Evaluation of Mycobacterium tuberculosis–Specific Antibody Responses in Populations with Different Levels of Exposure from Tanzania, Ethiopia, Brazil, and Denmark

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Background. New, simple, and better-performing diagnostic tools are needed for the diagnosis of tuberculosis (TB). Much effort has been invested in developing an antibody-based test for TB, but to date, no such test has performed with sufficient sensitivity and specificity. A key question remaining is the extent to which the disappointing performance of current tests is associated with a high background prevalence of latent TB.

Methods. We compared Mycobacterium tuberculosis–specific ESAT-6 and CFP-10 antibody responses in a total of 565 human serum samples from M. tuberculosis–uninfected donors and donors with latent infection, as well as samples from patients with active TB. Our study included samples from 4 countries, representing environments with low, intermediate, and high TB incidences.

Results. We demonstrated significant increases in antibody levels in latently infected contacts, compared with M. tuberculosis–uninfected individuals, and in patients with active TB disease, compared with latently infected contacts. Furthermore, we found a striking increase in the magnitude of the antibody responses in samples obtained from infected Ethiopian individuals (with and without disease), compared with Danish and Brazilian infected individuals; this was presumably the result of higher exposure levels.

Conclusions. Our study confirms the presence of ESAT-6 and CFP-10 antibodies in patients with TB, and we demonstrate that significant antibody responses are not restricted to active TB disease but can reflect latent infection, particularly in areas with high levels of exposure to M. tuberculosis. This finding is important for understanding the poor discriminatory power of current serodiagnostic tests in regions of endemicity, and it may have major implications on the future development of serologic tests.

According to the World Health Organization, the world witnessed >8 million new cases of tuberculosis (TB) in 2002, and approximately one-third of the global population today is thought to be infected with Mycobacterium tuberculosis [1, 2]. In countries with a heavy tuberculosis burden, the diagnosis of TB is based mainly on clinical examination and radiographic findings and is confirmed by sputum smear microscopic evaluation and/or bacterial culture. Unfortunately, the sensitivity of sputum smear microscopic evaluation, in particular, is low; thus, many contagious cases remain undetected, potentially allowing the spread of infection [3]. Recently, new diagnostic tools have emerged, including in vitro IFN-γ release assays (IGRAs). In parallel, much effort has been invested in developing a serologic test for TB, and because the humoral response has been seen to increase in magnitude as TB disease develops [4], a serodiagnostic test has been proposed as a test for active TB. The goal is a robust, cheap, fast, low-technology tool that can be used under conditions commonly encountered in countries with poor infrastructure and limited resources. Encouragingly, many laboratory-based studies have reported promising re-
Table 1. Characteristics of sample donors.

<table>
<thead>
<tr>
<th>Country, group</th>
<th>TB incidence, no. of cases per 100,000 persons&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of subjects</th>
<th>Age, median years (range)</th>
<th>No. (%) of male subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy volunteers</td>
<td>13</td>
<td>32</td>
<td>37.5 (19–64)</td>
<td>22 (69)</td>
</tr>
<tr>
<td>Community control subjects</td>
<td>80</td>
<td>17 (15–63)</td>
<td>31 (39)</td>
<td></td>
</tr>
<tr>
<td>TB contacts</td>
<td>24</td>
<td>18 (12–23)</td>
<td>19 (79)</td>
<td></td>
</tr>
<tr>
<td>Patients with TB</td>
<td>91</td>
<td>40 (21–84)</td>
<td>62 (68)</td>
<td></td>
</tr>
<tr>
<td>Brazil</td>
<td>62</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Community control subjects</td>
<td>23</td>
<td>27 (20–40)</td>
<td>12 (52)</td>
<td></td>
</tr>
<tr>
<td>TB contacts</td>
<td>27</td>
<td>44 (15–70)</td>
<td>7 (26)</td>
<td></td>
</tr>
<tr>
<td>Patients with TB</td>
<td>40</td>
<td>33.5 (16–58)</td>
<td>28 (70)</td>
<td></td>
</tr>
<tr>
<td>Ethiopia</td>
<td>370</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Community control subjects</td>
<td>18</td>
<td>29 (17–45)</td>
<td>1 (14)</td>
<td></td>
</tr>
<tr>
<td>TB contacts</td>
<td>25</td>
<td>22 (15–50)</td>
<td>5 (20)</td>
<td></td>
</tr>
<tr>
<td>Patients with TB</td>
<td>21</td>
<td>29 (15–60)</td>
<td>11 (52)</td>
<td></td>
</tr>
<tr>
<td>Tanzania: patients with TB</td>
<td>363</td>
<td>184</td>
<td>31 (15–75)</td>
<td>112 (61)</td>
</tr>
</tbody>
</table>

