Transmission of *Mycobacterium tuberculosis* Undetected by Tuberculin Skin Testing


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**Rationale:** The development of tuberculin skin test (TST) positivity following infection by *Mycobacterium tuberculosis* is not invariable and may depend on bacillary as well as host factors. **Objectives:** First, to compare the diagnostic performance of the TST and a form of *in vitro* IFN-γ release assay (IFNGRA) in the circumstances of a contact investigation prompted by an unusually severe index case of infectious pulmonary tuberculosis. Second, to investigate the ability of the strain of *M. tuberculosis* responsible to induce cytokine secretion from monocytes in *vitro*.

**Methods:** A routine TST-based tuberculosis-contact screening procedure supplemented by the use of an “in house” IFNGRA that assays the T-cell response to the *M. tuberculosis*-specific antigens ESAT-6, CFP-10 (presented as a fusion protein within the inactivated adenylate cyclase of *Bordetella pertussis*), and purified protein derivative of *M. tuberculosis*. Isolation and genetic typing of the strain of *M. tuberculosis* responsible, and investigation of its ability to induce cytokine secretion from monocytes in *vitro*.

**Measurements and Main Results:** TST screening suggested a low rate of transmission with just 2/75 unequivocally positive responses. By contrast, the IFNGRA suggested an infection rate of 16/75 (22%). When compared with two reference strains of *M. tuberculosis* (H37Rv and CDC1551), the outbreak strain induced lower levels of tumor necrosis factor-α and interleukin-12p40 (p < 0.04), cytokines associated with the development of delayed-type hypersensitivity.

**Conclusions:** These data suggest that infection by *M. tuberculosis* can be undetected by TST, and that this may partially relate to strain differences in immunogenicity.

**Keywords:** adenylate cyclase; diagnostic tests and procedures; ESAT-6 protein; *Mycobacterium tuberculosis*; tuberculin

Detection of tuberculosis (TB) transmission has, for many decades, relied on the cutaneous response to purified protein derivative of *Mycobacterium tuberculosis* (PPD) administered intradermally in various ways as the tuberculin skin test (TST) (1, 2). The induction of a positive response is thought to depend on both the ability of PPD to initiate a local inflammatory response and on T-cell recognition of mycobacterial antigens.

A significant advance in the routine immunodiagnosis of TB may soon be possible because of the identification of region-of-difference—1 encoded antigens ESAT-6 and CFP-10 and their use in various forms of the *in vitro* IFN-γ release assay (IFNGRA). These assays appear at least as sensitive and clearly more specific than the TST (3–10) because the ESAT-6 and CFP-10 antigens are restricted in distribution and thereby less likely to score positive responses when sensitization has arisen as a result of infection by nontuberculous mycobacteria. We have recently shown that the sensitivity of a whole-blood assay to detect latent TB infection (LTBI) is enhanced when CFP-10 is presented as a fusion protein within the genetically detoxified *Bordetella pertussis* adenylate cyclase (CFP-10–CyaA) (11). Importantly, ESAT-6 and CFP-10 are not present in bacille Calmette-Guérin (BCG), the vaccine strain of *Mycobacterium bovis* (12, 13), the previous administration of which is one of the commonest confounding factors when interpreting the TST result.

Failure to produce a cutaneous response to TB antigens (anergy) is well recognized in malnourished and immunosuppressed subjects, and during the course of overwhelming disease (14, 15). Conversely, unusually vigorous TST reactions have been reported in the context of an outbreak of strain CDC1551 (16). The ability of CDC1551 to give rise to pronounced TST reactions has been associated with its propensity to induce greater proinflammatory cytokine production from mononuclear phagocytes in *vitro* when compared with other strains (17, 18).

This report describes a sister and brother who developed severe TB due to the same strain. The resulting school-contact investigation was used to compare a whole-blood–based IFNGRA with the form of TST in use in the United Kingdom at that time (Heaf test). Very little evidence of transmission was detected by the TST but the IFNGRA results suggested extensive transmission. The strain of *M. tuberculosis* responsible was shown to be impaired in its ability to induce the secretion of tumor necrosis factor (TNF)-α and interleukin (IL)-12p40 from monocytes when compared with the reference strains H37Rv and CDC1551. These observations suggest that transmission of TB can be undetected by the TST and that one mechanism behind such transmission may be the decreased ability of certain strains to induce delayed-type hypersensitivity reactions.

Some of the results of these studies have been reported previously in the form of an abstract (19).

