Serodiagnostic Potential of Culture Filtrate Antigens of Mycobacterium tuberculosis

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Recent estimates by the World Health Organization suggest that approximately 30 million deaths were attributable to tuberculosis in the years 1990 to 1999 (21). A third of the world’s population is infected with Mycobacterium tuberculosis and about eight million individuals developed clinical tuberculosis last year (21). This global resurgence of tuberculosis has made formidable efforts to devise a sensitive and specific serodiagnostic test for tuberculosis (TB) have been made by researchers at several laboratories (7, 12). The most promising results for serodiagnosis of TB were obtained with the use of the 38-kDa PhoS protein of M. tuberculosis, which provides very high specificity (>98%) (3, 7). However, the sensitivity with this antigen varied from 45 to 80% for different cohorts, and studies have shown that anti-38-kDa protein antibodies are present primarily in patients with advanced, recurrent, and chronic disease (3, 6, 10). Moreover, the 38-kDa antigen was poorly recognized by serum antibodies from HIV-infected TB patients (28). Antigens (Ag) 85A and B have also been studied for development of serodiagnosis, but as was observed for the 38-kDa antigen, antibodies to both these proteins appear primarily in patients with extensive disease (24, 29). Also, Ag 85B is poorly recognized by antibodies from HIV-infected TB patients (18), although Ag 85A has been reported to be recognized by antibodies from some HIV-infected TB patients (30).

Based on 2-dimensional (2-D) fractionation of the culture filtrate proteins of M. tuberculosis grown in vitro in bacteriological medium and immunoblotting with TB patient sera, members of our group, along with others, recently defined the repertoire of antigens recognized by antibodies from TB patients (25). Our studies provided evidence that the profile of culture filtrate antigens recognized by antibodies from TB patients changes during disease progression. Thus, we demonstrated that of the >100 proteins present in the culture filtrates, only ~26 to 28 proteins were well recognized by patients...
with advanced cavitary disease who have anti-38-kDa protein antibodies (25). Patients who lack anti-38-kDa protein antibodies showed reactivity with only a subset of the above-mentioned immunogenic culture filtrate proteins. Thus, this subset of antigens can be expected to provide better sensitivities than the 38-kDa protein or other antigens that elicit antibodies only during advanced disease. Four of the proteins in this subset that are potential candidates for devising serodiagnosis for TB could be identified: Ag 85C, MPT32, an 88-kDa protein, and MPT51 (25).

Our observation that the profile of antigens recognized by patient antibodies is influenced by the stage of tuberculosis (17, 25) and the information that antibody responses to the 38-kDa antigen vary in different cohorts (5, 6) suggested that valid comparisons of potential serodiagnostic antigens can be made only if the same cohort is used for assessment of the different candidate antigens under study. In the present study, we report the reactivity of a cohort of 54 HIV-negative TB patients with three culture filtrate antigens, Ag 85C, MPT32, and an 88-kDa protein, which were previously identified to be strongly seroreactive (25). The reactivity of the same patient cohort with two antigens previously proposed as candidates for serodiagnosis for TB, the 38-kDa antigen and Ag 85A, was assessed for comparative purposes (7, 9). The same cohort was also evaluated for reactivity with two of these antigens expressed as recombinant proteins (Ag 85C and MPT32). We also report the reactivity of serum samples from 51 HIV-positive TB patients with four of the same antigens (Ag 85C, MPT32, and the 88-kDa and 38-kDa proteins).

MATERIALS AND METHODS

Subjects. The individuals from whom serum samples were obtained for this study belonged to the following groups (Table 1).

(i) HIV-negative TB patients. A total of 54 HIV-negative, culture-positive TB patients were included in this group. Of these, 42 patients were sputum smear positive for acid-fast bacilli (AFB), and 12 patients were sputum smear negative. Thirty-four of the 42 smear-positive patients had cavitary lesions, whereas the remaining 8 smear-positive patients lacked any evidence of cavitation.

