Combined Use of Serum and Urinary Antibody for Diagnosis of Tuberculosis


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Efforts to devise immunoassays for tuberculosis (TB) that can be adapted to rapid formats are ongoing. The present study was aimed at determining whether urinary anti-Mycobacterium tuberculosis antibodies are present in patients with TB, to evaluate the feasibility of developing a urine antibody–based diagnostic test. Urinary antibodies directed against the culture filtrate proteins of M. tuberculosis, MPT 32, and the 81-kDa GlcB protein were detectable in patients with TB, although the sensitivity of antibody detection was lower (53%–64%), compared with serum antibodies (68%–77%). Surprisingly, with all 3 antigens, the use of paired serum and urine samples provided higher sensitivities of antibody detection than either single specimen, and anti-GlcB antibodies were present in the serum and/or urine of 39 (90%) of 43 smear-positive patients with TB. Although, with the current methods and antigens, the level of sensitivity is insufficient to design a urinary antibody diagnostic test, these studies provide the foundation for further studies on the development of a urine antibody–based immunoassay for TB.

Estimates suggest that there will be 80 million new cases of tuberculosis (TB) and 20 million resulting deaths during the current decade. A majority of these patients (>90%) will be in developing countries where diagnosis of TB is primarily based on demonstration of acid-fast bacilli (AFB) in sputum smears, clinical symptoms, and results of chest radiograph [1]. The variable and low sensitivity of the AFB smear and the burden caused by the requirement for testing multiple smears result in delayed diagnosis and high patient drop-out rates [1]. More-sensitive diagnostic techniques, such as cultures of AFB, radiometric systems, and nucleic-acid probes, are too expensive and technologically complex for routine application in developing countries. Effective TB control in these countries requires the development of simple, low-cost, rapid diagnostic assays.

The ability to adapt immunoassays to inexpensive formats, such as dipsticks or lateral flow cassettes, has resulted in renewed interest in devising serodiagnostic tests for TB, and some rapid tests have been devised [2]. However, the sensitivities and specificities of the currently available tests, most of which are based on the use of either recombinant 38-kDa protein alone or in combination with other antigens, need significant improvement [2]. Of the new M. tuberculosis antigens cloned and evaluated in recent years, an 88-kDa culture filtrate protein (CFP), now recognized to be the 81-kDa GlcB protein of M. tuberculosis, was identified by us to be a serodominant antigen in both human immunodeficiency virus (HIV)–uninfected and HIV-infected patients with TB [3–5], providing ~75% sensitivity and >95% specificity. This sensitivity is equivalent to or greater than sensitivities obtained by use of additive responses to several other seroreactive antigens.
[4, 6–9]. The immunodominance of the GlcB protein also has been emphasized in studies from other laboratories [6, 7]. Thus, despite the availability of new immunodominant antigens, a major difficulty being encountered in the development of serodiagnosis for TB is that no antigen, or set of antigens, has provided sensitivities >75%. The absence of serum antibodies in ~20%–25% of the patients has been ascribed to dysfunctional antibody responses or to the formation of circulating immune complexes [10–12].

Even as efforts to develop serodiagnostic assays for TB proceed, the incidence of HIV is increasing in developing countries [13–15]. The presence of both HIV and TB in the same populations contributes an additional confounding factor for the diagnosis of TB, because most clinical settings in these countries lack the resources to procure disposable devices needed for obtaining serum specimens from HIV-infected persons, and safe disposal of these used, infected devices is difficult and costly. These difficulties would be overcome if an antibody-detection diagnostic test based on the use of body fluids such as urine, stools, or saliva could be developed.

Studies show that infections or antigen exposure at one mucosal site can lead to the presence of immune responses at different mucosal surfaces [16]. The presence of urinary antibodies has been demonstrated in patients with HIV-1 [17, 18], Shigella species [19], or Helicobacter pylori infection [20, 21]. Moreover, mice immunized with aerosols of bacille Calmette-Guérin (BCG) develop both mucosal and systemic immune responses [22]. Because M. tuberculosis infection also is initiated on a mucosal surface, we have assessed the feasibility of developing a urine antibody–based diagnostic test for TB. The current studies provide evidence that antimycobacterial antibodies are present in the urine of patients with TB and are directed against the same antigens, as those recognized by serum antibodies.

