Analysis of Antibody Responses to *Mycobacterium leprae* Phenolic Glycolipid I, Lipoarabinomannan, and Recombinant Proteins To Define Disease Subtype-Specific Antigenic Profiles in Leprosy

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A simple serodiagnostic test based on the *Mycobacterium leprae*-specific phenolic glycolipid I (PGL-I), for individuals with leprosy is nearly universally positive in leprosy patients with high bacillary loads but cannot be used as a stand-alone diagnostic test for the entire spectrum of the disease process. For patients with early infection with no detectable acid-fast bacilli in lesions or with low or no antibody titer to PGL-I, as in those at the tuberculoid end of the disease spectrum, this diagnostic approach has limited usefulness. To identify additional *M. leprae* antigens that might enhance the serological detection of these individuals, we have examined the reactivity patterns of patient sera to PGL-I, lipoarabinomannan (LAM), and six recombinant *M. leprae* proteins (ML1877, ML0841, ML2028, ML2038, ML0380, and ML0650) by Western blot analysis and enzyme-linked immunosorbent assay (ELISA). Overall, the responses to ML2028 (Ag85B) and ML2038 (bacterioferritin) were consistently high in both multibacillary and paucibacillary groups and weak or absent in endemic controls, while responses to other antigens showed considerable variability, from strongly positive to completely negative. This analysis has given a clearer understanding of some of the differences in the antibody responses between individuals at opposite ends of the disease spectrum, as well as illustrating the heterogeneity of antibody responses toward protein, carbohydrate, and glycolipid antigens within a clinical group. Correlating these response patterns with a particular disease state could allow for a more critical assessment of the form of disease within the leprosy spectrum and could lead to better patient management.

Leprosy is a chronic mycobacterial infection caused by *Mycobacterium leprae* that results in damage to cutaneous tissue and nerves, causing an extraordinary spectrum of skin lesions, peripheral neuropathy, and anemia, with the subsequent development of disfigurement, deformity, and disability if not properly treated (27). The efforts of leprosy control programs and multidrug therapy over the last 25 years have dramatically decreased the worldwide prevalence from approximately 5.4 million cases in 1985 to 212,802 registered cases at the start of 2008 (35, 36, 37). Despite reports of leprosy’s smaller global health impact, countries such as Brazil, Nepal, and East Timor still face high prevalence rates. Furthermore, local regions of high endemicity still exist in many countries, and the true number of cases may be underreported. For example, a population survey in Bangladesh revealed that the number of active leprosy cases was approximately six times higher than the actual number of registered cases despite effective leprosy control programs (21). Global new case detection declined only 3.5% between 2007 (126 countries reporting) and 2008 (121 countries reporting), but new case detection in children, a sign of continuing transmission, increased by 3.1% during this same period (37). It is generally agreed that the transmission of leprosy will be effectively interrupted only by earlier diagnosis, ideally in the period before clinical signs appear, and this would be practicable only with an easy-to-use serological test. The diverse disease spectrum of leprosy can be partitioned into a five-part classification scheme based on bacterial, histopathological, and clinical observations (24). Clinical manifestations range from a few well-organized granulomatous skin lesions with few or absent acid-fast bacilli (AFB) with the presence of robust cell-mediated immunity (termed polar tuberculoid [TT]/borderline tuberculoid [BT] or paucibacillary [PB]) and low or no titer to the *M. leprae*-specific phenolic glycolipid I (PGL-I) to disseminated infection with multiple lesions containing huge numbers of AFB, the absence of cell-mediated immunity, and high titers of antibody to PGL-I.