(ii) HIV-negative, smear-positive TB patients. This cohort of 51 HIV-negative, smear-positive TB patients is derived primarily from HIV-positive individuals who were being routinely monitored for their CD4 counts and developed TB during the course of HIV disease progression (16). As a result, multiple serum samples, obtained prior to clinical manifestation of tuberculosis (pre-TB) were available from several individuals, and are referred to herein as pre-TB serum samples. A total of 108 serum samples, of which 71 were obtained pre-TB and 37 were obtained at the time of clinical presentation with TB (at-TB) were tested in this study. For 28 patients, both pre-TB and at-TB serum samples were available.

Of the 37 HIV-positive TB patients for whom serum samples obtained at-TB were tested, 21 were AFB smear positive. However, in contrast to the HIV-negative, smear-positive TB patients, only 4 of 21 HIV-positive, smear-positive TB patients had cavitary disease. The remaining 17 smear-positive patients either showed signs of infiltration or no radiological changes upon chest X-ray. Sixteen HIV-positive TB patients were sputum smear positive, and none of them showed any evidence of cavitative disease.

TB-negative controls. The control populations included (i) 30 HIV-negative, TB-negative, purified protein derivative (PPD) skin test-positive, healthy individuals, of whom 16 were immigrants from countries where Mycobacterium bovis BCG vaccination is given at birth; (ii) 19 HIV-negative, TB-negative, smear-negative healthy individuals; and (iii) 34 HIV-positive, TB-negative, asymptomatic individuals with CD4 cell counts of < 800/mm^3 whose PPD reactivity was unknown.

Antigens. The details of the purification procedures used for obtaining purified Ag 85A and C have been described earlier (1). Briefly, late-log-phase culture filtrate proteins of _M. tuberculosis_ grown in glycerol-alanine salts (GAS) medium were precipitated with a saturated 40% (NH_4)_2SO_4 solution. The precipitated material was dialyzed against buffer containing 10 mM KH_2PO_4, 1 mM dithiothreitol (DTT), and 1 mM EDTA and applied to a phenyl-Sepharose column (Pharmacia Biotech, Uppsala, Sweden). The individual proteins of Ag 85 complex proteins were eluted using buffer A containing 0.1 M Tris-HCl (pH 8.6), 1 mM DTT, and 1 mM EDTA, followed by a linear gradient composed of 100% of buffer A to 100% of buffer B containing 50% ethylene glycol.

To obtain purified native MPT32 glycoprotein, the concentrated culture filtrate of _M. tuberculosis_ was dried and resuspended in loading buffer (50 mM KH_2PO_4, [pH 5.7], 500 mM NaCl, 1 mM MgCl_2, 1 mM CaCl_2, 20 mM sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] system (Prep Cell; Bio-Rad, Hercules, Calif.) on a 10% preparative tube gel containing a 6% stacking gel. The running buffer contained 25 mM Tris (pH 8.3), 192 mM glycine, and 0.1% SDS. The proteins were separated by using an increasing wattage gradient and eluted from the bottom of the tube gel with 5 mM sodium phosphate (pH 6.8). Individual fractions were assayed by 1-D SDS-PAGE and pooled accordingly. SDS was removed from the concentrated fractions by elution through an Extract gel (Pierce) column. The fractions were evaluated for reactivity with pooled sera from cavitary TB patients and PPD skin test-positive healthy controls. The fraction containing the highest-molecular-weight proteins contained the 38-kDa antigen, and this was the only strongly seroreactive antigen in this fraction (16, 17).

Recombinant antigens. Recombinant Ag 85C and MPT32 were produced in E. coli. The gene fragment encoding the mature Ag 85C was PCR-amplified with primers specific for the mature Ag 85C sequence (695 bp). The PCR product was subcloned into the pET 21a expression vector and transformed into _E. coli_ strain M15. The recombinant antigen (Ag 85C) was purified from _E. coli_ cells by nickel chelation affinity chromatography. The Ag 85C protein was purified to homogeneity from the inclusion bodies, and the recombinant Ag 85C was used for the construction of a vaccine candidate (26). The recombinant Ag 85C was expressed in _E. coli_ cells and purified from inclusion bodies by immobilized metal ion affinity chromatography (IMAC) using a His-Bind resin (Novagen). The recombinant Ag 85C was dialyzed against 0.1M Tris-HCl (pH 8.0) and concentrated by ultrafiltration to a concentration of approximately 1 mg/ml. The recombinant Ag 85C was then lyophilized and stored at -20°C.