**NOTE.** TB, tuberculosis.
<sup>a</sup> Data are from [1].

results with a wide range of antigens [5–9]. However, to date, no commercially available serologic test for TB has been able to perform with sufficient sensitivity and specificity when evaluated prospectively in a clinical setting [10–20]. Important for the interpretation of this finding is that most of the antigens used in first-generation serodiagnostic tests are widely shared within the *Mycobacterium* genus. Therefore, a key question remains: what is the extent to which the disappointing performance of current tests is the result of cross-reactivity to bacille Calmette-Guérin (BCG) and nontuberculous mycobacteria (NTM) from the environment or of the high prevalence of latent TB in these regions?

We addressed this question by focusing on the ESAT-6 and CFP-10 antigens, which are encoded in the RD1 region of the *M. tuberculosis* genome and which, therefore, are absent in all BCG vaccine strains and in the majority of NTM [21, 22]. Both antigens are currently used in the 2 commercially available IGRA s for detection of TB infection [4, 23, 24] and have also been evaluated as an ESAT6-CFP10 fusion protein—an approach that has several benefits, primarily with regard to its inexpensive price and easy large-scale production [25]. Several recent reports indicate that these antigens are frequently recognized by antibodies in patients with TB, both from regions where TB is not endemic [26–29] and from regions where TB is endemic [30, 31]. Evidence that ESAT-6 and CFP-10 are expressed during latent infection does exist [32, 33], but it remains unclear whether serorecognition is found in infected individuals with latent or subclinical TB.

We hypothesized that both patients who have active TB disease and individuals with latent *M. tuberculosis* infection might have high levels of circulating antibodies to *M. tuberculosis*-specific antigens. We constructed an ESAT6-CFP10 fusion protein and measured antibody responses to this reagent in a total of 565 human serum samples obtained from persons in 4 countries, which represent increasing *M. tuberculosis* incidences. Our study confirms the presence of ESAT-6 and CFP-10 antibodies in patients with TB and demonstrates that significant antibody responses are not restricted to active TB disease but can reflect latent infection (in particular, in areas with high *M. tuberculosis* exposure levels). This finding is important for the understanding of the poor discriminatory power of current serodiagnostic tests in regions of endemicity and may have major implications for the future development of serologic tests.

**METHODS**

**Study Populations and Definitions**

A total of 565 samples were collected from 4 different countries and are described in detail below and in table 1. HIV-infected individuals were excluded from the study.

**Samples from Denmark.** Two hundred twenty-seven samples were obtained from donors who reside in Copenhagen, Denmark. Written informed consent was obtained from the persons, and the study was approved by the Local Ethics Committee for Copenhagen and Frederiksberg (approvals KF 01-369/98 and KF 01–300471). These samples comprised (1) 91 samples obtained from patients with confirmed TB who resided in Denmark and who were recruited at Danish hospitals (DK-TB); the means of diagnosis of TB included sputum smear microscopic examination, culture, chest radiography, and, in
provided written consent, and the study was performed under a study protocol approved by the institutional and national ethics review boards. Sample breakdown was as follows: (1) 21 samples from sputum-positive patients with TB who were recruited from the rural towns of Hossana and Butajira, Ethiopia (ET-TB); (2) 25 ESAT-6–positive, asymptomatic household contacts of the sputum-positive index cases; all had normal radiograph findings and negative sputum test and culture results, and all tested positive for ESAT-6 using an in-house IGRA, as described elsewhere (ET-CC) [35]; (3) 18 samples from community control subjects who were randomly selected from the same areas as the patients with TB; recent TB contact was excluded on the basis of answers to a questionnaire, and all subjects had normal radiograph findings, had no symptoms, and had either negative results for all sputum tests and cultures or could not produce sputum (ET-CON). Fourteen of these 18 persons were ESAT-6 positive.

**Samples from Tanzania.** One hundred eighty-four samples were obtained from *M. tuberculosis* culture–positive donors who resided in Mwanza, Tanzania (TZ-TB). The samples were obtained from subjects involved in a randomized, controlled trial that investigated the role of micronutrients and coinfections in pulmonary TB that was conducted in Mwanza, Tanzania [36]. The study was approved by the Ethics Committee of the National Institute for Medical Research in Tanzania, and informed oral consent was obtained from all study participants.