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(termed borderline lepromatous [BL]/polar lepromatous [LL] or multibacillary [MB]). The criteria for leprosy diagnosis are based on clinical manifestations, including hypopigmented or reddish demarcated skin lesions that exhibit loss of sensation, involvement of peripheral cutaneous nerves detected by thickening, and the detection of AFB in either slit skin smears or biopsy specimens of skin or nerves using the Fite-Faraco modification of the carbol fuchsin stain (6). Definitive diagnosis relies on clinical observations and on time-consuming invasive tests to reveal immunopathological changes in the biopsy specimens of skin lesions or the presence of AFB in slit skin smears or stained histological sections. Due to the lack of skin smear or histological services in many areas of the world, the number of skin lesions and nerves involved is now used for clinical classification for the purposes of defining treatment regimens (fewer than six skin lesions for PB; six or more lesions for MB) (34). The reduction of trained leprologists and laboratory technicians has inadvertently increased the likelihood of misdiagnosis and delayed treatment, which averages around 2 years for most individuals in areas where leprosy is highly endemic (20).

Thus, the availability of a more sensitive and discriminatory serological test for *M. leprae* infection, particularly in the case of early leprosy and individuals at the TT/BT end of the spectrum, would greatly improve leprosy diagnosis.

With the completion of the *M. leprae* and *Mycobacterium tuberculosis* genome sequences (7, 8), attempts have been made to identify T cell-based biomarkers for detecting *M. leprae* infection using postgenomic approaches (1, 9, 10, 11, 12, 29). In a previous study, we identified *M. leprae*-specific recombinant proteins and peptides capable of stimulating a strong cell-mediated response in leprosy patients (29). Furthermore, many of these were not recognized by tuberculosis (TB) patients or endemic controls (EC). However, these hypothetical unknown proteins which do not have homologs in other mycobacteria were not well recognized serologically by the majority of BL/LL patients. For this reason, we decided to concentrate on six recombinant protein antigens that had previously been shown to be immunologically important and/or recognized by either cell-mediated or serological responses in leprosy patients. Several members of the mycolyltransferase Ag85 family (ML0097, ML2028, and ML2655) were identified by serological tests to reveal immunopathological changes in the biopsy specimens of skin lesions or the presence of AFB in slit skin smears or stained histological sections. One of the most highly expressed native proteins of the leprosy bacillus is ML0380 (13), a highly immunogenic protein that was shown by limiting dilution analysis to be recognized by about one-third of all *M. leprae*-reactive T cells in TT/BT patients or healthy contacts (19). In the current study, we have examined the serological responses to these recombinant proteins as well as to glycopilipid and carbohydrate antigens to determine whether they are predictive of a patient’s position in the disease spectrum (TT to LL), with an additional goal of developing more rapid, simple, and objective tests for *M. leprae* infection.

**MATERIALS AND METHODS**

**Subjects and samples.** Leprosy patients were diagnosed at the Leonard Wood Memorial Center for Leprosy Research, Cebu, Philippines. Leprosy was classified according to the Ridley-Jopling classification system based on bacterial index (BI; a measure of the number of acid-fast bacilli found in the dermis based on a logarithmic scale from 0 to 6+), and histological and clinical observations were carried out by experienced leprologists and a leprosy pathologist. All leprosy patient sera were collected at the time of initial diagnosis prior to beginning multidrug therapy. Serum samples were collected from healthy volunteers who did not have any known exposure to either TB or leprosy within their household or other contacts in a region where leprosy and TB are endemic (HEC sera). Serum samples from areas where leprosy and TB are nonendemic (NEC sera) were obtained from non-*Mycobacterium bovis* BCG-vaccinated, U.S.-born healthy individuals with no known exposure to either TB or leprosy from the area surrounding Colorado State University. Serum samples from 30 cavitary TB patients were from a cohort of newly diagnosed TB patients from the Tuberculosis Trials Consortium (TBTC) study group 22 (31). Eleven were sputum smear negative, while 19 were smear positive (ranging from 1+ to 4+; too many to count). The sera were kindly provided by William R. Mac Kenzie through a serum bank repository from the Centers for Disease Control in Atlanta, GA. Serum samples from all sources were anonymized and coded to protect donor identities and were obtained with informed consent and/or with permission from the local ethics committee or institutional review boards of the relevant countries and institutions involved. The characteristics of the individuals in the study groups are listed in Table 1.

