Immunodiagnosis of latent tuberculosis : new answers to an old question?

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INTRODUCTION

Background

Tuberculosis (TB) remains one of the most serious public health problems with one third of the word population latently infected with *Mycobacterium tuberculosis* (1). Estimates in 2006 of the World Health Organization indicate that yearly about 1.7 million people die because of TB (2). Most of these deaths occur in poor, underdeveloped countries where the HIV/AIDS epidemic is going hand in hand with TB disease (3). In the Netherlands, the incidence of TB is at its lowest point in history, with 6.2 cases per 100,000 inhabitants in 2006 (4). Like in many industrialized countries, in the Netherlands nearly 75% of all new TB cases occur among immigrants. Thus, a small part of the population contributes the most to the total number of TB cases. In contrast, the incidence of TB among native Dutch individuals is only 1.9 cases per 100.000 (4).

The pathogen responsible for TB is the *Mycobacterium tuberculosis*. Following inhalation of *M. tuberculosis* by the human host this intracellular pathogen is phagocytosed by macrophages that are present in the lung alveoli (5;6). In stead of being killed in the macrophage after fusion of the phagosome containing the bacteria with a lysosome, the bacteria escape from the immune system and remain safely hidden in the host macrophage (7-9). Since MTB is an intracellular pathogen protection against disease is predominantly dependant on the cellular immune system (10). Especially responses of T helper 1 (Th1) cells are believed to be crucial in containment of the bacteria which is shown both in humans as well as in animal studies (6;10-13). These TB specific $CD4^+$ cells will recognize specific antigenic components of the MTB bacteria presented on MHC class II receptors of infected macrophages (9) and will respond with, amongst others, the production of several cytokines like interferon-γ (IFN-γ) (10). The function of IFN-γ is multiple. Firstly, it will increase antimicrobial activity, and enhance antigen presentation of the macrophage in order to activate more cells (14;15). Secondly, IFN-γ will activate other cells both of the innate and adaptive immune system to join the battle. Thirdly, it will steer the immune system towards a Th1 response (16-18). Finally, it can activate infected macrophage to go into apoptosis (suicide of the cell), in this way eliminating its content including the tubercle bacilli (14;19).
The counterpart of the T helper cells are the cytotoxic $CD8^+$ T cells. Until recently, not much attention was paid on the potential role of $CD8^+$ cells in TB. However, studies in mice have shown that $CD8^+$ cells are important for control of TB infections and $CD8^+$ cells specific for mycobacterial components have been discovered (10;20-24). The proposed role of $CD8^+$ T cells in TB is the recognition of cells in which the MTB bacteria are no longer successfully suppressed (24;25).

The main function of these $CD8^+$ cells is to lyse the infected cells, but they can also produce IFN-γ. Other cells that are critical for controlling the infection with TB are Natural Killer (NK) cells and NK-like T cells which are reported to produce considerable amounts of IFN-γ early during infection. Another cell type involved in infection are the regulatory T cells (Tregs), these cells are suggested to suppress the immune response in order to prevent excessive tissue damage (26). After the initial infection with MTB, when the bacteria are contained, most of the short-living, activated T lymphocytes will go into apoptosis. However, some of these cells will differentiate further and become long-lived memory cells that can respond to a new infection more rapidly (27).

After inhalation of TB bacteria extensive influx of cells into the infected lung tissue is seen. Together with the infected tissue these cells will form a barrier to contain the TB bacteria called a granuloma. While specific T cell immunity has not yet developed, dissemination to the regional lymph nodes and to distant sites via blood stream occurs. Development of specific T cell immunity most often results in containment in granulomas and a state of latent infection; the subject is infected but the immune system is able to contain MTB and the individual does not have any clinical symptoms. However, in about 10% of latently infected individuals reactivation of LTBI occurs and active TB develops. Whether or not this will happen is not yet completely understood, but it is believed to be the result of the balance between host-, environmental- and pathogen factors (28). During reactivation, the immune response can be strong, but ineffective ultimately leading to tissue necrosis. Due to necrosis the content of the confluent granulomas containing live MTB will come into contact with the airways of the patients, which gives the possibility of transmission of the disease. Transmission can occur when live MTB are coughed up by a patient with 'open' active lung TB and aerosols containing the bacteria are inhaled by others.
TB reactivation can occur at any site where MTB is present and TB thus can affect most organs and tissues present in the body. The most common feature of active TB is pulmonary TB which is the manifestation responsible for keeping the transmission chain ongoing. Clinical symptoms of active TB often consist of persistent cough, night sweats, fever, and weight loss. The risk of reactivation of latent TB infection is even higher than 10% in individuals who are immuno-compromised, like those who have an HIV/AIDS infection or who are receiving TNF-α antagonist treatment (29-31). The reason for this enhanced risk of reactivation is a shift in the balance of the immune system in favor of the MTB. For example during anti-TNF-α treatment granulomas are disrupted and the bacteria can replicate and disseminate (32).

