Enzyme-Linked Immunospot Assay Responses to Early Secretory Antigenic Target 6, Culture Filtrate Protein 10, and Purified Protein Derivative among Children with Tuberculosis: Implications for Diagnosis and Monitoring of Therapy


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Background. The ability to detect tuberculosis-specific lymphocytes by enzyme-linked immunospot (ELISPOT) assay may have important implications for the diagnosis and monitoring of tuberculosis in children, for which routine methods lack sensitivity. We conducted a study to determine the presence and time course of ELISPOT responses in children with tuberculosis.

Methods. Blood samples were obtained from children with a clinical diagnosis of tuberculosis, and interferon-γ ELISPOT assays were performed using purified protein derivative (PPD), early secretory antigenic target 6 (ESAT-6), and culture filtrate protein 10 (CFP10) as stimulants. A subset of children were retested after 1, 3, and 6 months of therapy.

Results. Detectable responses to ESAT-6 or CFP10 were found in 49 of 70 children with clinical tuberculosis but were more frequently found in those with culture-proven disease ($P < .05$). The number of subjects with responses to PPD increased after 1 month of therapy ($P = .0004$) and decreased at 3 and 6 months.

Conclusion. Tuberculosis-specific ELISPOT testing is a promising tool that should be evaluated as a potential diagnostic test for childhood tuberculosis. We caution against the use of an early decrease in response as a marker of successful antituberculous chemotherapy.

Tuberculosis remains an important cause of morbidity and mortality in children from the developing world [1]. Children aged <5 years are highly susceptible to developing active tuberculosis. Because tuberculosis in children frequently produces negative smear and culture results, it poses a diagnostic and therapeutic challenge [2]. The ability to detect the presence of circulating tuberculosis-specific lymphocytes with use of a simple laboratory assay offers new possibilities for diagnosing tuberculosis and monitoring therapeutic efficacy. An advance has been the identification of a cluster of T cell antigens present in the Mycobacterium tuberculosis complex but absent from bacille Calmette-Guérin (BCG) and many environmental mycobacteria [3]. The most widely studied of these antigens are early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP10), which have been used in both whole blood–based and PBMC-based in vitro assays to determine the presence of specific cellular immune responses [4–6]. These have been found to be promising for the diagnosis of latent tuberculosis infection in adults [7–9].

The ELISPOT technique is a sensitive method of enumerating lymphocytes that secrete IFN-γ in response to specific antigens. ELISPOT responses to ESAT-6 and CFP10 appear to be more specific than
METHODS

Selection and description of participants. All children <14 years old presenting to the Red Cross Children’s Hospital (Cape Town, South Africa) from January 2002 through August 2004 who received an admission diagnosis of tuberculosis were eligible for inclusion in the study. Children were considered for inclusion if the admitting clinician determined that the child should receive antituberculous chemotherapy and if a parent or legal guardian was available to consent to the child’s enrollment. The cohort of healthy contacts was recruited by tracing the household contacts of adults who presented with newly diagnosed tuberculosis at an urban clinic in the Cape Town metropolitan area. Ethics approval was obtained from the Research Ethics Committee of the University of Cape Town. All participating children were tested for HIV infection by means of ELISA after extensive counseling and after giving consent. Children found to be HIV positive according to the results of ELISA were excluded from participation in the study and were referred to the hospital HIV clinic. A sequential subgroup of children was enrolled for evaluation of the time course of responses at 1, 3, and 6 months after commencing therapy.

Diagnostic investigations, including radiographic and microbiological tests and TST, were performed at the discretion of the children’s physician. Once the results of this testing were available, records and chest radiographs were reviewed in a blinded fashion by a study clinician with extensive experience in the diagnosis of childhood tuberculosis, and children were categorized as having definite, probable, or possible tuberculosis (table 1).

ELISPOT assays. After informed consent was received, 5 mL of blood was obtained from each subject and processed within 2 h. ELISPOT was performed as described elsewhere [11]. In brief, PBMCs were separated by means of Ficoll-Paque centrifugation. Cells were washed, resuspended, and counted.

