisons. The statistical significance level used was a *P* value of <0.05.

RESULTS

Selection of candidate proteins unique to *M. leprae*. The *M. leprae* genome contains 165 candidate genes with no orthologue in *M. tuberculosis* (10; <http://genolist.pasteur.fr/Leproma/>). Whereas 29 of these genes have attributable functions, the remaining 136 were previously described as belonging to "functional classification VI" (i.e., with unknown function) and contained no similarity to known genes at the time of selection (10).

Short hypothetical proteins encoding fewer than 70 amino acids were excluded from further analysis. The remaining 126 hypothetical proteins were analyzed by the TEPITOPE-2000 program (<http://www.vaccinome.com/>), a software package for the prediction of promiscuous HLA-class II binding peptides and human T-cell epitopes (23). A total of 24 hypothetical proteins that contained multiple T-cell epitopes predicted to bind $\geq 75\%$ of the tested HLA-DR alleles were scanned next for sequence homology with *M. tuberculosis* by using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). Hypothetical proteins showing an overall homology of $\geq 30\%$ were excluded, resulting in 10 candidate proteins that were unique to *M. leprae*. In

addition, seven hypothetical group VI proteins were added that were located in a single putative operon with one of the above selected proteins. This strategy yielded 17 candidate *M. leprae* antigens. In addition, two candidate *M. leprae* antigens, ML0007 and ML2649, for which homologs were found in several other mycobacteria (Table 2), were included as specificity controls to examine the level of cross-reactivity in tuberculosis patients.

During the course of the present study, information from other mycobacterial genome projects became available, notably the completed sequence of *M. bovis* (15) and the almost completed or draft genome sequences of *M. avium*, *M. smegmatis*, *M. marinum*, *M. paratuberculosis*, and *M. ulcerans*. In Table 2, we further analyzed the 17 hypothetical *M. leprae* proteins selected above against those mycobacterial genome sequences. The results indicated that ML0369, ML0840, ML0925, and ML1601, which do not have orthologues in *M. tuberculosis*, do have orthologues in *M. avium paratuberculosis*, whereas a gene with 67% homology to ML0126 is present in *M. ulcerans* (Table 2). It remains unknown, however, whether these hypothetical genes are indeed expressed in *M. avium paratuberculosis* (whereas our results indicate they are in *M. leprae* [see below]). Following these analyses, ML0573, ML0574, ML0575, ML0576, ML1602, ML1603, ML1604, ML1788, ML1989, ML1990, ML2283, and ML2567 remained unique to *M. leprae* (Table 2).

Expression of *M. leprae*-specific candidate genes in infected murine and human tissue. Since the 19 candidate genes selected above were of unknown function, we first assessed their expression in *M. leprae* bacilli isolated from nude mice. *M. leprae* RNA was extracted from the hind footpad tissue of nude mice 6 months postinfection with *M. leprae*. To analyze whether these genes are also expressed during *M. leprae* infection in humans, their transcriptional activity was also determined in *M. leprae* obtained from a skin lesion of a MB leprosy patient. Specific mRNA for all 19 genes could be detected by reverse transcription-PCR analysis in *M. leprae* from both sources (Table 2).

Grouping of individuals according to T-cell response to whole *M. leprae* extracts. PBMC of the individuals included in the study ($n = 127$) were tested first for IFN- γ responses to crude *M. leprae* extracts (Table 1). Of the 43 healthy control individuals, 30 did not show any detectable in vitro T-cell response to *M. leprae*. Since they also lacked detectable anti-PGL-I IgM levels (see above), they most likely had not been *M. leprae* infected and were therefore regarded as negative endemic controls in further data analysis (controls; Table 1 and Fig. 1). The other 13 healthy endemic controls showed strong responses to *M. leprae* and therefore were classified as potentially *M. leprae* exposed controls. These 13 individuals were combined with the 21 healthy household contacts of leprosy patients (HHC), of whom the majority ($n = 13$; 62%) were *M. leprae* responders; together, they were designated as (H)HC group (Fig. 1 and Table 1).