**Recombinant Protein Expression and Purification.** The plasmid encoding the ESAT6-CFP10 protein was generated by linkage of the coding region of the CFP-10 polypeptide to the coding region of the ESAT-6 polypeptide. The hybrid encoding ESAT6-CFP10 was linked to a region encoding an N-terminal histidine tag and was inserted in the expression vector pQE60 (Qiagen) using the Neol and BamHI sites of the vector. The final plasmid construct was transformed into *Escherichia coli* strain NF1830 for production of the ESAT6-CFP10 fusion protein, and expression was induced with 1 mmol/L isopropyl β-d-thiogalactopyranoside. The ESAT6-CFP10 fusion protein was then purified by metal chelate chromatography, as described elsewhere [5]. Recombinant proteins ESAT-6 and CFP-10 were expressed and purified, as described elsewhere [22, 37].

**Measurement of Serum IgG Antibodies by ELISA**

Ninety-six–well polystyrene microtiter plates (Nunc) were coated overnight at 4°C with 100 μL of antigen solution in 0.05 M carbonate buffer (pH, 9.6) in the following concentrations: ESAT-6, 1 μg/mL; CFP-10, 1 μg/mL; ESAT6-CFP10 fusion protein, 2 μg/mL; or Ovalbumin, 5 μg/mL. The plates were subsequently washed 3 times with PBS (pH, 7.2) plus 0.05% Tween 20 (PBS-T) and were blocked overnight at 4°C with carbonate buffer plus 2% bovine serum albumin (pH, 9.6). The serum samples were diluted to 1:400 in PBS plus 1% BSA and 0.2%...
Figure 2. Scatter graph showing the correlation between the highest optical density (OD) value for either ESAT-6 or CFP-10 single proteins against the OD value for the ESAT6-CFP10 fusion protein. A nonparametric test for correlation (Spearman) was performed (Spearman’s $r = 0.79$; $P < .001$).

 Tween 20, were added to the wells, and were incubated for 1 h at room temperature. After another wash with PBS-T, the plates were incubated for 1 h at room temperature with Horseradish peroxidase–conjugated mouse anti-human IgG monoclonal antibodies (BD Pharmingen; catalogue no. 555788) at a 1:4000 dilution. Enzyme activity was assayed by incubation for 30 min at room temperature with 100 µL of TMB plus substrate per well. To stop the reaction, 100 µL of 0.2 M sulphuric acid was added, and the optical density (OD) was measured at 450 nm and at 620 nm for background subtraction.

Data Analysis
The cutoff value for the ELISA assay was calculated on the basis of 32 samples from BCG-vaccinated, healthy Danish volunteers with no known TB risk factors (negative control subjects) using the mean value plus 3 SDs of the OD values. Statistical analyses were performed and graphs were designed using SAS statistical software, version 8.02 (SAS Institute), and GraphPad Prism software, version 4.0. Median OD values were compared between groups of samples using the nonparametric Mann-Whitney rank sum test with Bonferroni’s posttest correction.

RESULTS

Characterization of the ESAT6-CFP10 fusion protein.
We designed a fusion protein of ESAT-6 and CFP-10 that was produced and expressed in E. coli. The apparent molecular weight of the fusion protein was 24 kDa, and monoclonal antibodies specific for the ESAT-6 and for the CFP-10 component bound to the fusion protein in Western blot experiments (figure 1). One hundred ninety serum samples were tested for antibody responses specific to ESAT-6–CFP-0, ESAT-6 alone, and CFP-10 alone (figure 2). We tested for correlation between the highest OD value for either ESAT-6 or CFP-10 alone and for the ESAT6-CFP10 fusion protein, and we found a strong correlation (Spearman’s $r = 0.79$; $P < .001$), demonstrating that the fusion protein and the single components had similar activity.

ESAT-6–CFP-0–specific antibodies are associated with M. tuberculosis infection. We tested 184 samples obtained from patients with TB from a region where TB is endemic (Tanzania) and from 32 BCG-vaccinated community control subjects who had not been exposed to M. tuberculosis from a region where TB is not endemic (Denmark). On the basis of these data, we calculated a cutoff value for use in subsequent analyses. As shown in figure 3, 91% of the Tanzanian patients with TB had positive antibody responses to the ESAT6-CFP10 fusion protein, compared with none of the negative control subjects.

