Quantitative T Cell Assay Reflects Infectious Load of Mycobacterium tuberculosis in an Endemic Case Contact Model

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Background. Currently, reliable efficacy markers for assessment of new interventions against tuberculosis (TB) are limited to disease and death. More precise measurement of the human immune response to Mycobacterium tuberculosis infection may be important. A qualitative enzyme-linked immunospot assay (ELISPOT) result for early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) offers improved specificity over the purified protein derivative (PPD) skin test reaction in the detection of M. tuberculosis infection. We evaluated the quantitative ELISPOT and PPD skin test responses to recent M. tuberculosis exposure.

Methods. We studied quantitative PPD skin test and PPD ELISPOT results in 1052 healthy household contacts of index patients with cases of sputum smear–positive and culture-positive TB in The Gambia, according to a positive or negative ex vivo interferon-γ ELISPOT response to M. tuberculosis–specific antigens (ESAT-6/CFP-10). We then studied the quantitative PPD skin test and PPD ELISPOT results in patient contacts who had positive ESAT-6/CFP-10 results against a natural exposure gradient according to sleeping proximity to a patient with TB.

Results. The number of positive results was significantly greater for both PPD skin test and PPD ELISPOT in ESAT-6/CFP-10–positive subjects, compared with others (P < .0001). However, when quantitative PPD skin test and PPD ELISPOT results were compared in ESAT-6/CFP-10–positive subjects, only the ELISPOT count was sensitive to the exposure gradient, increasing significantly according to exposure (P = .009).

Conclusions. The quantitative ELISPOT response to PPD in specific-antigen–positive contacts of patients with TB reflects the infectious load of M. tuberculosis as a result of recent exposure. This finding offers new possibilities for assessment of the efficacy of new interventions, and adjustment should be made for it when relating the early immune response to progression to disease.

A significant barrier to the assessment of vaccines and other interventions against pathogens that undergo a latency period is the protracted time—often decades—before the onset of progression to disease. One alternative is to use a biomarker that reflects infection status. For Mycobacterium tuberculosis, the diagnosis of infection has been reliant on the conventional PPD skin test, which has unknown sensitivity and specificity and is subject to variation by age, sex, and latitude [1]. It is also subject to inhibition by orally ingested mycobacteria [2], which may explain the early reversion from “positivity” that occurs in tropical settings in which tuberculosis (TB) is endemic [3]. These properties make the PPD skin test problematic for setting entry criteria and infection end points for studies of new interventions.

It has been shown that a positive ex vivo T cell response to “early-secreted” antigens of M. tuberculosis (early secretory antigenic target 6 [ESAT-6] and culture filtrate protein 10 [CFP-10]) by enzyme-linked immunospot assay (ELISPOT) identifies individuals likely to be recently infected with M. tuberculosis as a result of recent exposure [4–6]. Recently, we reported that the use of ESAT-6 and CFP-10 by ELISPOT provides improved specificity over PPD by ELISPOT and skin test in the diagnosis of M. tuberculosis infection in The
Gambia (a tropical setting in which TB is endemic) by analysis of qualitative (i.e., yes or no) results [7]. Because the ELISPOT assay and PPD skin test are quantitative measures of cell-mediated immunity, the magnitude of the response may provide important information with respect to the relationship between infecting *M. tuberculosis* and the host, such as the likelihood of transition to latency versus progression to disease [8]. Therefore, in the present study, we extended our previous cohort of healthy household contacts of patients with cases of TB in The Gambia and evaluated the quantitative ELISPOT and PPD skin test responses to *M. tuberculosis* according to *M. tuberculosis* exposure.

**METHODS**

**Participants.** Index patients with sputum smear–positive cases of TB who were >15 years of age were recruited in Greater Banjul, an area housing 450,000 people, in which the incidence rate of newly diagnosed sputum smear–positive TB is ~80 cases per 100,000 population per year [9]. Patients who were included had 2 sputum samples that were positive for acid-fast bacilli by Ziehl-Neelsen staining and that grew *M. tuberculosis* on culture. Their household contacts were included in the study if they were ≥6 months of age and lived the majority of the time on the same compound as the case patient. They were not eligible if they had been treated for TB in the past year, and they were excluded from the study if they received a diagnosis of TB within 1 month after recruitment. The contacts were invited to give informed consent, interviewed, and examined, and a blood sample was obtained for ELISPOT and HIV testing. Fresh samples from all participants were processed onsite. A maximum of 12 contacts per day were screened; the other contacts were randomly excluded.