Ninety-six–well polyvinylidene fluoride–backed plates (MABP3510; Millipore) were coated with 15 μg/mL of anti–IFN-γ mAb 1-D1K (Mabtech). Cells (300,000 or 100,000 per well) were added to duplicate wells containing antigen or mitogen (table 2). No antigen was added to the background control wells. After 18 h of incubation, plates were washed, and 100 μL of 1 μg/mL of biotinylated anti–IFN-γ mAb, 7-B6-1-biotin (Mabtech), was added for 2 h. Plates were then washed and streptavidin-alkaline phosphatase toxoid (Mabtech) was added. After 1.5 h and additional washing, 100 μL of chromogenic alkaline phosphatase substrate (Biorad) was added. After 10–15 min, plates were washed, and spots were enumerated independently by 2 observers with use of a stereomicroscope. The mean values determined by the 2 observers (mean interobserver coefficient of variation, 8%) and both duplicate wells were used in all calculations. The number of spots in the background control wells was subtracted from the number in the test wells, and a response was considered positive if the number of spots per test well was ≥10 (i.e., 33 spots per million PBMCs) and at least twice the value found in the background control wells. The use of this value to determine a positive response was based on previous reports by Lalvani et al. [9, 13]. This value also corresponded to 2 SDs above the mean of the value for the unstimulated control wells (data not shown).

STATISTICAL ANALYSIS

Comparisons between ELISPOT results in clinical categories were made using the Kruskal-Wallis test. A χ² test for trend...
was used to compare the proportion of positive results in each clinical category. A 2-tailed Mann-Whitney test was used to compare unpaired results (from a single time point), and the Wilcoxon signed rank test was used to compare paired observations (from the acute phase and from 1 month after initiation of therapy). Repeated-measures analysis of variance (ANOVA) was used for evaluation of responses after >6 months of therapy.

**RESULTS**

A total of 70 children (median age, 32 months; range, 3–154 months) with a diagnosis of tuberculosis were recruited. After record review, 12 children were determined to have definite cases of tuberculosis, 47 to have probable cases, and 11 to have possible cases (table 1). To evaluate responses after 1 month of therapy, a subgroup of 42 patients underwent additional ELISPOT assays after 1 month of therapy. Of this subgroup, 25 children for whom follow-up was possible were restested after 3 months of therapy, and 10 of these were restested after 6 months of therapy. Of the 70 children, 52 received a TST with 2 TU of PPD RT23 (Statens Serum Institut). An additional 26 children (median age, 24 months) who were healthy contacts of adults with tuberculosis were recruited. The presence of active tuberculosis was excluded after radiographic and clinical examination. Of these children, 14 of 26 had a nonreactive TST result (i.e., one measuring 0 mm in diameter), and the remaining 12 children had TST results measuring ≥15 mm in diameter. Children with positive TST results were referred for chemoprophylaxis, in accordance with national guidelines.

**Responses to ESAT-6, CFP10, and PPD at diagnosis.** The distribution of responses to ESAT-6, CFP10, and PPD by diagnostic category at diagnosis is shown in figure 1. There was a nonsignificant trend for median ESAT-6 responses ($P = .0631$) and CFP10 responses ($P = .1968$) to be lower in the probable tuberculosis and possible tuberculosis groups. Positive responses to either ESAT-6 or CFP10 were found in 10 (83.3%) of 12 subjects with definite tuberculosis, 34 (72.3%) of 47 subjects with probable tuberculosis, and 5 (45.5%) of 11 subjects with possible tuberculosis ($\chi^2$, 3.77; $P = .05$). There was no significant difference between the median ages of children with positive responses and those with negative responses. There was no correlation between the response to ESAT-6 and PPD and the TST result (Spearman $\rho$, 0.1587). The response to PPD was significantly greater in subjects with positive responses to ESAT-6 or CFP10 (median response, 446 spots per million PBMCs) than in those with negative responses to both (median response, 144 spots per million PBMCs; $P < .0001$) (figure 2). There was no correlation between the response to any antigen and the weight-for-age Z score (PPD: Spearman $\rho$, $-0.070$; $P = .55$; ESAT-6: Spearman $\rho$, $-0.1718$; $P = .138$; CFP10: Spearman $\rho$, $-0.1721$; $P = .1372$).