All leprosy patients were tested similarly for responses to *M. leprae* extracts. Both PB leprosy patients and MB patients with a history of leprosy reactions (Rx) showed significant IFN- γ production in response to *M. leprae* extract. Since these two groups did not differ significantly in their IFN- γ response to *M. leprae*, they were combined (designated as PB/Rx) for further

TABLE 2. *M. leprae*-specific candidate antigens^a

Gene accession no.	Functional classification	Protein type	Length (bp)	Size (kDa)	Murine and human RT-PCR	% Identity ^b with:									
						<i>M. bovis</i> AF2122/97 (bIasp) ^d	<i>M. tuberculosis</i> H37Rv (bIasp) ^d	<i>M. paratuberculosis</i> K10 (bIasp) ^e	<i>M. microti</i> # (tblasn) ^d	<i>M. marinum</i> # (tblasn) ^d	<i>M. avium</i> # (tblasn) ^f	<i>M. ulcerans</i> # (tblasn) ^g	<i>M. smegmatis</i> MC2# (tblasn) ^f		
ML0007	II.C.4	Conserved membrane	912	32	+	53 (Mb0007)	56 (Rv0007)	53 (MAP0007)	48	59	59	56	38		
ML0126	VI	Hypothetical	822	31	+	<30	<30	<30	<30	<30	<30	67	<30		
ML0369	VI	Hypothetical	357	13	+	<30	<30	38 (MAP4250c)	<30	33	39	37	33		
ML0573	VI	Hypothetical	276	10	+	<30	<30	<30	<30	<30	<30	<30	<30		
ML0574	VI	Hypothetical	318	11	+	<30	<30	<30	<30	<30	<30	<30	<30		
ML0575	VI	Hypothetical	219	7	+	<30	<30	<30	<30	<30	<30	<30	<30		
ML0576	VI	Hypothetical	231	8	+	<30	<30	<30	<30	<30	<30	<30	<30		
ML0840	VI	Hypothetical	1,305	48	+	<30	<30	59 (MAP2122)	<30	<30	64	<30	47		
ML0927	VI	Hypothetical	309	11	+	<30	<30	36 (MAP1963c)	<30	36	35	33	<30		
ML1601	VI	Hypothetical	366	13	+	<30	<30	33 (MAP3249)	<30	<30	33	<30	<30		
ML1602	VI	Hypothetical	321	11	+	<30	<30	<30	<30	<30	<30	<30	<30		
ML1603	VI	Hypothetical	261	9	+	<30	<30	<30	<30	<30	<30	<30	<30		
ML1604	VI	Hypothetical	390	14	+	<30	<30	<30	<30	<30	<30	<30	<30		
ML1788	VI	Very hypothetical	465	17	+	<30	<30	<30	<30	<30	<30	<30	<30		
ML1989	VI	Hypothetical	351	13	+	<30	<30	<30	<30	<30	<30	<30	<30		
ML1990	II.C.5	Putative integral membrane	243	9	+	<30	<30	<30	<30	<30	<30	<30	<30		
ML2283	VI	Hypothetical	315	12	+	<30	<30	<30	<30	<30	<30	<30	<30		
ML2567	VI	Hypothetical	480	17	+	<30	<30	<30	<30	<30	<30	<30	<30		
ML2649	IV	Hypothetical ^f	765	28	+	60 (Mb0270c)	60 (Rv0264)	58 (MAP3709c)	60	60	63	59	58		

^a Annotations for gene accession number, functional classification, and protein are according to the Sanger database. Functional classifications II.C.4, conserved membrane proteins; VI, unknown; II.C.5, other membrane proteins; IV, other.
^b Blast reports were performed December 2004; bIasp, protein versus protein; tblasn, protein versus translated DNA. #, Unfinished genomes.
^c According to Leproma (<http://genolist.pasteur.fr/Leproma/>), ML2649 is a putative oligosaccharide deacetylase.
^d <http://www.sanger.ac.uk/Projects/>.
^e <http://www.ncbi.nlm.nih.gov/genomes/>.
^f <http://www.tigr.org>.
^g <http://genopole.pasteur.fr/Mulc/BurnList.html>.

