Identification of *Mycobacterium tuberculosis* Ornithine Carboamyltransferase in Urine as a Possible Molecular Marker of Active Pulmonary Tuberculosis

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Although the antigen detection assay has the potential to discriminate active tuberculosis from latent infection, development of such a test for the accurate diagnosis of this serious disease has only recently become a matter of interest. Here we present evidence that a *Mycobacterium tuberculosis* protein (ornithine carboamyltransferase, coded for by MT_1694; Rv1656 [argF]) is an interesting candidate molecule for this test development. The protein was initially discovered by mass spectroscopy in urine of patients with pulmonary tuberculosis and shown by Western blot analysis to be present in *M. tuberculosis* crude cell extract as well as in the culture supernatant (“secreted” protein). In addition, a recombinant ornithine carboamyltransferase (rMT1694) produced in *Escherichia coli* was recognized by immunoglobulin G (IgG) antibodies from patients with active tuberculosis but not by IgG from uninfected healthy subjects. Moreover, rMT1694 was strongly recognized by peripheral blood mononuclear cells from both healthy tuberculin purified protein derivative (PPD)-positive individuals and patients with pulmonary tuberculosis. More importantly, a capture enzyme-linked immunosorbent assay formatted with rabbit IgG antibodies specific to rMT1694 was able to identify the presence of this antigen in urine samples from 6 of 16 patients with pulmonary tuberculosis and in none of 16 urine samples collected from healthy PPD⁺ controls. These results indicate that an improved antigen detection assay based on *M. tuberculosis* ornithine carboamyltransferase may represent an important new strategy for the development of a specific and accurate diagnostic test for tuberculosis.

Despite the existence of anti-*Mycobacterium tuberculosis* drugs and the widespread application of the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine, global tuberculosis (TB) morbidity and mortality remain high and in many parts of the world are increasing due to coinfection with human immunodeficiency virus (6, 17, 23). It is estimated that one-third of the world’s population is infected with *M. tuberculosis* (18) and that every year 8 million new cases of TB are diagnosed, and up to 2.5 million deaths are attributed to the disease (15). BCG, the only commercially available vaccine, has been in use since the early 1920s. However, while this vaccine protects children from disseminated TB, it does not prevent adult or pulmonary disease (2, 4), the latter being the most common and contagious form of TB. Effective treatment of TB requires multiple medications that must be used over extended periods of time and is complicated by multidrug-resistant *M. tuberculosis* strains already affecting more than 50 million people around the world.

Another limitation to control of TB is the lack of a sensitive and reliable diagnostic procedure. Diagnosis of active TB still relies primarily on the direct finding of the tubercle bacilli either in sputum smears or in culture, procedures that are operator dependent and not sensitive enough to detect more than 65 to 70% of the disease burden.

Numerous novel diagnostic candidates are currently being pursued. The primary approaches to their discoveries have used the immune response of patients with TB as the readout of the antigen discovery strategies to select the candidate molecules. However, an interesting alternative approach to this strategy is the direct identification of *M. tuberculosis* antigens in the bodily fluids of humans with active disease.

Using this premise, we searched for *M. tuberculosis* proteins in the urine of patients with pulmonary TB and found four unique peptides that have identical sequence homologies with the deduced amino acid sequences of four different *M. tuberculosis* proteins. The initial biological, immunological, and clinical validation of one of these molecules (ornithine carboxamyltransferase) as a candidate for the development of an antigen detection assay is reported here.

**MATERIALS AND METHODS**

**Human samples.** A total of 96 blood samples were evaluated in this study. These samples were collected from three distinct groups of donors. Group 1 comprised 27 patients diagnosed with pulmonary TB based on the following criteria: a clinical course consistent with active TB (e.g., fever, cough, productive sputum, suggestive chest X ray), and culture of *Mycobacterium tuberculosis* from a specimen of either sputum or pleural fluid. Nine patients were from the University Hospital, Medical School of Triângulo Mineiro, Uberaba, Minas Gerais, Brazil, and 18 patients were enrolled from Lemuel Shattuck Hospital (LSH), Jamaica Plain, MA. Group 2 comprised 59 healthy tuberculin purified...
protein derivative (PPD) skin test-positive individuals with no previous history of treatment for TB; approximately 95% of these individuals were not born in the United States, and approximately 93% reported a possible history of BCG vaccination in early childhood. These subjects were employees at the Beth Israel Deaconess Medical Center, Boston, MA. Group 3 comprised eight healthy PPD-negative individuals with no previous history of either BCG vaccination or known contact with TB patients. These subjects were employees at the Forsyth Institute, Boston, MA. Approximately 10 ml of blood from each individual was collected in sodium heparin vacutainer tubes (BD, Franklin Lakes, NJ) and immediately processed for the preparation of mononuclear cells (see below). In addition to blood, urine samples were collected from the nine patients from the University Hospital, Medical School of Triângulo Mineiro, from the 16 TB patients at LSH, and also from 16 healthy PPD + and PPD − subjects. Approximately 200 ml of first morning urine was collected and centrifuged at 2,000 rpm to remove debris, followed by filtration on 0.2-μm-pore sterile filters. Urine samples were stored at 4°C until use. All donors were above 18 years of age and gave informed consent. The blood and urine donation protocols were approved by the Investigational Review Boards and Ethics Committees of the Medical School of Triângulo Mineiro, LSH, Beth Israel Deaconess Medical Center, and the Forsyth Institute.

