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REPLACING THE TUBERCULIN SKIN TEST WITH A SPECIFIC BLOOD TEST

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Abstract For almost 100 years has the tuberculin skin test (TST) been used for the support the diagnosis of active and latent TB infection. The TST test has, however, a number of limitations most notable low specificity in BCG vaccinated individuals due to cross-reactive components in PPD and the *M. bovis* BCG vaccine strain and an intensive search for new and more specific diagnostic antigens has therefore be ongoing. In this review we describe the discovery process leading to the identification of the *M. tuberculosis* specific antigens ESAT6 and CFP10; two low molecular weight proteins which are highly sensitive and specific for detection of a *M. tuberculosis* infection.

Key words: Tuberculosis, Diagnosis, Antigen discovery, Interferon-gamma assay, ESAT6, RD1 antigens, QuantiFERON-TB.

Introduction

The tuberculin skin test (TST) has been used for almost a century to support the diagnosis of active and latent TB infection. The TST is 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. The main drawback with the clinical use of the TST is the lack of specificity due to the high number of shared antigens in the other mycobacteria, such as the *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) vaccine strain and atypical mycobacteria such as *Mycobacterium avium* (Fig. 1). This can lead to positive reactions in individuals either vaccinated with the BCG vaccine or infected with *Mycobacterium avium* as demonstrated in animal models[1)2)] The limited specificity also characterizes *in vitro* assays based on PPD, as demonstrated by the development of a whole blood assay which uses PPD to induce a secretion of gamma interferon (IFN-γ) as a measurement for *M. tuberculosis* infection[3)].

For many years research has been focused on identification of novel antigens which are immunological active and can be used for a more specific detection of a *M. tuberculosis* infection.

Antigen Discovery from *M. tuberculosis*

M. tuberculosis is an intracellular pathogen, and the bacteria reside in the phagosomal compartment of macrophages. It is still not clear exactly what constitutes a protective immune response to TB, but it is demonstrated in both animals and humans that T-cell mediated immune responses rather than antibody-mediated immune responses are essential for control of TB. IFN-γ is the cytokine most strongly associated with

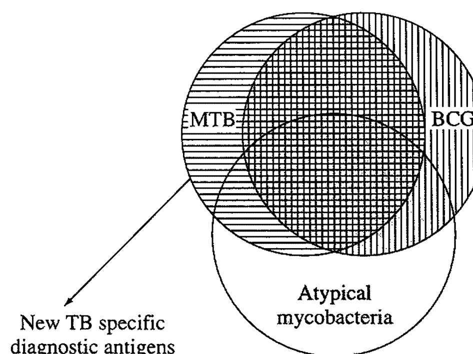

Fig. 1 Most mycobacterial proteins are shared between different species, and only a few proteins are specific for *M. tuberculosis* (eg. ESAT6 and CFP10) and not present in *M. bovis* vaccine strain BCG or in atypical mycobacteria.

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protective immunity to TB and mice with a disrupted IFN-γ gene as well as humans with mutated IFN-γ receptor genes are highly susceptibility to mycobacterial infections[4)~6)]. The ability to stimulate T-cell release of IFN-γ has therefore been used as a critical criterion for the identification of immunodominant antigens both for vaccine and diagnostic purposes.

The search for *M. tuberculosis* antigens that are recognized by the immune system of *M. tuberculosis* infected humans has shown to be a challenging task that requires a huge effort. Various experimental approaches have been developed during the past decades, and the sequencing of the *M. tuberculosis* genome has accelerated the discovery process substantially. The tendency has been that different laboratories have evaluated the recognition of the antigens by the human immune system during the course of infection. Peripheral blood mononuclear cells (PBMC) from PPD^+ non-vaccinated healthy donors, BCG vaccinated healthy donors, TB-contacts, and TB patients are used to assess candidate *M. tuberculosis* antigens. T-cell proliferation and/or production of IFN-γ from *in vitro*-stimulated PBMC are used to identify relevant antigens which are frequently recognized by a genetically heterogeneous population.