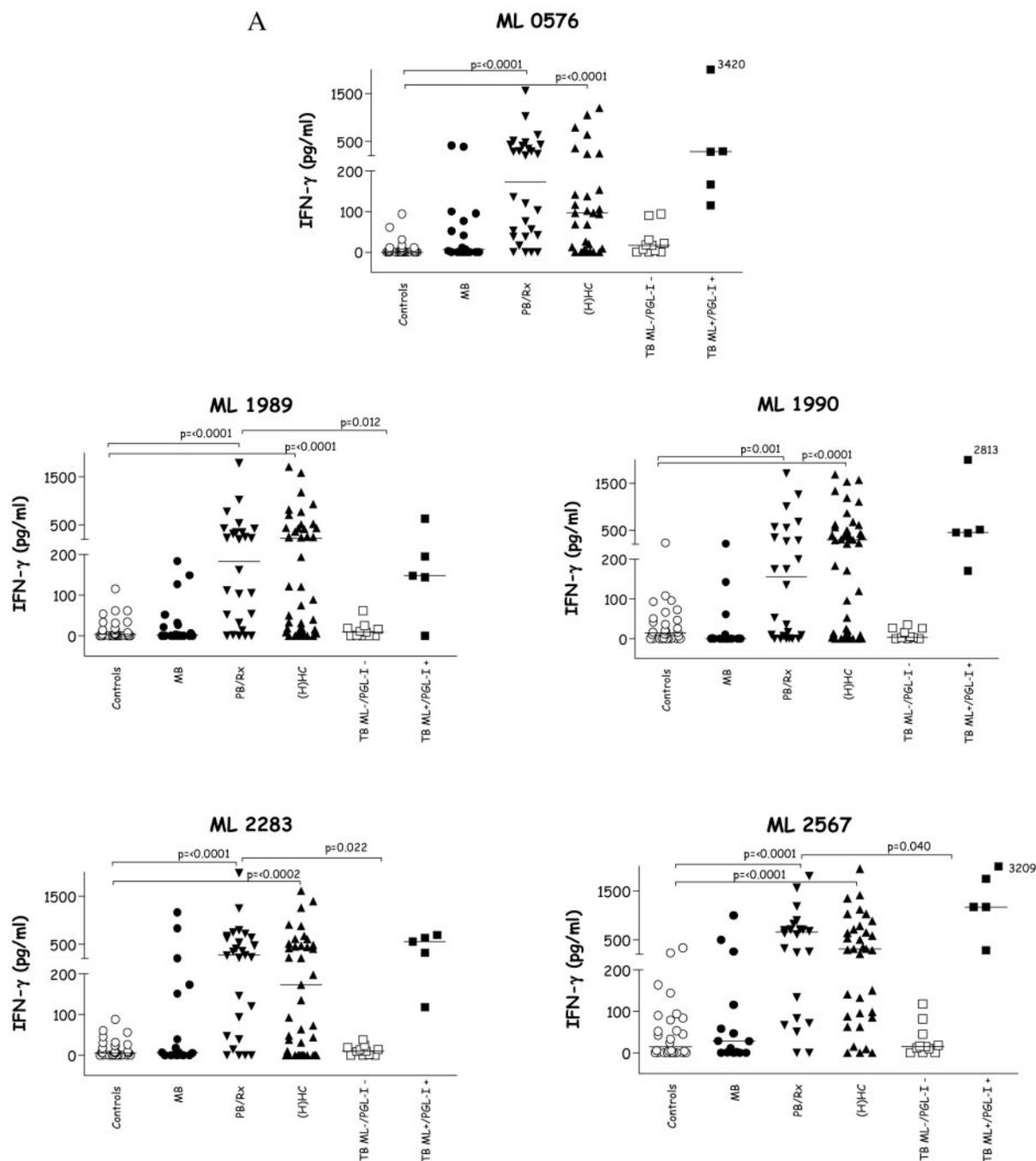


FIG. 1. (A) Ex vivo IFN- γ production against five unique *M. leprae* antigens by PBMC of healthy controls without T-cell responses to *M. leprae* extracts (Controls, $n = 30$) (\circ), MB patients (MB, $n = 19$) (\bullet), PB patients and MB patients with leprosy reactions (PB/Rx, $n = 28$) (\blacktriangledown), healthy household contacts and endemic controls with T-cell responses to *M. leprae* extracts [(H)HC, $n = 35$] (\blacktriangle), tuberculosis patients without anti-PGL-I antibody or *M. leprae*-reactive T cells (TB ML-, $n = 11$) (\square), and tuberculosis patients that show significant T-cell responses to *M. leprae* extracts and/or are seropositive for anti-PGL-I antibody (TB ML+, $n = 5$) (\blacksquare). Median values are indicated by horizontal lines. P values are calculated by the nonparametric Mann-Whitney U test. (B) Ex vivo IFN- γ production against two *M. leprae* antigens (ML0007 and ML2649) with homologues in *M. tuberculosis* (Rv0007 and Rv0264). Symbols are as defined for panel A.

