Cellular Immune responses during latent tuberculosis : immunodiagnosis and correlates of protection

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Chapter 2

Specific T cell epitopes for immune based diagnosis of *M. tuberculosis* infection

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ABSTRACT

The currently used method for immunological detection of tuberculosis infection, the tuberculin skin test, has low specificity. Antigens specific for *Mycobacterium tuberculosis* to replace purified protein derivative are therefore urgently needed. We have performed a rigorous assessment of the diagnostic potential of four recently identified antigens (Rv2653, Rv2654, Rv3873 and Rv3878) from genomic regions that are lacking from the *M. bovis* bacille Calmette-Guèrin vaccine strains, as well as from the most common non-tuberculous mycobacteria. The fine specificity of potential epitopes in these molecules was evaluated by sensitive testing of the T cell responses of peripheral blood mononuclear cells derived from BCG vaccinated healthy individuals to synthesized overlapping peptides. Three of the four molecules contained regions with significant specificity problems (Rv2654, Rv3873 and Rv3878). We selected and combined the specific peptide stretches from the four proteins, not recognized by BCG vaccinated individuals. These peptide stretches were tested with peripheral blood mononuclear cells obtained from patients with microscopy or culture confirmed tuberculosis and from healthy BCG vaccinated controls. The combination of the most promising stretches from this analysis showed a sensitivity level (57%) comparable to the level found with the two well-known *Mycobacterium tuberculosis* specific proteins ESAT-6 and CFP-10 (75 and 66% respectively). The combination of ESAT-6, CFP-10 and the novel specific peptide stretches gave an overall sensitivity of 84%, at a specificity of 97%. In a validation experiment, with new experimental groups, the sensitivities obtained were: 57% for the combination of peptides and 90% for the combination of the peptides, ESAT-6 and CFP-10. This combination gave a specificity of 95%.

INTRODUCTION

Tuberculosis (TB) is a major cause of morbidity and mortality throughout the world. It is estimated that nearly 1% of the worlds population is newly infected each year and that approximately one third of the worlds population is latently infected with *Mycobacterium tuberculosis*.

On average, immunocompetent individuals infected with *M. tuberculosis* have a lifetime risk of 10% of developing active TB, but this risk increases to a 10 percent yearly risk in persons co infected with HIV. If left untreated a patient with active pulmonary TB will transmit the infection to 10 to 15 contacts each year (44). Therefore, novel tools to detect, and subsequently treat, infected individuals before the disease progress to active contagious TB is an international research priority (10).

In addition to chest radiographs to detect pulmonary TB, the current diagnostic assays for the detection of infection with *M. tuberculosis* include culture, microscopy and PCR of relevant patient material and the tuberculin skin test (TST). The first three methods are based on the identification of the bacteria and hence depend on a certain bacterial load and access to the infection site. This makes them inapplicable for detection of extrapulmonary TB or early diagnosis of the preclinical latent stages of infection.

The standard tuberculin skin test (TST) represents a delayed type hypersensitivity reaction, based on immunological recognition of mycobacterial antigens in exposed individuals and is a simple and inexpensive assay. It employs intradermal injection of purified protein derivative (PPD) which is a crude and poorly defined mixture of mycobacterial antigens many of which are shared with proteins from the vaccine *M. bovis* bacille Calmette-Guèrin (BCG) and from non-tuberculous environmental mycobacteria (NTM) (4) (20). The broad cross reactivity of PPD results in the poor specificity of the TST, leading to a situation where BCG vaccination and exposure to NTM gives a test result that can be similar to that seen in individuals infected with *M.tuberculosis* (21) (15) (25) (18). To overcome the problem of sensitisation to NTM, comparative skin testing with PPD derived from two sources; *M. tuberculosis* and *M. avium*, has been used. A blind study showed that *M. avium* sensitin dominant skin tests can discriminate *M. avium* infection from TB with high specificity in humans (42) and furthermore, that infection with NTM is responsible for the majority of PPD reactions of 5-14 mm in the annual TST performed on US- born health care workers and medical students (41). The limited specificity of a test based on PPD also characterizes *in vitro* assays as was demonstrated by early attempts to develop serodiagnostic tests based on PPD (12), and more recently from the whole blood QuantiFERON®-TB test in which PPD is used to induce the secretion of IFN-γ from sensitised T-cells in whole blood (9).