The observation that immunization with live mycobacteria induces a higher degree of protection than with killed bacilli has had a major influence on the search for immunological relevant TB antigens. It has led to the hypothesis that proteins secreted by living bacilli in the phagosome are the first antigens to be presented to the immune system in an early phase of infection and consequently will be good candidates to be developed into diagnostic tools for the detection of an early infection[7) 8)]. In 1991 Andersen and colleagues defined a short term culture filtrate (ST-CF) enriched in secreted antigens with minimal amounts of proteins released from the cytoplasm[9)]. Antigens derived from ST-CF were found to be recognized in the very early phase of experimental TB infection in mice whereas antigens released from dead bacteria were recognized at a later stage of infection[10)]. These findings stimulated attempts to use culture filtrate antigens as a source for the search for new and immune reactive antigens and an extensive antigen discovery programs which aimed to identify key antigenic molecules in culture filtrate were initiated.

One approach was to systematically analyse the ST-CF proteins, divided into a manageable number of pools according to molecular mass. This was achieved by separating the proteins into narrow molecular mass regions by SDS-PAGE followed by eluting the proteins by the Whole gel Eluter[11)]. Using this method 15–30 proteins fractions were obtained and each pool contained only a few proteins bands all in the same molecular mass region (Fig. 2A). These fractions were used to stimulate cells obtained either from *M. tuberculosis* infected