**Cloning and purification of recombinant *M. leprae* proteins.** The DNA sequence coding for six full-length *M. leprae* proteins was cloned from genomic DNA (strain NHDP-63, single nucleotide polymorphism [SNP] type 3) using Vent Frag DNA polymerase (New England BioLabs, Beverly, MA). The proteins targeted for investigation were EF-Tu (ML1877), the major membrane protein I (ML0841), Ag85B (ML2028), bacterioferritin, the major membrane protein II (ML2038), CFP-10 (ML0050), and GroES (ML0380). PCR amplifications were carried out with primers designed to introduce NdeI and HindIII restriction sites, respectively, at the 5′ and 3′ ends of the open reading frame. The PCR product was digested with the restriction enzymes NdeI and HindIII and cloned into expression vector pET28a (+) (Novagen, Madison, WI). Sequence-verified products were transformed into the Escherichia coli expression host BL21(DE3) (Invitrogen, Carlsbad, CA) to produce the recombinant proteins as previously described (28). Recombinant His-tagged proteins were purified using a Talon nickel affinity chromatography resin (Clontech, Palo Alto, CA) and eluted with...
50 mM imidazole followed by dialysis. The purified protein concentration was quantified using the bicinchoninic assay (BCA; Pierce, Rockford, IL), and the quality of the recombinant protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Other peptides and antigens. Eighteen 15-mer peptides, each overlapping by five amino acids, covering the 100-amino-acid M. leprae CFP-10 protein (p1-p18) were synthesized by solid phase pin technology (Mimotopes, San Diego, CA) as previously described (28). Peptides were dissolved in sterile distilled water at 2 mg/ml and stored frozen at −70°C until use. The disaccharide epitope of PGL-I was synthesized and coupled to bovine serum albumin (ND-O-BSA) as previously described (5), with modifications (38). The native cytosolic subcellular fraction of M. leprae (MLSA) was prepared as previously described (18). Lipoarabinomannan (LAM) was purified from M. leprae whole cells as previously described (30). All of the recombinant proteins, native MLSA, and ND-O-BSA were supplied through the NIH/NI/AIDS Leprosy Research Contract N01 AL25469 from Colorado State University (these materials are now available through the Biodefense and Emerging Infections Research Resources Repository listed at http://www.beiresources.org/TBVRTResearchMaterials/tabid/1431/Default.aspx).

Western blot analysis. Approximately 0.25 μg of each of the six recombinant proteins was separated on 15% SDS-PAGE gels and transferred to nitrocellulose membranes. Native MLSA was loaded at 5 μg per lane. The membranes were blocked with 1% BSA in phosphate-buffered saline (PBS)−0.05% Tween 80 (PBS-T) and then incubated with individual serum samples at a 1:5,000 dilution (PBS-T) and then incubated with individual serum samples at a 1:5,000 dilution overnight with gentle rocking. The membranes were washed with PBS-T and then incubated with individual serum samples at a 1:5,000 dilution with PBS six times, 100 μl of 0.1 M sodium carbonate/bicarbonate buffer, pH 9.6, at 4°C overnight. Unbound antigen was washed away using PBS, pH 7.4, containing 1% BSA and 0.05% Tween 80 (blocking buffer) six times. Serial 2-fold dilutions of serum from 1:100 to 1:106 diluted in 100 μl of PBS, pH 7.4, containing 1% BSA and 0.05% Tween 80 (blocking buffer) six times. The reactivity patterns of leprosy patient sera demonstrated some clear-cut differences in the recognition patterns of recombinant proteins, particularly with ML2028, ML0050, and ML0380. In general, TT/BL patient sera showed overall lower responses to ML2028 and ML0050, while none of these 20 sera from this group showed any discernible reactivity against ML0380. As a group, nine out of 13 (69%) BL patients showed a weaker pattern of reactivity with the recombinant proteins than LL patients, which was similar in intensity with the pattern found with TT/BL individuals (compare BL30 and BL7).
TABLE 2. Reactivity of MB (LL, n = 37 samples; BL, n = 13 samples) and PB (TT, n = 9 samples; BT, n = 11 samples) leprosy patients toward the six recombinant proteins and LAM by Western blot analysisa

