Prospective Evaluation of a Whole-Blood Test Using Mycobacterium tuberculosis-Specific Antigens ESAT-6 and CFP-10 for Diagnosis of Active Tuberculosis

Pernille Ravn,1* Martin E. Munk,2† Åse B. Andersen,3 Bettina Lundgren,4 Jens D. Lundgren,5 Lars N. Nielsen,1 Axel Kok-Jensen,6 Peter Andersen,2 and Karin Weldingh2

Department of Infectious Diseases, Hvidovre University Hospital, Kettegårds Alle 30, 2650 Hvidovre, Denmark;1 Department of Infectious Disease Immunology, Statens Serum Institute, Artillerivej 5, 2300 Copenhagen S, Denmark;2 Department of Infectious Diseases, Rigshospitalet, Blegdamsvej 3, 2000 Copenhagen Ø, Denmark;3 Department of Microbiology, Hvidovre Hospital, Kettegårds Alle 30, 2650 Hvidovre, Denmark;4 Copenhagen HIV Programme, Hvidovre University Hospital, Copenhagen, Denmark; and Department of Pulmonary Diseases, Gentofte Hospital, Niels Andersensvej, 2300 Gentofte, Denmark5

Received 26 October 2004/Returned for modification 7 January 2005/Accepted 22 February 2005

A new immunodiagnostic test based on the Mycobacterium tuberculosis-specific antigens CFP-10/ESAT-6 (QFT-RD1) has been launched as an aid in the diagnosis of latent tuberculosis (TB) infection (LTBI). The aim of this study was to evaluate this test for the diagnosis of active TB. Eighty-two patients with suspicion of TB and 39 healthy BCG-vaccinated persons were enrolled. Forty-eight had active TB, 25 did not, and 9 were excluded. Sensitivity and specificity of the test for active TB were evaluated in a prospective blinded manner in patients suspected of TB. The sensitivity of the QFT-RD1 was 85% (40/48; confidence interval [CI], 75 to 96), and it was higher than the sensitivity of microscopy, 42% (20/48; CI, 27 to 56; \( P = 0.001 \)), and culture, 59% (27/46; CI, 44 to 73; \( P = 0.009 \)). Of patients with extrapulmonary TB, 92% (12/13) were QFT-RD1 positive, whereas only 31% (4/13) were positive by microscopy and 42% (5/12) by culture (\( P < 0.05 \)), and 87% (13/15) of those who were negative by both microscopy and culture were QFT-RD1 positive. By combining microscopy and culture with the QFT-RD1 test, sensitivity increased to 96% (CI, 90 to 102). Ten of 25 (40%) non-TB patients were QFT-RD1 positive, resulting in a specificity of 60%. However, 80% (8/10) of these had risk-factors for TB, indicating latent infection in this group. In healthy controls, only 3% (1/39) were QFT-RD1 positive. In conclusion, the QFT-RD1 test is sensitive for diagnosis of TB, especially in patients with negative microscopy and culture. The accuracy of the QFT-RD1 test will vary with the prevalence of LTBI. We suggest that the QFT-RD1 test could be a very useful supplementary tool for the diagnosis of TB.

Received 26 October 2004/Returned for modification 7 January 2005/Accepted 22 February 2005