Recombinant MPT32 was produced in _E. coli_ strain BL21 (DE3) and purified by IMAC. The recombinant MPT32 was dialyzed against 0.1M Tris-HCl (pH 8.0) and concentrated by ultrafiltration to a concentration of approximately 1 mg/ml. The recombinant MPT32 was then lyophilized and stored at -20°C.

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TABLE 1. Clinical details of subjects tested

<table>
<thead>
<tr>
<th>Infection status of subjects (n)</th>
<th>Cavitary</th>
<th>Smear positive</th>
<th>Smear negative</th>
<th>Total no. of serum samples</th>
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</thead>
<tbody>
<tr>
<td>HIV negative, TB positive, PPD skin test result unknown (54)</td>
<td>34</td>
<td>8</td>
<td>12</td>
<td>54</td>
</tr>
<tr>
<td>HIV positive, pre-TB, PPD skin test result unknown (42)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>71</td>
</tr>
<tr>
<td>HIV positive, TB-positive, PPD skin test result unknown (37)</td>
<td>4</td>
<td>17</td>
<td>16</td>
<td>37</td>
</tr>
<tr>
<td>HIV negative, TB negative, PPD skin test positive (30)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>30</td>
</tr>
<tr>
<td>HIV negative, TB negative, PPD skin test negative (19)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>19</td>
</tr>
<tr>
<td>HIV positive, TB negative, PPD skin test result unknown (34)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>34</td>
</tr>
</tbody>
</table>

*All smear-negative patients were noncavitary. NA, not applicable.

b 28 patients are included in both groups.
amplified by PCR using the primers CATATGTTCTTGATGCGGCGCTTT (forward) and TCGAGATGCTGTGGTGGCGCGCTT (reverse). The underlined sequence represents an NdeI site. The amplified gene product was ligated into a small site of pBluescript II SK-(−) and recovered by digestion with NdeI and SalI. This DNA fragment was ligated into the NdeI-SalI site of the E. coli expression vector pBACE (4) and transformed into E. coli DH5α. The Ag 85C gene was expressed in E. coli using phosphate minimal medium (4). E. coli cells producing the recombinant Ag (Ag 85C) were harvested and lysed by passing through a French press cell. The rAg 5C was purified from the lysate by precipitation with a 60% saturated (NH4)2SO4 solution, followed by solubilization and separation by hydropathic interaction chromatography using the procedure employed for the native Ag 85C (1).

The gene fragment encoding the mature MPT32 was amplified by PCR using the primers CATATGTTCTTGATGCGGCGCTTT and TCGAGATGCTGTGGTGGCGCGCTT. This DNA fragment was ligated into the NdeI-XhoI restriction sites for the forward and reverse primer, respectively. The amplified gene product was ligated into the pBluescript II SK-(−) and recovered by digestion with NdeI and XhoI. This DNA fragment was ligated into the NdeI-XhoI site of pET22b and the recombinant plasmid was transformed into E. coli BL21(DE3) (27). The recombinant gene was expressed via isopropyl-β-D-thiogalactopyranoside (IPTG) induction for 4 h. The E. coli cells were harvested, lysed, and centrifuged at 10,000 × g for 15 min. The supernatant was precipitated with a 40% saturated solution of (NH4)2SO4. This precipitate was suspended in 50 mM KH2PO4 (pH 7.5)–500 mM (NH4)2SO4–1 mM DTT, dialyzed against the same, and applied to a 2.5- by 20-cm phenyl-Sepharose column and eluted with a linear gradient of a decrease in concentration of (NH4)2SO4 (500 to 0 mM). Fractions containing the recombinant MPT32 (rMPT32) were pooled, dialyzed against 10 mM Na2CO3, and lyophilized. This material was suspended in a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, and 0.02% NaN3; applied to a 2.5-cm by 20-cm phenyl-Sepharose column and eluted with the same buffer. A final purification step was performed by preparative SDS-PAGE using a 15% polyacrylamide gel and the Whole Gel Eluter (Bio-Rad, Hercules, Calif.). Electrophoresis was performed in 10 mM Na2CO3, ELISA with MPT32 antigens. The enzyme-linked immunosorbent assay (ELISA) was used to detect reactive antibodies prior to use in any ELISA (17). Briefly, 100 µl of E. coli Y1900 (Promega, Madison, Wis.) lysates (suspended at 500 µg/ml) were used to coat wells of E. coli (Immunol; Immuno, Brea, Calif.). Plates were covered with 5% bovine serum albumin (BSA) at 1:10 in phosphate-buffered saline (PBS; Tween 20) and 20 µl of serum samples from the same 39 smear-positive patients and 10 out of 12 smear-negative patients were exposed to eight cycles of absorption against the E. coli lysates.