<table>
<thead>
<tr>
<th>Protein or LAM</th>
<th>Patient type</th>
<th>No. of samples with a reactivity of:</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>ML1877</td>
<td>MB</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>ML0841</td>
<td>MB</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>ML2028</td>
<td>MB</td>
<td>22</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>ML2038</td>
<td>MB</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ML0050</td>
<td>MB</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ML0380</td>
<td>MB</td>
<td>3</td>
<td>13</td>
</tr>
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<td>0</td>
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<tr>
<td>LAM</td>
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<td>14</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

* Grading was dependent on the relative bandwidth and intensity, from strong (3+) to weak (±) to negative (–).

with TT and BT blots in Fig. 2B). In terms of responses against ML0050, 76% of BL/LL sera demonstrated a positive reaction, in contrast to only 25% of TT/BT sera. The response to ML2028 was consistently the strongest in both patient groups, while responses to ML0050, ML0380, and ML2038 showed the most variability, from strongly positive to completely negative. Interestingly, BL/LL individuals with the strongest responses toward one of these three proteins did not automatically have high reactivity with the other two. The heterogeneity of the responses toward the recombinant proteins and MLSA in leprosy patients characterized as LL, BL, TT, or BT is illustrated in Fig. 2A and B.

The cytosolic protein fraction was used to examine antibody responses to native proteins and to LAM. BL/LL individuals generally had very high antibody responses to LAM, which was visible as an intense smear between 20 and 40 kDa. Approximately 72% of BL/LL patient sera showed a very strong reaction to LAM (≥2+ intensity). Less than half (45%) of TT/BT sera demonstrated this intensity, whereas about one-third of individuals in this group showed weak or no reactivity. The intensity of this smear correlated very well with the antibody titer to purified M. leprae LAM in the ELISA. A minority of BL/LL patients gave weaker LAM responses, so protein banding patterns were discernible in this region. The only native antigen that was reliably recognized in this fraction was ML0380, which was in a region that was devoid of any reactivity to LAM or other proteins and when positive was always found aligned with the recombinant protein. Collectively, these results indicate that unique response patterns to various M. leprae antigens are generated in each individual and that responses to some antigens are predictive of the course of clinical disease status.

Seroreactivity of TB patient, HEC, and NEC sera by blot analysis. The dominant antigen recognized by individuals with cavitary TB was LAM, with 14 of 30 individuals (47%) responding with a ≥2+ intensity. However, the intensity of the anti-LAM response had no correlation with the sputum AFB count. This is illustrated with four highly smear-positive TB patients showing large differences in the intensity of the LAM smear by blot analysis (Fig. 2C). Conversely, three smear-negative cavitary TB patients had an intensely strong recognition of LAM (data not shown). Cross-reactivity to heterologous ML2028 was surprisingly weak in light of the 83% identity of this protein with the Rv1886c homolog in M. tuberculosis, with only two of 30 individuals showing a 2+ reaction. Only one weakly positive response to ML0380 was detected, while there was no detectable cross-reactive response to ML0050 by any of the TB sera, consistent with our previous observations (28). Responses to both ML2038 and ML0841 were weak or negative in all cases. Twenty-five percent of healthy endemic control individuals from the Philippines (6 out of 24) showed a ≥2+ reactivity pattern toward LAM, while responses to the six recombinant proteins were weak or negative, except for one individual giving a 2+ reaction to ML0841. This same individual had a 1+ reactivity toward LAM. None of the 23 healthy nonendemic U.S. controls reacted with the recombinant proteins, although there was an occasional very weak smear with MLSA by blot analysis, suggesting a possible weak response to LAM. Taken together, the strongest responses overall in the leprosy patient groups were against ML2028 and ML2038, with little evidence of cross-reactivity with TB sera and weak to negative responses in the HEC group, indicating that these protein antigens would be good candidates to augment leprosy diagnosis with PGL-I.

