Rapid Detection of *Mycobacterium tuberculosis* Infection by Enumeration of Antigen-specific T Cells

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There is no reliable means of detecting latent *M. tuberculosis* infection, and even in patients with active tuberculosis, infection is often unconfirmed. We hypothesized that *M. tuberculosis* antigen-specific T cells might reliably indicate infection. We enumerated peripheral blood-derived interferon γ (IFN-γ)-secreting T cells responding to epitopes from ESAT-6, an antigen that is highly specific for *M. tuberculosis* complex but absent from BCG, in four groups of individuals. Forty-five of 47 patients with bacteriologically confirmed tuberculosis had ESAT-6-specific IFN-γ-secreting T cells, compared with four of 47 patients with nontuberculous illnesses, indicating that these T cells are an accurate marker of *M. tuberculosis* infection. This assay thus has a sensitivity of 96% (95% confidence interval [CI] 92–100) for detecting *M. tuberculosis* infection in this patient population. By comparison, of the 26 patients with tuberculosis who had a diagnostic tuberculin skin test (TST), only 18 (69%) were positive (p = 0.003). In addition, 22 of 26 (85%) TST-positive exposed household contacts had ESAT-6-specific T cells, whereas zero of 26 unexposed BCG-vaccinated subjects responded. This approach enables rapid detection of *M. tuberculosis* infection in patients with active tuberculosis and in exposed asymptomatic individuals at high risk of latent infection; it also successfully distinguishes between *M. tuberculosis* infection and BCG vaccination. This capability may facilitate tuberculosis control in nonendemic regions.

There are approximately 8 million new cases of tuberculosis worldwide every year, and it is estimated that one-third of the world's population is latently infected with *Mycobacterium tuberculosis* (1, 2). The ability to accurately detect *M. tuberculosis* infection will be crucial for global control of this epidemic (3) and for improved contact tracing and outbreak control, yet there is no accurate and reliable means of detecting infection. A rapid means of detecting *M. tuberculosis* infection would also help to accelerate initial diagnosis and allow early treatment in patients with clinically suspected tuberculosis.

Mycobacterium tuberculosis is often difficult to recover from infected subjects; even with good microbiological facilities, as in the United Kingdom, only 50% of tuberculosis cases are bacteriologically confirmed (4, 5). However, *M. tuberculosis* infection evokes a strong cell-mediated immune response, and we reasoned that detection of *M. tuberculosis*-specific T cells might reliably signal the presence of infection. This approach has been hampered by the lack of a defined antigen that is specific for *M. tuberculosis.* We therefore selected ESAT-6, a secreted antigen (6), which is expressed in *M. tuberculosis* complex (*M. tuberculosis*, *bovis*, and *africanum*), but is absent from all strains of *M. bovis* BCG (7–9) and the majority of environmental mycobacteria (8, 10). In animal models of tuberculosis, ESAT-6 is a target of interferon γ-secreting $CD4^+$ T cells (11–13) and *in vitro* stimulation of human peripheral blood mononuclear cells (PBMCs) by ESAT-6 induces interferon γ (IFN-γ) detectable by enzyme-linked immunosorbent assay (ELISA) in the supernatant from about half of tuberculosis patients (14–16). This T cell immunogenicity, together with its species specificity, make ESAT-6 a promising candidate antigen.

We hypothesised that circulating ESAT-6-specific T cells would indicate *M. tuberculosis* infection. We therefore employed an assay that enumerates IFN-γ-secreting T cells directly from peripheral blood, the *ex vivo* enzyme-linked immunospot (ELISPOT) assay: one of the most sensitive methods for detecting antigen-specific T cells (17–19). First, we studied subjects known definitively to be overtly infected with *M. tuberculosis*; that is, patients with bacteriologically confirmed active disease. To be clinically useful, an assay for *M. tuberculosis* infection should not cross-react with BCG vaccination or nontuberculous conditions that cause nonspecific activation of the cellular immune system. We therefore included a series of control patients with a range of infectious, inflammatory, and granulomatous conditions, the majority of whom were BCG vaccinated. Finally, to assess whether this approach could identify exposed latently infected individuals and not just patients with active disease, we also studied a series of tuberculin skin test (TST) positive healthy household contacts (HHCs) of cases of sputum smear-positive pulmonary tuberculosis and a series of BCG-vaccinated healthy individuals with no known exposure to *M. tuberculosis.*

METHODS

Patients

All participants were recruited prospectively in London and Oxford over a 16-mo period from October 1997 through January 1999. A heparinized blood sample was drawn after obtaining informed consent. Ethical approval for the study was granted by the Harrow and Central Oxford Research Ethics Committees.