Fifty microliters of the individual antigens, suspended at a concentration of 2 µg/ml in coating buffer (except for the purified 38-kDa antigen, which was used at 6 µg/ml), were added to bind overnight to wells of ELISA plates. After three washes with PBS, the wells were blocked with 7.5% fetal bovine serum (FBS; Hyclone, Logan, Utah)–2.5% BSA in PBS for 2.5 h at 37°C. Fifty microliters of each serum sample was added per well at predetermined optimal dilutions (1:50 for Ag 85C and Ag 85A, 1:150 for the MPT32, and 1:200 for both the semipurified 88-kDa antigen and the purified 38-kDa antigen). The antigen-antibody binding was allowed to proceed for 90 min at 37°C. The plates were washed six times with PBS-Tween 20 (0.05%) and 50 µl of alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG; Zymed, Calif.), diluted 1:2,000 in PBS-Tween 20, was added per well after wash. After 30 min the plates were washed six times with PBS-Tween 20. Fifty microliters of tetramethylbenzidine (50 mM Tris, 150 mM NaCl) and the Gibco BRL Amplification System (Life Technologies, Gaithersburg, Md.) was used for development of color. The optical density (OD) at 490 nm was read after the reaction was stopped with 50 µl of 0.3 M H2SO4. The cutoff in all ELISA assays was determined by using the mean OD for the TB-negative control group (PPD skin test-positive; PPD skin test-negative; and HIV-infected, asymptomatic individuals) plus 3 standard deviations (SD).

RESULTS

Reactivity of sera from HIV-negative TB patients with native antigens of M. tuberculosis. The reactivity of serum samples from 54 HIV-negative TB patients and 83 healthy controls with MPT32, Ag 85C, and the 88-kDa protein was compared to their reactivity with Ag 85A and the 38-kDa protein. Regardless of the PPD skin test status or HIV infection status the three groups of controls (HIV-negative, TB-negative PPD skin test-positive individuals; HIV-negative, TB-negative PPD skin test-negative individuals; and HIV-positive, TB-negative individuals) showed similar reactivity with each of the antigens and were therefore considered as one group for calculating the cutoff (Fig. 1). Except for MPT32, with which 1 out of 83 healthy control serum samples showed reactivity, none of the healthy control serum samples were reactive with any of the antigens, providing specificities ranging from 98 to 100% with each of the antigens (Fig. 1). Of the 42 smear-positive TB patients, serum samples from 69% (29 out of 42) of patients had antibodies to MPT32, serum samples from 79% (33 out of 42) of patients had antibodies to Ag 85C, and serum samples from 74% (31 out of 42) of patients had antibodies to the semipurified 88-kDa antigen. In the same cohort, only 62% (26 out of 42) of the patients had antibodies to the Ag 85A, and 38% (16 out of 42) of the patients had antibodies to the 38-kDa antigen (Fig. 1). Thus, all three of the antigens identified to be seroreactive in TB patients in our previous studies provided higher sensitivities in the smear-positive TB patients (Table 2).

In the cohort of smear-negative TB patients, 33% (4 out of 12 patients) possessed anti-MPT32, 33% (4 out of 12 patients) had anti-Ag 85C, and 33% (4 out of 12 patients) had anti-88-kDa protein antibodies (Fig. 1). In contrast, only 2 out of 12 (17%) serum samples from the same cohort had antibodies to Ag 85A antigen, and serum from only 1 out of 12 patients (8%) was reactive with the 38-kDa antigen (Table 2).