Presence of ESAT6-CFP10–specific antibodies increases with infection and with high exposure to M. tuberculosis. Antibody levels were compared among 9 groups of samples collected from Denmark, Brazil, and Ethiopia, representing environments of low, intermediate, and high incidences of TB, respectively. In addition, the samples from each country were divided into community controls (CC), M. tuberculosis–infected contacts without disease (CON), and patients with confirmed TB (TB; table 1). The M. tuberculosis infection status
for the subjects was determined by measurement of cellular mediated immune (CMI) response at all sites, by IFN-γ release assays for the Danish and Ethiopian subjects, and by TST for Brazilian subjects. All individuals included in the DK-CC and BZ-CC groups had negative CMI results; in contrast, only 4 of 18 ET-CC subjects had a negative CMI response to ESAT-6. Although this makes the ET-CC group less comparable to the Danish and Brazilian equivalents, it reflects the high level of infection among the general population in Ethiopia. All TB contacts included from the 3 countries had positive CMI results and were presumed to be infected.

Figure 4A shows the antibody OD values obtained from 80 persons from the DK-CC group, 24 from the DK-CON group, and 91 from the DK-TB group. Because Denmark has a low overall incidence of TB, a very low background rate of exposure to *M. tuberculosis* must be presumed in all 3 groups. We found a highly significant difference between the median antibody OD values for the *M. tuberculosis*-uninfected DK-CC group and the *M. tuberculosis*-infected (QFN-positive) DK-CON group, and although a similar trend was present, no significant differences between the DK-CON group and the DK-TB group were observed. The median antibody OD values and exact *P* values are shown in table 2. Among the Brazilian samples (figure 4B), the differences between the BZ-CC and BZ-CON groups were significant, as were the differences between the BZ-CON and BZ-TB groups. Brazil has an intermediate incidence of TB, but the Brazilian samples had OD values similar to those for their Danish equivalents (table 2). In contrast, figure 4C depicts a remarkable increase in the magnitude of the antibody responses in Ethiopia, possibly reflecting the high incidence of TB. Here, a significant difference was also observed between the ET-CC, ESAT-6–positive ET-CON, and ET-TB groups (figure 4C). Of particular interest was that the antibody responses were clearly higher in the ET-CON group than in the DK-CON and BZ-CON groups, even though all subjects also in the Danish and Brazilian groups had latent *M. tuberculosis* infection, as determined on the basis of their CMI responses. The same was observed for the groups of patients with TB.

Two persons from the BZ-CC group and 10 from the ET-CC group had antibody levels that were greater than the cutoff value. Of the latter, 2 tested negative for ESAT-6. Exclusion of ESAT-6–positive persons from the ET-CC group did not change the median OD value significantly (data not shown).

**DISCUSSION**

Antibody-based diagnosis of TB is a close-to-ideal point-of-care test, but it has proven to be difficult to implement it, because it has inadequate sensitivity and specificity when evaluated by independent researchers [20]. It is striking that antibody-based tests, which were developed and initially evaluated in countries where TB is not endemic, have consistently per-
Table 2. Antibody responses in subjects from Denmark, Brazil, and Ethiopia.

<table>
<thead>
<tr>
<th>Country, group</th>
<th>No. of subjects</th>
<th>Median OD</th>
<th>P</th>
<th>Positive samples, % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Community control subjects</td>
<td>80</td>
<td>0.023</td>
<td></td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>TB contacts</td>
<td>24</td>
<td>0.128</td>
<td>&lt;.001</td>
<td>33 (14–52)</td>
</tr>
<tr>
<td>Patients with TB</td>
<td>91</td>
<td>0.158</td>
<td>.321</td>
<td>43 (33–53)</td>
</tr>
<tr>
<td>Brazil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Community control subjects</td>
<td>23</td>
<td>0.075</td>
<td></td>
<td>9 (0–20)</td>
</tr>
<tr>
<td>TB contacts</td>
<td>27</td>
<td>0.120</td>
<td>.043</td>
<td>22 (7–38)</td>
</tr>
<tr>
<td>Patients with TB</td>
<td>40</td>
<td>0.194</td>
<td>.006</td>
<td>60 (45–75)</td>
</tr>
<tr>
<td>Ethiopia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Community control subjects</td>
<td>18</td>
<td>0.238</td>
<td></td>
<td>56 (33–79)</td>
</tr>
<tr>
<td>TB contacts</td>
<td>25</td>
<td>0.272</td>
<td>.038</td>
<td>96 (88–100)</td>
</tr>
<tr>
<td>Patients with TB</td>
<td>21</td>
<td>0.593</td>
<td>.004</td>
<td>100 (100–100)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are based on a cutoff value calculated as described in Methods. TB, tuberculosis.