data analysis. None of the 19 MB leprosy patients showed a significant IFN- γ response to *M. leprae* extract as expected.

Of note, 5 of the 16 tuberculosis patients responded significantly to *M. leprae* extracts, whereas the remaining 11 showed

no significant response (Table 1). These five *M. leprae*-responsive tuberculosis patients are indicated separately in Fig. 1.

T-cell recognition of *M. leprae* antigens in Brazilian leprosy patients and controls. T-cell recognition of all 19 *M. leprae*

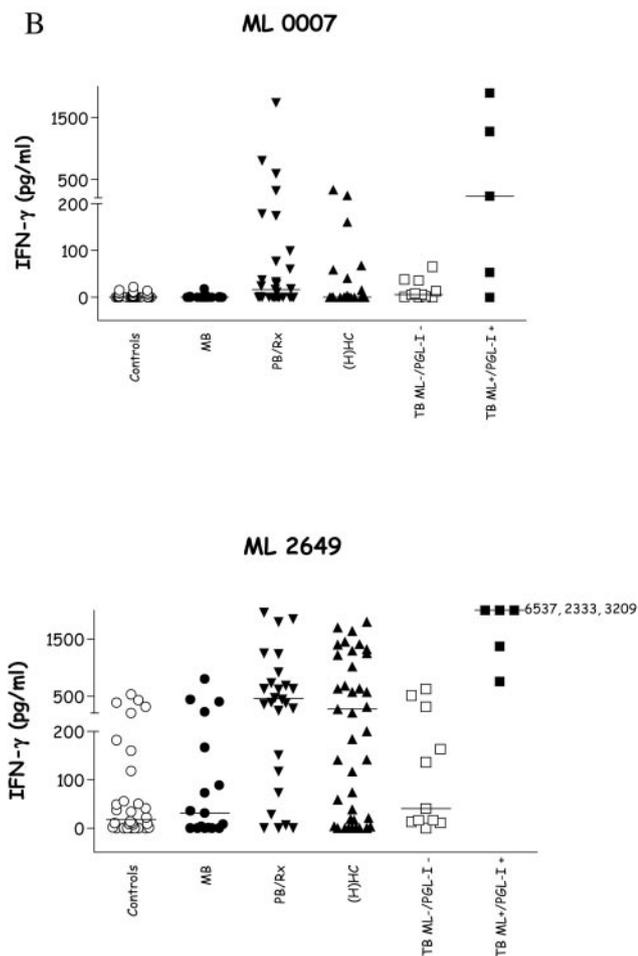


FIG. 1—Continued

candidate antigens was evaluated by measuring IFN- γ production by PBMC in response to each individual recombinant protein (data for seven proteins are shown in Fig. 1A and B). As expected, most MB patients did not respond to any of the

19 tested antigens (median, <30 pg/ml). Similar results were found for the negative control group (median, <25 pg/ml in response to any of the antigens with the exception of ML1602 [data not shown]). In contrast, the PBMC of PB/Rx patients were highly responsive to several of the *M. leprae* hypothetical antigens examined (Fig. 1 and Table 1).