Contacts underwent a PPD skin test (2 TU, PPD RT23; Statens Serum Institut). Induration was recorded at 48–72 h. Subjects with a positive skin test result (mean induration diameter, ≥10 mm) were offered the opportunity to undergo chest radiography, and those with symptoms underwent a clinical assessment. Those with TB disease were referred to the National Programme for free treatment. There is no current practice of preventive treatment in The Gambia. This study was approved by the Gambia Government/Medical Research Council Joint Ethics Committee.

**Laboratory procedures.** Sputum smears were prepared and stained with auramine-phenol [10], and the results were confirmed by Ziehl-Neelsen staining. Decontaminated specimens were inoculated into 1 slope each of Lowenstein-Jensen medium containing glycerol and sodium pyruvate respectively and 1 vial of BACTEC 9000 MB media for isolation of *M. tuberculosis*. All mycobacterial cultures were identified and confirmed by standard procedures.

The ex vivo ELISPOT assays for IFN-γ were performed as described elsewhere [11]. For this study, synthetic, sequential peptides spanning the length of ESAT-6 and CFP-10 (ABC; Imperial College) were used. Each peptide was 15 amino acids long and overlapped its adjacent peptide by 10 residues. ESAT-6 and CFP-10 peptides, used at 5 μg/mL, were each divided equally and sequentially into pools of peptides. PPD (*M. tuberculosis*, RT49; Statens Serum Institut) was used at 10 μg/mL. The positive control was phytohaemaglutinin (PHA; Sigma-Aldrich). All antigens were tested in duplicate wells.

Assays were scored by an ELISPOT counter (AID-GmbH). Positive test wells were predefined as containing ≥10 spot-forming units (SFUs) more than—and at least twice as many as—negative control wells. For a positive ESAT-6/CFP-10 result, it was necessary for ≥1 pool of overlapping peptides to have a positive result. PHA-positive control wells were set to ≥150 SFUs above negative control wells. Negative control wells were required to have <30 SFUs. Quantitative counts were represented as SFUs above the negative control well. Testing for HIV-1 or HIV-2 infection was by competitive ELISAs (Wellcome Laboratories) and Western blot test (Diagnostics Pasteur).

**Ascertainment of exposure.** As per previous study designs in The Gambia [12], TB contacts were categorized according to where they slept: in the same bedroom as the patient, in a different bedroom in the same house, or in a different house.

**Data management and analysis.** The number of SFUs in each well were automatically entered into a relational Access database (Microsoft) [13] using Matlab software (MathWorks). Supplementary details were added by double data entry by 2 immunologists who were blinded to subject details, and the entries were checked for errors. All other data were entered using double data entry and were checked for errors. A linear mixed model, taking into account household clustering as a random effect, was fitted to the square root–transformed quantitative measures. All other variables were fitted as fixed effects, and the significance across proximity was quantified, taking into account the effects of possible confounding factors. All statistical analyses were conducted using Stata software, version 8 (Stata).

**RESULTS**

From 2 May 2002 through 30 November 2003, 1382 healthy contacts of 188 patients with sputum smear–positive cases of TB were recruited for our study. Of these, 1187 were selected for ELISPOT for IFN-γ, and 1052 (89%) of these had results that met inclusion criteria. Of these, 1018 (97%) had PPD skin test results. The median age of the case patients was 32 years (range, 15–75 years), 130 (70%) of the patients were male, and 12 (7%) of 184 tested for HIV infection had positive results. The median age of the contacts was 17 years (range, 6 months to 100 years), and 473 (45%) were male (table 1). We found no evidence that subjects who suspected that they were HIV
positive excluded themselves from the study, because a positivity rate of ~2% is consistent with sentinel surveillance studies for the population [14]. Overall, 313 subjects (30%) had positive ESAT-6/CFP-10 ELISPOT results.

Quantitative PPD skin test and PPD ELISPOT results, according to evidence of recent M. tuberculosis infection (figure 1), revealed that subjects who had positive ESAT-6/CFP-10 results tended to have a higher PPD ELISPOT count and a larger skin test reaction induration than did those who had negative results (P < .0001 for both analyses). These data show an association between recent M. tuberculosis exposure and an elevated immune response to PPD as measured by ELISPOT count or skin test reaction.