Tuberculosis (TB) remains an important and potentially fatal infection in humans, and it is estimated that one-third of the world population is infected with Mycobacterium tuberculosis (30). The most powerful tools in any TB control program are prompt diagnosis and successful treatment of patients with active contagious disease. Diagnosis of TB is often based on clinical suspicion and appropriate response to anti-TB therapy. While culture confirmation is optimal, this is frequently not possible. Detection of acid-fast bacilli or granulomatous lesions is not optimal means of diagnosing TB but are strongly indicative. The diagnosis of patients with microscopy- and culture-negative or extrapulmonary TB is complicated and is often delayed due to the need for invasive diagnostic procedures, and no definitive diagnostic test can exclude infection with M. tuberculosis. The tuberculin skin test (TST) has been used for almost a century to support the diagnosis of active and latent TB infection (LTBI) (1). The main drawback with the clinical use of the TST is the lack of specificity due to cross-reactivity with proteins present in other mycobacteria, such as the Mycobacterium bovis bacillus Calmette-Guérin (BCG) vaccine strain and Mycobacterium avium (1, 18). Identification and characterization of the two M. tuberculosis-specific antigens ESAT-6 and CFP-10 (4, 5, 26) has led to the development of new specific diagnostic tests for infection with M. tuberculosis (2). ESAT-6 and CFP-10 are contained within the RD1 region of the mycobacterial genome, which is absent from M. bovis BCG, M. avium, and most other nontuberculous mycobacteria (2, 12). A number of studies have demonstrated a high sensitivity and specificity when detecting gamma interferon (IFN-γ) responses to these specific antigens in patients with active TB (6, 17, 20, 21, 25, 28), nonexposed healthy people (3, 6, 7, 13, 17, 20, 21, 25, 28), and recently M. tuberculosis-infected healthy TST converters independently of their BCG vaccination status (3, 7, 10, 16, 27). A high percentage of healthy individuals in TB high-endemicity areas respond to the RD-1 antigens, indicating a high prevalence of LTBI (8, 15, 25, 29). Recently, a simple whole-blood test, QuantiFERON-TB (Cellestis Limited, Australia), for in vitro detection of M. tuberculosis infection in low-endemicity regions, has been approved by the Food and Drug Administration (19). The test is simple to perform, results can be obtained within 24 h, it takes only one patient visit, and it does not boost subsequent tests. The QuantiFERON-TB test, however, is based on purified protein deriv-
ative (PPD), and its specificity is impaired by prior BCG vaccination, just as is the case with the TST (6, 11, 13). The aim of the present study was to evaluate the \textit{M. tuberculosis}-specific RD1 antigens (ESAT-6 and CFP-10) using the QuantiFERON test system (QFT-RD1) for the diagnosis of active TB. The prospective nature of our study allowed a direct comparison between QFT-RD1 test and microscopy and culture.

(Part of this work was presented orally at the 14th European Congress of Clinical Microbiology and Infectious Diseases in Prague, 4 May 2004, abstr. 411.)

\textbf{MATERIALS AND METHODS}

\textbf{Antigens.} Recombinant CFP-10 and ESAT-6 were produced as previously described (5, 23). PPD and phytohemagglutinin (PHA) were provided by Cellestis.

\textbf{Whole-blood assay.} Peripheral blood was collected in tubes coated with lithium-heparin and kept at room temperature for a maximum of 12 h before assaying. Blood samples were collected before or within the first week of chemotherapy. Whole-blood stimulation was performed essentially according to the instructions by Cellestis, with the difference that the specific antigens ESAT-6 and CFP-10, produced at Statens Serum Institute, were used along with PPD. Five 1-ml aliquots of heparinized blood were incubated in 24-well culture plates (Nunc, Roskilde, Denmark) at 37°C in a humidified atmosphere for 24 h in the presence of 3 drops of saline (nil control), 5 \(\mu\)l PPD (mitogen control), 5 \(\mu\)g/\(\mu\)l PPD, 5 \(\mu\)g/\(\mu\)l ESAT-6, or 5 \(\mu\)g/ml CFP-10. Supernatants were harvested and immediately frozen at \(-20°C\) until further analysis. The amount of IFN-\(\gamma\) released was determined using a sensitive ELISA, and results were expressed as IU/ml, as determined from a standard curve run on each plate. IFN-\(\gamma\) produced in the saline control well was subtracted from the respective antigen-stimulated wells. The cutoff value for a positive response was set at 0.35 IU/ml for ESAT-6 and CFP-10 based on receiver-operator-characteristic curve analysis of 39 healthy nonexposed controls and 26 culture-confirmed TB patients. The cutoff value for a positive response to PPD or PHA was arbitrarily set at 1.5 U/ml, and patients with IFN-\(\gamma\) responses below 1.5 U/ml to both PPD and PHA were considered inconclusive on suspicion of anergy or failures during processing.

\textbf{Microscopy and culture.} Microscopy for acid-fast bacilli and culturing for mycobacteria were performed at the International Reference Laboratory of Mycobacteriology, Statens Serum Institut, Denmark, which is accredited according to the European standard EN45001.

The study was approved by the local ethical committee of Frederiksberg and Copenhagen KF 01-033/00. Written informed consent was always obtained.