ELISA reactivity of leprosy patient, TB patient, HEC, and NEC sera to ML2028, ND-O-BSA, and LAM. ELISAs were used to gain an understanding of the relationships between the various antibody responses against protein, glycolipid, and carbohydrate antigens within the various groups. These assays were employed to detect antibodies binding to ML2028, ND-O-BSA, and LAM, and the OD values for the various groups are shown in Fig. 3. For responses to ML2028, there was very good correlation between the band intensity and thickness in the blot and the OD value. BL/LL samples that gave a 3+ reaction by blot analysis gave a correspondingly lower median OD of 0.57. BL individuals had a median OD of 0.47 for ML2028, which was not statistically different from that of TT/BT individuals (P = 0.66). There was a positive result toward ML2028 in 90% of BL/LL and 65% of TT/BT individuals, with a specificity of 89%. As expected, a high BI correlated well with levels of antibody toward ND-O-BSA. LL and BL individuals gave a median OD of 1.35 and 1.27, respectively, while TT/BT individuals showed a median OD of 0.58, which was significantly different from either the LL (P < 0.0001) or BL (P = 0.0002) group values. The numbers of positives for ND-O-BSA were 96% for BL/LL and 80% for TT/BT, the latter of which was much higher than the rate of seropositivity generally reported for this group in previous studies, while the specificity was 93%. The reactivity to LAM was the highest in all of the patient groups, with 100% of BL/LL, 90% of TT/BT, and 87% of TB individuals being pos-
itive. However, 79% of EC individuals were also positive, indicating that specificity for LAM would be rather low (21%). TT/BT individuals that showed a positive reactivity to LAM in the Western blot analysis (9/20 [45%]) gave a median OD of 1.42, while TT/BT individuals that had a positive reaction by blot analysis (11/20 [55%]) gave a median OD of 0.77. The median OD and 25% to 75% interquartile range (IQR) for each group toward the three antigens are shown in Table 3. In summary, the ELISA results showed that PGL-I antibody was detected at slightly higher numbers in leprosy patient groups than ML2028, showing high specificity for both, while the high positive responses to LAM in the endemic control group indicated low specificity for this antigen.

Serological recognition of ML0050 peptides. Since our previous analysis of the reactivity of leprosy patient sera to ML0050 peptides involved a small sample size ($n = 4$), we now wanted to validate those results with a larger set of individuals. The responses of the strongest ML0050-reactive BL/LL sera ($n = 16$) to 18 overlapping ML0050 15-mer peptides by ELISA are shown in Fig. 4. Of the 16 BL/LL leprosy patient sera examined in the current study, 15 reacted with one or more peptides (maximum of seven, with an average of three peptides recognized per individual), and reactions with a number of peptides were seen repeatedly. The reactivity toward certain peptides with some serum samples was very strong, particularly to peptides p2, p6, p8, p10, p15, p16, and p17. Seven peptides did not elicit any positive reaction in this group of individuals (p1, p4, p5, p9, p12, p13, and p18). Since these results involved only patients from the Philippines, HLA responses are biased in this study, but overall, the strong responses to a number of peptides in several individuals suggest that peptide pools could potentially be used in serological assays for leprosy, as ML0050 peptides did not show any cross-reactivity in individuals infected with $M. tuberculosis$.