METHODS

Antigens. The lipoarabinomannan (LAM)–free CFP of M. tuberculosis H37Rv have been used in all our previous studies of humoral responses to M. tuberculosis [3–5, 23] and is available from the National Institutes of Health/National Institute of Allergy and Infectious Diseases Tuberculosis Research Materials and Vaccine Testing, Colorado State University (Fort Collins). This preparation has been used recently by us to delineate the profiles of serodominant antigens recognized by antibodies from patients with TB at different stages of disease progression [5, 24].

Purified M. tuberculosis protein MPT 32 was obtained from M. tuberculosis, as described elsewhere [25]. For preparation of purified recombinant GlcB (Rv1837c), a 2.6-kb region of the M. tuberculosis H37Rv genome that contains glcB was amplified by polymerase chain reaction (PCR), using the oligonucleotide primers 5′-CTTCTGAGCCGCTAGCCGAGTA-3′ (forward) and 5′-GTCGCCGATGATAGTATCGT-3′ (reverse). The purified 2.6-kb PCR product was used as the template for the PCR amplification of GlcB, using the primers 5′-CATATGACAGATCGCCGTTGCGGTG-3′ (forward) and 5′-AAGCTTGGGGCGCGCATC-3′ (reverse). The resulting 2.2-kb PCR product was cloned into the pCR-Blunt plasmid vector (Invitrogen), and the recombinant plasmid was amplified in E. coli DH5α. The glcB gene was digested out of the recombinant plasmid with the restriction endonucleases NdeI and HindIII and ligated into appropriately digested pET23b plasmid (Novagen). The resulting plasmid (pMRLB8) was used to transform E. coli BL21(DE3) pLysS, and gene expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG). The recombinant GlcB possessing a C-terminal hexa-histidine tag was purified by nickel affinity chromatography using the His·Bind Resin (Novagen). The recombinant and culture filtrate–derived Glc proteins showed comparable reactivity with serum samples from several patients with TB and HIV-infected patients with TB (data not shown).

Subjects and samples. Paired serum samples and random, single void urine samples were obtained after informed consent was obtained from different groups of patients and control subjects (table 1). Thirty-eight of the 43 HIV-uninfected, smear-positive patients with TB were from the Lala Ram Sarup Hospital for TB and Allied Chest Diseases (LRSH; New Delhi), and 5 were from the Manhattan Veterans Affairs Medical Center or Bellevue Hospital (VA/Bellevue; New York). Samples from 4 HIV-uninfected, smear-negative, AFB culture–positive patients with TB also were obtained from the VA/Bellevue site. Eleven HIV-infected patients with TB, 8 from the VA/Bellevue site and 3 from the Laboratoire De Sante Hygiene Mobil Yaonde (Cameroon) also were tested. Paired specimens from 41 healthy individuals, 26 of whom were purified protein derivative (PPD) skin-test positive (>10-mm induration), were included as control specimens. These individuals were mostly immigrants from countries where TB is endemic, and none had received treatment for TB. Paired samples from 19 clinically asymptomatic individuals who were household contacts of smear-positive patients with TB were also obtained from the LRSH. The PPD reactivity of these individuals is not known. The urine samples were filter-sterilized, aliquotted into 2-mL aliquots, and stored at −20°C; the serum samples were stored at −70°C.

ELISA for detection of antibodies. For detection of urinary antibodies to the LAM–free CFP and MPT 32 by ELISA, 125 μL/well of LAM–free CFP (4 μg/mL) or MPT 32 (5 μg/mL) were allowed to bind to wells of ELISA plates (2 h at 37°C followed by overnight incubation at 4°C), washed with PBS, and blocked with 7.5% fetal calf serum (FCS; Hyclone) in 2.5% bovine serum albumin (BSA) for 2.5 h. After a second washing, 125 μL of undiluted urine was added to each well, the plates
Table 1. Groups of patients with tuberculosis (TB) and control subjects examined in this study.