The sequenced genomes of not only *M. tuberculosis* but also BCG and *M. avium* provide a blueprint for the rational design of the next generation of specific immunodiagnostic

reagents (http://www.pasteur.fr/english.html), and allowed the identification of the RD regions (regions of deletion) representing genomic deletions in the *M. bovis* BCG vaccine strain compared to the virulent *M. tuberculosis* strain (7). Proteins from these regions, and in particular the subset of proteins which are also lacking in *M. avium* and other NTM, represent an excellent source of candidate antigens for a highly specific TB diagnostic test. A number of antigens from these regions have recently been characterized and have undergone initial evaluation as potential diagnostic reagents for human or cattle TB (3). The antigens characterized in most detail are ESAT-6 and CFP-10, which have already shown great potential for TB diagnosis (5) (40) (43) (17), but a number of more recently identified candidate molecules have also been reported. In the present study we have evaluated four of these recently characterised antigens; Rv3873 and Rv3878 encoded in the RD 1 region, in addition to Rv2653 and Rv2654 encoded in the RD 11 region (32) (1) (47).

We report that although these molecules are encoded in the deleted regions, BCG vaccinated individuals recognized epitopes in three of the four molecules. By excluding these cross-reactive parts of the molecules, we demonstrate that it is possible to compose a highly specific cocktail of peptides for sensitive and specific TB diagnosis. The potential of combining this cocktail with ESAT-6 and CFP-10 is addressed.

MATERIALS AND METHODS

Recombinant proteins and synthetic peptides

Recombinant CFP-10 and ESAT-6 were produced as previously described (8) (33).

The overlapping peptides (18 or 20- mers) from the four proteins Rv2653, Rv2654, Rv3873 and Rv3878, were synthesized by standard solid-phase methods at Schafer-N, Copenhagen, Denmark. The peptides were purified by reverse phase HPLC. Purified peptides were lyophilized and stored dry until reconstitution in PBS. Sequences for the peptides synthesized are shown in Figure 1.

Subjects

Derivation panel

The healthy BCG vaccinated subjects (controls, n=29) were all Danish born adults between 25-67 years of age (mean age: 40 years; 8 male, 21 female) who had received BCG vaccination during their childhood 20 to 62 years before venapuncture. They were recruited through advertisement. All controls answered a questionnaire to reveal potential exposure to TB: travel history, contact with TB patients or occupational exposure to *M. tuberculosis*, and all controls with possible exposure were excluded. Vaccination status was

Figure 1. Amino acid sequence of the four diagnostic antigens.
Synthetic 18-20 mer peptides spanning the proteins are indicated by horizontal double lines. Peptide numbers are used throughout the paper.

determined through questionnaire. Thirty-four healthy BCG vaccinated individuals were recruited and six were excluded based on information revealed in the questionnaire.

PBMC from 60 TB patients 14-72 years old (mean age: 39 years; 40 male, 20 female) with microscopy- or culture-proven infection were used. Patients were recruited from hospitals in the Netherlands and Denmark. Blood samples were drawn from TB patients

shortly after diagnosis and treatment initiation (0-6.6y; median 0.12y), except for five of the patients who had their blood drawn two years after (n=1), six years after diagnosis (n=2), and six months after diagnosis (n=2). About 1/3 of the patients were of Caucasian Danish or Dutch origin, 2/3 were immigrants born in North Africa, Central and South Africa, South East Asia, Central Asia, South-west Asia, South America and Eastern Europe. Thirty-six of the patients had pulmonary TB, two patients had milliary TB while the remaining patients had extra pulmonary TB e.g. of the lymph nodes (neck, axillae and groin), of the pericardia, the spine, thoracic spine, pleura, mammae, joint, or peritoneum.

Results from one of the patients were excluded based on a low response to phytohaemagglutanin (PHA) (< 150 pg/ml) suggestive of a low survival rate of the peripheral mononuclear cells (PBMC) after freezing and thawing of the cells. Three of the patients had *in vitro* anergy (low response to PPD).

Validation panel

For validation of the findings PBMC from 43 individuals were used, 22 BCG vaccinated controls and 21 individuals with *M. tuberculosis* infection, comprising 13 individuals with latent TB and 8 patients with active TB.

The latently infected individuals (male/female: 10/3; mean age: 17 y) were Danish born high school students with close contact to a TB patient with sputum microscopy and culture positive pulmonary tuberculosis. They were BCG non-vaccinated, had a strongly positive TST (> 15mm induration) and therefore received prophylactic treatment according to the TST results. They were recruited from a local TB outbreak.