Forty-seven tuberculosis patients, bacteriologically confirmed with positive cultures for *M. tuberculosis* from one or more clinical specimens, were recruited prospectively. The patients were ethnically diverse and represented the broad clinical spectrum of tuberculosis, with 22 cases of extrapulmonary disease (Table 1). Twenty-nine of 47 (62%) had received less than 1 mo of therapy or were untreated at the time of venipuncture for ELISPOT assays; the remaining 18 patients were at later time points in their treatment course.

Control patients were group matched for ethnicity, age (within 4 yr) and sex and 36 of 47 (77%) were BCG vaccinated (33 had a BCG scar, and three gave a history of BCG vaccination) (Table 1). Control patients had the following diagnoses: pneumonia (n = 6), sarcoidosis (n = 3), infective endocarditis (n = 3). The following diagnoses were present in two control patients each: lymphoma, lung cancer, chronic osteomyelitis, ulcerative colitis, Crohn's disease, infective enterocolitis, malaria, cirrhosis, cellulitis, hemoglobinopathy. The following diagnoses were present in one control patient each: pulmonary *As-*

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TABLE 1. DEMOGRAPHIC CHARACTERISTICS OF PATIENTS WITH TUBERCULOSIS AND CONTROL PATIENTS WITH NONTUBERCULOUS ILLNESSES

	Patients with Tuberculosis* (%)	Control Patients with Nontuberculous Illnesses (%)
Ethnicity		
ISC†	24 (51)	24 (51)
African	14 (30)	14 (30)
White	8 (17)	9 (19)
Asian	1 (2)	0 (0)
Total	47	47
Sex M/F	30/17	27/20
Age, yr: mean (range)	35 (18–74)	39 (17–75)

* The patients with tuberculosis were representative of the broad clinical spectrum of tuberculosis: 25 patients had pulmonary tuberculosis, of whom 19 were sputum smear positive, and 22 patients had extrapulmonary tuberculosis. This group comprised patients with tuberculous lymphadenitis (n = 6), musculoskeletal tuberculosis (n = 6), miliary tuberculosis (n = 3), gastrointestinal tuberculosis (n = 3), pleural tuberculosis (n = 3), and one patient with miliary and meningeal tuberculosis.

† ISC = Indian subcontinent

caris lumbricoides infection, acute pancreatitis, Dengue fever, urinary schistosomiasis, systemic lupus erythematosus (SLE), acute bronchitis, meningococcemia, tonsillitis, sickle cell crisis, gastric ulcer, dermatitis herpetiformis, venous thrombosis, nephrotic syndrome, cardiac failure. Patients with a past history of tuberculosis or recent contact with a case (n = 2) were excluded. No tuberculosis or control patients had any features of human immunodeficiency virus (HIV) infection.

Twenty-six HHCs were recruited on the basis of the following criteria. All had lived in the same household as an index case of untreated sputum smear-positive pulmonary tuberculosis within the last 6 mo. In addition, all HHCs were asymptomatic, had normal chest X-rays (CXRs), and had a positive tuberculin skin test (Heaf grades 3–4). Over 12 mo of follow-up, no HHCs developed active tuberculosis. Twenty-six healthy unexposed control subjects with no history of tuberculosis and no known contact with a case of tuberculosis were also recruited. All had a BCG scar or history of BCG vaccination.

ESAT-6-derived Peptides

Seventeen peptides spanning the length of the ESAT-6 molecule were purchased (Research Genetics, Huntsville, AL). Each peptide was 15 amino acids long and overlapped its adjacent peptide by 10 residues. A response to one or more of the eight broadly immunogenic peptide epitopes in Table 2 was scored as indicative of *M. tuberculosis* infection.

