Detection of Active Tuberculosis Infection by T Cell Responses to Early-Secreted Antigenic Target 6-kDa Protein and Culture Filtrate Protein 10

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Tuberculosis (TB) remains a major global public health problem. Timely diagnosis of active pulmonary cases is important for TB control, yet isolation of Mycobacterium tuberculosis (MTB) can take up to 6 weeks, and cultures may remain falsely negative. The sensitivity of the purified protein derivative (PPD) skin test for detection of active TB does not exceed 75%, and negative results are often associated with extensive disease [1]. In individuals vaccinated with Mycobacterium bovis bacillus Calmette-Guérin (BCG) or exposed to environmental mycobacteria, PPD skin testing is unreliable because of cross-reactive responses to nonspecific constituents of PPD. T cell responses to early-secreted antigenic target 6-kDa protein (ESAT-6) and the newly identified culture filtrate protein 10 (CFP-10), 2 proteins specifically expressed by M. tuberculosis (MTB) but not by BCG strains, were evaluated. Most TB patients responded to ESAT-6 (92%) or CFP-10 (89%). A minority of BCG-vaccinated individuals responded to both ESAT-6 and CFP-10, their history being consistent with latent infection with MTB in the presence of protective immunity. No responses were found in PPD-negative controls. The sensitivity and specificity of the assay were 84% and 100%, respectively, at a cutoff of 300 pg of interferon-γ/mL. These data indicate that ESAT-6 and CFP-10 are promising antigens for highly specific immunodiagnosis of TB, even in BCG-vaccinated individuals.

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H37Rv and BCG [3] have identified 1 region of difference, designated RD1, that was found to be present in all MTB and pathogenic M. bovis strains but lacking in all BCG vaccine strains and almost all environmental mycobacteria. One antigen encoded by RD1 is the early-secreted antigenic target 6-kDa protein (ESAT-6) [4, 5], which is immunodominant in mice [6]. T cell responses to ESAT-6 can discriminate between cattle infected with M. bovis and cattle sensitized to environmental mycobacteria [7]. In previous studies of human pulmonary TB, T cell responses to ESAT-6 were observed in approximately half of the patients [8–10]. Recently, a second RD1-encoded protein, designated culture filtrate protein 10 (CFP-10), was identified [11]. Recent results indicate that this antigen is strongly recognized by T cells [12, 13]. In the present study, we evaluated T cell responses to ESAT-6 and CFP-10 in patients with TB in comparison with various control groups without TB, including BCG-vaccinated individuals.

Subjects and Methods

Subjects. Thirty-seven TB patients aged 45.9 ± 16.1 years (mean ± SD; range, 12–70 years) were recruited at the Leiden University Medical Center in Leiden or at the Rijnland Hospital in Leiderdorp, The Netherlands. The patients originated from Western Europe (n = 20), Africa (n = 8), Asia (n = 4), South America (n = 4), or the Middle East (n = 1). The localization of TB was pulmonary (n = 20), pleural (n = 6), lymphatic (n = 5), skeletal (n = 5), urogenital (n = 4), peritoneal (n = 3), adrenal (n = 1), cerebral (n = 1), cutaneous (n = 1), or subcutaneous (n = 1). TB was...
present at >1 site in 9 patients. We included patients before treat-
m ent was started, as well as patients during and after treatment, to
evaluate the time course of immune responses. Patients infected
with human immunodeficiency virus were excluded because cell-
mediated immunity to mycobacteria has been shown to be defective
in the presence of low CD4+ cell counts.

Twelve individuals with documented PPD conversion after con-
tact with contagious TB, 14 BCG-vaccinated subjects (10 of whom
were PPD positive), and 8 healthy, PPD-negative, non–BCG-vac-
cinated subjects, including medical, nursing, and technical hospital
personnel and relatives of TB patients, were recruited from various
sources.

Antigens. Recombinant ESAT-6 (batch p432) and CFP-10
(batches 98-2 and 99-1) were expressed in Escherichia coli, as
described elsewhere [4, 5, 11]. MTB H37Rv sonicate was provided by
Dr. D. van Soolingen (National Institute of Public Health and
Environment, Bilthoven, The Netherlands). The production of
short-term culture filtrate (ST-CF) has been described elsewhere
[14].

Cellular stimulation assays. Peripheral blood mononuclear
cells (PBMC) were isolated from heparinized venous blood by Fi-
coll-Hypaque density gradient centrifugation. Cells were frozen in
RPMI 1640 (Gibco, Paisley, Scotland) supplemented with 0.04
mM/mL glutamine, 20% fetal calf serum, and 10% dimethyl sul-
foxide. For the experiments, PBMC (1.5 × 10^6 per well) were in-
cubated in round-bottom microtiter wells in the presence or absence
of antigen in 200 μL of Iscoves modified Dulbecco’s medium
(Gibco), supplemented with 10% pooled human AB serum, 40 U/
ml penicillin, and 40 μg/mL streptomycin in triplicate at 37°C in
humidified air containing 5% CO2. The final concentrations of the
antigens used were as follows: ESAT-6, 1 and 10 μg/mL; CFP-10,
0.5 and 5 μg/mL; MTB sonicate and ST-CF, 0.1 and 1 μg/mL each.
Supernatants for interferon-γ (IFN-γ) determinations, as the read-
out of T cell activation, were collected at days 3 and 6 (50 μL/well)
and pooled per triplicate. Responses at day 6 were optimal and
were used for the analysis.