The patients with active TB (male/female: 6/2 age: 17-46y, mean: 37y.) were recruited from a Danish hospital and all had culture or microscopy positive TB and, their diagnosis confirmed within 1 month before or after venapuncture. One patient was Danish born and seven were immigrants from Pakistan, North Africa, Central and South Africa and Greenland. Seven patients had pulmonary TB and one patient had extra pulmonary TB (psoas abscess). One patient was also HIV positive and one patient received immunosuppressive treatment.

The control group in the validation panel (n=22; male/female: 12/10; mean age: 45y) comprised healthy Danish adults having low risk of exposure to *M. tuberculosis,* and were BCG vaccinated in childhood.

All participating individuals gave their informed written consent before blood sampling. The study was approved by the Local Ethical Committee for Copenhagen and Frederiksberg (RH 01-282/96 and KF 01-369/98), and by the Institutional Review Board of the Leiden University Medical Center (protocol P136/97).

Lymphocyte preparations and cell culture

PBMC were freshly isolated by gradient centrifugation of heparinized blood on Lymphoprep (Nycomed, Oslo, Norway) and stored in liquid nitrogen until use. The viability and number of cells was determined by Nigrosin staining. Cell cultures were established in triplicates of 1.25 x 10^5 PBMCs in 100µl in microtitre plates (Nunc, Roskilde, Denmark) and stimulated with 5 µg/ml PPD, 5 µg/ml recombinant CFP-10, 5 µg/ml recombinant ESAT-6 and 10 µg/ml of the individual peptides. Peptide mixtures were used in the concentration that was found to be optimal for the individual mixtures upon testing in preliminary experiments. Optimal concentrations were 10 µg/ml for each individual peptide in the mixture except for peptide mixture Rv3978 A and B which had optimal performance at 5 µg/ml for each individual peptide in the mixture. For all analysis standard error was generally below 30% of the mean on analysis of the triplicates.

Cell cultures without antigen were included as negative controls and PHA (2 µg/ml) was used as a mitogenic positive control (results not shown). Cell cultures were incubated for 5 days at 37 °C in a humidified (5%CO_2, 95% air) incubator. Cell free supernatants were harvested and kept frozen at -20 °C until use.

Cytokine analysis

IFN-γ was detected with a standard sandwich ELISA technique using a commercially available pair of monoclonal antibodies (Endogen, MA, US) and used according to the manufacturer's instruction. Recombinant IFN-γ (Endogen, MA, US) was used as a standard. Cytokine levels are given as picograms (pg) of protein/ml in supernatants. The detection limit of the assay was 20 pg/ml. For all individuals values from the unstimulated control well, 0-400 pg/ml (median 2.0; CI 9.9-33.6), were subtracted from stimulation wells if detectable. Cell culture and cytokine analysis for the selection of specific peptide stretches and validation of the diagnostic performance were performed by trained laboratory assistants and conducted in a random blinded fashion, so that the clinical status of the tested subjects was unknown.

Statistical methods

Comparisons of proportions were made using a two-sided Chi-square test. Calculations of diagnostic performance were made using SISA statistical analysis: http://home.clara.net/ sisa/diagnos.htm. Confidence intervals are given as 95 % CI throughout the text.

RESULTS

Fine specificity of T cell responses to diagnostic antigens

The main purpose of the current study was to identify specific molecules or parts of molecules for a future diagnostic peptide cocktail. The four antigens Rv2653, Rv2654, Rv3873 and Rv3878 are encoded in the regions of the *M. tuberculosis* genome deleted in BCG and lacking in most NTM. These antigens have all previously been described to be well recognised by TB patients, but also some recognition in healthy controls was observed (1) (47) (32). Therefore, the fine specificity of these antigens was evaluated in detail by studying the responses in healthy BCG vaccinated individuals using panels of overlapping peptides covering the entire sequences of these proteins (Figure 1), and stimulating PBMC *in vitro* (Figure 2A).

In general, most individual peptides induced low IFN-γ responses with maximum responses below 300 pg/ml in the BCG vaccinated individuals. However, some peptides did induce IFN-γ release above this background in several individuals, in particular for Rv3873 where recognition in controls was seen scattered throughout the molecule and peptide 1, 8, 12 and 28 were recognized with high level of IFN γ release in controls. For Rv3878, especially peptides 2, 20 and 22 from the c-terminal end of the molecule, was recognised by controls, while for Rv2653 a strong recognition of the central part of the molecule (especially peptide 6) was seen. On the other hand the peptides covering the Rv2654 molecule were not recognized by any of the BCG vaccinated individuals.