The additive reactivity of sera with different antigens was computed from the above data (Table 2). For smear-positive patients, the maximum sensitivity of antibody detection (81%) was achieved by combining the reactivity with Ag 85C, the 88-kDa antigen, and MPT32 (Table 2), although this was only slightly higher than the results obtained with Ag 85C or the 88-kDa protein alone. All patients who had circulating antibodies to Ag 85A or to the 38-kDa protein also had antibodies to Ag 85C, MPT32, and/or the 88-kDa protein, and the sensitivity of antibody detection did not increase by taking reactivity with the former antigens into account. However, for the smear-negative patients, the combined reactivity with the MPT32, Ag 85C, or the 88-kDa protein raised the sensitivity to 50% (Table 2). There was no further increase in the sensitivity of antibody detection in the smear-negative patients if reactivity of sera with Ag 85A or the 38-kDa antigen was taken into consideration (Table 2).

Reactivity of sera from HIV-negative TB patients with recombinant MPT32 and Ag 85C. In view of the reactivity of native Ag 85C and MPT32 with the TB patient sera, the reactivity of the same sera with the recombinant versions of these antigens was evaluated. Serum samples from 39 out of 42 smear-positive-patients were tested for reactivity with the rAg 85C expressed in E. coli (Fig. 2). In contrast to the reactivity observed with native Ag 85C (Fig. 1), serum samples from only 4 out of 39 smear-positive patients and from none of the smear-negative patients showed reactivity with recombinant Ag 85C (Fig. 2). Serum samples from the same 39 smear-positive patients and 10 out of 12 smear-negative patients were also assessed for reactivity with the rMPT32 expressed in E. coli (Fig. 2). Only 11 out of 39 (28%) serum samples from the smear-positive patients showed reactivity with the recombinant MPT32 expressed in E. coli (Fig. 2).

Reactivity of sera from HIV-positive TB patients with native antigens of M. tuberculosis. Pre-TB sera from 74% of the HIV-positive TB patients possessed anti-88-kDa protein antibodies (16) (Table 2). Sera from the same cohort were tested for reactivity with MPT32, Ag 85C, and the 38-kDa antigen. In contrast to the results obtained with the 88-kDa antigen, pre-TB sera from only 29% (12 out of 42) of the HIV-positive TB patients had anti-MPT32, 36% (15 out of 42) had anti-Ag 85C, and 12% (5 out of 42) had anti-38-kDa protein antibodies (Table 2).

Members of our group and others have earlier reported that at -TB, ~65% of the patients have anti-88-kDa protein antibodies (16). When the reactivity of the serum samples from
smear-positive and smear-negative patients in this cohort was calculated separately, 66% of the former and 62% of the latter possessed anti-88-kDa protein antibodies (Table 1). In contrast to the results obtained with the 88-kDa antigen, sera from the HIV-positive, smear-positive TB patients showed poor reactivity with MPT32, Ag 85C, and the 38-kDa protein. Moreover, in contrast to the HIV-negative TB patients, the HIV-positive, smear-positive and smear-negative patients showed similar responses (Table 2).

DISCUSSION

Our earlier studies, in which the immunogenic culture filtrate proteins of *M. tuberculosis* were mapped, identified several proteins that were predicted to have strong serodiagnostic potential on the basis of their reactivity with sera from patients at different stages of disease progression (25). In the present study, we evaluated the reactivity of three of these antigens, an 88-kDa protein, Ag 85C, and MPT32, with a cohort of smear-positive and smear-negative TB patients in order to compare the antibody assay with the sputum smear test. Our results show that in the HIV-negative, smear-positive TB patients, antibodies to Ag 85C, MPT32, and the 88-kDa antigen are detectable in more than 80% of the patients. This is a significant improvement over the sensitivities obtained for the same cohort with the 38-kDa protein or Ag 85A, both of which were previously shown to be the most successful candidates for developing serodiagnosis for TB (7, 9). In the smear-negative
cohort, although antibodies were detectable in only a small proportion of patients with the use of individual antigens, combining the reactivity with all three antigens raised the sensitivity to \( \approx 50\% \). Although this is significantly lower than the sensitivity in the smear-positive patients, it is higher than sensitivities achieved with any other antigen studied so far and represents an improvement over AFB smear-based diagnosis.