<table>
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<tr>
<th>Infection status, origin</th>
<th>No. of subjects</th>
<th>Sex</th>
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<th>Smear negative</th>
<th>Culture positive</th>
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<td>19</td>
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NOTE. Data are no. of subjects, unless otherwise indicated. NA, not available; ND, not done.

were incubated overnight at 4°C, washed and exposed to 125 μL/well of alkaline phosphate–conjugated anti–human IgG (1:2000; Zymed Laboratories). After 60 min, the plates were washed with Tris-buffered saline (50 mmol Tris and 150 mmol NaCl), and the Gibco-BRL amplification system (Life Technologies Gibco BRL) was used for the development of color.

The ELISA with the serum antibodies from the same patients was performed by use of cross-reactive antibody-depleted serum, as described elsewhere [4, 26]. LAM-free CFP and MPT 32 were used at 5 and 2 μg/mL, respectively, and serum at 1:1000 and 1:150, respectively.

Western blots. Western blots prepared from a 10% SDSPAGE–fractionated LAM-free CFP (10 μg/lane) or purified Histagged GlcB (0.25 μg/lane) were washed with PBS, blocked with 3% BSA in PBS, washed with PBS-Tween (0.05%), and exposed overnight to undiluted urine or to 1:100 diluted, E. coli lysate–absorbed serum samples from patients and control subjects. Subsequently, the blots were exposed to alkaline phosphatase–conjugated anti–human IgG (1:2000) for 1.5 h and washed extensively, and the 5-bromo-4-chloro-indolyl-phosphatase–nitroblue tetrazolium substrate (Kirkegaard & Perry Laboratories) was used for visualizing the antigen-antibody reactivity. The Western blots were scanned by use of an Agfa Horizon Plus Scanner (Agfa USA Graphics), and the Kodak 1D Image analysis software (Eastman Kodak) was used to determine the net intensity of the GlcB band.

Statistical analysis. The cutoff in the ELISA assays was determined by use of the mean OD + 3 SD of the control subject group. To confirm the reproducibility of results obtained from ELISAs on different occasions, the percentage of agreement between the ELISAs for each specimen (serum or urine) with each antigen (LAM-free CFP or MPT 32) were computed. The ELISAs were performed 3–5 times with each specimen, and samples that were positive in at least 2 of 3 or 4 of 5 ELISAs were considered to be positive. For the evaluation of positivity with the GlcB protein on Western blots, the mean net intensity of the background reactivity with the control serum (+3 SD) was used as cutoff. To determine the correlation between the presence of antibodies against the LAM-free CFP and the MPT 32 antigen in the urine of patients with TB, and the correlation...
between the presence of antibodies in serum and urine from the same individuals, the McNemar’s paired test was used. The GraphPad Prism software was used to plot the dot plots.

RESULTS

Previous studies from our laboratory identified an 88-kDa protein, which was subsequently recognized as the 81-kDa GlcB, to be one of the dominant targets of antibody responses both in cavitary and noncavitary HIV-uninfected patients with TB and in HIV-infected patients with TB [3–6, 26]. Another CFP, MPT 32, also was identified to be a prominent target of antibody responses in smear-positive HIV-uninfected patients [4]. The studies of assessment of urinary antibodies in patients with TB described below were based on our experience with humoral immune responses in patients with TB when serum antibodies were studied.

Assessment of the presence of antimycobacterial antibodies in urine samples from patients with TB. To determine whether urinary antimycobacterial antibodies are present in patients with TB, urine samples from 22 HIV-uninfected, smear-positive patients with TB and 21 healthy PPD–skin test–positive control subjects were initially assessed for reactivity with the LAM-free CFP preparation and purified MPT 32 (figure 1). By use of the mean optical density of the control urine samples (+3 SD as cutoff), anti–LAM-free CFP antibodies were detectable in urine samples from 12 (55%) of 22 patients with TB, but not in urine samples from any of the control subjects (figure 1). In the same cohort, serum anti–LAM-free CFP antibodies were present in 17 (77%) of 22 patients. Urinary anti–MPT 32 antibodies were detectable in the 12 patients with anti–LAM-free CFP antibodies and in 2 additional patients (total, 14 [64%] of 22 patients), and in none of the control subjects. Serum anti–MPT 32 antibodies were present in 15 (68%) of 22 patients. When positivity was defined as reactivity in either the urine or serum sample from the same patient, 20 (90%) of 22 patients possessed anti–LAM-free CFP antibodies, and 17 (77%) of 22 patients had anti–MPT 32 antibodies (data not shown).