MS. Individual human urine samples (15 ml) were loaded onto 15-ml Vivaspin filters with a molecular weight cutoff of 5,000 and centrifuged at 3,000 × g at 4°C to reduce the retentate volume to <2 ml. After appropriate reduction and alkylation of cysteine residues, 300 μl of urine from each patient was used for gel analysis and protein identification. These procedures were conducted at the Harvard Medical School Partners Health Care Center for Genomics, Genomics, and Proteomics in Cambridge, MA. Protein bands of interest were removed and digested in situ with MS-compatible proteases by trypsinization in 12-μl reaction volumes. Acetonitrile–0.1% formic acid, and by mass spectrometry on an LCQ DECA XP plus Proteome X workstation (ThermoFinnigan). For each run, 10 ml of each reconstituted sample was injected on a 75- by 18-cm column packed with C18 and run at a flow rate of 235 μl/min with a flow splitter gradient of 5 to 60% water–0.1% formic acid and acetonitrile–0.1% formic acid over the course of a 90- to 400-min run. The LCQ was run in top five configurations with one mass spectrometer in MS mode and five tandem MS scans. Dynamic exclusion was set to 1 with a limit of 30 s. Sequence analyses were performed using Sequest through the Bioworks Browser 3.1. Sequential database searches were made using the NCBI RefSeqHuman Database using differential carbamidomethyl-modified cysteines and oxidized methionines, followed by further searches using differential modifications. Secondary searches were performed using Sequest with the RefSeqHuman Gnomon predicted protein database. In this fashion, known and theoretical protein hits are found without compromising the statistical relevance of all the data. Peptide score cutoff values were chosen at Xcorr of 1.8 for singly charged ions, 2.5 for doubly charged ions, and 3.0 for triply charged ions, along with deltaCN values of 0.1 and rank of preliminary score (RSP) values of 1. The cross-correlation values chosen for each peptide assure a high confidence match for the different charge states, while the deltaCN cutoff ensures the uniqueness of the peptide hit. The RSP value of 1 ensured that the peptide matched the top hit in the preliminary scoring and that the peptide fragment file only matched to one protein hit.

**M. tuberculosis antigens.** The recombinant antigen used in these studies was produced and purified as described previously (10, 19). Briefly, oligonucleotide PCR primers were designed to amplify the full-length open reading frame derived from the gene. The reverse primer contained a BamHI restriction site followed by a stop codon and sequences derived from the gene. The resultant PCR products were digested with the respective restriction enzymes and subcloned into a PET11b expression vector similarly digested for directional cloning. Ligated PET14b was subsequently used to transform Escherichia coli BL-21(DE3)pol-lysS host cells (Novagen, Madison, WI) for expression. Recombinant protein was purified from 1,000 ml of IPTG (isopropyl-β-D-thiogalactopyranosyl)-induced batch cultures by affinity chromatography using the one-step, QIAexpress Ni-nitrilotriacetic acid (NTA) agarose matrix (Qiagen, Chatsworth, CA) as described previously (19). The yields of recombinant proteins were between 20 and 100 μg of protein per liter of induced bacterial culture, and purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining. Endotoxin contamination was removed using immobilized protamine B (Detox-Gel; Pierce, Rockford, IL). Endotoxin levels in purified recombinant protein were all <10 endotoxin units/mg protein, as indicated by the Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD). M. tuberculosis culture filtrate proteins were kindly provided by John Belisle and Karen M. Dobos (NIH contract HHSN266200400091C/ADB contract NO1-AF-40091, Tuberculosis Vaccine Testing and Research Material contract). PPD for in vitro tests was prepared as described previously (3).