...formed poorly when tested in regions where TB is endemic [10–19]. Our study reports an antigen that is highly specific and sensitive (figure 3) but that generated a surprisingly high percentage of positive antibody responses in healthy control subjects (ET-CC and ET-CON) from a region where TB is endemic (table 2)—but that also generates responses that follow a clear gradient from community control subjects, to contacts, to patients with TB in every setting.

The simplest solution to this puzzle is that the responses that we observed reflect exposure to and infection with *M. tuberculosis*—even in healthy individuals from regions where TB is highly endemic. On the basis of data showing that circulating *M. tuberculosis*-specific antibodies can be found not only in individuals with active TB disease, but also in individuals with latent *M. tuberculosis* infection, we suggest that the poor performance may at least partially reflect the high prevalence of latent TB in these regions. It is important to emphasize that not all IGRA-positive individuals have *M. tuberculosis*-specific antibodies. For example, we observed that, in the Danish QFN-positive contact group, only 33% had a positive response with a low OD value, suggesting that a positive serologic response has either different developmental kinetics or different requirements (e.g., it may arise from boosting by frequent exposure).

Our findings are in accordance with previous findings from The Gambia. Greenaway et al. [30] found that 49% of Gambian community control subjects had specific antibodies to ESAT-6, and 45% had specific antibodies to CFP-10. Among household contacts, the numbers were slightly higher (49% and 54%, respectively), with the highest percentage of responders noted among patients with active TB disease (67% and 63%, respectively).

Some reports have been published regarding antibody responses during different stages of infection [27, 29, 38]. A study from Canada by Silva et al. [27] found that 53% of patients with inactive TB had a positive humoral response to ESAT-6. Davidow et al. [29] later repeated this finding and suggested that “immunoprofiling” (i.e., use of different *M. tuberculosis* antigens) may have the potential to distinguish between clinical states of tuberculosis. These results are consistent with our results, because they demonstrate the presence of *M. tuberculosis*-specific antibodies without active disease, suggesting that the antibodies may arise during latency. However, Davidow and colleagues found that a response to ESAT-6 was associated with inactive TB rather than active TB disease. Our findings do not support this observation, because the highest levels of ESAT-6 and CFP-10 antibodies were found among patients with TB.

In this study, we also observed that individuals who live in an area where TB is endemic have elevated levels of *M. tuberculosis*-specific antibodies, compared with individuals who live in areas of nonendemicity and intermediate endemicity. This indicates that the TB incidence in a given country has a marked influence on antibody responses to *M. tuberculosis* antigens, most likely reflecting repeated exposure to the pathogen. Another possibility is that the elevated antibody levels seen in the Ethiopian samples could have resulted from infection with NTM or *Mycobacterium leprae* or from unspecific binding of irrelevant antibodies. However, ESAT-6 and CFP-10 were chosen for this study, because these antigens are present in very few of the NTM, making cross-reaction with NTM antigens unlikely as a major confounder. Moreover, representative samples from the Ethiopian study cohorts were tested for antibody responses to an irrelevant antigen (Ovalbumin), and we found only minor elevations and no difference in the antibody levels between persons in the ET-CC group, contacts, and patients with TB (data not shown), indicating that the observed difference between the Ethiopian groups is not due to unspecific binding of irrelevant antibodies. With regard to *M. leprae* infection, it is unlikely to be a significant confounder because of...
the low incidence, compared with *M. tuberculosis* infection (the incidence of *M. leprae* infection is estimated to be perhaps 5% of the incidence of *M. tuberculosis* infection [39]), and because *M. leprae* infection appears to induce only weak anti–ESAT-6 responses, compared with *M. tuberculosis* infection [40].

We observed that 2 TST-negative Brazilian subjects and 2 ESAT-6–negative persons from the ET-CC group had positive antibody levels. This was unexpected, but neither an ESAT-6–based IGRA nor the TST has a sensitivity of 100%. Therefore, we cannot determine whether these subjects were indeed infected, as suggested by their antibody levels.

Because ESAT-6 and CFP-10 have consistently proven to be the antigens with the most consistent association with *M. tuberculosis* infection, our data suggest that the very high prevalence of latent TB infection in countries where TB is endemic may reduce the specificity of any antibody-based test intended to identify active TB. On the other hand, the data from Brazil imply that this may not be the case in countries with intermediate TB incidences—countries where TB still has a major impact on public health. It is debatable whether the criterion used to define success for an antibody-based test exclusively for TB should be specific identification of active TB in populations with a very high prevalence of latent TB.

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**Potential conflicts of interest.** PA, a named coinventor on patents relating to the discovery of the ESAT-6 protein; all rights have been assigned to the Statens Serum Institut. All other authors: no conflicts.

**References**


