Enoyl-Coenzyme A Hydratase and Antigen 85B of *Mycobacterium habana* Are Specifically Recognized by Antibodies in Sera from Leprosy Patients

J. Serafín-López,¹ M. Talavera-Paulin,¹ J. C. Amador-Molina,² M. Alvarado-Riverón,¹ M. M. Vilchis-Landeros,³ P. Méndez-Ortega,¹† M. Fafutis-Morris,⁴ V. Paredes-Cervantes,⁵ R. López-Santiago,¹ C. I. León,²,⁶ M. I. Guerrero,²,⁶ R. M. Ribas-Aparicio,² G. Mendoza-Hernández,³ C. Carreño-Martínez,⁶ S. Estrada-Parra,¹ and I. Estrada-García¹*

Departamento de Inmunología¹ and Departamento de Microbiología,² Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México, D.F., México; Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, México, D.F., México;² CHINDE Departamento de Fisiología, CUCS, Universidad de Guadalajara, Guadalajara, México;³ Grupo de Micobacterias-Investigación, Instituto Nacional de Salud, Bogotá, Colombia; and Centro Nacional de Programas Preventivos y Control de Enfermedades (CENAPRECE), Secretaría de Salud, México, D.F., México⁶

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Leprosy is an infectious disease caused by *Mycobacterium leprae*, which is a noncultivable bacterium. One of the principal goals of leprosy research is to develop serological tests that will allow identification and early treatment of leprosy patients. *M. habana* is a cultivable nonpathogenic mycobacterium and candidate vaccine for leprosy, and several antigens that cross-react between *M. leprae* and *M. habana* have been discovered. The aim of the present study was to extend the identification of cross-reactive antigens by identifying *M. habana* proteins that reacted by immunoblotting with antibodies in serum samples from leprosy patients but not with antibodies in sera from tuberculosis (TB) patients or healthy donors (HDs). A 28-kDa antigen that specifically reacted with sera from leprosy patients was identified. To further characterize this antigen, protein spots were aligned in two-dimensional polyacrylamide gels and Western blots. Spots cut out from the gels were then analyzed by mass spectrometry. Two proteins were identified: enoyl-coenzyme A hydratase (lipid metabolism; ML2498) and antigen 85B (Ag85B; mycolyltransferase; ML2028). These proteins represent promising candidates for the design of a reliable tool for the serodiagnosis of lepromatous leprosy, which is the most frequent form in Mexico.

Intradermal immunization with killed *Mycobacterium leprae* renders mice immune to infection with viable *M. leprae* (28). This protection is long lasting and systemic. However, when other mycobacteria are used to immunize mice against infection with viable *M. leprae* bacilli, they have been shown to be either ineffective (i.e., *Mycobacterium duvalii*) or to confer only partial protection (i.e., *M. bovis* BCG) (29). In 1985 and then 1989, *Mycobacterium habana* TMC 5135 was found to be as effective as *M. leprae* in protecting mice against footpad infection (32, 33). This was surprising, since Shepard et al. found that among a large panel of mycobacteria tested, only *M. leprae* and BCG were able to confer protection (30).

*M. habana* was first described following its isolation from 35 cases of pulmonary tuberculosis (TB) (40); subsequently, it was found to be closely related to the species *Mycobacterium simiae* serovar 1 and is now known as *M. simiae* serotype 1 (20). In 1996, Khoo et al. demonstrated that *M. habana* TMC 5135 and several *M. simiae* strains differed in their polar glycopeptidolipid (GPL) compositions, conferring sufficient specificity for identification of *M. habana* as a distinct serotype of *M. simiae* (14).

*M. habana* is a cultivable organism, protects mice against *Mycobacterium ulcerans* challenge, and offers consistent protection against infection with *Mycobacterium tuberculosis* H37Rv and other strains of *M. tuberculosis* (11, 12). Some studies suggest that the secretory antigens released by actively growing *M. habana* bacilli are protective against *M. tuberculosis* infections (6, 8). Recent experimental data indicate that *M. habana* TMC 5135 and *M. habana* 1PK-220 strains, which differ in the fine structure of their mycolates, are not equally immunogenic or virulent in a BALB/c mouse model of progressive pulmonary tuberculosis (38, 39).