\textbf{Statistical methods.} Median values and 25th to 75th percentiles are shown. Chi-square and McNemar tests were used to compare proportions using Med Calc and Sigma Stat version 1.01 software. \(P\) values of <0.05 were considered significant.

\textbf{RESULTS}

Baseline information for all 82 patients is shown in Table 1. None had inconclusive results due to no responses to both PPD and PHA and were excluded; thus, 73 TB suspect patients were evaluated. Active TB was diagnosed in 48 (66\%) subjects,

\begin{table}
\centering
\caption{Baseline data for the patients included in the study}
\begin{tabular}{lcccc}
\hline
Variable & Active TB & No TB & All evaluable patients & Inconclusive results\(^a\) & Healthy control \\
\hline
No. of patients & 48 & 25 & 73 & 9 & 39 \\
Age (median and 25th to 75th percentile) & 41 (34–51) & 47 (34–55) & 42 (34–53) & 38 (27–41) & 36.5 (29–45) \\
\hline
Sex [no. (%)] female & 16 (33\%) & 10 (40\%) & 26 (36\%) & 6 (67\%) & 32 (82\%) \\
No. [\(\%\)] HIV positive & 3 (6\%) & 4 (16\%) & 7 (10\%) & 3 (33\%) & 0 \\
Individual CD4 cell count (10\(^3\))/liter & 15, 100, 133 & 200, 500, 650, 730 & 28/44 (64\%) & 5, 44, 159 & ND\(^c\) \\
Tuberculin skin test results >12 mm, no./total screened [\(\%\)] & 20/24 (88\%) & 8/20 (40\%) & 2/2 (100\%) & ND & ND \\
Immigrant [no. \(\%\)] & 27 (56\%) & 9 (36\%) & 36 (49\%) & 2 (22\%) & 0 \\
IVDU [no. \(\%\)] & 2 (4\%) & 3 (12\%) & 5 (7\%) & 1 (11\%) & 0 \\
Alcohol/shelter [no. \(\%\)] & 8 (16\%) & 3 (12\%) & 11 (15\%) & 0 & 0 \\
Recent exposure [no. \(\%\)] & 5 (8\%) & 5 (20\%) & 10 (14\%) & 0 & 0 \\
Patients with one or more risk factors [no. \(\%\)] & 33 (69\%) & 17 (64\%) & 51 (68\%) & 3 (33\%) & 0 \\
\hline
\end{tabular}
\textsuperscript{a} Intrahepatic drug user.
\textsuperscript{b} Three were HIV seropositive, one was bone marrow suppressed following immunosuppressive treatment, one had severe advanced pulmonary TB, and one had disseminated TB.
\textsuperscript{c} ND, not done.
\end{table}
median age was 41 years (range, 34 to 51 years), 33% were female, and 56% had emigrated from TB high-endemicity regions into Denmark, 3 (6%) were human immunodeficiency virus (HIV) antibody positive, and 69% had one or more risk factors. Among the 25 patients without TB, the median age was 47 years (34 to 55), 40% were female, 36% were immigrants from TB high-endemicity regions, 4 (16%) were HIV antibody positive, and 64% had one or more risk factors. Of 48 patients with active TB, 33 (69%) were positive by either microscopy or culture. Of 20 microscopy-positive patients, 15 were positive by microscopy of acid-fast bacilli and 5 of granulomatous lesions. Five were positive by microscopy only, and 13 were positive by culture only. Twenty-five (34%) of the 73 TB suspect patients were classified as non-TB patients, and in 15 of these patients another diagnosis was obtained: bacterial infections responding to antibiotics (n = 10), leukemia (n = 1), vasculitis plus diabetes mellitus (n = 1), HIV positive with fever (n = 1), and M. avium infection (n = 2). No specific diagnosis was obtained despite thorough investigation in 10 patients with respiratory (n = 4) or gastrointestinal (n = 4) symptoms, fever and lymphadenitis (n = 1), or possible sarcoidosis (n = 1).

Recognition of the ESAT-6/CFP-10 antigens and PPD in vitro. Figure 1 shows the individual IFN-γ responses to PPD and either of the RD1 antigens ESAT-6 or CFP-10 (E6/C10) (for each patient the highest IFN-γ responses) are shown. In the left panel, the responses of the TB suspect population (n = 73); and in the right panel, the responses for the healthy control population (n = 39). The cutoff value for a positive response was set at 0.35 IU/ml for ESAT-6 and CFP-10 and arbitrarily at 1.5 IU/ml for PPD. The dotted lines represent the cutoff for the specific antigens and PPD.