**Western blot.** Crude M. tuberculosis antigens (100 ng) were fractionated by SDS-PAGE (4 to 20% gradient gel) and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). The blots were blocked overnight at 4°C with Tris-buffered saline–0.1% Tween 20 containing 5% bovine serum albumin (BSA) and subsequently incubated with either human or rabbit antisera. Following several rinses in Tris-buffered saline–0.1% Tween 20, either Staphylococcus aureus protein A or goat anti-rabbit immunoglobulin G (IgG) labeled with horseradish peroxidase at a 1:30,000 dilution (Pierce) was added for 1 h. After additional washings, bound conjugates were detected using the ECL enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ) and proteins were visualized by exposing the blot to autoradiography film (Kodak BioMax, Rochester, NY).

**Proliferation assay.** Human peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation (12). PBMC (1 × 10⁶/well) were incubated in 96-well round-bottom plates (Costar) in medium only (RPMI with 10% pooled human AB serum and penicillin-streptomycin (100 μg/ml) or in medium containing antigens. Plates were cultured for 6 days at 37°C in 5% CO₂ and were pulsed with 0.5 μCi of [³²P]thymidine (Amersham, Arlington Heights, IL) for an additional 8 h. Cells were harvested onto filter mats and counted using a Matrix 9600 scintillation counter (Packard, Meriden, CT). Results are expressed in a stimulation index (SI), which is the ratio of the cpm of [³²P]thymidine incorporation by cells cultured in the presence of antigens to the cpm incorporation by cells cultured in the presence of medium alone (nonstimulated cells).

**Antigen detection assay.** A capture enzyme-linked immunosorbent assay (ELISA) was developed using purified IgG anti- M. tuberculosis recombinant antigen obtained from antisera produced in two different rabbits. Briefly, wells of 96-well ELISA plates (high-binding enzyme immunoassay/radioimmunoassay plate; Corning International, Corning, NY) were coated overnight at 4°C with 0.2 μg purified IgG (obtained from one of the immunized rabbits) diluted in bicarbonate buffer (pH 9.0). Wells were washed with phosphate-buffered saline (PBS) plus 0.1% Tween 20 (Sigma Chemical Co., St. Louis, MO) and blocked at room temperature with PBS plus 0.1% BSA plus 1% Tween 20 (PBS/BSA/Tween) for 2 h. After washing, human urine samples (10⁵× 100% concentrated) were added and incubated overnight at 4°C. Plates were washed followed by incubation for 1 h with biotin-labeled IgG (obtained from the second immunized rabbit) 1/2,000, a dilution previously determined by conventional sandwich ELISA. Following several rinses in PBS/BSA/Tween, peroxidase-labeled streptavidin at 1/2,000 dilution (BD Biosciences) was added for 30 min. The plates were then washed, and reactions were developed with tetramethylbenzidine substrate and read at 450 nm.

**Statistical analysis.** Statistical significance was determined by unpaired t test for comparisons between two groups. Comparisons were performed using the Mann-Whitney rank sum test. (P values of <0.05 were considered statistically significant.)

**RESULTS**

**Characterization of M. tuberculosis antigens present in the urine of patients with pulmonary TB.** Vis-à-vis our previous results, which defined the presence of M. tuberculosis in the urine of infected mice (11, 12), we searched for such antigens from urine of patients with pulmonary TB. Urine was obtained from nine patients with active, culture-confirmed disease registered at the University Hospital, Medical School of Triângulo Mineiro (Uberaba, Minas Gerais, Brazil). The enrolled patients had no clinical signs or symptoms or laboratory findings compatible with renal or urinary tract abnormalities, therefore supporting the proposed lung (but not kidney) origin of the M. tuberculosis antigens present in the patients' urine. Individual urine samples were analyzed by MS, generating a total of approximately 400 peptide sequences. As expected, most sequences of the identified peptides had identical sequence homologies to those of human proteins. However, four peptides had identical sequence homologies to those of the deduced sequences of four different M. tuberculosis proteins (Table 1). One of them (ornithine carboxyltransferase, coded for by the...
gene MT_1694) was selected for further studies because this enzyme is essential for the in vivo survival of M. tuberculosis (7) and therefore is an interesting target molecule for the development of diagnostic tests designed to detect active TB. Moreover, the protein is conserved among the species of Mycobacterium (Table 2), with percentages of amino acid sequence identity with M. tuberculosis CDC 1551 ranging from 88.3% (M. bovis) to 72% (M. smegmatis).