It is not clear which characteristics of *M. habana* render it capable of protecting mice against infection with *M. leprae*, particularly in view of the large degree of antigenic cross-reactivity found throughout the genus. Previous work showed that *M. habana* and *M. leprae* share some antigenic epitopes. Using a panel of monoclonal antibodies raised against *M. leprae*, Lamb et al. were able to show that the specific 18-kilodalton antigen of *M. leprae* is also present in *M. habana*, as is the 65-kDa antigen (16). In 1996, Bisht et al. described the puri-
fication and immunochemical characterization of a major 23-kDa cytosolic protein antigen of the vaccine candidate \textit{M. habana} (4).

Although a remarkable decrease in world leprosy prevalence from ~5.4 million patients in 1985 to ~0.2 million in the first quarter of 2010 was observed (http://www.who.int/wer/2010/wer8535/en/), the incidence of the disease has remained almost unchanged for the last years. In Mexico, according to official reports, the number of new cases detected during 2010 was 171; the incidence in 1989 was 0.30, and that in 2008 it was 0.187 (http://www.dgepi.salud.gob.mx/boletin/2011/sem1). The major reported cases have the multicellular (MB) form, therefore presenting a large amount of specific antibodies which may be useful for detecting infection with \textit{M. leprae}.

To our knowledge, there are only three reports showing antigens in \textit{M. habana} which are recognized by leprosy patient sera (7, 24, 34). In this study, we have analyzed antigens from \textit{M. habana} TMC 5135 that specifically reacted with sera from leprosy patients (with the tuberculosis leprosy [TT] or lepromatous leprosy [LL] form) but not with sera from healthy household contacts (HHCs) or TB patients. In this manner, we detected two antigens with a molecular mass of 28 kDa that were identified as enoyl-coenzyme A hydratase (enoyl-CoA; ML2498) and antigen 85B (Ag85B; ML2028). These proteins from \textit{M. habana} could be used as the basis of a serodiagnostic test for the multicellular forms of the disease.

\section*{MATERIALS AND METHODS}

\textbf{Mycobacteria.} \textit{M. habana} TMC 5135 was kindly provided by the late M. J. Colston (National Institute for Medical Research, Mill Hill, London, United Kingdom) and was grown under stationary conditions for 2 to 4 weeks in Proskauer and Beck medium, modified by Youman (PBY) (1). The cell mass was then harvested under aseptic conditions by centrifugation at 8,000 \(\times\) g at 4°C (Sorvall RCB5; DuPont, Newtown, CT).

\textbf{MHSE.} Briefly, a \textit{Mycobacterium habana} soluble extract (MHSE) was prepared as follows: cells were washed in saline. This suspension was sonicated from an area with leprosy endemicity similar to that where patients were recruited: 23 from active pulmonary TB patients and 33 from healthy donors (HDs) analyzed 66 serum samples from HHCs of Colombian leprosy patients (42 from the Ridley-Jopling criteria (25) and/or the operational classification of the World and in Colombia. The characteristics of the individuals in the study groups are listed in Table 1.

| Sample category (no. of individuals) | Median (range) age (yr) | Male/female ratio | Bacterial index range |
|--------------------------------------|-------------------------|--------------------|---------------------|
| LL (96) | 30 (8–64) | 1:1 | 2–6 |
| TT (11) | 35 (16–55) | 1:2 | 0 |
| I (22) | 40 (16–69) | 1:3 | 0 |
| HHC-PB (24) | 17 (2–76) | 1:1 | NA* |
| HHC-MB (42) | 22 (1–66) | 1:1 | NA |
| HD (26) | 21 (16–50) | 1:1 | NA |

* NA, not applicable.