**METHODS**

**Study Population**

A total of 120 school contacts (staff and pupils) of the index case were screened by TST Heaf screening with PPD (Evans Vaccine Ltd., Liverpool, UK) at a strength of 10,000 units delivered by a UniHeaf six-needle disposable-head Heaf gun (Owen Mumford, Oxford, UK) according to U.K. guidelines. Cutaneous induration, grades 1–4, was measured 1 wk later (20, 21). Students with Heaf grades 0 or 1, or Heaf grade 2 with a BCG scar or documented history of BCG vaccination, were scored as uninfected. Students with Heaf grade 2 and no evidence of prior BCG vaccination or those with Heaf grade 3 or 4 with or without evidence of prior BCG were scored as infected. All students who agreed to the additional blood test were recruited at the time of Heaf screening with informed consent. A total of 75 students (52% female, 48% male; median age, 16.4 yr) thereby underwent *in vitro*...
detection of T-cell reactivity to *M. tuberculosis*-specific antigens. Of these, 59 (79%) had previously received BCG vaccine as established either by medical records or the presence of a BCG scar. The ethnic distribution of those enrolled into the study was as follows: 43 (57.4%) of white ancestry; 18 (24%) of Indian subcontinent ancestry; 10 (13.2%) of black African/Afro-Caribbean ancestry; two (2.7%) of southeast Asian ancestry; and two (2.7%) of eastern Mediterranean ancestry. As control subjects for the IFNGRA, 12 *M. tuberculosis*-unexposed and BCG-unvaccinated individuals (75% female, 25% male; median age 26.5 yr), who had recently undergone Heaf testing by the same investigating team and had been found to be negative, were included in the study. The ethnic distribution of the control subjects was as follows: nine (75%) were of white ancestry, three (25%) were of Indian subcontinent ancestry, and one (8.3%) was of Afro-Caribbean ancestry. Only one control subject had a history of overseas travel to a country in which TB is endemic.

**Whole-Blood IFNGRA**

The Harrow Local Research Ethics Committee approved the use of the Harrow Local Research Ethics Committee to supplement the contact investigation (EC 03169). The whole-blood IFNGRA and IFN-γ ELISA were set up as previously described (11). Antigens included recombinant ESAT-6 at 250 nM, CyaA–CFP-10 at 5 nM, and PPD (RT23) at 1,000 U/ml. Phytohemagglutinin at 5 μg/ml was included as a positive control. Appropriate background values (unstimulated wells for ESAT-6 and “empty” CyaA toxoid carrier for CFP-10) were subtracted in each case. Negative cut-off values for each antigen were determined as above the highest value of IFN-γ production obtained from screening the 12 TB-negative control subjects.

**Culture of *M. tuberculosis* Strains and Infection of Monocytes**

*M. tuberculosis* strains H37Rv, CDC 1551, and NPH4216 were cultured to midlog phase, frozen at −80°C in aliquots, and defrosted before use. Monocytes from seven buffy coats were isolated by adherence and infected at 1:1 bacillus:cell as previously described (22). Supernatants were aspirated 72 h later and the IL-12p40, IL-1β, and TNF-α cytokine levels were determined by ELISA using the DuoSet ELISA Development Systems (R&D Systems, Minneapolis, MN). The sensitivity of the assays was 15 pg/ml for IL-12p40, 10 pg/ml for IL-1β, and 50 pg/ml for TNF-α. IL-10 was determined using the human IL-10 kit (Mabtech AB, Nacka, Sweden). The sensitivity of this assay was 30 pg/ml. IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were measured using the human Quantikine ELISA kits (R&D Systems), with sensitivities of 2 and 4 pg/ml, respectively. A test of normality was applied to cytokine data that indicated all values approximated a Gaussian distribution. Therefore data are quoted ± SD. Comparison of cytokine levels between strains was made by Student’s paired *t* test. Values were multiplied by 2 (n = 1, Bonferroni correction) to reflect the comparison of three strains.

**RESULTS**

In late 2003, a healthy, HIV-uninfected, 15-yr-old girl (index) was diagnosed with infectious pulmonary TB in a North London school. She was a second-generation West African and had been unwell for 3 mo before the establishment of the diagnosis. Sputum examination was strongly (3+) smear positive for acid-fast bacilli, and a fully drug-susceptible *M. tuberculosis* was subsequently identified (NPH4216). Despite full adherence to antituberculous therapy, the index suffered a complex clinical course with several episodes of deterioration during therapy. Ultimately, a pneumonectomy became necessary because of refractory stenosis of the left main bronchus that was unrelieved by high-dose corticosteroids and endobronchial stenting. The radiograph shows marked loss of volume in the left hemithorax and the arrow indicates the site of the stenosed left main bronchus. L = left side of body.