Exposure of case contacts to M. tuberculosis was classified by sleeping proximity to a patient with a case of TB. Of the 313 contacts with positive ESAT-6/CFP-10 ELISPOT results, 97 (31%) slept in the same room as a patient with TB, 144 (46%) slept in a different room in the same house, and 72 (23%) slept in a different house on the same compound. When quantitative PPD ELISPOT and PPD skin test values were analyzed according to exposure in contacts with positive ESAT-6/CFP-10 ELISPOT results (figure 2), PPD ELISPOT counts increased significantly according to the level of exposure to M. tuberculosis (figure 2A; P = .009, adjusting for the effects of ethnicity, sex, age, and household clustering), but no such relationship across the exposure gradient was found for the PPD skin test results (figure 2B).

There were no significant interactions found between variables. With respect to the quantitative responses to individual M. tuberculosis antigens in those subjects with positive results, a similar trend was observed for subjects with positive responses to ESAT-6 (subjects with positive results, 252; P = .08; data not shown), but there were too few subjects with positive responses to CFP-10 for such an analysis (subjects with positive results, 124). For completeness, we also assessed quantifiable PPD skin test data according to exposure history in those with a positive PPD skin test result (with use of a cut-off point of 10 mm induration) and found no trend (median indurations of 15.5 mm, 16.5 mm and 16 mm, respectively, across the gradient of increasing exposure; data not shown).

### DISCUSSION

The results presented here provide an analysis of the quantitative immune response to natural M. tuberculosis exposure in an endemic setting in >1000 healthy, largely (97.6%) HIV-negative household contacts of patients with sputum smear–positive cases of TB. These results provide new information that adds to the qualitative analyses published in our earlier report [7]. The quantitative PPD test results were significantly higher for those subjects who were ESAT-6/CFP-10 positive than for those who were ESAT-6/CFP-10 negative. This was true whether the response to PPD was measured by ELISPOT or by skin test. When the quantitative PPD test results of ESAT-6/CFP-10–positive subjects were compared across a natural exposure gradient, the ELISPOT assay revealed a positive dose–immune response relationship, whereas the skin test showed no such relationship. To our knowledge, this is the first time that a dose–immune response relationship has been demonstrated in a natural model for any infecting organism, and these results provide evidence for the utility of the ELISPOT assay beyond a gain in specificity for the diagnosis of M. tuberculosis infection.

The concept of utilizing sleeping proximity as a measure of exposure dates back at least to a classic report by Glover [15], published in 1920, that addressed meningococcal carrier rates. The sleeping proximity gradient has been found to be both reproducible and reliable with respect to tuberculosis transmission in The Gambia [7, 16]. Because the model is natural, there will be a number of environmental variables [17]. We overcome such background “noise” by analysis of large numbers of case contacts.

To give close quantitation of the response to the whole organism without losing specificity, we conducted the ELISPOT analyses using an “inverse” combination that combined qualitative, antigen-specific responses with those that were quantitative and nonspecific. The first combination identified individuals likely to be infected with M. tuberculosis (i.e., those showing a positive response to ESAT-6/CFP-10 peptides), and

### Table 1. Characteristics of 1052 contacts of patients with tuberculosis.

<table>
<thead>
<tr>
<th>Demographic or clinical characteristic</th>
<th>Contacts (n = 1052)</th>
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<tbody>
<tr>
<td>Age, years</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>21</td>
</tr>
<tr>
<td>Median (range)</td>
<td>17 (0–100)</td>
</tr>
<tr>
<td>Male sex</td>
<td>473 (45)</td>
</tr>
<tr>
<td>Ethnic group</td>
<td></td>
</tr>
<tr>
<td>Mandinka</td>
<td>294 (28)</td>
</tr>
<tr>
<td>Jola</td>
<td>269 (26)</td>
</tr>
<tr>
<td>Wolof</td>
<td>187 (18)</td>
</tr>
<tr>
<td>Fula</td>
<td>72 (7)</td>
</tr>
<tr>
<td>Other</td>
<td>230 (22)</td>
</tr>
<tr>
<td>Proximity to patient with tuberculosis</td>
<td></td>
</tr>
<tr>
<td>Same room</td>
<td>232 (22)</td>
</tr>
<tr>
<td>Different room</td>
<td>494 (47)</td>
</tr>
<tr>
<td>Different house</td>
<td>326 (31)</td>
</tr>
<tr>
<td>BCG scar presenta</td>
<td>419 (47)</td>
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<tr>
<td>HIV positiveb</td>
<td>25 (2.4)</td>
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</tbody>
</table>