\textbf{Soluble extracts.} Soluble extracts were prepared using phosphate buffered saline (PBS) and centrifuged at 4°C for 10 min. Finally, they were incubated with 5 ml of equilibrium buffer containing 250 mg of DTT with 125 mg of iodoacetamide for 10 min.

The strips were overlaid onto 10% SDS-polyacrylamide gels, and after electrophoresis, proteins were stained with 0.2% Coomassie brilliant blue R-250 in 10% acetic acid–20% methanol and used to perform mass spectrometry (MS) or reequilibrated with 5 ml of equilibrium buffer containing 250 mg of DTT for 10 min. The sample was then desalted in a Sephadex G25 column (NAP-10; GE Healthcare, Pittsburgh, PA), and the eluate was concentrated by ultrafiltration and requantified by a microtiter plate Bradford assay (Bio-Rad, Hercules, CA). The protein pellet was resuspended and adjusted to 130 mg/ml with 1% formic acid. MS analysis was carried out on a 3200 Q Trap hybrid liquid chromatography/electrospray ionization tandem mass spectrometer (LC/ESI-MS/MS). Antigen spots in Western blots were matched with the corresponding protein spots in the Coomassie-stained gel and were selected for MS analysis. The protein spots were carefully excised from the 2-DE gel, digested with 1% trypsin (Promega, Madison, WI), and extracted as previously described by Bienvenut et al (3). The volume of the extracts was reduced by evaporation in a vacuum centrifuge at room temperature and then adjusted to 20 μl with 1% formic acid. MS analysis was carried out on a 3200 Q Trap hybrid tandem mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with a nanoelectrospray ion source (NanoSpray II) and a MicroIonSpray II head.

Data interpretation and protein identification were performed with the MS/MS spectrum data sets using the MASCOT search algorithm (version 1.6.69; Matrix Science, London, United Kingdom). Searches were conducted using the \textit{Actinobacteria} subset of the National Center for Biotechnology Information nonredundant database (NCBI; http://www.ncbi.nlm.nih.gov). Trypsin was used as the proteolytic enzyme.
the specific protease, and one missed cleavage was allowed, with tolerances of 0.5 Da for the precursor and 0.3 Da for the fragment ion masses. A protein “hit” was accepted as a valid identification when at least one MS/MS spectrum matched at the 95% confidence level ($P < 0.05$). Protein identifications based on a single peptide required a matching probability of $>95\%$ and were manually verified. Manual validation of MS/MS spectra was based on mass accuracy of precursor ions and the presence of a consecutive $b$ or $y$ ion series of three or more amino acids.

**RESULTS**

**Antibody response to** *M. habana* **proteins in leprosy patients.** The humoral reactivity to soluble extracts of *M. habana* was assessed by WB. This was done using a small number of samples from the serum bank: LL ($n = 25$), TT ($n = 4$), and pulmonary TB ($n = 13$) patients and HDs ($n = 13$). All the samples were diluted 1:100. Reactivities to MHSE antigens in leprosy patient samples are shown in Fig. 1. Patients with the LL form of the disease (either nodular or diffuse) showed reactivity with a band with a molecular mass of 28 kDa. None of the sera from TT or TB patients or HDs showed antibodies against this band.

**Seropositivity rates of leprosy patients, TB patients, HHCs, and HDs.** To confirm that only LL sera and not TB patients or HDs had antibodies against the 28-kDa band of MHSE, a pool of sera from each group was used in a WB assay using MHSE proteins separated in a 13.5% polyacrylamide gel. Figure 2A shows this result, where a doublet is now visible in the lane developed with the LL sera (arrows). Once we confirmed the specificity of the reaction between MHSE and LL patient sera, we evaluated the seropositivity and specificity of the MHSE 28-kDa antigen; for this, all serum samples collected from different groups were used in WB assays: 96 LL, 11 TT, 22 I, and 23 TB patients; 66 HHCs; and 26 HDs. Results for LL, TT, and I patient and HHC sera are shown in Fig. 2B. LL patient samples showed an 83% (80/96) positive response to the 28-kDa antigen from *M. habana*, while samples from TT and I patients showed 36.3% (4/11) and 41% (9/22) seropositivity rates, respectively. Only 3% of the samples (2/66) from HHC sera reacted with the 28-kDa antigen.