Statistical analysis of the data showed that the results of the ELISAs performed on different days were fairly consistent, with percentage of agreement ranging from 82% to 100% for different combinations. There was a strong correlation \((P = .001)\) between the presence of anti–LAM-free CFP and anti–MPT 32 antibodies in the urine samples of patients with TB. However, there was no statistically significant correlation between the presence of antibodies in the urine and serum samples from the same individuals \((P = .87\) for antibodies to LAM-free CFP and \(P = .27\) for MPT 32).

Profile of antigens recognized by urinary antibodies. The ELISA results described above confirmed that antibodies to antigens of \(M. tuberculo\)sis are present in a sizable proportion of the smear-positive patients with TB. To compare the profile of antigens recognized by the urinary and serum antibodies, paired samples from 3 patients with TB and 2 control subjects were used to probe SDS-PAGE–fractionated LAM-free CFPs (figure 2). The 2 body fluids contained antibodies to a similar profile of antigens. Prominent among the antigens recognized by the serum and urine antibodies was an ~81-kDa protein. The antibody titers in urine samples were lower, because undiluted samples provided visible responses, whereas the serum samples from the same individuals were used at a 1:100 dilution.

Reactivity of urinary and circulating antibodies with recombinant GlcB. Because antimycobacterial antibodies are detectable in the urine samples of patients with TB, the antigen profile recognized by urinary and circulating antibodies is similar, and an 81-kDa protein is well-recognized by the urinary antibodies, reactivity of the specimens from the same 22 patients with TB, as well as additional 21 smear-positive patients with purified recombinant 81-kDa GlcB, was evaluated by use of Western blot (figure 3). Urine and serum samples from 36 healthy individuals (25 PPD–skin test positive and 11 PPD–skin test negative) were included as control subjects. The testing was done by use of Western blot, because the ultimate goal is to use the antigen on a membrane-based format. The large numbers of specimens required the use of several blots; thus, each experiment included approximately equal numbers of test and control specimens. By use of the mean net intensity of the background reactivity with the control serum in each blot (+3 SD as cutoff), all 36 control subjects were negative for anti–GlcB antibodies (100% specificity). In contrast, of the 43 patients, anti-GlcB antibodies were present in urine samples from 23 (53%) patients, and serum antibodies were present in 33 (77%) patients (figure 3). Additive reactivity with both the samples resulted in the detection of anti–GlcB antibodies in 39 (90%) of the 43 smear-positive TB patients (figure 3).

Dual specimens obtained from HIV-uninfected smear-negative patients with TB and from HIV-infected smear-positive and smear-negative patients with TB also were assessed for the presence of anti–GlcB antibodies (figure 3). Urinary antibodies were present in patients from all groups of patients with TB, although the numbers of patients in each individual group were too small to determine whether the use of dual samples would result in an increased sensitivity of antibody detection in these categories of patients. No anti–GlcB antibodies were detectable in either of the samples from the 19 household contacts tested (data not shown).

DISCUSSION

The present study provides evidence that antibodies directed against the CFPs of \(M. tuberculo\)sis are present in the urine sam-

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Figure 1. ELISA reactivity of undiluted urine samples from patients with tuberculosis (TB) and healthy control subjects with lipoarabinomannan-free culture filtrate protein (A) and purified native MPT 32 (B). Mean OD + 3 SD was obtained by use of urine samples from healthy control subjects; OD + 3 SD was used as the cutoff to determine positive reactivity. Panels C and D show reactivity of serum antibodies from the same individuals with the same 2 antigens.