One of the things most needed in control of TB is an assay to detect those 10% of all latent infected individuals who will develop active TB. That would allow to give prophylactic treatment with e.g. isoniazide (INH) only to those who would actually benefit from it and prevent overtreatment of individuals who are not at risk.

Diagnosis of tuberculosis

Active TB

Active TB can, as discussed above, present itself with many different faces. Lung TB is the most common form of active TB and can be detected by making a radiographic image of the lungs. Furthermore, if it is possible to obtain material, the bacteria can be directly visualized by an acid-fast staining dye like auramine-rhodamine or Ziehl-Neelsen or by performing PCR to detect the bacteria and/or bacterial DNA (33). A third option is to culture the bacteria, although this is a quite time-consuming procedure that does not allow a rapid diagnosis. Examples of material used to directly demonstrate the presence of MTB are sputum, bronchial lavage fluid or tissue biopsy. A drawback of these procedures is that sometimes it is difficult to obtain material and some are invasive, without guarantee of success. A different assay that can be used to diagnose active TB is the tuberculin skin test (TST). However, the TST might be false negative in patients with active TB, due to anergy of the T cells (34-37). Furthermore, the TST is not able to make a distinction between active and latent TB infection (LTBI) (33).

Latent TB infection

The diagnosis of latent TB infection relied until recently solely on the century old TST. The TST is performed by intradermal injection of 0.1 ml purified protein derivative (PPD) in the left forearm of the patient. The delayed type (type IV) hypersensitivity response to PPD results in local influx of immune cells. After 48-72 hours, induration is measured as an indication of delayed type hypersensitivity. Several guidelines propose different cut-offs for positivity as based on the setting, ranging from 5 mm in children and immuno-compromised individuals, 10 mm in contact investigations and in the clinic, up to 15 mm in BCG vaccinated subjects (33;38;39). In the Netherlands, the guidelines for testing and treatment of LTBI revised in 2005 also used graded cut- off values depending on origin, age, immune status and setting (40). The advantages of the TST are that the test is cheap and has been studied extensively in the past. A disadvantage of the TST is that PPD is an ill-defined crude extract of killed MTB. PPD contains antigens also present in other mycobacteria, such as the *M. bovis* bacillus Calmette–

Guérin (BCG) vaccine strain and environmental mycobacterial species (non-tuberculous mycobacteria (NTM)) and is therefore not specific for MTB (35;41). However, it has been described that if BCG-vaccination is given before the first year of life this will not have influence on the TST 10 years later (42-44), and TST indurations due to vaccination are generally smaller than those cause by true infection. To complicate things further, it has been described that repeated TSTs can induce boosting in BCG vaccinated TB naïve individuals, leading to false positive responses (45;46). This makes determination by skin test of the presence of a latent infection difficult. Furthermore once positive, the TST as a rule remains positive for a lifetime excluding the possibility to detect re-infections with this test (35;45;47). Other disadvantages of the TST are that the healthcare worker performing the test needs adequate training before being able to execute the test correctly and two patient visits are required, one for administration of the TST and one for reading. Taken all limitations into account it is surprising that no alternatives to the TST were developed.