The QFT-RD1 test identifies patients with active TB. The final results of the QFT-RD1 test and clinical and microbiological investigations are depicted in Fig. 2. The QFT-RD1 test was positive in 51 persons of whom 41 were diagnosed with active TB, and the QFT-RD1 test was negative in 22 persons of whom 7 had active TB. Sensitivity of the QFT-RD1 for the diagnosis of TB was 85% (41/48; CI, 75 to 96%). In the group of TB patients who were negative by both microscopy and culture, sensitivity was 87% (13/15) (data from Fig. 2), and in the patients with either culture-negative or microscopy-negative TB, the sensitivity of QFT-RD1 was 85% (17/20) and 89% (25/28), respectively (data not shown). Not all patients had a TST done, but where the TST was performed, we found that 8/20 (40%) non-TB patients and 20/24 (88%) TB patients had a positive TST (>12 mm).

The prospective design of this study allowed us to compare the sensitivity of the QFT-RD1 test with that of microscopy and culture, and we found that the sensitivity of the QFT-RD1 test for the diagnosis of TB of 85% (41/48; CI, 75 to 96%) was significantly higher than that of microscopy, 42% (20/48; CI, 27 to 56; P = 0.001, McNemar test), and culture, 59% (27/46; CI, 44 to 73%; P = 0.009) (Table 2). Pulmonary TB is often easily recognized by typical infiltrates on X-ray and a positive sputum smear, whereas the diagnosis of extrapulmonary TB and smear-negative TB is more difficult. In the present study, we found an equally high proportion of patients with pulmonary TB and extrapulmonary TB responding to QFT-RD1, 83% (29/35; CI, 70 to 96%) versus 12/13, 92% (CI, 78 to 107%), respectively (P > 0.05, χ² test) (Table 2). The sensitivity of the QFT-RD1 test was then compared with microscopy and culture results in patients with pulmonary and extrapulmonary TB. In patients with pulmonary TB, the QFT-RD1 test was more sensitive (83%; CI, 70 to 96%) than microscopy (46%; CI, 29 to 63; P = 0.009), but there was no difference between the sensitivity of the QFT-RD1 test and culture at 63% (CI, 47 to 79; P = 0.121). For extrapulmonary TB, the QFT-RD1 test was more sensitive (92%; CI, 78 to 107%) than both microscopy (31%; CI, 5 to 56; P = 0.013) and culture (42%; CI, 13 to 70; P = 0.041) (Table 2). The combined sensitivity of microscopy and/or culture was 69% (33/48; data are shown in Fig. 2), and by combining microscopy and culture with the QFT-RD1 test for the diagnosis of active TB, overall sensitivity increased to 96% (CI, 90 to 102%) (Table 2).

The potential influence of LTBI on specificity. The QFT-RD1 test has previously been shown to discriminate patients with active TB from healthy unexposed individuals, and in this
study, only 1 of the 39 healthy unexposed Danish controls recognized CFP-10 or ESAT-6 in the QFT-RD1 test, resulting in a specificity of 97% (CI, 94 to 108) (Fig. 1) in this group of healthy unexposed individuals. However, the primary aim of this study was to evaluate the performance of the QFT-RD1 test for the diagnosis of active TB in a clinical setting, and in this situation we found that 40% (10/25; CI, 20 to 60%) of the 25 non-TB patients were QFT-RD1 positive compared to 85% of the patients with active TB ($P < 0.01$). Thus, specificity for the diagnosis of active TB was calculated to be 60% (CI, 40 to 80). The data on TB risk factors for the QFT-RD1-positive non-TB patients were examined as part of the initial screening, and of the 10 QFT-RD1-positive non-TB patients, 8 (80%) were found to have TB risk factors suggesting possible LTBI.

Table 3 shows the individual risk factors for each of the QFT-RD1-positive non-TB patients. Only two patients had no known TB risk factors, one was diagnosed with acute myeloid leukemia, and one had granulomatous lesions in the liver interpreted as sarcoidosis, but self-limiting $M. tuberculosis$ infection could have been an alternative diagnosis.