DISCUSSION

The presence of IgM serum antibody to $M. leprae$-specific PGL-I correlates well with BI in BL/LL patients, thereby fa-
cilitating the development of particle agglutination, dipstick, ELISA, and lateral flow (ML Flow) test formats to detect their levels, which has assisted in the classification of patients for various treatment regimens (3, 4, 14). These tests are almost universally positive, with BL/LL patient sera indicative of higher Th2 responses, whereas the majority of TT/BT patients are seronegative. One study comparing the ELISA and ML Flow format showed a high level of sensitivity and concordance using both tests, with the lateral flow test correctly diagnosing 97.4% of BL/LL individuals with a specificity of 90.2% (4), while only 40% of individuals from the TT/BT group were seropositive. Results from the analysis of responses to additional antigens would likely improve the sensitivity of detecting TT/BT individuals. The variable patterns of recognition of these antigenically distinct components of *M. leprae* in this work describe a unique approach to better defining the complex nature of the immune response across the leprosy disease spectrum. Such an approach is important to providing a better clinical assessment as well as a prognostic tool predicting the course of disease progression. By Western blot analysis, four of the recombinant proteins, ML1877, ML0841, ML2028, and ML2038, were recognized by sera from all BL/LL and TT/BT patients. The bacillary indices and ELISA results for ML2028 correlated well with the intensity of reactivity by Western blot analysis, with 84% of LL patients showing a reaction with ML2028, while 69% of BL patients showed a reaction to this antigen. The lower blot and ELISA values for ML2028 in

![ML2028 (Ag85B)](image)

![ND-O-BSA](image)

![M. leprae LAM](image)

using both tests, with the lateral flow test correctly diagnosing 97.4% of BL/LL individuals with a specificity of 90.2% (4), while only 40% of individuals from the TT/BT group were seropositive. Results from the analysis of responses to additional antigens would likely improve the sensitivity of detecting TT/BT individuals. The variable patterns of recognition of these antigenically distinct components of *M. leprae* in this work describe a unique approach to better defining the complex nature of the immune response across the leprosy disease spectrum. Such an approach is important to providing a better clinical assessment as well as a prognostic tool predicting the course of disease progression. By Western blot analysis, four of the recombinant proteins, ML1877, ML0841, ML2028, and ML2038, were recognized by sera from all BL/LL and TT/BT patients. The bacillary indices and ELISA results for ML2028 correlated well with the intensity of reactivity by Western blot analysis, with 84% of LL patients showing a reaction with ML2028, while 69% of BL patients showed a reaction to this antigen. The lower blot and ELISA values for ML2028 in

![FIG. 3. Reactivity of sera from clinically diagnosed LL (n = 37), BL (n = 13), and BT/TT (n = 20) leprosy patients and TB patients (n = 30) and HEC (n = 42) and NEC (n = 23) sera toward recombinant protein ML2028, the synthetic PGL-I antigen ND-O-BSA, and native *M. leprae* LAM by ELISA. Optical density readings were performed using a 1:200 dilution for each serum sample, with the mean denoted by the solid line within each set. Statistical differences between groups were calculated using the Mann-Whitney U test (2-tailed).](image)

![FIG. 4. Reactivity patterns of 16 leprosy patient serum samples to recombinant protein CFP-10 from *M. leprae* (ML0050) and *M. tuberculosis* (Rv3874) and to 18 individual overlapping ML0050 peptides by ELISA. The sera were selected on the basis of high reactivity to recombinant ML0050 by Western blot analysis. The intensity of the reaction was graded: black, very strong (OD, >1.00); gray, midrange (OD between 0.50 and 0.99); dotted, weakly positive (OD, 0.20 to 0.49); and white, negative.](image)

| Table 3. Reactivity of leprosy patient sera, cavitary TB sera, and HEC and NEC sera to ML2028, ND-O-BSA, and LAM* |
|---|---|---|---|
| Sample category (total no.) | ML2028 Median OD (IQR) | ND-O-BSA Median OD (IQR) | LAM Median OD (IQR) |
| LL (37) | 1.11 (0.85–1.47) | 1.35 (1.10–1.56) | 2.26 (1.92–2.57) |
| BL (13) | 0.47 (0.33–0.94) | 1.27 (0.75–1.52) | 1.98 (1.80–2.34) |
| BT/TT (20) | 0.57 (0.31–0.71) | 0.58 (0.36–0.64) | 0.94 (0.76–1.42) |
| TB (30) | 0.36 (0.32–0.51) | 0.36 (0.30–0.43) | 0.78 (0.51–1.28) |
| HEC (42) | 0.36 (0.30–0.42) | 0.28 (0.24–0.31) | 0.61 (0.49–0.73) |
| NEC (23) | 0.13 (0.12–0.14) | 0.13 (0.13–0.14) | 0.14 (0.13–0.16) |

*The medians and 25% to 75% interquartile ranges (IQR) were determined from OD values obtained from a 1:200 dilution of serum in an ELISA.*
the BL group are similar to the overall pattern we observed in the TT/BT group, which gave a uniformly low positive reaction (≤1+) to this protein. Thus, the titer of antibodies to ML2028 appears to correlate well with the BL of the patient and may provide a discriminatory marker related to disease status.