TABLE 2. $CD4^+$ T CELL EPITOPES IN ESAT-6 THAT ARE BROADLY RECOGNIZED IN PATIENTS WITH ACTIVE TUBERCULOSIS AND ASYMPTOMATIC HEALTHY HOUSEHOLD CONTACTS*

Peptide	Position	Sequence	Percentage of Patients with TB (n = 47) and HHCs (n = 26) Responding to Individual Peptides
ES1	1–15	MTEQQWNFAGIEAAA	57
ES2	6–20	WNFAGIEAAASAIQG	40
ES4	16–30	SAIQGNVTSIHSLLD	21
ES7	31–45	EGKQSLTKLAAAWGG	15
ES11	51–65	YQGVQQKWDATATEL	27
ES12	56–70	QKWDATATELNNALQ	25
ES14	66–80	NNALQNLARTISEAG	26
ES15	71–85	NLARTISEAGQAMAS	36

Definition of abbreviation: HHC = healthy household contact.

* Sequence homology searches of the SwissProt and translated GenBank databases confirmed that the peptide sequences are uniquely restricted to the ESAT-6 protein of the *M. tuberculosis* complex. T cell lines were generated against each of these peptides *in vitro*, and peptide-specific responses were abrogated by immunomagnetic depletion of CD4 T cells, identifying these peptides as CD4 T cell epitopes. ESAT-6-specific CD8 T cells were also detected by certain peptides in the panel (e.g., ES14) that contain CD8 epitopes (20).

Ex Vivo ELISPOT Assay for Single-Cell IFN-γ Release: Rapid Enumeration of Circulating ESAT-6 Peptide-specific T Cells from Peripheral Blood

The procedure was carried out and scored as previously described (17, 20, 21). *See* online data supplement at www.atsjournals.org.

Tuberculin Skin Testing

Twenty-six patients with tuberculosis underwent tuberculin skin testing, while being investigated for tuberculosis, with 1 tuberculin unit of PPD (Evans Medical, Liverpool, UK), in accordance with standard UK practice. Cutaneous induration of 5 mm or more at 72 h, measured with a ruler, was scored as positive, as per convention.

All 26 HHCs underwent standardized multiple puncture tuberculin skin testing (Heaf test) with a six-needle disposable head Heaf gun (Bignall 2000; Bignall Surgical Instruments, UK) and concentrated PPD (100,000 tuberculin units/ml; Evans Medical, Liverpool, UK) in accordance with UK guidelines for contacts.

RESULTS

Rapid Diagnosis of *Mycobacterium tuberculosis* Infection in Patients with Active Tuberculosis by Detection of ESAT-6-specific T Cells in Peripheral Blood

Eight ESAT-6-derived peptides were frequently recognized epitopes (Table 2). A response to one or more of the peptides in this panel was scored as indicative of *M. tuberculosis* infection, and 45 of 47 (96%) patients with tuberculosis had circulating IFN-γ-secreting T cells specific for one or more of these eight peptides, with many donors recognizing several peptides. Thus, the sensitivity of the assay in this patient population was 96% (95% confidence interval [CI], 92–100). Frequencies of ESAT-6 peptide-specific IFN-γ-secreting T cells were generally high, with a median of 200 ESAT-6-specific T cells per million PBMCs (interquartile range, 105–596) (Figure 1A). Of the 17 tuberculosis patients tested against recombinant ESAT-6 in addition to the ESAT-6 peptides, all responded to the whole antigen, indicating the peptide epitopes are naturally processed and presented to T cells. For the most broadly recognized peptides (ES1, ES2, ES11 and ES15), we used anti-HLA-DR, -DQ, and -DP monoclonal antibodies to block presentation to $CD4^+$ T cells in ELISPOT assays. In all patients with tuberculosis and contacts where this was tested (n = 14), the responses were MHC class II restricted (data not shown).