Nine patients were excluded due to no response to PPD and PHA. One non-TB patient and two TB patients did respond to ESAT-6 or CFP-10. It could be claimed that exclusion of these patients might have resulted in altered sensitivity of the assay; however, while recalculating the sensitivity and specificity of the QFT-RD1 test and including these nine patients, sensitivity and specificity were unchanged (84% [43/51] and 36% [11/31], respectively).

FIG. 2. Results of the QFT-RD1 test, clinical, and microbiological investigations of all 73 TB suspects are shown in this diagram. Patients with a response in the QFT-RD1 test above 0.35 IU/ml were considered positive. The 48 patients were classified as having TB if either culture was positive for $M. tuberculosis$ or histology and clinical findings indicated mycobacterial infection (i.e., granulomatous necrosis or identification of acid-fast bacilli) or the physician decided that the patient suffered from TB based on clinical findings, patient history, and/or X-ray, and the patients responded appropriately to a full course of anti-TB treatment. In total, 33 TB patients were positive by microscopy and/or culture and 15 had negative microscopy and culture and were diagnosed by clinical criteria. The final classification of a non-TB patient was based on the fact that $M. tuberculosis$ was not found by conventional screening and another diagnosis was found ($n = 15$) or no other diagnosis was found ($n = 10$), but the patient recovered without an anti-TB treatment and TB was not diagnosed during follow-up.

TABLE 2. Diagnostic sensitivities of QFT-RD1, microscopy, and culture

| Patient group (n) | QFT-RD1 | Microscopy | Culture | Microscopy, culture, and QFT-RD1 |
|-------------------|---------|------------|---------|----------------------------------|
|                   | No. (%) | CI         | No. (%) | CI     | $P$ value | No. (%) | CI     | $P$ value | No. (%) | CI     |
| Pulmonary TB (35) | 29/35 (83) | 70–96     | 16/35 (46) | 29–63 | 0.009 | 22/35 (63) | 47–79 | 0.121 | 34/35 (97) | 92–103 |
| Extrapulmonary TB (13) | 12/13 (92) | 78–107     | 4/13 (31) | 5–56 | 0.013 | 5/12 (42) | 13–70 | 0.041 | 12/13 (92) | 78–107 |
| All TB patients (48) | 41/48 (85) | 75–96     | 20/48 (42) | 27–56 | 0.001 | 27/48 (59) | 44–73 | 0.009 | 46/48 (96) | 90–102 |

a Sensitivity of the QFT-RD1 test, microscopy, or culture for $M. tuberculosis$ or combined sensitivity of microscopy, culture, and the QFT-RD1 test. For each group the numbers and percentages of responders are shown together with the 95% confidence intervals. Differences in sensitivity between QFT-RD1 and either microscopy or culture were calculated using the McNemar test, and differences in proportions between the patient groups were calculated using a chi-square test; a $P$ value of $<0.05$ was considered significant.
b $n$, no. of patients.
c The extrapulmonary manifestations were distributed as meningitis ($n = 1$), lymphadenitis ($n = 9$), appendicitis ($n = 1$), osteomyelitis ($n = 1$), erythema nodosum, and a strong clinical suspicion of TB ($n = 1$).
find that 10 of the 25 non-TB patients (40%) were QFT-RD1 positive, resulting in a lower specificity of 60% for the diagnosis of active TB. Thus, although the QFT-RD1 test detected most patients with active TB, it was also positive in a number of patients in whom active TB could not be demonstrated. ESAT-6 and CFP-10 have not been shown to be able to discriminate between active and LTBI, and the non-TB patients had one or several risk factors for TB, suggesting that the positive QFT-RD1 responses were due to the presence of LTBI.