Cloning of the gene, protein expression, and characterization of the rMT1694 protein. The open reading frame of the full-length MT_1694 gene was amplified by PCR and subcloned into the pET-14b expression vector. This expression vector contains a histidine tag (His tag) sequence before the NdeI cloning site, thus generating a recombinant protein containing a sequence of six His (His6) residues at the N terminus to facilitate its purification by affinity binding to an Ni-NTA agarose matrix. Expression of the recombinant protein was consistently achieved at yields ranging from 10 to 20 mg/liter of E. coli culture. Coomassie blue staining of gels (Fig. 1) performed under reduction conditions revealed the presence of a distinct induced protein migrating at a position corresponding to a molecular mass of ~41 kDa. This migration is slightly higher than the molecular mass of the native molecule, which is expected because of the addition of the His tag sequence and a thrombin site added to the recombinant protein to facilitate purification. To validate the expressed protein as a genuine M. tuberculosis protein, Western blot analysis was carried out using a polyclonal rabbit anti-MT1694 antiserum and two crude preparations of M. tuberculosis antigens (whole bacterial cell extract and the culture filtrate) and the recombinant protein. Figure 2 indicates that the antiserum recognizes a band of ~39 kDa in both the mycobacterial lysate and culture filtrate preparations (lanes 1 and 2) as well as (as expected) the recombinant protein (lane 3). These results confirm that the overexpressed recombinant protein is a genuine M. tuberculosis protein. In addition, because the protein was strongly detected in the microbial culture filtrate the results indicate that ornithine carbamylytransferase is an M. tuberculosis “secreted” protein.

Immunological recognition of rMT1694 by sera and by PBMC of TB patients, healthy PPD+ individuals, and PPD− individuals. To evaluate the potential use of rMT1694 as a diagnostic candidate molecule, experiments were performed to verify if this molecule is actively produced in vivo during infection with M. tuberculosis. To investigate this requisite, we tested the recognition of this protein by serum antibody and PBMC from various PPD skin test-positive subjects, including healthy subjects and patients with pulmonary TB, as well as healthy PPD skin test-negative individuals. The anti-rMT1694 antibody response of the IgG isotype was measured by Western blot analysis. The IgG fraction was purified from a pool of sera from six patients with confirmed pulmonary TB and from a pool of commercial normal human gammaglobulin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Figure 3A shows that IgG antibodies from patients with TB clearly recognized rMT1694. In contrast, IgG from healthy control individuals did not recognize the recombinant antigen (Fig. 3B). This pattern of response has also been observed with M. tuberculosis α-crystalline (22). Recognition of the recombinant molecule by human PBMC is depicted in Fig. 4. PBMC from

### TABLE 1. M. tuberculosis proteins identified in the urine of patients with pulmonary TB

| Putative identification                  | TIGR gene annotation | Cellular role(s) (TIGR)                      |
|-----------------------------------------|----------------------|---------------------------------------------|
| Ornithine carboamyltransferase          | MT_1694              | Amino acid biosynthesis, glutamate family   |
| MoaA-related protein                    | MT_1721              | Unknown function                            |
| Phosphoadenosine phosphosulfate reduc-tase | MT_2462              | Amino acid biosynthesis, serine family, central intermediary metabolism, sulfur metabolism |
| Homoserine O-acetyltransferase          | MT_3444              | Amino acid biosynthesis, aspartate family   |

![FIG. 1. Purification and characterization of the recombinant protein coded for by the gene MT_1694. Recombinant protein containing His6-tagged amino-terminal residues was expressed in E. coli BL21(DE3)/pLysS followed by purification by affinity chromatography using Ni-NTA agarose matrix. Expression and purity were evaluated by SDS-PAGE (4 to 20% gradient polyacrylamide gel) and Coomassie blue staining. Numbers on the left are the molecular masses of the markers (MWM) in kDa. Lane 1, E. coli lysate from noninduced cultures; lane 2, E. coli lysate from IPTG-induced cultures; lane 3, purified rMT1694 protein.](http://cvi.asm.org/)

### TABLE 2. Percentage of amino acid sequence identity of M. tuberculosis ornithine carbamylytransferase to representative members of the Mycobacterium genus

| Species                  | % Identity | p value  |
|--------------------------|------------|----------|
| M. bovis                 | 88.3       | 8.9×10⁻⁴⁰ |
| M. avium                 | 72         | 7.1×10⁻¹⁵ |
| M. smegmatis             | 72         | 4.9×10⁻¹⁶ |
| M. leprae                | 75.8       | 1.2×10⁻¹² |
| Mycobacterium sp.        | 71.6       | 1.5×10⁻¹¹ |