Although 36 of 47 (77%) control patients with nontuberculous illnesses were BCG vaccinated, only four of 47 (8%) responded to one or more of the panel of eight ESAT-6-derived peptides (and only one of these was BCG vaccinated), which gives the assay a specificity of 92% (95% CI, 86–97) in this population. Two had acute pneumonia, one had acute bronchitis and the fourth had cellulitis. All four also responded to PPD by *ex vivo* ELISPOT. In all remaining controls, there was a complete lack of response to all peptides (Figure 1A). The expanded set of 17 peptides spanning ESAT-6 gave the same results as the panel of eight broadly immunogenic epitopes.

The two of 47 tuberculosis patients who did not respond to ESAT-6 had advanced pulmonary disease and, interestingly, were lymphopenic, but both were HIV negative and responded to therapy. Advanced tuberculosis causes depression of peripheral blood T cell responses, which might account for the lack of detectable ESAT-6-specific IFN-γ-secreting T cells in these patients, but all four patients with miliary disease, often associated with cutaneous anergy, nonetheless had ESAT-6-specific T cells by *ex vivo* ELISPOT.

In contrast to other assays of the cellular immune response in tuberculosis (22, 23), the strength of the ESAT-6-specific *ex vivo* ELISPOT response did not rise with increasing duration

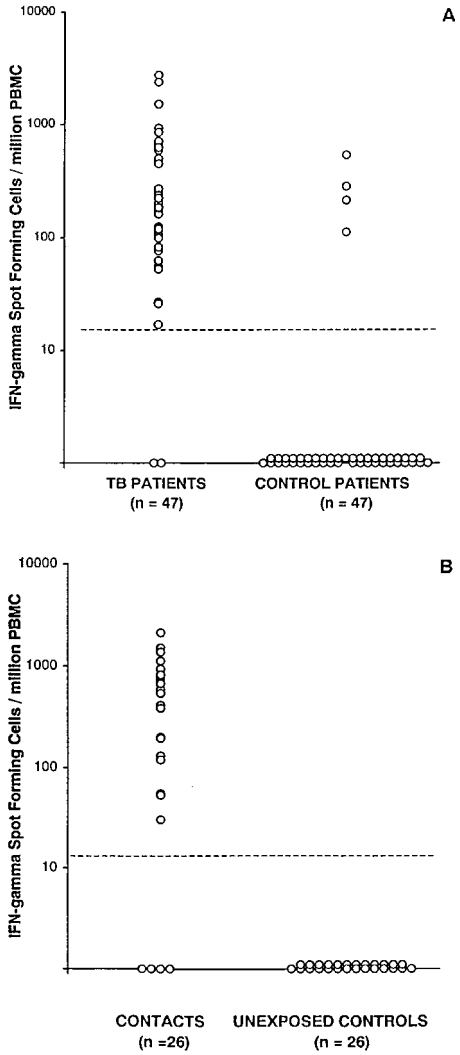

Figure 1. Distribution of frequencies of ESAT-6-specific IFN-γ-secreting T cells in all subjects. (*A*) Frequencies of ESAT-6-specific IFN-γ-secreting T cells for all 47 patients with tuberculosis (TB patients) and 47 control patients (77% of whom are BCG vaccinated). (*B*) Frequencies of ESAT-6-specific IFN-γ-secreting T cells for all 26 tuberculin skin test-positive healthy household contacts and 26 BCG-vaccinated unexposed controls. For (*A*) and (*B*), each circle represents an individual subject; the frequency of IFN-γ-secreting T cells to each peptide (Table 2) was summated to give the total number of ESAT-6 peptide-specific T cells. Circles on the baseline represent individuals with no response to any of the ESAT-6 peptides. The broken horizontal line represents the predefined cutoff point (5 IFN-γ SFCs per 3×10^5 PBMCs, which translates into a lower threshold of detection of 17 peptide-specific T cells per million PBMCs).

of therapy. Rather, the frequency of ESAT-6 peptide-specific T cells actually declined progressively with treatment in patients who were followed up longitudinally (A. A. Pathan and A. Lalvani, unpublished data, 2000). Thus, one would not expect the sensitivity of the ESAT-6-based *ex vivo* ELISPOT assay for detecting *M. tuberculosis* infection to be lower in patients who are untreated or early in the course of therapy.