Since the QFT-RD1 test detects both active TB and LTBI, the applicability of the test for active TB will depend on the risk—in the study population—of being exposed to M. tuberculosis. The present study comprised patients with risk factors, such as immigrants from high-endemicity regions, homeless people, and patients with intravenous drug abuse, possibly latently infected and thus QFT-RD1 positive but suffering from non-TB diseases. In low-risk groups, i.e., patients with chronic obstructive lung diseases or patients under investigation for tumor suspect infiltrates on X-ray, the accuracy of this test should be higher, and preliminary data have shown that only 17% of such patients with no known M. tuberculosis exposure were positive by QFT-RD1 (unpublished data). Thus, the positive and negative predictive value of the test for active TB will vary with the sample size and the prevalence of LTBI in the study population. The latter may be a particular problem in high-TB-endemicity regions, where there is a high prevalence of both active and LTBI and where 30 to 50% of healthy individuals have been shown to respond to the RD-1 antigens (8, 15, 25, 29). However, a negative test result for healthy individuals may be useful as a tool to exclude TB infection, and a positive result may help in identifying persons who are candidates for preventive chemotherapy or intensified clinical follow-up.

At present we cannot predict who or how many of the ESAT-6- or CFP-10-responsive non-TB patients will develop TB. We have previously shown an association between ESAT-6 responsiveness and later progression to TB in a group of healthy exposed contacts (9). In the present study, 10 QFT-RD1 responsive non-TB patients did not develop active TB within the observation period of almost 2 years, but 1 patient with pneumonia and pericarditis, who was initially categorized as a non-TB patient, progressed clinically and developed miliary TB after having received intensive immunosuppressive treatment. The patient who developed TB was QFT-RD1 positive when presenting with symptoms 3 months before progressing to miliary TB, but at that time active TB was not found despite intensive investigations (24). This case suggests that RD1-responsive patients may be at risk of progressing to active TB during immune suppression. The use of immune suppression with biological agents like anti-tumor necrosis factor alpha antibodies is increasing, and reactivation of LTBI is one of the most feared side effects (14). Screening for LTBI with the QFT-RD1 test may turn out to be extremely useful in this situation.

Immunosuppression due to HIV or severe TB is a question of potential concern with any assay based on cellular immune responses. In the present study, seven TB patients were QFT-RD1 negative. Four of these were positive by microscopy and culture. Interestingly, three of these four individuals suffered from severe TB, and the fourth was HIV positive with a low

TABLE 3. Risk factors for QFT-RD1-positive non-TB patients

| Pt. no.: | History of: | Immigrant | IVDU/HIV | No known risk factor |
|---------|-------------|-----------|---------|---------------------|
|         |             | Alcohol/  |         |                     |
|         |             | shelter   |         |                     |
| 1       |             | +         | +       |                     |
| 2       |             |           | +       |                     |
| 3       |             |           | +       |                     |
| 4       |             |           | +       |                     |
| 5       |             |           | +       |                     |
| 6       |             |           | +       |                     |
| 7       |             |           | +       |                     |
| 8       |             |           | +       |                     |
| 9       |             |           | +       |                     |
| 10      |             |           | +       |                     |

a The clinical diagnoses for patients (Pt.) no. 1 to 3 were bacterial infection with S. pneumoniae, S. aureus, or Streptococcus species. No. 4 had acute myeloid leukemia with nontuberculous mycobacterial pneumonia; patient no. 5 had diabetes mellitus with vasculitis, and no diagnosis was obtained for patients no. 6 to 10.

b The patients were immigrants from Pakistan, Nigeria, Somalia, Turkey, and Rwanda, respectively.
c Both patients had present or recent IVDU, and in addition, one was HIV positive.

compare the QFT-RD1 test with conventional microbiological diagnostic methods. Our study showed a high sensitivity of the QFT-RD1 test (85%) compared to microscopy (42%) and culture (59%), with a positive predictive value of 80% (41/51). Interestingly, we found that as many as 87% of the patients with negative microscopy and negative culture were QFT-RD1 positive, and by combining microscopy and culture with the QFT-RD1 test, the overall sensitivity increased to 96%, together suggesting that the QFT-RD1 may be of use for the diagnosis of active TB.

Diagnosis of extrapulmonary TB and culture-negative TB remains a challenge; our study demonstrates that the QFT-RD1 test has a particular advantage in this group of patients and may greatly improve the diagnosis of extrapulmonary TB and microscopy- and culture-negative TB. In line with previous studies (21, 22), we found no difference in the sensitivity of the QFT-RD1 test between patients with pulmonary TB and extrapulmonary TB, whereas the sensitivity of the QFT-RD1 test (85%) was significantly higher than microscopy (42%) and culture (59%). In addition, we found that 87% of the patients who were negative by culture or microscopy were detected by the QFT-RD1 test, suggesting the QFT-RD1 test could improve diagnosis of active TB. Taken together, our findings suggest that the RD1 test may be a useful supplementary tool in the diagnosis of extrapulmonary TB and microscopy- and culture-negative TB. Studies based on larger groups of well-characterized TB patients are warranted to confirm these findings.