**NOTE.** Data are no. (%) of subjects, unless otherwise indicated. BCG, bacille Calmette-Guérin.

*a* Data are for 883 subjects; 169 subjects had uncertain BCG scar status.

*b* Data are for 1047 subjects.
Figure 1. Enzyme-linked immunospot assay (ELISPOT) counts and PPD skin test induration diameter, by response to Mycobacterium tuberculosis-specific peptides. A, ELISPOT counts (spot-forming units [SFUs]/well/×10^5) in response to PPD in subjects with negative responses to early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) peptides (n = 739). B, PPD skin test induration diameter for subjects negative responses to ESAT-6 and CFP-10 peptides (n = 717). C, ELISPOT counts in subjects with positive responses to ESAT-6 and CFP-10 peptides (n = 313). D, PPD skin test induration diameter for subjects with positive responses to ESAT-6 and CFP-10 peptides (n = 301). The quantitative ELISPOT results were cut off at 250 SFUs per well above background; therefore, the results are not presented for 10 subjects with counts >300 SFUs per well (9 of these subjects had positive responses to ESAT-6 and CFP-10 peptides).

the second enumerated these by a response to PPD. The evaluation of PPD responses also enabled a direct comparison between the immune responses of the skin and blood compartments using the same antigenic challenge.

The ex vivo ELISPOT is reported to be specific for recently activated lymphocytes with an effector/memory phenotype that wanes as antigen is cleared [18]. The specific transient nature of ex vivo responsive cells may be key to the sensitivity to recent infectious load that is lacking in the skin test reaction. Such a function, beyond a qualitative (i.e., yes or no) test for infection, has several important possible applications. For example, a relative overall difference in ELISPOT frequency between intervention recipients and placebo recipients could provide an early indication of protection in vaccine efficacy trials, either against new infection in those with known TB exposure after prophylactic vaccination or against existing infection (i.e., therapeutic intervention).

Furthermore, we hypothesize that the quantitative ELISPOT frequency will decrease rapidly and reliably with successful intervention against M. tuberculosis infection, and we have commenced a randomized trial to assess ELISPOT reversion with isoniazid treatment in 300 contacts of patients with TB in The Gambia. A small study involving patients with TB has shown promising results in this regard [19]. A surrogate marker of efficacy would assist greatly in the assessment of new interventions.

The complexities of the organism-host interaction include organism factors, such as differences in virulence between organism strains [20] and, as shown in this study, differences in the infectious load received. It has been suggested that the strength of the immune response to recent infection could be a predictor of progression to disease over time [8]. Our results dictate that the infectious load must now be taken into account in longitudinal studies that address this question. For example,
Figure 2. Quantitative PPD enzyme-linked immunospot assay (ELISPOT) results (A; n = 313) and PPD skin test results (B; n = 301), by Mycobacterium tuberculosis exposure category, in subjects with positive reactions to early secretory antigenic target 6 and culture filtrate protein 10. Horizontal lines, median values.
in a patient contact study, a measure of the amount of exposure to an index patient should be obtained when assessing the quantitative immune response to recent \textit{M. tuberculosis} infection in relation to the later development of secondary disease. As the immunological features of protection from tuberculosis are delineated, it is important that the best tools available are utilized. Improved precision in the quantitative measurement of the immune response to recent infection is possible through a functional T cell assay that measures a transient component of the immune response. This provides new possibilities for the assessment of the efficacy of new interventions against TB and new considerations for those evaluating the initial immune response to \textit{M. tuberculosis} in relation to the progression to disease. These results are also of relevance to studies of interventions against other major infectious diseases.

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\section*{Potential conflicts of interest.}
R.H.B. has a patent relating to ex vivo ELISPOT that is licensed through Oxford University. All other authors: no conflicts.

\section*{References}