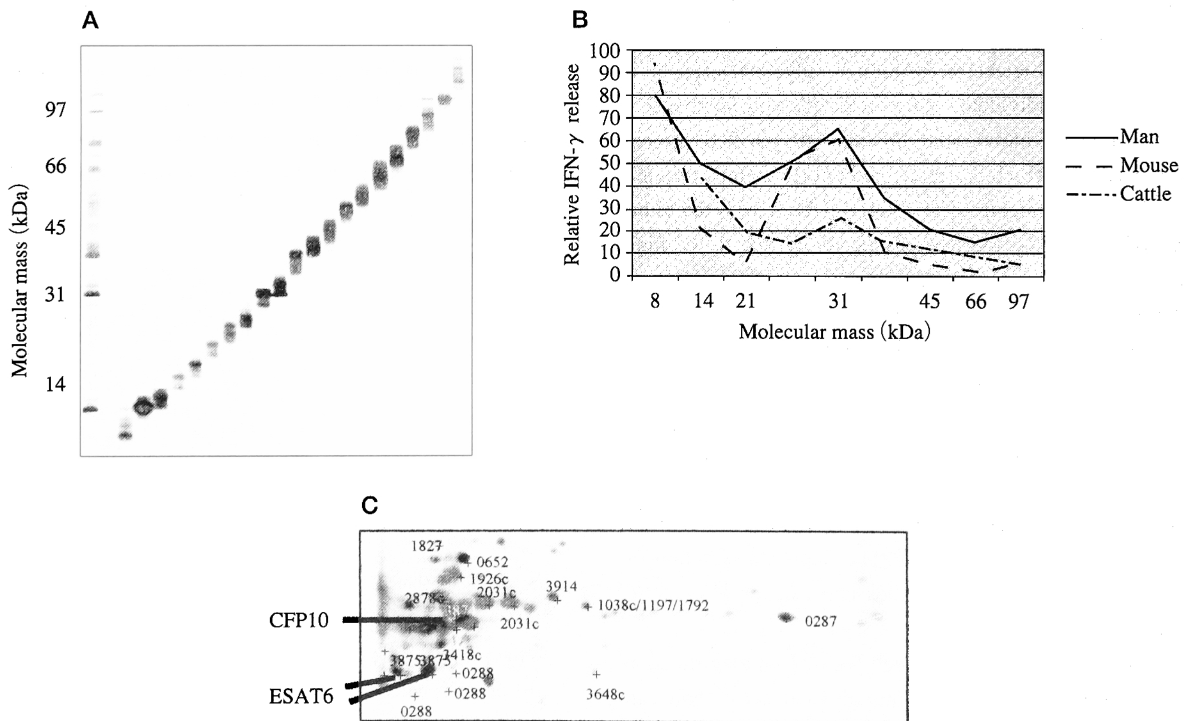

Fig. 2 Illustration of the discovery process leading to the identification of the immune reactive low mass antigens ESAT6 and CFP10. A: Size fractionation of the short term culture filtrate (ST-CF) antigen into narrow molecular weight fractions. B: Relative IFN-γ release after using the ST-CF fractions to stimulate PBMCs from TB patients, *M. tuberculosis* infected mice and cattle. C: Mapping of the positions of ESAT6 (Rv3875) and CFP10 (Rv3874) in a 2-dimensional gel electrophoresis of the ST-CF low molecular mass region.

mice, cattle or human TB patients and the amount of IFN-γ release was determined as a marker for immunological relevance (Fig. 2B). The low molecular mass fraction (6–12 kDa) of culture filtrate was consistently found to induce the highest levels of IFN-γ release in the early phase of infection in animal models and in cells from *M. tuberculosis* infected individuals[12) 13)]. Column chromatography of the low mass antigen fraction resulted in the purification of one of the active components in the low molecular mass fraction, a 9.8 kDa protein, subsequently named Early Secreted Antigen Target (ESAT6)[14)] This protein was found to be an immunodominant target for IFN-γ producing T cells from infected mice, and contains numerous epitopes that are frequently recognized by *M. tuberculosis* infected individuals[15) 16)]. By two dimensional electrophoresis the proteins present in the low molecular mass region of ST-CF were dissected and the positions of the ESAT6 protein as well as a number of other proteins were mapped (Fig. 2C). Most importantly the immunological active component CFP10 was also identified in this molecular weight region and the gene encoding this protein is located in the same operon as the ESAT6 gene.

Searching for Diagnostic Proteins That Are Absent in BCG

The genome sequence data is now available not only for *M. tuberculosis* but also for BCG and *M. avium* and this information enables a precise selection of specific antigens for the next generation of specific immunodiagnostic reagents. It has 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[17)]. 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. One such region; the RD1 region; is missing from all *M. bovis* BCG vaccine strains and is believed to be involved in bacterial virulence and possible cell to cell spreading[18) 19)]. The RD1 region encodes nine proteins including the ESAT6 and CFP10 which are the antigens characterized in most detail and are the only members from the immunoreactive *esat-6* gene family which are absent from BCG and most other environmental mycobacteria. ESAT6 and CFP10 have shown great potential for diagnosis of human TB[20)~22)]. A number of more recently identified human diagnostic molecules have also been reported such as the TB27.4 (Rv3873) and TB37.6 (Rv3873) encoded in the RD1 region as well as the TB12.3 (Rv2653) and TB7.7 (Rv2654) encoded in the RD11 region[23) 24)]. An unexpected and important finding, 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 homologous to genes outside the deleted regions, or even contain limited regions homologous to genes outside the mycobacterial genus[25)]. This can cause immunological cross-reaction in healthy control individuals and result in decreased specificity. In the study by Brock et al. the proteins containing cross reactive epitopes were produced synthetically and tested as overlapping peptides which enabled the identification of the particular peptide stretches that were cross reactive in BCG vaccinated healthy controls. Specific peptides stretches could hereafter be selected and combined. This resulted in increased sensitivity without loss of specificity[25)].