The specificity of this test was considered to be 100%, since
serum samples from TB patients and HDs did not show any reactivity against the 28-kDa antigen.

Identification of M. habana antigens that are recognized by antibodies in sera from lepromatous leprosy patients. Direct identification of antigens from 1-dimensional SDS-PAGE gels is not possible because of the complexity of the protein mixtures. Therefore, the MHSE proteins were separated by 2-DE and transferred onto PVDF membranes for WB analyses. These membranes were prepared to be individually probed with the sera of three LL patients. As a negative control, sera from TB patients and HDs were also tested. In Fig. 3A we can see representative results obtained with a serum sample from each group. Antigen spots were observed only with the sera from the three LL patients.

The identified spots are indicated with arrows and numbered in the WB membrane (Fig. 3A). They correspond to the spots that could be seen in the Coomassie blue-stained gel (Fig. 3B). All three spots corresponding to the assigned antigens were excised from the gel, destained, and subjected to digestion with trypsin. The resulting tryptic fragments were analyzed by tandem mass spectrometry using the MASCOT software. As the M. habana genome is not available, protein identification was performed using the predicted peptides with BLAST searches against the nonredundant NCBI database, comprising annotated proteins of the Actinobacteria complex.

Two proteins were identified in the three spots, and these corresponded to enoyl-CoA hydratase (ML2498) and Ag85B (ML2028); the results are listed in Table 2. Both antigens were in the pH range of 5.2 to 5.6, and the theoretical and experimental molecular masses were similar for enoyl-CoA hydratase (i.e., 28 and 27.2 kDa, respectively). In contrast, differences were observed between the experimental and theoretical molecular masses of Ag85B (i.e., 28 and 34.9 kDa, respectively) (Table 2).

Although a match for Ag85A was also found in spot number 1, this protein was not considered to be relevant because its theoretical pI of 7.66 do not coincide with the pI of the spot. The matching peptides identified by mass spectrometry were aligned to their corresponding M. leprae counterparts (Fig. 4).

| Spot no. | Protein name, source (accession no.) | Exp/theo* molecular mass (kDa) | Exp/theo pl | Mowse score | No. of matched peptides | % sequence coverage |
|---------|-------------------------------------|-------------------------------|------------|-------------|------------------------|-------------------|
| 1a      | Enoyl-CoA hydratase, Mycobacterium kansasii (gi|240170297) | 28/27.22 | 5.25/5.22 | 98 | 4 | 17 |
| 1b      | Ag85A, fibronectin-binding protein, Mycobacterium gordonae (gi|13431271) | 28/35.62 | 5.25/7.66 | 70 | 1 | 5 |
| 1c      | Ag85B, Mycobacterium leprae (gi|44381) | 28/34.97 | 5.25/5.17 | 59 | 1 | 6 |
| 1d      | Ag85B, extracellular alpha antigen, Mycobacterium avium (gi|728748) | 28/34.88 | 5.25/5.33 | 53 | 1 | 5 |
| 2a      | Enoyl-CoA hydratase, Mycobacterium kansasii (gi|240170297) | 28/27.2 | 5.33/5.22 | 101 | 7 | 29 |
| 2b      | Ag85B, extracellular alpha antigen, Mycobacterium avium (gi|728748) | 28/34.9 | 5.33/5.35 | 57 | 5 | 19 |
| 3a      | Enoyl-CoA hydratase, Mycobacterium kansasii (gi|240170297) | 28/27.2 | 5.6/5.22 | 124 | 8 | 30 |
| 3b      | Ag85B, extracellular alpha antigen, Mycobacterium avium (gi|728748) | 28/34.9 | 5.6/5.33 | 56 | 4 | 17 |