In order to get an overview of the regions within Rv3873, Rv3878 and Rv2653 with non-specific activity in BCG vaccinated individuals; the IFN-γ responses shown in Figure 2A were converted into recognition frequencies (% of individuals with responses >100 pg/ml to the individual peptides) (Figure 2B). On the basis of these data the stretches of the molecules without specificity problems were selected by excluding peptides recognized (>100 pg/ml) by more than one individual out of the groups of 28 individuals (corresponding to more than 4 %). Additionally, peptides that gave rise to recognition with an IFN-γ release of over 500 pg/ml in any control subject were excluded. For the Rv3873 protein three major regions were identified that were not recognized by cells from BCG vaccinated individuals. These regions designated A, B and C contain peptides 2 to 6 (Rv3873A), 9-11 (Rv3873B) and 15-18 (Rv3873C). Furthermore, to explore the specific peptides in the c-terminal part of the molecule, a number of specific single peptides (peptides 22, 24, 29, 32, 33 and 35) were combined into one cocktail designated Rv3873D.

In protein Rv3878, two specific regions were selected; peptides 3-9 (Rv3878A)) and peptides 11-15 (Rv3878B).

The recognition frequency for single peptides spanning the Rv2653 protein showed that this protein contains a cross-reactive stretch of approximately 36 amino acids (peptides 4, 5 and 6) in the central part of the protein. The two specific regions (peptides

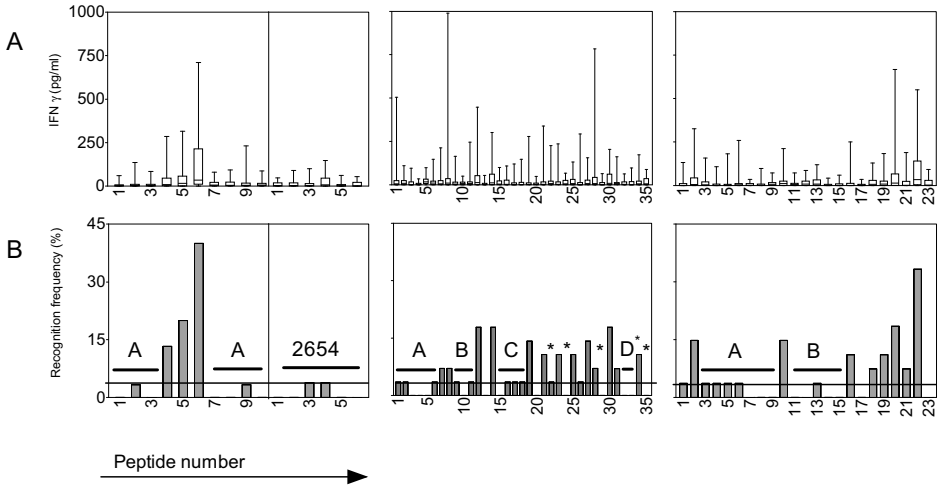

Figure 2. Recognition of the single peptides in BCG vaccinated individuals

A: Level of recognition for the single peptides spanning the four proteins in BCG vaccinated individuals: Synthetic peptides 18-20 mer spanning the whole proteins were tested by measuring the IFN-γ release after stimulation of PBMC from healthy BCG vaccinated individuals (n=28). The peptide numbers refer to Figure 1 and are used throughout the paper.

Boxes represent the 25 to the 75 percentiles, whiskers the maximum response.

B: Recognition frequencies of single peptides in BCG vaccinated individuals. Recognition frequencies (% of individuals with responses >100 pg/ml to the individual peptides) of the IFN- γ responses in BCG vaccinated individuals. Horizontal bars indicate the regions selected and pooled as diagnostic cocktails. Asterisks indicate the single peptides included in the Rv3873D cocktail.

1-3 and peptides 7-10) were chosen, combined and designated Rv2653A. The Rv2654 molecule did not contain non-specific segments and was therefore used as a cocktail of all 6 peptides covering the sequence.

Diagnostic potential of peptide stretches

To evaluate the diagnostic potential of the selected specific regions from the four proteins the peptide mixtures were tested in *in vitro* assays on a panel of PBMC from TB patients with culture/microscopy proven TB, and in parallel in BCG vaccinated healthy controls. These control PBMC was derived from the same individuals as used in the selection of specific peptide stretches. They had a second venupuncture, and PBMC from one additional individual was included. The eight different peptide mixtures were compared with the well known *M. tuberculosis* specific proteins CFP-10 and ESAT-6 in order to determine not only the diagnostic potential of each of the peptide cocktails, but also the additive value of the new proteins combined with ESAT-6 and CFP-10.