When individually analyzed, four types of responses could be distinguished. First, five of the *M. leprae* proteins (ML0576, ML1989, ML1990, ML2283, and ML2567; Fig. 1A) induced IFN- γ production in PB/Rx leprosy patients that was significantly greater than that seen in negative controls ($P < 0.001$) or in MB patients ($P < 0.05$). Median values differed between PB/Rx and tuberculosis patients for all five proteins (250 versus ≤ 30 pg/ml), and differences reached significance for ML1989, ML2283, and ML2567 (Fig. 1). Of the 16 tuberculosis patients, 11 (62%) did not show a clear response to the *M. leprae* antigens tested, a finding in agreement with their lack of response to *M. leprae* extracts (see above). In contrast, the five tuberculosis patients that responded to the *M. leprae* proteins also responded to *M. leprae* extracts. Three of them had antibodies to the *M. leprae* specific PGL-I (Table 1 and Fig. 1), suggesting previous exposure to *M. leprae*. Importantly, all five proteins unique to *M. leprae* were recognized by the (H)HC group. Two of these, ML1989 and ML1990, induced higher responses among the latter group compared to PB/Rx leprosy patients, but these differences did not reach statistical significance.

Second, three antigens—ML1602, ML1604 (data not shown), and the control antigen ML2649 (shown as a representative example in Fig. 1B), which has a homologue in *M. tuberculosis* (Rv0264)—induced responses that were not specifically associated with *M. leprae* infection: ML1602 and ML1604 were recognized well by a majority of the individuals in the negative control group that did not respond to *M. leprae* extracts, whereas ML2649 induced responses in 63% of the tuberculosis patients. These results identify ML2649, and likely also its *M. tuberculosis* ortholog (Rv0264), as a new T-cell stimulatory antigen in tuberculosis.

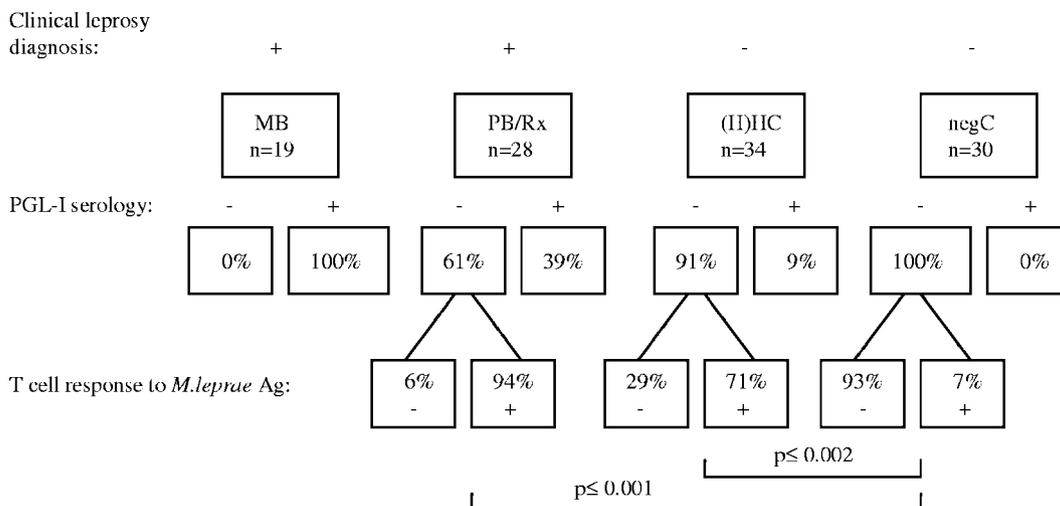


FIG. 2. Added value of the use of in vitro T-cell responses to selected *M. leprae* antigens ML0576, ML1989, ML1990, ML2283, and ML2567. Study subjects T-cell responses were considered positive if IFN- γ production in response to one of these antigens was >200 pg/ml.