Figure 2. Reactivity of SDS-PAGE–fractionated lipoarabinomannan-free culture filtrate proteins with serum and urine from purified protein derivative (PPD)–positive control subjects and from patients with tuberculosis. Lanes 2 and 4 were probed with serum, and lanes 3 and 5 were probed with urine from 2 PPD-positive control subjects. Lanes 6, 8, and 10 were probed with serum, and lanes 7, 9, and 11 were probed with urine from 3 patients with tuberculosis. Lane 1 contains molecular weight markers.
Figure 3. Reactivity of recombinant GlcB (rGlcB) with paired serum and urine samples from 43 human immunodeficiency virus (HIV)–uninfected smear-positive patients (HIV– smear+ TB), 4 HIV-uninfected smear-negative patients (HIV– smear− TB), 6 HIV-infected smear-positive patients (HIV+ smear+ TB), and 5 HIV-infected smear-negative patients with TB (HIV+ smear− TB), as determined by Western blots. None of the serum and urine samples from 36 healthy control subjects (25 purified protein derivative [PPD]–skin test positive and 11 PPD–skin test negative) were positive (data not shown). Black bars, additive reactivity obtained when reactivity with both serum and urine samples was calculated. TB, tuberculosis.

present study were recent immigrants from countries where TB is endemic and were all antibody negative. However, these individuals could have become skin test positive because of BCG vaccination or exposure to M. tuberculosis. The 6 PPD–skin test–positive individuals from the US had not received BCG vaccination and, therefore, were likely to be PPD–skin test positive because of previous infection and also were all antibody negative. Moreover, none of the 19 household contacts of smear-positive patients with TB had anti–GlcB antibodies present. Although larger cohorts need to be tested to validate the high specificity obtained with GlcB, our data, as well as studies from other laboratories, suggest that the presence of anti–GlcB antibodies is associated with clinical disease [4–7, 26].

The presence of urinary antibodies in patients lacking detectable serum antibodies suggests that circulating immune complexes may occlude the detection of free antibody in serum [10–12]. It is known that plasma cells generated at one mucosal site can populate remote mucosal sites, and studies show that the urothelial surface is an integral component of the mucosal immune system [16, 29]. Immunohistochemical studies have shown that CD4 and CD8 T cells, macrophages, dendritic cells, and plasma cells are present in the urothelium and/or the submucosa [29]. Mice immunized with aerosols of BCG have been shown to develop both mucosal and systemic immune responses [22], and urinary antiphenolic glycolipid IgG antibodies were reported to be present in 85% of the tested patients with lepromatous leprosy [30]. The molecular size of the immunoglobulin molecules is significantly greater than the limit of filtration through glomeruli in the kidney, which suggests that the urinary antibodies are a product of the local immune responses; however, no studies to validate this hypothesis have been done so far.

The current sensitivity of detection of anti–GlcB antibodies in urine (~55%) is too low to be useful for devising a urinary antibody–based diagnostic test. However, design of assays that can detect lower titers of antibodies should help to improve the sensitivity. Moreover, urinary and circulating antibodies appear to be directed against the same M. tuberculosis antigens, and several serodominant culture filtrate antigens are currently being cloned [5]. In addition, antibodies to the total cell wall–derived protein preparation of M. tuberculosis also are present in the urine samples of patients with TB (data not shown). It is possible that antibodies to some of these other antigens may exist in higher titers, or that the combination of ≥2 serodominant antigens will enable achievement of acceptable sensitivities of detecting antibodies in the urine.

In view of the current explosion of the HIV epidemic in developing countries, a urine antibody–based diagnostic test would be an important contribution to TB control programs. Studies have shown that urinary antibodies to other pathogens are stable for weeks at room temperature, that urine samples do not have to be processed in any manner before testing, and
that urine-based tests can be developed to achieve sensitivities and specificities that are comparable to serological assays [31]. This preliminary study provides the foundation for further studies of a urine antibody-based immunoassay that could provide the on-the-spot diagnosis that is critical for TB control.

Acknowledgments

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References