In healthy BCG-vaccinated controls without known exposure to M. tuberculosis, an IFN-γ response to the RD1 antigens was detected in only 1 out of 39 (3%), indicating a high M. tuberculosis specificity. In contrast to the RD1 antigens, IFN-γ responses to PPD were present in both patients and healthy controls, confirming the lack of specificity of PPD in healthy BCG-vaccinated individuals (6, 7, 11, 13, 18, 20, 21, 28). High specificity of the QFT-RD1 is a consistent finding in populations selected because they have a very low risk of exposure to M. tuberculosis (3, 6, 17, 20, 21, 25, 28). Given the previously reported very high specificity of the RD1-based tests in low-risk populations (3, 6, 17, 20, 21, 25, 28), it was interesting to...
CD4 cell count. These data indicate that more-advanced disease may be associated with weak T-cell responses, which has been suggested in previous studies (22, 25). The performance of this test in immunocompromised patients is under investigation. Together, these findings underline the necessity of combining the QFT-RD1 test with conventional microscopy and culture for maximal diagnostic sensitivity.

In conclusion, our data suggest that the QFT-RD1 test could be a very useful supplementary tool for the diagnosis of active TB, especially in patients with microscopy- and culture-negative and extrapulmonary TB. Together with negative microscopy and culture, a negative QFT-RD1 test may be used to exclude TB in immunocompetent individuals.

ACKNOWLEDGMENTS
We thank Vita Skov (Statens Serum Institute, Copenhagen, Denmark) for excellent technical support, Eskild Pedersen (University Hospital, Skejby, Denmark) and Kåre Mølbak (Statens Serum Institut, Copenhagen, Denmark) for discussions on the design of the study, Søren Hoff (Statens Serum Institute, Copenhagen, Denmark) for assistance in the clinic, and Klaus Larsen (University Hospital, Hvidovre, Denmark) for statistical counseling.

Peter Andersen and Aase Bengaard Andersen are inventors of patents and patent applications files by Statens Serum Institut relating to the discovery of the 6-T. tuberculosis antigen ESAT-6. The rights have been assigned to Statens Serum Institute, and Statens Serum Institute owns and controls the rights to the reagents.

Quantiferon TB reagents were supplied by Cellestis Limited, Victoria and were used by Kåre Mølbak in the Copenhagen Tuberculosis Study, Søren Hoff (Statens Serum Institute, Copenhagen, Denmark) and Kåre Mølbak (Statens Serum Institute, Copenhagen, Denmark). The Quantiferon TB assay was used in the Copenhagen Tuberculosis Study, in which the main author, Søren Hoff (Statens Serum Institute, Copenhagen, Denmark), is principal investigator. The test was not used in the study by Søren Hoff as a tool for the study of QFT-RD1.