* Exp/theo, experimental/theoretical.
To identify additional antigens that might serologically detect leprosy patients, in this work we examined the reactivity of a soluble extract from *M. habana*. After collecting a large number of serum samples from untreated leprosy patients, two proteins with a molecular mass of 28 kDa which induced a potent humoral immune response were identified. These immunodominant antigens were recognized in WB assays by 83% of LL, 36.3% TT, and 41% of I patient sera. It was remarkable that neither active TB patients nor healthy individuals showed antibodies against these antigens, and only 3% of leprosy patient HHCs showed a positive reaction. This figure of 3% is similar to that of contacts that may develop active disease, which in Mexico is less than 10% (http://www.cenavece.salud.gob.mx/descargas/pdf/lepra.pdf). Therefore, it is possible that this assay may have a potential use to select HHCs with a higher risk of developing the disease.

Although the seropositivity rate for the TT and I groups is higher than that obtained using IgM, anti-*M. leprae* phenolic glycolipid I (PGL-I) in some regions of the world, for example, 6.9% in Zaire (10), the number of serum samples in these groups should be increased before any firm conclusions are reached.

In a previous study, *M. habana* was used to detect leprosy patients in the sera of leprosy patients (24). This cross-reactivity was confirmed when *M. habana* sonicates used to detect leprosy cases showed a better performance than *M. leprae* sonicate or *M. leprae* PGL-I, increasing the number of cases that were detected (34).

Others authors have shown that the *M. habana* 18-kDa protein shares a cross-reactive epitope with the *M. leprae* 18-kDa protein that is not present in *M. tuberculosis* (16). These findings, together with our results, indicate that *M. habana* contains several cross-reactive epitopes with *M. leprae* which are not shared with *M. tuberculosis*.

The two *M. habana* antigens described here, enoyl-CoA hydratase and Ag58B, had an expected molecular mass of 28 kDa. Enoyl-CoA hydratase was identified in more than one spot, an indication of the likely presence of isoforms within the preparation. Similarly, an enoyl-CoA hydratase from *M. avium subsp. paratuberculosis* detected in a two-dimensional Western blot with sera from Crohn’s disease patients was also identified in two spots (31).

Enoyl-CoA hydratase is a key protein in the biosynthesis of fatty acids. In mycobacteria, this enzyme is involved in the biosynthesis of major and essential lipids such as mycolic acids. Sequence analysis predicted that these enzymes are synthesized as intracellular proteins and belong to the hydratase/isomerase superfamily (27, 36).

According to our results, *M. habana* enoyl-CoA hydratase could be considered a candidate antigen for serodiagnosis of leprosy. This is not the first time that this molecule is being proposed as a useful antigen for detection of *M. leprae* antibodies in the sera of leprosy patients. Having access to the complete genome sequences of *M. leprae*, *M. tuberculosis* complex (principal members), and four environmental mycobacteria, Araoz et al. used comparative genomics to establish a list of candidate *M. leprae* antigens that could ultimately serve as the basis for an immunodiagnostic test for leprosy (2). Twelve genes from *M. leprae* were cloned and purified, and the immunogenicity of each recombinant protein was then investigated in leprosy patients by measuring the reactivity of circulating antibody and gamma interferon (IFN-γ) production.

Remarkably, only two proteins showed marked humoral responses. One of these was the enoyl-CoA hydratase (ML2498); this protein is present in *M. leprae* and has orthologues in organisms besides mycobacteria (2).

Moreover, in a recent report in which *M. leprae* restricted or unique proteins were identified by MS, only 13 appeared to be expressed at all. Of these, three were expressed in quantities...
large enough to make them suitable for diagnostic purposes: ML2346, ML2347, and ML2498 (enoyl-CoA hydratase) (42). This adds further evidence to how \textit{M. habana} enoyl-CoA can be recognized by antibodies in sera from leprosy patients in a cross-reactive manner.