IFN-γ production. IFN-γ was measured with a standard
ELISA technique (U-CyTech, Utrecht, The Netherlands). The de-
tection limit of the assay was 20 pg of IFN-γ/mL. IFN-γ values in
unstimulated cultures were typically undetectable, except in 15
(11%) of 136 unstimulated triplicates with a median concentration
of 62 pg/mL. Detectable values were subtracted from the value in
stimulated cultures.

Statistical analysis. Differences between responses were tested
with the nonparametric Kruskal-Wallis and Mann-Whitney tests.
Correlation of individual responses to different antigens was an-
alyzed by Spearman correlation. Receiver-operator characteristic
ROC curves were constructed to describe the relation between the
sensitivity and specificity at varying cutoff levels. All statistical
analyses were 2 sided, and P values <.05 were considered statistically
significant.

Results

Responses to MTB sonicate and ST-CF. T cell responses to
MTB sonicate in TB patients (figure 1A) were significantly higher than those in PPD-negative controls without BCG vac-
cination but were not different from those in BCG-vaccinated or
PPD-converted individuals. T cell responses to ST-CF of
TB patients (figure 1B) were significantly higher than the re-
sponses of the other groups.

TB patients and control groups respond differently to ESAT-
6 and CFP-10. Responses to ESAT-6 (figure 1C) and CFP-
10 (figure 1D) differed highly significantly between the study
groups (P < .0001 for comparison of all groups). Individual
responses to ESAT-6 and CFP-10 were highly correlated (r =
.79; 95% confidence interval, 0.67–0.86; P < .0001 [data not
shown]). Most patients given a diagnosis of TB produced IFN-
γ in response to ESAT-6 (34/37 [92%]), CFP10 (33/37 [89%]),
or both (32/37 [86%]), and 35/37 (95%) responded to >1 of
both antigens. Responses to ESAT-6 and CFP-10 were inde-
pendent of age and sex and were similar in patients with pul-
monary or extrapulmonary TB and in those with TB at ≥1 site.
The geometric mean ± SD of IFN-γ responses was lower at the
time of diagnosis than during or after treatment (639 ± 2550 vs.
1228 ± 4407 pg/mL for ESAT-6, P = .26; 326 ± 938 vs. 990 ± 6104 pg/mL for CFP-10, P = .04), but the range of responses was similar.

Nine of 12 individuals with documented PPD conversion
after contact with contagious TB responded to ESAT-6; 4 of
those 9 individuals also responded to CFP-10. Five (36%) of
14 BCG-vaccinated persons responded to ESAT-6, and 5 (36%)
of 14 responded to CFP10, with concordant responses in 4, all
of whom were PPD positive and had a history compatible with
past exposure to MTB. None of them had ever had clinical signs suggestive of TB. None of the BCG-vaccinated individuals
who were nonresponsive to ESAT-6 and CFP-10 had recog-
nized exposure to MTB, but all had been vaccinated with BCG
because they were in high-risk jobs. None of the PPD-negative, non–BCG-vaccinated controls responded to ESAT-6 or CFP-
10.

Cutoff level to define a positive test result. ROC curves were
constructed from the responses to MTB sonicate (figure 2A),
ST-CF (figure 2B), and the individual maximum of responses
to ESAT-6 and CFP-10 (figure 2C), comparing all 37 TB pa-
patients with BCG-vaccinated and PPD-negative, non–BCG-vac-
cinated controls, taken together as a group without TB. At a
cutoff level of 300 pg/mL, the sensitivity of the individual maxi-
mean of the responses to ESAT-6 and CFP-10 was 84%, and
the specificity was 100%. ROC analysis limited to TB patients
who were tested before anti-TB treatment was initiated (n =
13) resulted in similar ROC curves as those based on all 37 TB
patients (figure 2D through 2F), indicating that the postinfec-
tion interval was not a major determinant of the overall test
performance.

Discussion

In the present study, T cell responses to ESAT-6 and the
newly identified RD1-encoded antigen CFP-10 are shown to
Figure 1. T cell responses of patients with tuberculosis (TB; □) to *Mycobacterium tuberculosis* (MTB) sonicate (A) were significantly higher than those of controls negative for purified protein derivative (PPD) and without *M. bovis* bacillus Calmette-Guehrin (BCG) vaccination (○; P = .0005) but not different from those of BCG-vaccinated (△) or PPD-converted (▲) individuals. T cell responses of TB patients to short-term culture filtrate (ST-CF) (B) were significantly higher than those of each of the other groups (P = .0002, P = .01, and P = .007 for the comparisons with PPD-negative, BCG-nonvaccinated; BCG-vaccinated; and PPD-converted subjects, respectively). Interferon-γ (IFN-γ) production in response to early-secreted antigenic target 6-kDa protein (ESAT-6) (C) and culture filtrate protein-10 (CFP-10) (D) was significantly higher in TB patients than in BCG-vaccinated and PPD-negative, non-BCG-vaccinated controls (P < .0001 for all comparisons). Comparison of TB patients and individuals with documented PPD conversion after exposure to MTB demonstrated significant differences in responses to CFP-10 (P = .02) and differences of borderline significance in responses to ESAT-6 (P = .07). Statistical comparison between groups was performed using the Mann-Whitney U test. Short lines represent geometric mean responses for the groups. Dotted lines represent detection limit of the IFN-γ ELISA (20 pg/mL).