Two protein antigens, ML0380 and ML0050, gave markedly diverse responses across the clinical leprosy spectrum and varied greatly from individual to individual. Within the BL/LL group, roughly 60% of patients recognized ML0380, whereas 76% recognized ML0050, with 32% and 18%, respectively, categorized as strong (≥2+). This is interesting, because ML0380 is the most highly expressed protein produced by *M. leprae*, while ML0050 was much more difficult to detect in the native subcellular fractions (28). Despite the disparity in expression or detection, it is known that both ESAT-6 and ESAT-10 elicit very potent and early cell-mediated responses in individuals infected with TB (2, 22). Clearly, the immune response to this virulence factor at the cell-mediated and humoral level is disproportionate to protein expression, as indicated by the detection of responses to this protein in 25% of TT/BT individuals, a much higher response than that seen in this group toward ML0380.

There was limited cross-reactivity of TB patient sera to all of the recombinant proteins, even those such as ML2028, the homolog to *M. tuberculosis* Rv1866c, which shares 83% identity at the amino acid level. This would be desirable to discriminate between individuals with leprosy or TB in a setting such as the Philippines or Brazil, where exposure or infection to both of these pathogens is high. We chose to examine individuals with cavitary TB, thinking that those patients with higher smear positivity reflecting a higher bacterial load would produce higher titers of antibodies to protein and other antigens such as LAM, as is the case with MB leprosy patients. However, there was a broad range in reactivity to LAM in TB patients, even among those with the highest AFB count, suggesting that factors other than bacterial load determine the strength of the response to this antigen in TB patients. One of the most interesting findings was the number of LAM-positive individuals in the HEC population. Approximately one-third of the individuals gave a ≥1+ LAM reactivity pattern by Western blot analysis, while none of the NEC individuals gave this type of reaction. There is a significant amount of LAM in native MLSA, and the characteristic pattern of LAM on SDS-PAGE gels was shown to be present between 20 and 40 kDa, the region of most intense staining for almost all of the leprosy patient sera. This staining pattern was very similar to that shown when using an anti-LAM-specific mouse monoclonal antibody, CS-35, when MLSA was probed in a Western blot. The higher reactivity in HEC individuals toward LAM could be due to an increased exposure to pathogenic as well as avirulent environmental mycobacteria (for example, in the soil and water) in a setting where leprosy is endemic, or it could also be partially due to BCG vaccination. Whether antibodies to LAM provide any protection or might be considered a risk factor, as is the case with being seropositive to PGL-I, is unknown, but we are currently examining this in contacts of BL/LL patients as well as in a larger group of healthy endemic controls to determine its role in mycobacterial infection.

In this work, we have examined the relationships between antibodies to protein, PGL-I, and LAM in leprosy and TB patients and in HEC and NEC sera. The responses of leprosy patients to ML028 and ML0380 were generally strong, even in individuals in the TT/BT group, while cross-reactive responses in TB individuals and HEC sera were very low. The combination of such protein antigens with more sensitive detection methods, such as the dual-path platform (DPP) lateral flow (LF) device (10) that, compared to one-dimensional LF devices, has been reported to increase the threshold of detecting antibody reactivity in TT/BT sera by 100-fold, could greatly improve current technology for diagnosing and categorizing leprosy patients. Increasing the sensitivity and specificity in a cheap and simple serological test using a combination of protein antigens could lead to earlier diagnosis and more rapid treatment, reduce the transmission rate, and decrease nerve damage and disability, all of which would lead to a decrease in the incidence of leprosy and complications.

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REFERENCES