*Figure 1.* Chest radiograph during treatment of the index case, a 15-yr-old girl with fully drug-sensitive *Mycobacterium tuberculosis* infection. Despite full adherence to antituberculous therapy, the index suffered a complex clinical course with several episodes of deterioration during therapy. Ultimately, a pneumonectomy became necessary because of refractory stenosis of the left main bronchus that was unrelieved by high-dose corticosteroids and endobronchial stenting.

BCG vaccination); a repeat at 6 wk showed a decrease to grade 1. Thus, transmission from sister to brother was thought unlikely. However, at 3 mo, the brother developed culture-confirmed mililiary TB and the isolate (NPH4216) was confirmed to be genetically identical to that of the index case by 12 locus MIRU-VNTR and spoligotyping (23). The 12-locus MIRU-VNTR pattern was 223226173323 and the Octal spoligotype was 777777607760731 (24). These analyses showed the strain to belong to the Latin American and Mediterranean 4 clade, an unusual cause of TB in the United Kingdom (25).

The school-contact investigation presented the opportunity to compare the TST Heaf results with the whole-blood IFN-γ responses to ESAT-6 and CyaA–CFP-10. Unequivocal Heaf positivity and thus LTBI could only be inferred in 2 of the 75 school contacts screened by Heaf testing: both were BCG-unvaccinated individuals with grade 2 Heaf reactions (Table 1). In contrast, the ESAT-6– and/or CyaA–CFP-10–induced IFN-γ levels were greater than the highest value of control subjects in 22% of contacts (Figure 2): 11 (14.6%) positive for ESAT-6, 13 (17.3%) positive for CyaA–CFP-10, and eight (11.1%) positive for both. The mitogen phytohemagglutinin produced significant responses, ranging from 3,818 to 11,213 pg/ml (mean, 8,034 pg/ml) in all contacts and control subjects.

In the absence of a gold standard for LTBI, proximity to the index case together with duration of exposure may be used as a measure of the likelihood of infection. We documented the duration of contact and proximity between the index case and contacts according to school timetables but found no clear relationship between these factors and the risk of a positive IFNGRA result. Nevertheless, the prolonged symptoms and
TABLE 1. RESULTS OF HEAF SCREENING OF THE 75 SUBJECTS IN THE STUDY

<table>
<thead>
<tr>
<th>Heaf Grade</th>
<th>Mantoux Equivalent (mm)</th>
<th>No. Contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>0</td>
<td>47</td>
</tr>
<tr>
<td>1</td>
<td>1–5</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>&gt;15</td>
<td>8</td>
</tr>
<tr>
<td>Positive</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>&gt;15</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

Values in the second column are the estimated equivalent results with Mantoux testing.

* unvaccinated.

infectivity of the index suggested that transmission of, and sensitization by, *M. tuberculosis* had occurred but had not been detected by the TST.

The infrequency of Latin American and Mediterranean 4 strains in the United Kingdom, the unusual clinical progression of our index and her sibling, and the low skin-test conversion rate suggested the possibility that the causative organism might be biologically distinct from common infecting strains. There is evidence that the capacity to induce cytokines from mononuclear phagocytes varies among strains of *M. tuberculosis*. For CDC1551, it has been related to the strain’s capacity to induce marked TST reactions (17). We therefore compared the ability of strain NPH4216 to elicit the secretion of cytokines from human monocytes with that of the reference strains H37Rv and CDC1551. The mean IL-12p40 and TNF-α secretions in response to NPH4216 were 434 ± 566 and 4,179 ± 2,445 pg/ml, respectively. These values were significantly lower than the corresponding values for both H37Rv (2,374 ± 603 pg/ml IL-12p40 and 8,802 ± 1,882 pg/ml TNF-α) and CDC1551 (1,959 ± 647 pg/ml IL-12p40 and 8,368 ± 1,657 pg/ml TNF-α; *p*corr ≤ 0.04). In addition, NPH4216 induced less IL-10 than H37Rv (572 ± 131 and 877 ± 147 pg/ml, respectively; *p*corr ≤ 0.05). The production of GM-CSF was also markedly reduced after exposure to the outbreak strain (476 ± 79 pg/ml) compared with the control strains (1,473 ± 467 pg/ml for H37Rv and 1,257 ± 346 pg/ml for CDC1551), although this difference did not reach significance. The difference between CDC1551 and NPH4216 was significant with respect to TNF-α (*p*corr = 0.033) (Figure 3).