Third, intermediate responses (median, 100 to 150 pg/ml) were found in PB patients to ML0575 and ML1603 (data not shown).

Finally, IFN- γ responses to the six remaining proteins (*M. tuberculosis* homologue ML0007 (shown as a representative example in Fig. 1B), ML0126, ML0369, ML0573, ML0574, and ML1788 (data not shown) overall appeared to be low in all groups, with median IFN- γ responses of <100 pg/ml for ML0573 and ML0574 and <50 pg/ml for all other antigens tested. None of these antigens displayed any inhibitory activity in control cellular stimulation assays (see Materials and Methods).

***M. leprae*-specific anti-PGL-I IgM antibodies.** All 43 endemic control individuals were seronegative for *M. leprae*-specific anti-PGL-I IgM. Anti-PGL-I IgM antibodies were present in all 19 MB patients, in 2 of the 15 PB patients, in 9 of the 13 Rx patients, in 3 of the 21 household contacts, and in 3 of the 16 Brazilian tuberculosis patients (Table 1; OD₄₅₀ values of >0.200 [range, 0.210 to 3.922]).

T-cell responses to *M. leprae* antigens in relation to anti-PGL-I IgM titers. A relevant question is whether the five most promising *M. leprae* proteins studied above (Fig. 1A) may provide any added value in detecting *M. leprae* exposure/infection, compared to currently available assays that measure *M. leprae* specific humoral immunity, such as anti-PGL-I IgM titers. T-cell reactivity to these five *M. leprae* proteins was therefore analyzed versus humoral immune responses to *M. leprae* as assessed by IgM antibody levels to *M. leprae*-specific PGL-I (Fig. 2). In the (H)HC group, 31 of 34 individuals did not have detectable levels of anti-PGL-I IgM. Interestingly, 22 (71%) of these exposed individuals responded to at least one of the five proteins unique to *M. leprae* (Fig. 2). In the PB/Rx group, 17 of the 28 patients did not have detectable levels of anti-PGL-I IgM antibody, whereas 16 of these 17 individuals (94%) recognized one to five of these antigens. Only two individuals in the negative control group responded to *M. leprae* antigens: one to ML1990 and ML2567 the other only to ML2567 (data not shown).

DISCUSSION

Using comparative mycobacterial genomics and immunological assays, we have selected several antigens that are potentially unique to *M. leprae* and that have potent T-cell stimulatory activity. Such antigens may provide a first generation of postgenomic tools that may offer added value in identifying *M. leprae* infection. Better diagnostic tests that detect early *M. leprae* infection have been an important goal in leprosy research since the beginning of the 20th century (19). Although assays have been developed that detect *M. leprae*-specific IgM antibodies to PGL-I which are able to identify MB leprosy patients (who develop strong humoral immunity to *M. leprae*), they fail to detect most PB leprosy patients and leprosy patients' contacts (who typically develop strong cellular but not humoral immunity [reviewed in reference 20]). Furthermore, MB leprosy is hard to detect at an initial stage when the patient is already an active source of infection.

Tests that measure cellular immunity to *M. leprae* historically have relied on the use of *M. leprae* extracts or purified complex mixtures of *M. leprae* components. The diagnostic value of

these tests, however, is compromised by the presence of conserved, immunologically cross-reactive components that are shared with other mycobacteria, which results in low test specificity. For leprosy, such cross-reactivity is particularly problematic in countries with high incidence rates of tuberculosis, routine BCG vaccination practice, and high levels of exposure to nonpathogenic environmental mycobacteria.