In contrast to the HIV-negative, smear-positive TB patients, sera from the HIV-positive, smear-positive TB patients showed poor reactivity with MPT32, Ag 85C, and the 38-kDa antigen (Table 2). Earlier studies with HIV-positive TB patients have also reported poor reactivity of these patients with the 38-kDa protein (28). Since \( \approx 70\% \) of the same HIV-positive TB patients possess anti-88-kDa protein antibodies, and since the presence or absence of these antibodies did not correlate with either the CD4 numbers or the CD4/CD8 ratios (16), the lower reactivity of HIV-positive TB patient sera with MPT32, the 38-kDa protein, and Ag 85C is probably unrelated to immune dysfunction caused by HIV infection. One obvious difference between the smear-positive, HIV-negative and HIV-positive TB patients was the lack of cavitary lesions in a vast majority of the latter group. It is known that extensive extracellular bacterial replication occurs during growth in cavities, and the expression of antigens like the 38-kDa protein, Ag 85C, and MPT32 by the in vivo bacteria is possibly enhanced in the cavitary environment. This hypothesis is further strengthened by the observation that the anti-88-kDa protein antibodies are present \( \approx 75\% \) of serum samples obtained from HIV-positive TB patients during the pre-TB stages of the disease (16). The presence of anti-88-kDa protein antibodies from sera of patients lacking anti-38-kDa protein antibodies and from pre-TB sera of HIV-positive TB patients, suggests that this protein is expressed in vivo prior to the production of cavitary lesions. Surprisingly, sera from smear-negative HIV-positive TB patients showed better reactivity with the 88-kDa protein than sera from smear-negative, HIV-negative TB patients (Fig. 1 and Table 2). The reasons for this are not clear, but it has been shown that spum smear-negative, HIV-positive TB patients can have very high levels of bacillary replication in their lungs even in the absence of any cavitary lesions (8). Possibly, the differences in the alveolar bacterial loads between smear-negative HIV-negative TB patients and HIV-positive TB patients may account for the better anti-88-kDa protein responses seen in the latter group of patients.

Although the antigens employed in this study provide significantly greater sensitivities than those achieved with antigens studied by other investigators, even the combined reactivity with all three proteins failed to diagnose \( \approx 20\% \) of the HIV-negative, smear-positive TB patients and \( \approx 50\% \) of the smear-negative patients. Moreover, except for the 88-kDa protein, none of the antigens tested showed significant reactivity with the sera of HIV-positive TB patients. 2-D and 1-D immunoblot analyses of reactivity of culture filtrate proteins with sera of patients at different stages of disease progression show that besides the three antigens tested, there are additional proteins (that are not yet characterized) that are recognized by antibodies from patients in the relatively early stages of the disease (25) and from those with HIV coinfection (16). It is possible that inclusion of one or more additional seroreactive antigens will further enhance the sensitivity of antibody detection, and we are currently involved in identifying and obtaining

\[ \text{TABLE 2. Proportion of specimens from patients and controls containing antibodies to various antigens of } M. \text{ tuberculosis} \]

<table>
<thead>
<tr>
<th>Infection status (n)</th>
<th>Native 38-kDa Ag</th>
<th>Native Ag 85A</th>
<th>Native MPT32</th>
<th>Native Ag 85C</th>
<th>Native 88-kDa Ag</th>
<th>MPT32-, Ag 85C, and 88-kDa Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV negative, TB positive (smear positive)</td>
<td>38 (16/42)</td>
<td>62 (26/42)</td>
<td>69 (29/42)</td>
<td>79 (33/42)</td>
<td>74 (31/42)</td>
<td>81</td>
</tr>
<tr>
<td>HIV negative, TB positive (smear negative)</td>
<td>8 (1/12)</td>
<td>17 (2/12)</td>
<td>33 (4/12)</td>
<td>33 (4/12)</td>
<td>33 (4/12)</td>
<td>50</td>
</tr>
<tr>
<td>HIV positive, Pre-TB (42)</td>
<td>12 (5/42)</td>
<td>ND</td>
<td>29 (12/42)</td>
<td>36 (15/42)</td>
<td>74 (32/42)</td>
<td>ND</td>
</tr>
<tr>
<td>HIV positive, at-TB (smear positive) (21)</td>
<td>14 (3/21)</td>
<td>ND</td>
<td>24 (5/21)</td>
<td>19 (4/21)</td>
<td>66 (14/21)</td>
<td>ND</td>
</tr>
<tr>
<td>HIV positive, at-TB (smear negative) (16)</td>
<td>19 (3/16)</td>
<td>ND</td>
<td>12 (2/16)</td>
<td>19 (3/16)</td>
<td>62 (10/16)</td>
<td>ND</td>
</tr>
<tr>
<td>TB negative (controls) (83)</td>
<td>0 (0/83)</td>
<td>0 (0/83)</td>
<td>2 (1/83)</td>
<td>0 (0/83)</td>
<td>0 (0/83)</td>
<td>2 (1/83)</td>
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<tr>
<td>Specificity</td>
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<td>100</td>
<td>98</td>
<td>100</td>
<td>100</td>
<td>98</td>
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</table>