**DISCUSSION**

We have investigated the transmission and ability to induce cytokines from monocytes of a strain of *M. tuberculosis* that caused severe disease in two healthy, HIV-uninfected siblings. Our data show that transmission of, and sensitization by, *M. tuberculosis* can be undetected by the TST. The form of TST in use (Heaf test) was insufficiently discriminatory in the presence of prior BCG vaccination to detect infection in a family contact with the consequence that the disease was disseminated. In addition, the Heaf-based contact-screening procedure also suggested that transmission had been minimal. By contrast, the results of the IFNGRA suggested that there had in fact been transmission within the school. We were unable to completely ascertain proximity to and duration of contact with the index case; this study was completed at a sixth form college in which students’ time is less structured. We were also unable to account for nonrandom social interaction. However, the findings are consistent with the known infectivity and duration of symptoms of the index case. Our results therefore support a number of reports that the IFNGRA is more sensitive than the TST for the detection of tuberculous infection (4, 8, 26, 27). A limitation of both assays is that they document sensitization, but cannot inform whether live mycobacteria are present in the individual.

To define a cutoff for a positive IFNGRA, we recruited TB-unexposed control subjects with no known TB exposure who had been tuberculin skin tested 7 d before IFNGRA was performed. We and others have observed a transient increase of in vitro IFN-γ response to antigens of *M. tuberculosis* after skin testing (28). The cutoff for positivity for each stimulating antigen was conservatively defined as the highest value obtained from screening 12 controls with the assay and is represented by the *dashed line.*

![Figure 2. IFN-γ responses (pg/ml) in the IFN-γ release assay to the antigens ESAT-6, CyaA–CFP-10, and purified protein derivative (PPD) in school contacts of the index TB case. The negative cut-off value for each antigen was determined as the highest value obtained from screening 12 controls with the assay and is represented by the dashed line.](Image 327x337 to 542x738)
exposure with these reagents and found a similarly low level of reactivity (11).

Some previous studies comparing IFNGRA and the TST have tended to demonstrate a relationship between skin test and in vitro positivity (6, 15), whereas others note discrepancies (29). A major decrement in the sensitivity of the TST in use (Heaf Test) under the conditions of this study was apparent. This feature, together with the unusual and severe clinical manifestations in both the index and secondary case, predicated investigation of the infecting strain NPH4216. The possibility that the infecting strain of M. tuberculosis may influence the TST response was first illustrated by the clinical isolate CDC1551 (16). Because CDC1551 induced vigorous TST reactions despite occasionally minor exposure, it was initially considered to be a virulent strain. Further analysis revealed CDC1551 to be of no greater virulence in animals than other strains in terms of its growth in vitro and in vivo (30). However, it was found to induce a marked proinflammatory cytokine response in mononuclear phagocytes: a mechanism proposed to underlie the marked TST responses that were observed (17). NPH4216 induced significantly less TNF-α and IL-12p40 than either CDC1551 or H37Rv. Both cytokines are implicated in the protective and proinflammatory response to M. tuberculosis. This cytokine phenotype resembles that induced by strain HN878, which is reported to be highly virulent in animal models (31). A more in-depth analysis of the immunologic response to NPH4216 might better delineate any propensity it has to subvert the innate immune response.

Although the occurrence of TB in siblings raises the possibility of Mendelian susceptibility to mycobacterial disease (32, 33), both the index case and her brother were otherwise healthy, thriving, and immune-competent individuals with no family history of consanguinity. Of the documented cases of single-gene defects accounting for Mendelian susceptibility to mycobacterial disease, the majority of individuals have presented with either disseminated BCG disease after vaccination, become infected with poorly pathogenic environmental mycobacteria (e.g. Salmonella sepsis), or, on rare occasions, have presented in childhood with M. tuberculosis disease (34).

Our findings illustrate the well-known phenomenon that M. tuberculosis may infect and cause progressive disease that is not detected by skin testing. Our study further suggests that transmission to healthy people in the absence of skin-test conversion can occur and that this may relate to the differing capacity of strains to elicit delayed-type hypersensitivity reactions. If such a phenomenon is widespread, it is a cause for some concern, as the true global burden of TB, as estimated by TST, is likely to be an underestimate.

**Conflict of Interest Statement**: S.T.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.J.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.R.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.M.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.S. is a co-inventor of a patent on the use of CyaA for diagnostic purposes for detection of antigen-specific T cells in infected subjects. M.P.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.S. is a co-inventor of a patent on the use of CyaA for diagnostic purposes for detection of antigen-specific T cells in infected subjects. M.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.J.W. is a co-applicant on two patents filed in the European Union and United States relating to the in vitro diagnostic use of CyaA-CFP-10. K.A.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

**Acknowledgment**: The authors thank Marion Davis and members of the TB nursing team at Northwick Park Hospital for their help with patient recruitment, Dr. Rob Davidson for referring the patient, and Dr. Robert Wall for providing us with the isolate NPH4216.

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