Comparative genomic analyses of the *M. tuberculosis* and BCG genomes have provided a blueprint for the rational design of a new generation of specific proteins to assess *M. tuberculosis* specific cellular immunity in blood tests. *M. tuberculosis* "RD1" proteins that were found to be lacking from the BCG genome were shown to have considerable value as potential diagnostic reagents for tuberculosis in humans or cattle (3, 5). The antigens characterized in most detail are ESAT-6 (Rv3875) and CFP-10 (Rv3874). Recently, several other candidate molecules were reported, which in combination with ESAT-6 and CFP-10 provided enhanced specificity and sensitivity (2, 8, 18). Scrutiny of the *M. leprae* genome revealed the presence of two candidate genes, ML0049 and ML0050, that encode the *M. leprae* homologs of ESAT-6 and CFP-10, respectively (16, 17, 21, 22). We recently reported that recombinant *M. leprae* ESAT-6 and CFP-10 proteins were efficiently recognized by T cells from the majority of *M. leprae* responsive leprosy patients (12, 16). Despite limited sequence identity with their *M. tuberculosis* homologues Rv3875 and Rv3874 (36 and 40%, respectively), however, significant immunologic cross-reactivity with *M. tuberculosis* proteins was found for human T cells. This clearly limits the diagnostic potential of ML0049- and ML0050-encoded proteins in leprosy areas of endemicity with a high prevalence of tuberculosis.

Based on these observations, we hypothesized that candidate antigens that are unique to *M. leprae* may provide superior targets to measure *M. leprae*-specific cellular immunity. Using the recently published genome sequences of *M. leprae* (10) and of *M. tuberculosis* and *M. bovis* (11, 13, 15), we identified and studied a series of *M. leprae* proteins that *M. tuberculosis* lacked. Published comparative genomic analyses of the *M. leprae* and *M. tuberculosis* genomes had previously revealed the presence of 165 *M. leprae* genes with no homologue in *M. tuberculosis*. Based on the size and the presence of promiscuous HLA-class II binding-motifs, we selected 12 hypothetical proteins that are unique to *M. leprae* and 2 with homologues in *M. tuberculosis* for gene expression analyses, cloning, and expression as recombinant proteins.

In addition, five antigens were analyzed (ML0126, ML0369, ML0840, ML0927, and ML1601) that have homologues in several other mycobacteria, particularly *M. avium* and *M. paratuberculosis*, but not in *M. tuberculosis*.

Since these genes have no known functions and thus encode hypothetical proteins, we first examined whether they are expressed at the transcriptome level. Our results show that mRNA derived from these hypothetical *M. leprae*-specific genes is indeed transcribed in *M. leprae*, both in the mouse footpad and in MB leprosy patients' lesions. These results strongly suggest that the selected *M. leprae* genes are expressed, lending support to the hypothesis that they could encode functional proteins with a potential for use in *M. leprae*-specific tests.

The *M. leprae* genes evaluated were selected partly based on

the presence of amino acid motifs predicting high-affinity binding to multiple HLA class II molecules, thus increasing the chance that single antigens could be recognized by T cells from multiple donors.

The results from our antigen discovery approach show that several of the *M. leprae* proteins thus selected are recognized efficiently by T cells from *M. leprae*-responsive donors but not from *M. leprae*-unresponsive individuals, as evaluated by IFN- γ production by PBMC. PB and reactional leprosy patients and healthy household contacts of leprosy patients produced significant levels of IFN- γ in response to the five unique *M. leprae* antigens encoded by ML0576, ML1989, ML1990, ML2283, and ML2567. These proteins were not recognized by most negative endemic controls, MB patients, or *M. leprae*-unresponsive tuberculosis patients from the same area of endemicity or by healthy, *M. leprae*-unexposed, and BCG-negative donors. Only tuberculosis patients that likely had been exposed to *M. leprae*, as evidenced by the presence of T-cell responses to *M. leprae* and/or IgM antibodies to *M. leprae* PGL-I, responded well in T-cell assays to these *M. leprae*-specific proteins. These responses thus are likely due to coinfection with *M. leprae*, although we cannot exclude possible cross-reactivity with as-yet-unknown environmental antigens.