In confirmation of earlier results (5) (9) (30) (40), both ESAT-6 and CFP-10 were recognised by a large proportion of the patients (75 and 66 % respectively) and induced the release of high levels of IFN-γ in this group, while only low levels were induced in BCG vaccinated controls (Figure 3). The mean IFN-γ release for ESAT-6 for all patients was 1130 pg/ml and 25 pg/ml for controls. Also CFP10 showed an excellent discrimination between patients and controls, with a mean IFN-γ release for patients of 1249 pg/ml and 23 pg/ml for controls. PPD was recognized by all except one individual and induced high levels of IFN-γ, but in contrast to the two specific proteins did not discriminate between patients and controls.

The peptide cocktails were each recognized by cells from between 14 and 43 % of TB patients with responses above the established cut-off (100 pg/ml). However, for some of the cocktails the discrimination between BCG vaccinated individuals and TB patients was not as clear-cut as would have been expected based on the results from the individual peptides. One example was Rv3873C, which despite being recognized by 33 % of the patients was also recognized by 10% of the BCG vaccinated individuals. Four of the peptide mixtures (Rv2654, Rv3873A, Rv3878A and Rv3878B), on the other hand, were recognized in a highly specific fashion with no more than one responder out of the 29 BCG vaccinated individuals. Of these specific peptide cocktails, Rv2654 and Rv3873A were the most frequently recognised with 41 and 43 % of the patients responding above the cut-off level. Low levels of IFN-γ were found in the control wells for both the BCG vaccinated group (median 2.0; CI 9.9-33.6 pg/ml) and the TB patients (median 3.0; CI: 5.5-34.0). Also for the PHA wells no significant difference was found for the patients and BCG vaccinated individuals (median 10530 and 9533 pg/ml, respectively).

Combining peptide cocktails for optimal diagnostic performance

The diagnostic performance of the most promising peptide cocktails (2654, 3873A, 3878B) was evaluated and the sensitivity calculated based on the cut off values established by analysis of ROC curves at a specificity level of 97% in the derivation panel of subjects (Table 1).

The two epitope cocktails 2654 and 3873A both gave a sensitivity of approximately 50% while Rv3878B had a sensitivity of 32%. To maximise the sensitivity of a future diagnostic reagent we investigated whether it would be advantageous to combine the selected peptide cocktails. By combining 2654, 3873A and 3878B peptides a sensitivity of 57% (CI: 42-72) was obtained (Table 1). This sensitivity level is not significantly different from the sensitivity obtained with ESAT-6 or CFP-10 (75 and 66% respectively) when tested in a two-sided Chi-square test: (peptide mixture (57%) compared to ESAT-6 (75%p=0.0898), and peptide mixture (57%) compared to CFP-10 (66%; p=0.4714). Furthermore, there was a trend of an additive, but not statistically significant (p=0.8958), value of the combination of ESAT-6, CFP-10 and the novel specific peptide cocktails resulting in a