### Table 1. Clinical classification of patients with cases of tuberculosis (TB).

<table>
<thead>
<tr>
<th>Classification</th>
<th>No. of patients</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definite TB</td>
<td>12</td>
<td>Isolation of <em>Mycobacterium tuberculosis</em> in culture of sputum, gastric aspirate, or CSF sample</td>
</tr>
<tr>
<td>Probable TBa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>15</td>
<td>Residence in an area with a high prevalence of TB; symptoms consistent with TB; known close contact with a patient with TB; tuberculin skin test results ≥15 mm in diameter; AND abnormal chest radiograph findings</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>Residence in an area with a high prevalence of TB; symptoms consistent with TB; no known close contact with a patient with TB; tuberculin skin test results ≥15 mm in diameter; AND abnormal chest radiograph findings</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>Residence in an area with a high prevalence of TB; symptoms consistent with TB; known close contact with a patient with TB; AND either tuberculin skin test results ≥15 mm in diameter OR abnormal chest radiograph findings</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>Residence in an area with a high prevalence of TB; symptoms consistent with TB; AND 1 of the following: positive results of gastric aspirate smear (1 patient), CSF and cranial CT findings consistent with tuberculous meningitis (4 patients), OR abdominal ultrasound and CT findings suggestive of TB abdomen and response to therapy (1 patient)</td>
</tr>
<tr>
<td>Possible TB</td>
<td>7</td>
<td>Residence in an area with a high prevalence of TB; symptoms consistent with TB; no known close contact with a patient with TB; AND either tuberculin skin test results ≥15 mm in diameter OR abnormal chest radiograph findings</td>
</tr>
</tbody>
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a To be classified as having probable TB, a patient must fulfill all criteria in A, B, C, or D.
Table 2. Antigens and cell concentrations used in the enzyme-linked immunospot assay.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PHA</th>
<th>PPD$^a$</th>
<th>PPD</th>
<th>ESAT$^b$</th>
<th>CFP10$^b$</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of stimulant, µg/mL</td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>2.5</td>
<td>...</td>
</tr>
<tr>
<td>Cells per well</td>
<td>100,000</td>
<td>300,000</td>
<td>100,000</td>
<td>300,000</td>
<td>300,000</td>
<td>300,000</td>
</tr>
</tbody>
</table>

**NOTE.** CFP10, culture filtrate protein 10; ESAT-6, early secretory antigenic target 6; PHA, phytohemagglutinin antigen.

$^a$ Tuberculin PPD provided by Evans Vaccines.

$^b$ Provided by Lionex Diagnostics and Therapeutics.

**Responses to ESAT-6, CFP10, and PPD after 1 month of treatment.** Additional ELISPOT testing was performed after 1 month of treatment for 42 subjects. Nine patients were classified as having definite tuberculosis, 6 as having probable tuberculosis, and 27 as having possible tuberculosis. The acute-phase and 1-month responses are shown in figure 3. The median PPD response was significantly greater after 1 month of treatment (419 spots per million PBMCs) than at diagnosis (190 spots per million PBMCs; $P = .0004$). There was no significant difference between the acute-phase visit and the visit after 1 month of therapy in median response to ESAT-6 (58 and 85 spots per million PBMCs, respectively) or CFP10 (57 and 59 spots per million PBMCs, respectively). Acute-phase results and results after 1 month of treatment were highly correlated for CFP10 (Spearman $\rho$, 0.827; $P < .0001$) and ESAT-6 (Spearman $\rho$, 0.6289; $P < .0001$).

After 1 month of treatment, median responses to ESAT-6, CFP10, and PPD were different between diagnostic groups ($P = .0133$, $P = .0118$, and $P = .0079$, respectively, by Kruskal-Wallis test), with significantly greater responses among the group with probable tuberculosis, compared with the group with possible tuberculosis. There was a trend for responses in the definite tuberculosis group to be greater than those in the possible tuberculosis group, but this trend failed to reach statistical significance.

**Responses to ESAT-6, CFP10, and PPD after 3 and 6 months of treatment.** Additional responses were evaluated in a consecutive subset of 10 patients. The evolution of responses to PPD and ESAT-6 is shown in figure 4. In this subset, an initial increase in response to these antigens after 1 month of treatment was followed by a decrease at 3 and 6 months of treatment (repeated-measures ANOVA for PPD, $P = .003$; for ESAT, $P = .039$; and for CFP10, $P = .0697$).