Using ESAT6 and CFP10 as Diagnostic Antigens

ESAT6 and CFP10 are the most well documented diagnostic antigens and many studies have demonstrated high sensitivity and specificity of tests based on IFN-γ responses to these antigens in patients with active or latent TB. The assay formats evaluated so far for ESAT6 and CFP10 are; measurement of amount of IFN-γ release in either whole blood or PBMCs by ELISA assays[16) 3)], ex-vivo ELISPOT for the detection of the number of IFN-γ producing T-cells[26)] and FACS analysis for the activation of specific cells[27) 28)]. Two commercially available kits containing ESAT6 and CFP10 are now on the market; the T SPOT-TB assay (Oxford Immunotec, Oxford, UK) and the QuantiFERON-TB GOLD (Cellestis Limited, Victoria, Australia). The T SPOT-TB assay is an ELISPOT assay which uses fresh PBMCs and detects the number of IFN-γ producing T-cells in response to stimulation with ESAT6 and CFP10. The QuantiFERON-TB GOLD test is a whole blood based assay that measures the IFN-γ response to ESAT6 and CFP10 and this test has recently been approved by the FDA and the Japanese Ministry of Health, Labor and Welfare for the detection of *M.tuberculosis* infection.

The diagnostic performance of ESAT6 and CFP10 has been evaluated extensively in the QuantiFERON-TB GOLD test format for both latent and active TB. For latent TB we have used the QuantiFERON-TB GOLD test to detect subclinical infections in a recent TB outbreak in a Danish school[21)]. As part of a contact tracing operation we analysed blood samples from 45 BCG unvaccinated Danish high school students and 40 BCG vaccinated individuals whom all had a possible contact to a patient with pulmonary TB. All the BCG unvaccinated individuals were young Danish high school students who had no other known exposure to *M. tuberculosis* and the conventional PPD based Mantoux skin test could therefore be used as a gold standard for the detection of latent TB in this group of persons. We investigated the IFN-γ response in whole blood samples to ESAT6, CFP10 and PPD and compared the results with the Mantoux skin test results from the same individuals. All of the individuals which were positive in the QuantiFERON-TB GOLD assay were also positive in the skin test and a 94% agreement between these two tests was observed. Two persons were only positive in the PPD skin test and this can either be due to lower specificity of the skin test (false positives) maybe caused by exposure to environmental

mycobacteria or due to higher sensitivity (true positives) of the skin test compared to the QuantiFERON-TB GOLD ESAT6/CFP10 test. Unfortunately, no true gold standard exists for the detection of latent infection with *M. tuberculosis* and it is therefore not possible to rule out any of these possibilities.

For active TB we have used the QuantiFERON-TB GOLD assay in a prognostic setting and analysed 82 patients suspected of TB[29]. Using this assay we found a sensitivity of 85% with a specificity of 97% . In particular, was the whole blood test very efficient to detect TB disease in patients with extra pulmonary TB which is an important observation as this disease form can be very difficult to diagnose with traditional methods. The sensitivity in these patients using the QuantiFERON-TB GOLD assay was 92% whereas only 31% were positive by microscopy and 42% by culture. Another recent study by Ferrara et al. used the QuantiFERON-TB GOLD test for the diagnosis of tuberculosis infection in a hospital routine setting and concluded than the test is practical and provides more correct results that the TST test in BCG vaccinated individuals[30] As with the TST, immunosuppression may negatively affect test performance giving indeterminate results which highlights the importance of the inclusion of the positive cells control (e.g. PHA) in the QuantiFERON-TB GOLD assay.

In Japan Mori et al. have investigated the use of QuantiFERON-TB GOLD for the detection of active TB in 188 patients with culture-confirmed TB and compared to 216 healthy BCG vaccinated persons[20]. The QuantiFERON-TB GOLD assay was shown to be highly specific (98%) in the BCG vaccinated healthy group, which was a markedly higher specificity than observed with the PPD based TST (35%). Also impressive sensitivity was found in this study; (ESAT6 and CFP10: 89%) compared to the TST (66%).

In conclusion, the use of the *M. tuberculosis* specific antigens ESAT6 and CFP10 in novel diagnostic assays has provided an urgently needed specific diagnostic tool to replace the TST for the detection of both active and latent TB.

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