Comparison of ESAT-6-specific T Cell Responses with Skin Test and ELISPOT Responses to Purified Protein Derivative

Twenty-six patients with tuberculosis underwent tuberculin skin testing (TST); only 18 of these (69%) were positive. By comparison, one-third more, 24 of 26 (92%), were positive to ESAT-6 by *ex vivo* ELISPOT for IFN-γ and, overall, 45 of 47 (96%) responded to ESAT-6 by *ex vivo* ELISPOT ($p = 0.003$, Fisher exact test). Although the control patients were not subjected to TST, 26 of 47 (55%) responded to PPD by *ex vivo* IFN-γ ELISPOT, indicating prior *in vivo* sensitization to PPD, probably as a result of BCG vaccination. All 47 tuberculosis patients responded to PPD by *ex vivo* IFN-γ ELISPOT.

Rapid Detection of Latent *Mycobacterium tuberculosis* Infection in Asymptomatic Healthy Subjects

Twenty-two of 26 tuberculin skin test-positive HHCs had IFN-γ-secreting T cells specific for one or more of the eight ESAT-6 peptides (Figure 1B). By contrast, none of 26 healthy control subjects with no history of exposure to tuberculosis responded to ESAT-6 peptides in the *ex vivo* ELISPOT assay (Figure 1B). All unexposed control subjects were BCG vaccinated, indicating that the ESAT-6-based ELISPOT assay for IFN-γ successfully distinguishes *M. tuberculosis*-exposed contacts from unexposed, but BCG-vaccinated, individuals. By contrast, 11 of 14 unexposed control subjects, and all the household contacts, responded to PPD in the IFN-γ ELISPOT assay. The median number of ESAT-6 peptide-specific IFN-γ-secreting T cells in HHCs was 338 per million PBMCs (interquartile range, 63–634).

DISCUSSION

We report a novel approach to accurately and rapidly detect infection with *M. tuberculosis.* By studying a series of patients, contacts, and control subjects with a sensitive method for detecting antigen-specific T cells, combined with an immunogenic antigen that is highly specific for *M. tuberculosis*, we have identified ESAT-6-specific IFN-γ-secreting T cells as an accurate marker for *M. tuberculosis* infection.

The high frequency of T cells specific for this single 6-kD antigen, given that almost 4,000 open reading frames are encoded in the *M. tuberculosis* genome (24), distinguishes ESAT-6 as a major target of the human immune response to *M. tuberculosis.* The genetic diversity of the study population indicates, moreover, that *M. tuberculosis* infection induces ESAT-6-specific $CD4^+$ T cells in the context of a wide variety of different HLA class II haplotypes. Interestingly, a single peptide, ES1, is a target of IFN-γ-secreting $CD4^+$ T cells in more than half the patients with tuberculosis and healthy contacts.

Forty-five of 47 (96%) patients with active tuberculosis had IFN-γ-secreting ESAT-6-specific T cells, indicating that this assay identifies overtly infected individuals with active tuberculosis with a sensitivity of 96% (95% CI, 92–100). This high sensitivity was achieved in a patient group that included critically ill patients with disseminated disease and patients with extrapulmonary tuberculosis, who can be difficult to diagnose by conventional means. The absence of ESAT-6-specific T cells in 43 of 47 (92%) control patients indicates that these cells are highly specific for *M. tuberculosis* infection. The high specificity of the assay, despite the fact that 77% of control patients were BCG vaccinated, makes it possible to distinguish successfully between BCG-vaccinated and *M. tuberculosis*-infected patients. Furthermore, the presence in the control patients of a variety of infectious, inflammatory, and granulomatous diseases indicates that the assay is not confounded by nonspecific activation of the cellular immune system, which often accompanies conditions that clinically mimic tuberculosis.

The current method for determining infection status, the TST, measures a delayed-type hypersensitivity response to intradermal inoculation of PPD, a crude precipitate of *M. tuber-*

culosis culture supernatant containing more than 200 proteins widely shared among mycobacteria other than *M. tuberculosis*, including *M. bovis* BCG and many environmental mycobacteria. This antigenic cross-reactivity accounts for the poor specificity of the TST (25–28). Sensitivity is also low at 75–90% (29, 30) and falls to less than 50% in critically ill patients with disseminated tuberculosis (29). Moreover, administration and reading of the TST are both highly operator dependent and patients must make a return visit. The sensitivity of the TST among the 26 tuberculosis patients tested by this method was 69%, significantly less than the 96% sensitivity of the ESAT-6-based *ex vivo* ELISPOT ($p = 0.003$), suggesting that the latter may be a superior means for the rapid detection of *M. tuberculosis* infection.