Responses to ESAT-6 were significantly greater among patients with probable tuberculosis than among patients with possible tuberculosis after 3 months of treatment ($P = .0406$, by Kruskal-Wallis test). The trend for patients with definite or probable cases of tuberculosis to have greater responses to CFP10 and PPD, compared with patients with possible cases of tuberculosis, was still present after 3 months of treatment, but it was nonsignificant ($P = .1097$ for CFP10 and $P = .0797$ for PPD, by Kruskal-Wallis test).

Figure 1. Response to early secretory antigenic target 6 (ESAT-6) (A), culture filtrate protein 10 (CFP10) (B), and PPD (C) by clinical category at diagnosis. Results are mean values determined by 2 independent observers, given as spots per million PBMCs, less the values in the background control wells. A dotted horizontal line is included to show the cut-off value for a positive response (33 spots per million PBMCs) for ESAT-6 and CFP-10. Definite, definite tuberculosis group; probable, probable tuberculosis group; possible, possible tuberculosis group.
DISCUSSION

It is likely that there will be increasing use of in vitro assays that detect the presence of specific cellular responses to tuberculous antigens. Several studies have suggested that ELISPOT and whole-blood-based assays detecting responses to RD1-coded antigens are more specific than PPD skin testing in diagnosing latent infection in both adults and children [8, 9]. ELISPOT has been shown to be sensitive in detecting active tuberculosis infection in small groups of adults [7]. Sequential monitoring of ELISPOT responses may also be useful in monitoring response to antituberculous chemotherapy [14]. The role of ELISPOT in diagnosis and monitoring of acute-phase tuberculosis in children has not been reported.

We have defined IFN-γ ELISPOT responses to ESAT-6 and CFP10 in a group of children with a clinical diagnosis of tuberculosis. The findings of this study have a number of important implications for the potential use of RD1-based ELISPOT for the diagnosis and monitoring of tuberculosis in childhood.

Responders and nonresponders. It is clear that a proportion of children with a clinical diagnosis of tuberculosis have negative ELISPOT responses to ESAT-6 and CFP10 at diagnosis (21 [30%] of 70). This may be because of impaired sensitivity of the ELISPOT assay in these circumstances, poor specificity of the clinical diagnosis of tuberculosis in children, or both. Of note, positive responses at diagnosis were found among fewer patients with possible tuberculosis (45.5%) than patients with probable (72.3%) or definite (83.3%) cases of tuberculosis. The observation that those children in the possible tuberculosis group who had negative responses to ESAT-6 and CFP10 at
diagnosis had persistently negative responses after 1, 3, and 6 months of treatment (despite positive responses to PPD) suggests that this subset may well represent children with a diagnosis other than tuberculosis. The clinical diagnosis of tuberculosis may therefore lack specificity in the present study. Overdiagnosis of tuberculosis is important, given the expense, prolonged duration, and potential for adverse effects of therapy. This hypothesis will remain difficult to prove, given the difficulty in confirming tuberculosis in children, as no appropriate gold-standard comparator with high sensitivity and specificity is available.

Because there were 2 patients with culture-confirmed tuberculosis who had negative ELISPOT results, we retested 42 consecutive subjects 1 month after initial testing to evaluate whether repeating the ELISPOT test would improve the diagnostic yield. There was no significant difference in the proportion of patients who responded to either antigen at the time of diagnosis, compared with the proportion who responded 1 month later. However, 6 children had a positive response to at least 1 antigen at the 1-month follow-up visit who had not had a positive response at diagnosis, including 1 child with culture-confirmed tuberculosis. Only 1 of the 9 children with culture-
Responses to early secretory antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP10), and PPD among healthy children who were contacts of adults with tuberculosis. Results are mean values determined by 2 independent observers, given as spots per million PBMCs, less the values in the background control wells. Neg, negative; pos, positive; TST, tuberculin skin test.

Proven disease had persistently negative responses at 1 month after diagnosis. The strategy of repeating tests for which there were borderline or negative results at 1 month after initial testing might be a useful approach. The data obtained at 1 month after diagnosis may, however, be confounded by the possible effect of TST on ELISPOT responses, and additional work is required in this area.