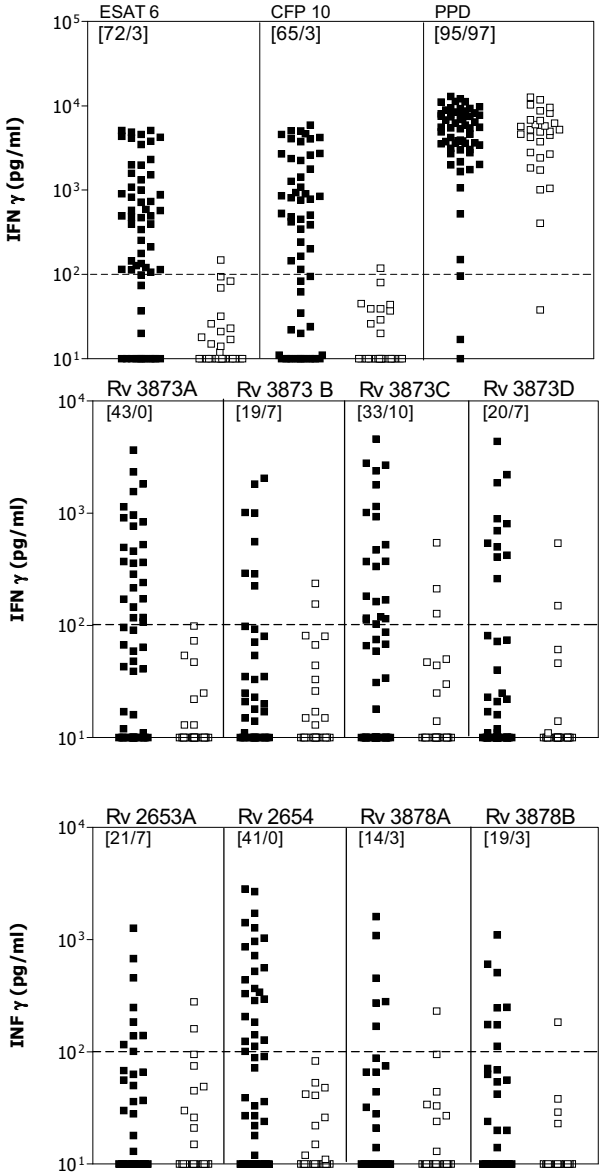

Figure 3. T cell responses to ESAT-6, CFP-10, PPD and the peptide cocktails.

The proteins and peptide mixtures were tested in a panel of PBMC from TB patients and BCG vaccinated healthy controls. Horizontal lines indicate the cut off value of 100 pg/ml used for the initial selection of peptides. Filled squares represent patients (n=44: Rv2653A, Rv3873B, Rv3878A, Rv3878B; n=59: ESAT-6, CFP-10, PPD, Rv3873A, Rv3873C, Rv3873D, Rv2654) and open squares represent the 29 BCG vaccinated controls tested. For each antigen/peptide cocktail the recognition (>100 pg/ml) is given in percent of tested patients/BCG vaccinated controls and is indicated in the upper right hand corner [% recognition in patients/ % recognition in controls].

Table 1. DERIVATION PANEL: Diagnostic performance of the novel peptide mixtures.

Antigen	Recognition[a]	Sensitivity[b] %(CI)[d]	Specificity[c] %(CI)[d]
Rv2654	28/59	47 [35-60]	97 [90-103]
Rv3873 A	27/59	46 [33-57]	97 [90-103]
Rv3878 B	14/44	32 [18-46]	97 [90-103]
Combination:			
Rv2654 + Rv3873 A + Rv3878 B	25/44	57 [42-72]	97 [90-103]
ESAT-6	44/59	75 [64-86]	97 [90-103]
CFP-10	39/59	66 [54-78]	97 [90-103]
Combination:			
ESAT-6 + CFP-10	48/59	81 [71-91]	97 [90-103]
Combination:			
ESAT-6 + CFP-10 + Rv2654 + Rv3873 A + Rv3878 B	37/44	84[73-95]	97 [90-103]

[a]Recognition: No. of patients responding / No. of patients tested. Cut off determined by ROC curve analysis; ≥97% specificity for the single proteins/ peptide cocktails: ESAT-6; 94 pg/ml, CFP-10; 80 pg/ml , Rv2654; 53 pg/ml , Rv3873 A; 73 pg/ml and Rv3878 B; 38pg/ml.
[b]Sensitivity: percentage of responding TB patient individuals out of all TB patient individuals tested.
[c]Specificity: percentage of true negative control individuals out of all control individuals tested.
[d] CI 95% confidence interval [percentage]

combined sensitivity of 84% (CI: 73-95) compared to the 81% (CI: 71-91) sensitivity of the combination of ESAT-6 and CFP-10 alone (Table 1).

For the sensitivities it is noteworthy that three of the included patients had *in vitro* anergy and were negative to all antigens tested, including PPD. Two of these patients received immunosuppressive treatment and one was severely ill with extensive disease.

Validation of the findings

To validate the findings a new panel of 21 *M. tuberculosis* infected individuals (13 latent TB and 8 active TB) and control subjects were investigated. Sensitivity and specificity was calculated using the 5 antigens and the 5 cut off values established in the derivation panel of subjects (Table 2). The results from the group of latently infected individuals and the group of individuals with active TB were very similar with no significant differences and will in the following be reported together.

The combination of the 3 new peptide stretches gave a sensitivity of 57% (CI: 36-78) and the specificity was 95% (CI: 87-104). The combination of ESAT-6 and CFP-10 gave for all

Table 2. VALIDATION PANEL. Diagnostic performance of the novel peptide mixtures.