be highly sensitive and specific for discrimination between patients with TB disease and noninfected individuals and to be significantly more specific than responses to the complex antigens MTB sonicate and ST-CF. Most patients with active or treated TB in our study responded to the specific antigens ESAT-6 or CFP-10 or both. The higher sensitivity of responses to ESAT-6 than that found in previous studies [8–10] could be related to differences in test conditions. Our results strongly support larger prospective studies to establish the actual predictive value of T cell responses to ESAT-6 and CFP-10 in patients suspected to have TB disease, and we recently started such a study. Moreover, ongoing follow-up studies of healthy, untreated, PPD-positive TB contacts indicate that responses to ESAT-6 are associated with the risk of developing active TB (authors’ unpublished observations). Factors contributing to the observed variability of responses to ESAT-6 and CFP10 in the TB patients could include mycobacterial load, antigen expression level of MTB strains, and genetically based characteristics of antigen processing and immune responsiveness in the host, including cytokine secretion profiles or polymorphisms of HLA or cytokine receptors.

The majority of the PPD-converted individuals and several of the BCG-vaccinated subjects in our study responded to ESAT-6, and some also responded to CFP-10, presumably as a result of latent infection with MTB. Latent infection with MTB is 10 times more frequent than active TB, with global prevalence rates estimated to amount to one-third of the world’s population. Subclinical or latent infection with MTB was the presumed cause of T cell responses to ESAT-6 observed in many Ethiopian control subjects in a previous study [8]. Today, half
Figure 2. Receiver-operator characteristic (ROC) curves were constructed from responses to *Mycobacterium tuberculosis* (MTB) sonicate (A), short-term culture filtrate (ST-CF) (B), and the individual maxima of responses to early-secreted antigenic target 6-kDa protein (ESAT-6) and culture filtrate protein–10 (CFP-10) (C) in 37 tuberculosis (TB) patients and in 22 control subjects (14 *M. bovis* bacillus Calmette-Guérin [BCG]-vaccinated and 8 PPD-negative, non-BCG-vaccinated individuals). Several potential cutoff levels are indicated between brackets. Including only those TB patients from whom samples were taken before anti-TB chemotherapy had been instituted (D–F) yielded ROC curves (D–F) similar to those including all 37 patients. The ROC curves end at a cutoff value equal to the detection limit of the ELISA (20 pg/mL), because interferon-γ production was below the detection limit in a number of subjects.

of all TB patients in Western countries originate from areas with highly endemic TB where BCG vaccination is routine practice. It must therefore be expected that latent infection with MTB, accompanied by T cell responses to MTB-specific antigens, can occur in relevant control groups.

The PPD skin test is technically simple, can be produced at low cost, and is more or less reproducible if performed by skilled personnel [1]. Yet the PPD skin test lacks specificity in BCG-vaccinated individuals, as was confirmed in the present study, in which responses to MTB sonicate were similar and those to ST-CF were only moderately different in TB patients and BCG-vaccinated individuals. Skin tests with PPDs derived from nontuberculous mycobacteria were of variable use for discrimination between TB and infection with atypical mycobacteria [15]. The requirements for an in vitro T cell assay are more complex and include highly purified MTB-specific antigens and facilities for cell-culture and cytokine assays, limiting its use as a routine diagnostic tool. However, antigens that appear specific in an in vitro T cell assay could be evaluated in a skin test. This preferably would be a test based on a combination of several specific antigens; in animal models of TB, the sensitivity of a skin test based on recombinant antigens was reported to increase with the number of antigens [16].

The functions of ESAT-6 and CFP-10 remain to be elucidated, but conceivably they contribute to virulence, because RD1 was deleted early during the process of attenuation of pathogenic *M. bovis* to essentially avirulent BCG [3]. Immunization of mice with DNA encoding for ESAT-6 conferred moderate protection against airborne experimental TB, although substantially less than that achieved by BCG vaccination [17]. ESAT-6 and CFP-10 probably have related functions, because both genes are part of the same operon, and
transcription is regulated under the same promoter gene [11]. An indirect indication of coexpression of ESAT-6 and CFP-10 is our observation that individual responses to ESAT-6 and CFP-10 were highly correlated (P < .0001).

In conclusion, in vitro T cell responses to ESAT-6 and CFP-10 appear to be highly sensitive and specific for detection of infection with MTB and to be significantly more discriminative than responses to the complex antigens, especially in BCG-vaccinated individuals. ESAT-6 and CFP-10 are therefore interesting and highly promising antigens to include in future studies aimed at specific immunodiagnosis of TB.

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