REFERENCES
1. American Thoracic Society. 2000. Targeted tuberculin testing and treatment of latent tuberculosis infection. Morb. Mortal. Wkly. Rep. Recomm. Rep. 49(RR-6):1–51.
2. Andersen, P., M. E. Munk, J. M. Pollock, and T. M. Doherty. 2000. Specific immune-based diagnosis of tuberculosis. Lancet 356:1099–1104.
3. Arend, S. M., A. C. Engelhard, G. Groot, K. de Boer, P. Andersen, T. H. Ewer, K., J. Deeks, L. Alvarez, G. Bryant, S. Waller, P. Andersen, P. Monk, and A. Lalvani. 2000. Evidence for occurrence of the ESAT-6 protein in Mycobacterium tuberculosis and for its absence in Mycobacterium bovis BCG. Infect. Immun. 68:16–22.
4. Johnson, P. D., R. L. Stuart, M. L. Grayson, D. Olden, A. Clancy, P. Ravn, P. Andersen, W. J. Britton, and J. S. Rothel. 1999. Tuberculin-purified protein derivative-, MPT-64-, and ESAT-6-stimulated gamma interferon responses in medical students before and after Mycobacterium bovis BCG vaccination and in patients with tuberculosis. Clin. Diagn. Lab. Immunol. 6:934–937.
5. Kuzmyk, J., S. Gershon, R. P. Wise, E. Tifariley-Levens, J. Kasznica, W. D. Schwieterman, J. N. Siegel, and M. M. Braun. 2001. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N. Engl. J. Med. 345:1098–1104.
6. Lein, A., P. Nagvenkar, Z. Udwadia, A. A. Pathan, K. A. Wilkinson, J. S. Shastri, K. Ewer, A. V. Hill, A. Mehta, and C. Rodrigues. 2001. Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent Mycobacterium tuberculosis infection in healthy urban Indians. J. Infect. Dis. 183:469–477.
7. Lelave, A., A. A. Pathan, H. Durkan, K. A. Wilkinson, A. Whelan, J. A. Deks, W. H. Reece, M. Latif, G. Pasvol, and A. V. Hill. 2001. Enhanced contact tracing and spatial tracking of Mycobacterium tuberculosis infection by enumeration of antigen-specific T cells. Lancet 357:1523–1527.
8. Lein, A., A. A. Pathan, M. ScHansE, R. M. LunDenGrEn, L. N. Alexander, and P. Andersen. 1999. Cellular immune responses to ESAT-6 discriminate between patients with pulmonary disease due to Mycobacterium avium complex and those with pulmonary disease due to Mycobacterium tuberculosis. Clin. Diagn. Lab. Immunol. 6:506–509.
9. Mazurek, G. H. M. E. Villarino, et al. 2003. Guidelines for using the Quantiferon-TB test for diagnosing latent Mycobacterium tuberculosis infection. Morb. Mortal. Wkly. Rep. Recomm. Rep. 52(RR-2):15–18.
10. Mori, T., M. SakaMani, F. Yamagishi, T. Takashima, K. Kagao, E. Shigeto, N. Harada, S. MitaraRi, M. Okada, K. Suzuki, Y. Inoue, K. Tsuyuwaychi, Y. Sasaki, G. H. Mazurek, and I. Tsuyuguchi. 2004. Specific detection of tuberculosis infection: an interferon-gamma based assay using new antigens. Am. J. Respir. Crit. Care Med. 170:59–64.
11. Munk, M. E., S. M. Arend, J. Brock, T. H. Ottenhoff, and P. Andersen. 2001. Use of ESAT-6 and CFP-10 antigens for diagnosis of extrapulmonary tuberculosis. J. Infect. Dis. 183:175–176.
12. Pathan, A. A., K. A. Wilkinson, P. Kaiserman, H. McShane, N. R. Davidson, G. Pasvol, A. V. Hill, and A. Lavalani. 2001. Direct ex vivo analysis of antigen-specific IFN-gamma-secreting CD4 T cells in Mycobacterium tuberculosis-infected individuals: associations with clinical disease state and effect of treatment. J. Immunol. 167:2127–2132.
13. Pollock, J. M., J. M. McNair, H. Bassett, J. P. Cassidy, E. Costello, H. Aggerbeck, I. Rosenkrands, and P. Andersen. 2003. Specific delayed-type hyper-sensitivity responses to ESAT-6 identify tuberculosis-infected cattle. J. Clin. Microbiol. 41:1856–1859.
14. Ravn, P., M. E. Munk, A. B. Andersen, L. N. Nielsen, T. LilbeckaE, I. J. Soeersen, P. Andersen, and K. Weldingh. 2004. Reactivation of tuberculosis during immunosuppressive treatment in a Quantiferon-RD1 positive patient. Scand. J. Infect. Dis. 36:499–501.
15. Ravn, P., A. Demissie, T. Egualle, H. Wondwosson, D. Lein, H. A. Amoudy, A. Lalvani, A. A. Pathan, H. Durcan, K. A. Wilkinson, and C. Rodrigues. 2004. Reactivation of tuberculosis during immunosuppressive treatment in a Quantiferon-RD1 positive patient. Scand. J. Infect. Dis. 36:499–501.
16. Schwieterman, J. N. Siegel, and M. M. Braun. 2001. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N. Engl. J. Med. 345:1098–1104.
17. World Health Organization. 2000. Tuberculosis, fact sheet no. 104. [Online]. http://www.who.org.