Antigen	Recognition[a]	Sensitivity[b] %[CI][d]	Specificity[c] %[CI][d]
Rv2654	9/21	42 [22-64]	100 [100-100]
Rv3873 A	8/21	38 [17-59]	95 [87-104]
Rv3878 B	12/21	57 [36-78]	100 [100-100]
Combination:			
Rv2654 + Rv3873 A + Rv3878 B	12/21	57 [36-78]	95 [87-104]
ESAT-6	18/21	86 [71-101]	100 [100-100]
CFP-10	15/21	71 [52-91]	100 [100-100]
Combination:			
ESAT-6 + CFP-10	18/21	86 [71-101]	100 [100-100]
Combination:			
ESAT-6 + CFP-10 + Rv2654 + Rv3873 A + Rv3878 B	19/21	90 [78-103]	95 [87-104]

[a]Recognition: No. of infected individuals responding / No. of infected individuals tested.
Cut off determined by ROC curve analysis in the derivation set: ESAT-6; 94 pg/ml, CFP-10; 80 pg/ml , Rv2654; 53 pg/ml, Rv3873 A; 73 pg/ml and Rv3878 B; 38pg/ml.
[b]Sensitivity: percentage of responding TB infected individuals out of all TB infected individuals tested.
[c]Specificity: percentage of negative individuals out of all (22) BCG vaccinated individuals categorised as having low risk of exposure to *M. tuberculosis*.
[d] CI 95% confidence interval (percentage)

infected 86% (CI: 71-101), and a specificity of 100% (CI: 100-100). The combination of all five antigens gave a sensitivity of 90% (CI: 78-103) for all infected and a specificity of 95% (CI: 87-104).

DISCUSSION

A next generation PPD in the form of selected antigens specific to *M. tuberculosis* has been on the research agenda for many years. In the seventies attempts were made to extract *M.tuberculosis* specific antigens by absorption with cross-reacting antibodies (39). Since then most attempts have been based on affinity purification with monoclonal antibodies (45) (46), or conventional biochemical purification (31). These efforts have more recently resulted in the discovery of ESAT-6 (2) (38) and CFP10 (8) (13), which in a number of studies from different laboratories have demonstrated a great potential for immune based TB diagnosis (5) (40) (9) (24). However, although highly specific for TB

infection the underlying problem has been the insufficient sensitivity often obtained with single antigens and therefore attempts have been made to discover more antigens with diagnostic potential (11) (26). In the present study we have conducted a post genomic antigen discovery programme focused on antigens recently identified in the regions in the *M. tuberculosis* genome that are not present in the *M. bovis* BCG genome (27) (7) (19). We demonstrate that it is possible to combine carefully selected specific peptides and antigens as ingredients in a sensitive and highly specific diagnostic CMI based test. Future studies will reveal whether these ingredients can be mixed into one single reagent. We have evaluated four recently characterized antigens with reported diagnostic potential; Rv3873, (32) a PPE family protein and Rv3878 (1) both encoded in the RD1 region and the two RD11 region proteins Rv2653 and Rv2654 (47).

An unexpected finding in the present investigation was the discovery that although an antigen in itself may be absent from BCG and other strains of mycobacteria, it may still contain stretches with T cell epitopes that are homologous to genes outside the deleted regions, or may even contain limited regions homologous to genes outside the mycobacterial genus. This is in agreement with recent attempts to develop TB diagnostic reagents for cattle. In a recent study by Cockle (11) this cross reactivity was observed for several antigens e.g. 17% of the BCG vaccinated cattle recognized the Rv3873 protein. Rv3873 was also found in the present study to contain many epitopes recognized by BCG vaccinated individuals. This protein belongs to the large PPE family, which contain members residing both inside and outside the RD regions and new data (32) indicates that one of the main epitopes recognized by BCG vaccinated individuals in fact represents a highly conserved epitope shared among the different PPE family members. For the other proteins in this study no homology to other mycobacterial proteins was found in database searches and therefore there is no obvious explanation for the observed "cross reactivity" (1) (47). In the present study, instead of abandoning the non-specific molecules as diagnostic reagents, we have dissected the proteins by testing overlapping peptides spanning their sequence and divided them into specific and non-specific moieties. Also for leprosy a peptide based diagnostic approach has been attempted, but although high specificity was demonstrated the selected specific peptides gave a relatively low sensitivity (14). We have made the same observation in the present study; selecting only the regions of the protein without cross reactive epitopes decreased the sensitivity. However, by combining peptides from different molecules we have obtained a combined sensitivity at the same level as the reference reagents ESAT and CFP10, without jeopardising specificity.