In the case of Ag85B, there was a discrepancy between the expected molecular mass (34.9 kDa; Table 2) and the actual cross-reactive manner. It could be recognized by antibodies in sera from leprosy patients in a cross-reactive manner.

Ag85B is a protein antigen secreted by live mycobacteria and plays an important role in humoral and cellular immune responses (17, 18, 23). This protein family is formed by three members, antigens 85A, 85B, and 85C, with molecular masses of 32, 30, and 33 kDa, respectively, in \textit{M. bovis} BCG, each encoded by three distinct genes (43).

Since the \textit{M. bovis} BCG Ag85 complex presents high sequence identity with the \textit{M. leprae} Ag85 complex, these antigens have been used to analyze T cell responses in leprosy patients (17, 18, 22). Moreover, quantification of antibody responses in leprosy sera against each member of the \textit{M. bovis} Ag85 complex showed that Ag85B was recognized by 64% of LL patient sera but not by control sera. These results suggest that \textit{M. bovis} Ag85B contains one or several epitopes specifically recognized by sera of LL patients (9). A recent report demonstrated that \textit{M. leprae} recombinant Ag85B (ML2028) is an ideal candidate for use for leprosy serodiagnosis, as it is recognized by sera from all leprosy patients, regardless of their clinical classification (35).

Although the degrees of identity between enoyl-CoA hydratase and Ag85B from \textit{M. leprae} and \textit{M. tuberculosis} are high, 83% and 84%, respectively, there are possible explanations for the fact that tuberculosis patients do not have antibodies against \textit{M. habana} homologues. One of these is that the native \textit{M. leprae} proteins expressed inside leprosy patients must have epitopes that are similar or identical to those present in the \textit{M. habana} proteins but that are not present in \textit{M. tuberculosis}. Using the algorithm of Welling et al. (41) to identify antigenic regions in both proteins, we could confirm that there are putative epitopes in both \textit{M. leprae} proteins which are not present in the \textit{M. tuberculosis} homologues (data not shown). The identification of the proteins described here was done using MASCOT software and the \textit{Actinobacteria} database, and the alignments of those peptides which produce a valid hit are shown in Fig. 4.

Currently, \textit{M. leprae} PGL-I is used as a specific antigen for leprosy serodiagnosis, but in practice, its sensitivity and specificity are not as high as expected. Previous studies reported that the detection rates for MB and PB patients were 57% and 20%, respectively (13); another study showed that the rates of percent positivity (anti-PGL-I IgM) observed with the MB patient sera were 79.6% for the dipstick assay and 74.1% for the enzyme-linked immunosorbent assay (5). In a systematic review by Moura et al., they propose that serology for \textit{M. leprae} using PGL-I might positively influence leprosy control programs. After an extensive analysis of published data, they identified that the range of rates of seropositivity for the MB form varied from 51.2% to 97.4% and that for the PB form ranged from 6.9% to 57.3%, and they concluded that this variation could be related to the differences in immunological responses of different populations (21). In Mexico, data for PGL-I are not available, making it impossible to compare this test with seropositivity for the \textit{M. habana} antigens. In Mexico, PGL-I has not been approved for use as part of a diagnostic test for leprosy, on the basis of results published by Rojas et al., in which it was demonstrated that sera from tuberculosis patients had cross-reactive antibodies against PGL-I, therefore limiting the usefulness of this antigen (26).

In conclusion, \textit{M. habana} enoyl-CoA hydratase and Ag85B may be used to develop serological diagnostic tests for the lepromatous form of leprosy. This is particularly relevant since \textit{M. leprae} cannot be grown in vitro. The facts that \textit{M. habana} TMC 5135 is an \textit{in vitro}-cultivable mycobacterium and that both identified antigens are recognized in their native form make them attractive candidates for the development of serological tests, which so far have proven to be the easiest to use and cheapest diagnostic tool for leprosy.

Certainly, using the already available PGL-I test with the detection of antibodies against the \textit{M. habana} antigens described herein could increase the overall sensitivity of the test, increasing, in turn, the detection of leprosy cases.

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