*a Data in parentheses are the number of specimens containing antibodies to the indicated antigen/the number of specimens tested.

*b ND, not done.

![FIG. 2. Reactivity of sera from TB patients and healthy controls with recombinant Ag 85C and recombinant MPT32.](http://cvi.asm.org/.../17.pdf)
these antigens. Another possibility is that antibodies in some patients are complexed to free antigen, leading to their occlusion in antibody detection assays. Other investigators have shown that immune complexes are present in the sera of TB patients, although besides the 38-kDa antigen, it has not so far been possible to identify the antigens in these complexes (2, 19, 20). Assays in which the serum immune complexes can be dissociated to release antibodies prior to testing are also being developed.

In parallel experiments using native and recombinant antigens, the results obtained with the recombinant forms of the antigen 85C and the MPT32 are disappointing. Our studies suggest that native mycobacterial proteins possess B-cell epitopes that elicit antibodies during natural infection and are absent from the recombinant versions. Similar problems were encountered when efforts to use recombinant 12-, 16-, and 38-kDa proteins for serological studies were made (31, 32). Differences between the recombinant and native MPT64 in recognition by T cells have also been reported (22). Recent studies have also shown that deglycosylation of MPT32 decreases its capacity to elicit in vivo or in vitro cellular immune responses in guinea pigs (23). Since the recombinant MPT32 used in this study was expressed in E. coli, the poor reactivity of the sera with this protein may be due to the lack of glycosylation. However, Ag 85C was reported not to be glycosylated (14), and the recombinant Ag 85C possesses the same enzymatic activity as the native protein (1). Also, Ag 85C is recognized by human sera after being run on SDS-PAGE gels, suggesting that conformational epitopes are not important for human antibody recognition. The difference in reactivity between native and recombinant Ag 85C is not explained, but given that purification of large quantities of native proteins from M. tuberculosis for production of serodiagnostic tests is difficult and expensive, cloning into mycobacterial hosts with homology to M. tuberculosis may be necessary.

Despite several attempts, success in the development of serodiagnosis for TB has been limited. In most earlier studies, the choice of antigens used was based on the availability, ease of purification, or immunodominance of the antigens in animal models (15). Although further studies are required, the results with the antigens identified in our studies so far provide evidence that use of antigens selected after a rational analysis of the humoral response of human TB patients should enable the design and development of a specific and sensitive serodiagnostic assay for TB. It will also be necessary to ensure that the antigens are able to distinguish between TB and pulmonary infections caused by other pathogens.

The current requirement for three AFB smears to confirm the diagnosis of TB is a major bottleneck in the TB control programs in developing countries. Smear evaluation is time-consuming, requires a sophisticated infrastructure, and contributes to delay in initiation of treatment. In addition, since results cannot be made available immediately, repeated patient visits are required to obtain specimens and provide results (11). These factors contribute to delayed diagnosis and high dropout rates, with subsequent increased spread of infection.

The development of antibody or antigen detection assays based on simple and inexpensive formats such as dipstick assays or flow-through cassettes that are easy to interpret are urgently required. Such assays, which would permit on-the-spot rapid diagnosis of tuberculosis in the absence of laboratory infrastructure, would make a significant contribution to early treatment and control the spread of TB, especially in the developing countries.

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REFERENCES


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