An observation of potential interest was that 71% (22 of 31) of the (H)HC group that lacked detectable levels of anti-PGL-I IgM responded to at least one of the five proteins. Only 9% of these likely *M. leprae*-exposed individuals would have been detected by the anti-PGL-I IgM test. This result suggests that these newly identified *M. leprae* antigens may offer added value in identifying *M. leprae* infection in addition to existing humoral assays.

In contrast to the five antigens discussed above, the proteins encoded by ML1602, ML1604, and the control ML2649 (homologue to *M. tuberculosis* Rv0264) were not specifically recognized by *M. leprae*-exposed individuals: ML1602 and ML1604 gene products were often recognized by *M. leprae* unresponsive controls, perhaps due to cross-reaction with unknown antigens, whereas that of ML2649 induced responses in 63% of the tuberculosis patients. These results identify ML2649, likely together with its *M. tuberculosis* ortholog Rv0264, as a new T-cell stimulatory antigen in tuberculosis.

Despite the presence of multiple HLA class II binding motifs, six other candidate *M. leprae* proteins (encoded by ML0126, ML0369, ML0573, ML0574, and ML1788 and the *M. tuberculosis* homologue ML0007) induced no significant IFN- γ responses. Although these results underscore the specificity of recognition of the five proteins identified above, it is possible that the former antigens induce alternative T-cell responses, such as Th2-associated IL-4/IL-13 production, or regulatory T cells that may preferentially produce IL-10. Further studies are required to investigate this possibility.

In spite of the selective recognition of the five *M. leprae* antigens, the question remains as to whether these antigens are really unique to *M. leprae*. Most (myco)bacterial sequences remain unknown, and cross-reactivity to other environmental antigens cannot be ruled out. As discussed above, the T-cell reactivity seen in some Brazilian tuberculosis patients may be due to previous *M. leprae* exposure, but cross-reactivity with other unknown antigens cannot be formally excluded, analogous to our previous observation in tuberculosis (1). In that

study one phiRv2 region-derived *M. tuberculosis* antigen that was selected on the basis of its unique presence in *M. tuberculosis* induced detectable IFN- γ responses in *M. tuberculosis*-nonexposed, BCG-vaccinated individuals. This phenomenon seemed to be antigen related since it was not observed for other *M. tuberculosis* antigens such as Rv2654 (1). This also holds true for most unreactive *M. leprae* antigens evaluated in our study. Alternatively, such unexpected T-cell responses may be due to molecular mimicry, which can induce cross-reactivity despite a total lack of sequence homology (27). Irrespective of the precise explanation to account for these observations, further studies need to be performed, in order to define epitopes that are uniquely recognized by *M. leprae*-exposed individuals, by using relevant amino acid stretches of these *M. leprae* antigens to identify peptides or peptide mixes with better diagnostic performance.

The results of the present study may provide a first generation of postgenomic tools that may eventually facilitate the development of more specific tests to detect *M. leprae* infection. Such tests may not only contribute to our understanding of *M. leprae* transmission in affected populations but also help to diagnose infection at early stages before immunopathological sequelae occur. Furthermore, longitudinal studies in healthy, well-documented *M. leprae*-infected contacts may help to develop new biomarker profiles that discriminate between resistance versus susceptibility to developing clinical disease, based on specific T-cell response profiles to unique *M. leprae* antigens. Finally, since the *M. leprae* antigens described here were recognized well by several leprosy patients with leprosy reactions, it may be of interest to monitor T-cell responses to *M. leprae* antigens over time and determine their temporal relationship with the development of typical leprosy reactions.

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