*The percentages of identity (and respective p values) of the deduced amino acid sequences coded for by the identified gene in M. tuberculosis CDC1551 with genes present in the indicated members of the Mycobacterium genus were obtained from the TIGR website (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi).*
40 out of 54 healthy, PPD skin test-positive (>10 mm of induration) individuals were stimulated by rMT1694 to proliferate with an SI of 5, although to a lesser extent, PBMC from 7 out of 16 patients with pulmonary TB also responded to stimulation with the recombinant antigen. In contrast, PBMC from eight PPD-negative healthy controls did not proliferate when stimulated with rMT1694. A similar pattern of response has also been described for antigens encoded by genes of the dormancy regulon cluster of Mycobacterium tuberculosis (9).

Detection of M. tuberculosis native antigen MT1694 in patients' urine samples using conventional capture ELISA. Because the Western blot analysis indicated that M. tuberculosis ornithine carboamyltransferase is a "secreted" antigen, we reasoned that this molecule would normally be released in vivo from the tubercle lesions, absorbed in the blood circulation, and eliminated in the urine, consistent with its detection in the urine of TB patients by high-performance liquid chromatography/MS. Given this and the above data showing that the molecule is intimately involved in the host-pathogen interaction, we considered ornithine carboamyltransferase to be an interesting candidate molecule for the development of an antigen detection assay for the diagnosis of active TB. This type of assay is in high demand to accurately diagnose TB disease and to distinguish it from latent infection. To begin to evaluate the utility of ornithine carboamyltransferase as a possible marker of active TB in clinical settings, an antigen capture ELISA was developed. Two rabbits were used for the production of two antisera to rMT1694. Purified IgG antibodies were obtained from the individually immunized rabbits using standard Staphylococcus protein G affinity chromatography. The IgG preparations were used either as capture antibody (unlabeled molecule) or as detection antibody (biotin labeled) of a sandwich capture ELISA. Thus far, 16 urine samples from well-characterized pulmonary TB patients from LSH (Jamaica Plain, MA) and 16 urine samples from healthy PPD-positive control individuals have been tested. Urine samples from six of the TB patients provided positive ELISA signals that were significantly higher than the signals given by the normal control urine samples (Fig. 5). All of the TB patients had had between 0 and 14 days of anti-TB therapy at the time of enrollment into our study and initial urine collection. Interestingly, two positive urine samples collected at the beginning of the therapy no longer tested positive for the antigen after 12 weeks of therapy (not shown). These results clearly open interesting possibilities for the development of a sensitive and specific antigen detection assay for the diagnosis of active TB.

DISCUSSION

We have recently described an interesting alternative approach for the direct identification of M. tuberculosis antigens in bodily fluids of mice infected with this organism (11, 12). Using this approach, we identified two M. tuberculosis antigens in the urine of infected mice and found that the recombinant protein version of one of these antigens, when tested as a vaccine candidate, induced protection similar to that induced by BCG (12). The second antigen proved to be of diagnostic interest (11).

The translation of this strategy for antigen discovery to humans was easily achieved. Pooled urine samples collected from patients with well-characterized pulmonary TB yielded over
PPD-positive subjects (n/H11005 native subjects (n/H11005 PBMC. The proliferative response of PBMC from healthy PPD-negative subjects, as well as to compare the results obtained for TB patients (7). Therefore, we reasoned that this molecule would be an interesting possible molecular marker of active disease and infection was done by Western blot analysis. The demonstration that the molecule was produced in vitro was done using a rabbit antiserum specific for the recombinant protein. This antiserum detected a single band of the expected molecular mass of M. tuberculosis ornithine carboamyltransferase in both bacterial cells and the culture supernatant (“secreted protein”). Conversely, using sera from patients with TB the Western analysis clearly demonstrated the presence of specific anti-M. tuberculosis rMT1694 IgG antibodies. In contrast, IgG from uninfected subjects did not recognize the antigen. Therefore, this observation indicates that ornithine carboamyltransferase is a relevant microbial molecule produced during disease, thus supporting the hypothesis that rMT1694 is an interesting target for diagnostics development. The results obtained with human PBMC further support this idea and suggest that ornithine carboamyltransferase is of particular interest in that this molecule is immunogenic in humans subsequent to sensitization with M. tuberculosis. Together, the results indicate that ornithine carboamyltransferase is actively produced in vivo and is involved in the host-pathogen interaction during infection, a sine qua non condition for the development of a successful antigen detection assay.