Combining TB specific antigens to improve diagnostic performance does not represent a new idea. In one study, TB infected guinea pigs were skin tested with MPT64 and ESAT-6. All infected animals responded to the combination of the two antigens, however a number of non-responders were found when the antigens were employed separately

(16). Furthermore, combinations of ESAT-6 and CFP-10 have been used in IFN-γ based assays resulting in an increased sensitivity compared to the sensitivity provided by the individual antigens, without jeopardising specificity (40). Very recent data confirm the strong potential of this combination for detection of latent *M. tuberculosis* infection (17). The data in the present study demonstrate that it may be possible to improve the diagnostic performance of ESAT-6 and CFP-10 even further by combining these two antigens with the new highly selected peptides. The additive effect seen was not statistically significant using the 44 individuals in the derivation panel of patients; but the trend was also seen in the validation set of *M. tuberculosis* infected individuals. This trend could very well be relevant and have advantages if tested in a broader scale. Recent data from a study in cattle indicated that combining antigens widens the repertoire of responsive individuals, and is not only due to the genetic restriction of responses to individual epitopes but interestingly also to a very pronounced variation in T cell responses to the individual antigens over the course of infection (48). By analogy with the present study, Rv3878 was in that study found to be a candidate molecule, which supplemented ESAT-6 and CFP10 for diagnosis of cattle TB, and interestingly this antigen was recognized at time points when animals were non-responsive to the other antigens. This factor may be of particular importance as ESAT-6 and CFP-10 both belong to the ESAT-6 gene family (37) and reside within one operon. Therefore, both antigens may be expressed and available for immune recognition in the same phase of infection. Evidence for such a sequential appearance of antigen responses has recently been provided by Gennaro and colleagues, both by monitoring gene expression (35), and most recently by the sequential appearance of antibody responses (36). These findings suggest that even though the ESAT-6 family members are unusually rich in T cell epitopes, and would potentially be recognized by genetically heterogeneous populations (23) (34) (6), a multiantigen cocktail for TB diagnosis would have the potential advantage of enabling the detection of patients in different phases of infection. The present study was based on cells from TB patients as well as individuals with latent TB infection indicating that the new specific peptides have potential both for the diagnosis of active and latent TB.

In the present study the cell mediated immune (CMI) responses were monitored using *in vitro* IFN-γ assays with PBMC isolated from whole blood and frozen until use. This is a very convenient research method but not a practical diagnostic assay for clinical application. Currently, two different methods are under development for CMI based TB diagnosis measuring IFN-γ: the ELISPOT and the QuantiFERON-TB® whole blood test. The QuantiFERON technology is currently based on PPD and has been demonstrated to correlate nicely with the TST in its ability to detect latent TB (28) (29). However, being based on PPD, QuantiFERON-TB® has a low specificity (9). Attempts to adapt this methodology to include specific antigens have provided promising data (22) (9), and very recently the second generation QuantiFERON TB test the so called QuantiFERON-TB

Gold assay, which uses ESAT-6 and CFP-10, has been released to the market for clinical application. Also the use of ESAT-6 and CFP-10 for an ELISPOT based diagnosis of latent TB, the so-called CLINISPOT-TB assay has recently attracted a lot of interest. In a contact tracing study it was found that although the ELISPOT results showed an overall concordance of 89% when compared with the TST, recognition of ESAT-6/CFP-10 correlated significantly more closely to the level of exposure to the index case than did TST (17). Both the QuantiFERON-TB® whole blood assay and the ELISPOT assay are based on fresh blood and may be difficult to implement in the third world for practical and logistical reasons. In this setting an improved skin test in which PPD is replaced by a cocktail of specific antigens would be an attractive alternative to the classic TST. ESAT-6 and CFP-10 have previously been demonstrated to induce strong skin test responses in guinea pigs (40) (16) (26), and a recent demonstration of the potential of ESAT-6 as a skin test reagent for the diagnosis of bovine TB (33) looks promising for its potential in a human skin test as well. Rv2654 has also proven to be able to induce skin test responses in *M. tuberculosis* infected guinea pigs (47). This antigen is therefore an obvious diagnostic candidate to be combined with CFP-10 and ESAT-6.

We have now demonstrated that it is possible to combine carefully selected specific peptides and antigens as ingredients in a highly sensitive and specific CMI based diagnostic test. The next generation skin test or blood based diagnostic test for TB infection shall discriminate individuals sensitised not only by BCG and NTM but also by any new vaccine developed for tuberculosis in the future. It is therefore important that a new diagnostic reagent is carefully aligned with the efforts to develop a new vaccine in the coming years.

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