In this series of patients, sputum microscopy would have detected only 76% (19 of 25) of the pulmonary tuberculosis cases, compared with 92% (23 of 25) for the *ex vivo* ELISPOT, which also detected all six cases of sputum smear-negative pulmonary tuberculosis. Furthermore, sputum microscopy cannot easily differentiate between *M. tuberculosis* and atypical mycobacteria. Because the *esat-6* gene is restricted to *M. tuberculosis* complex, *M. kansasii*, *marinum*, *flavescens* and *szulgai* (8) (of these only *M. kansasii* can cause disease clinically similar to tuberculosis), this ESAT-6-based test may prove to be more specific, as well as more sensitive, than sputum microscopy.

Interestingly, the four of 47 control patients with nontuberculous illnesses who responded in the ESAT-6-based *ex vivo* ELISPOT assay had all recently arrived from tuberculosis endemic countries (Kenya and Ethiopia). In contrast, none of the 22 UK-born control patients gave a positive response. These four responders were thus at increased risk of exposure to *M. tuberculosis* and might have been latently infected.

To determine whether we could detect latent *M. tuberculosis* infection in asymptomatic subjects, we studied a series of 26 TST-positive HHCs; active tuberculosis was clinically and radiographically excluded, so *M. tuberculosis* infection in these individuals would, by definition, be latent rather than active. ESAT-6 peptide-specific IFN-γ-secreting T cells were detected in 22 of 26 (85%) contacts (Figure 1B). By contrast, none of the 26 BCG-vaccinated healthy unexposed subjects had ESAT-6 peptide-specific IFN-γ-secreting T cells (Figure 1B), indicating that ESAT-6 peptide-specific T cells represent an accurate marker of individuals at high risk of latent *M. tuberculosis* infection that is not confounded by BCG vaccination. The four TST-positive contacts who were negative by *ex vivo* ELISPOT may not have been infected with *M. tuberculosis*; their positive TSTs may have resulted from prior BCG vaccination. This possibility cannot be formally tested as there is no other means of definitively confirming or excluding latent *M. tuberculosis* infection in asymptomatic exposed contacts.

In conclusion, we have developed a T cell-based assay that accurately detects *M. tuberculosis* infection in TST-positive healthy exposed contacts, as well as patients with active tuberculosis and that successfully distinguishes *M. tuberculosis* infection from BCG vaccination. Clearly, the usefulness of the assay will depend on the prevalence of latent *M. tuberculosis* infection in a given population. In nonendemic regions, this approach could help to improve contact tracing, outbreak control, and targeting of chemoprophylaxis. In addition, for individual patients with clinically suspected tuberculosis in low-prevalence populations, the ESAT-6-based *ex vivo* ELISPOT assay holds promise as a useful adjunct for the rapid presumptive diagnosis of tuberculosis allowing early initiation of therapy. However, before entering widespread clinical use, the assay will need to be further validated in a double-blind study in which a larger number of patients with clinically suspected tuberculosis is prospectively evaluated, and the results of the assay compared against the final clinical and bacteriological diagnoses; this would also generate positive and negative predictive values pertinent to the participating population. In tuberculosis-endemic regions, the high proportion of latently infected people would preclude application of this assay for the presumptive diagnosis of active tuberculosis, but it could nonetheless facilitate epidemiological studies investigating the basic parameters and pathways of *M. tuberculosis* transmission and might help to evaluate the impact of tuberculosis control measures (31). The assay generates results in less than 24 h, requires no specialized laboratory facilities or radioisotopes, and is amenable to automation; it is thus potentially well suited to routine diagnostic hospital laboratories. Appropriate application of this novel T cell-based approach could potentially make a significant contribution to tuberculosis control.

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