Magnitude of responses. There was no significant difference between the magnitudes of the responses to either CFP10 or ESAT-6 at diagnosis, compared with those at 1 month after diagnosis. In contrast, PPD responses were significantly greater at 1 month after diagnosis. A number of potential mechanisms could underlie this finding.

First, this may reflect variation between patients in the time to diagnosis. Second, TST might prime the ELISPOT response to PPD. The effect of TST on responses to ESAT-6 and CFP10 is not yet clear, although Mawa et al. [15] have shown a transient increase in IFN-γ production in response to culture filtrate proteins of M. tuberculosis after TST in HIV-infected individuals. Wilkinson et al. [16] have shown that the ratio of ESAT-6 to PPD responsive cells in central compartments is higher than that in blood. Such sequestration during active infection may explain the paucity of specific cells found by ELISPOT in some subjects. Finally, severe tuberculosis has been associated with decreased levels of IFN-γ production by PBMC in a proportion of patients with tuberculosis [17].

It has been previously shown that ELISPOT responses to PPD are unable to adequately distinguish between earlier BCG vaccination and exposure to tuberculosis [5]. The median responses to PPD at diagnosis were significantly greater in the group of children who had responses to either of the specific antigens, compared with children who did not have responses. PPD responses were almost universal, however, and there was no single threshold value that corresponded with a positive response to the specific antigens.

Carrara et al. [14] have demonstrated the disappearance of ELISPOT responses in patients who received 3 months of successful therapy but persistent responses in patients who experienced failure of therapy. Sequential testing has thus been advocated as a biomarker of successful chemotherapy. In the present study, an increase in ELISPOT responses at the 1-month visit was documented in 45% (for ESAT-6), 40% (for CFP10), and 69% (for PPD) of patients with definite or probable cases of tuberculosis. This cautions against the use of an early decrease in ELISPOT responses as a surrogate for successful treatment. Results among the 10 patients for whom 3-month and 6-month data were available suggest that an early increase in responses to PPD and ESAT-6 after 1 month of therapy is followed by a decrease in responses at 3 months and 6 months of therapy to a level similar to that seen at initial presentation.

Responses among healthy contacts. ELISPOT responses to PPD discriminated between children with positive TST results and children with negative results. Healthy children with positive TST results had responses that were similar to those of children with active tuberculosis. Healthy children with negative TST results (who were presumably uninfected) had negative responses to the specific antigens in 12 of 14 cases. Therefore, ELISPOT appears to be useful in identifying children infected with tuberculosis, but it is unable to discriminate between active tuberculosis and latent or subclinical infection.

Limitations of this study. This study was designed to document ELISPOT responses in children with a clinical diagnosis of tuberculosis, whose diagnosis is, at best, imperfect. We acknowledge that a number of the children included in this study, particularly those in the possible tuberculosis group, may have an alternative diagnosis. Indeed, the likelihood of overdiagnosis of childhood tuberculosis is an important conclusion of this study. We did not intend to conduct an evaluation of ELISPOT as a diagnostic tool, but rather, to document responses in children with a clinical diagnosis of tuberculosis. We believe that these data suggest that ELISPOT merits formal evaluation as a diagnostic tool for childhood tuberculosis, ideally with an additional test at 1 month after initial presentation, and that an early decrease in tuberculosis-specific immune responses cannot be used as a marker of successful therapy.

The profound effect of HIV infection on cellular immunity may affect the sensitivity of the ELISPOT assay, although in a small study, uncontrolled for CD4+ cell count, 90% of adults coinfected with tuberculosis and HIV were shown to have detectable responses [7]. The present study excluded HIV-infected children in an attempt to evaluate the ELISPOT assay without...
the confounding effect of HIV infection. Additional work is needed to explore the use of the assay in patients with HIV coinfection.

CONCLUSION

ESAT-6– or CFP10-specific IFN-γ–producing lymphocytes were detectable at diagnosis in two-thirds of children presenting with a clinical diagnosis of tuberculosis; however, responses were more frequently positive in patients with culture-proven disease. Additional testing after 1 month showed a similar pattern of results; however, median responses to PPD were significantly greater at the second visit. Responses tended to increase during the first month after diagnosis and then decrease by 3–6 months after diagnosis. ELISPOT is a promising tool for the clinical evaluation of childhood tuberculosis and merits additional assessment in a diagnostic trial.

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