The concept of detection of microbial molecules in human bodily fluids of infected individuals for diagnostic purposes has strong precedent. For example, molecules from numerous viruses, bacteria such as Streptococcus pneumoniae or Legionella pneumophila, and parasites such as Entamoeba histolytica have long been described in various human samples (e.g., blood, mucous secretions, and feces) from patients suffering from the diseases caused by these microorganisms. Interestingly, many of these molecules were successfully used either as vaccines (e.g., for hepatitis A and B) or as tools for the development of antigen detection-based diagnostics. Perhaps the most successful example of such tests is the commercially available test to detect Streptococcus pyogenes (group A) in patients with tonsillitis (Signify Strep A; Abbot Diagnostics, Abbot Park, IL). This rapid test has been universally used as a routine diagnostic of S. pyogenes pharyngitis for more than 10 years (5, 13, 14).

FIG. 4. Recognition of purified M. tuberculosis rMT1694 by human PBMC. The proliferative response of PBMC from healthy PPD-negative subjects (n = 8), tuberculosis patients (n = 16), and healthy PPD-positive subjects (n = 54) was evaluated following stimulation with purified rMT1694 (5 μg/ml). Proliferation was measured by [3H]thymidine incorporation, and results are expressed as the SI. Dots represent individual donors. The dashed line represents an arbitrary cutoff (SI of ≥5), which was defined using the results obtained with PBMC from PPD-negative donors. Unpaired t tests were used to compare the SI obtained for both healthy PPD-positive individuals (*) and TB patients (**) with the SI obtained for healthy PPD-negative individuals, as well as to compare the results obtained for TB patients with healthy PPD-positive subjects (***) P values were <0.001, <0.03, and <0.05, respectively.

FIG. 5. Detection of native MT1694 antigen by capture ELISA in urine of TB patients and controls. The test was assembled with two batches of purified rabbit IgG anti-rMT1694. An aliquot of purified IgG was used as the capture antibody, and a second aliquot was biotin labeled and used as the detection antibody. The reaction was developed with peroxidase-labeled streptavidin. Dots represent individual donors. The dashed line represents an arbitrary cutoff value, which was calculated using the mean of the optical density (OD) at 490 nm given by the healthy PPD+ control urine samples plus 3 standard deviations of the mean.
lipoarabinomannan (LAM) in the urine of TB patients is under clinical validation. Despite some conflicting results regarding the sensitivity and specificity of this test, a significant correlation between the mycobacterial burden in sputum and LAM antigen concentrations in the patients’ urine samples has been found (1, 16, 20, 21). More recently, the *M. tuberculosis* antigen Ag85 has been tested as a candidate molecule for the development of an antigen detection assay in sera of patients with TB (8). The sensitivity of the test obtained with this antigen was similar to that obtained with LAM. However, the specificity of the test was poor as 14% of sera from infected negative healthy controls were positive as well. Therefore, the highly purified recombinant proteins identified in the present studies are of great interest as candidates to either replace or complement the sensitivity and specificity of the previously described tests. This possibility is supported by our findings that the native antigen MT1694 could be detected in the urine samples from 6 of 16 patients with well-characterized active pulmonary TB. Although these results point to a low sensitivity of the antigen/test, we believe that they are encouraging because the capture ELISA in our studies was assembled with whole-IgG fraction from the immunized rabbits and not with antigen-purified antibody. We are currently preparing anti-MT1694 monoclonal antibodies as well as large quantities of the recombinant molecule to facilitate the purification of large quantities of both monoclonal and polyclonal antibodies to assemble a more sensitive capture ELISA as well as a rapid lateral immunochromatography test. Moreover, we are also assembling tests to detect the other three *M. tuberculosis* proteins that we identified in urine from patients with TB. These new reagents will allow us to test larger numbers of subjects, particularly TB patients prior to and posttherapy as well as children and human immunodeficiency virus-infected patients.

Finally, these results strongly support the premise of the approach used in these studies, confirming that we have developed a powerful and reliable antigen discovery strategy to directly identify *M. tuberculosis* diagnostic candidate antigens in human bodily secretions. This approach should be broadly applicable to several other infectious diseases, particularly those caused by organisms that already have their genome completely sequenced.

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