Interferon-γ release assays (IGRA)

Not until the beginning of this century new tools became available to detect TB infection, to which the deciphering of the *M.tuberculosis* genome contributed importantly (48). It was discovered that a specific region of the MTB genome, region of difference 1 (RD-1) was present in all MTB isolates, but absent in BCG-strains and most NTM (49-51). The discovery of this region offered the potential to specifically detect patients with TB infection. The RD-1 region codes for multiple proteins, those most extensively evaluated are Early Secreted Antigenic Target 6 (ESAT-6) and Culture Filtrate Protein 10 (CFP-10) (52-58). The function of ESAT-6 and CFP-10 is not yet completely understood, but they appear to be essential virulence proteins that are secreted early during infection by MTB bacteria. It was demonstrated that they inhibit NFκB transactivation by downregulation of reactive oxygen species (ROS) in the infected cell and in this way manipulate the immune response (59). ESAT-6 also inhibits TLR signaling in macrophages (60) indicating that these proteins might interfere with a protective immune response and thereby assist in the evasion of MTB from eradication. Both ESAT-6 and CFP-10 were found to be strongly immunogenic and broadly recognized by interferon-gamma (IFN-γ) producing T lymphocytes which make them excellent candidates for diagnostic assays. Identification of MTB-specific antigens provided the potential to develop an assay to specifically detect individuals infected with MTB (61).

At the time that the work described in this thesis was started there were two commercially available formats using ESAT-6 and CFP-10: QuantiFERON-TB® Gold and T-SPOT.*TB*®. The assays are T cell based in-vitro assays and determine the production of IFN-γ as a tool to measure T cell recognition after stimulation with the TB specific antigens. Both assays are also widely known as interferon-gamma release assays (IGRA). QuantiFERON-TB Gold is a whole blood assay based on the detection of IFN-γ production by ESAT-6 and CFP-10 specific T cells. This assay determines the concentration of the IFN-γ produced by antigen specific T cells with enzyme linked immunosorbent assay (ELISA), after an incubation period of 24 hours. This assay exists in two different formats, in the first format the antigens need to be added to 1 ml of blood in a 48-well plate. The second is an in-tube format (QuantiFERON- TB® Gold in-tube (QFT-GIT)) in which 1 ml of blood is drawn directly to a tube coated with the TB specific antigens. The in-tube format incorporates a peptide of TB7.7, which is an additional TB specific antigen encoded by RD11 and that was found to increase sensitivity (54). During the time span of the work described in this thesis an additional tube containing PHA became available, the positive control allowing to assess overall T cell responsiveness as this can be decreased in patients with impaired immune status or those with active TB. However, in immuno-competent individuals the in-tube format can still be used without a positive control.

T-SPOT.*TB* is based on the ELISPOT technique using stimulation with ESAT-6 and CFP-10 in separate wells for a maximum of 20 hours. The test requires that blood cells are first isolated and counted as a predefined number of cells are incubated in 96 well microtiter wells, the bottom of which is composed of a membrane precoated with anti-IFN-γ antibodies. During incubation IFN-γ is bound to the antibodies just below that particular cell. Following incubation cells are washed away and bound IFN-γ is stained resulting in a visible spot for each IFN-γ producing cell. This assay thus allows quantification of the number of antigen-specific T cells producing IFN-γ. Besides a different technique, the assays differ also in the fact that QFT-GIT stimulates with a combination of ESAT-6, CFP-10 and TB7.7, while T-SPOT.TB stimulates with ESAT-6 and CFP-10 separately. Cut-offs for both IGRA have been determined by the analysis of responses in a healthy population of uninfected subjects compared to those in a population of patients with proven active TB. It should be noted that various other mycobacterial species also encode RD1, including *M. leprae*, *M. kansasii*, *M. marinum* and *M. szulgai* (62). This could interfere with the detection of TB in for example an M. leprae endemic region.

It has been postulated that IGRA rely on the presence of effector T cells (63) directed towards the TB specific antigens ESAT-6 and CFP-10 and therefore they might also be able to detect LTBI. This can be regarded both as an advantage as well as a limitation of the IGRA (64). A test more specifically identifying those at risk of developing active TB is badly needed, but on the other hand it would limit the ability to diagnose active TB infection in a patient when it is impossible to make the discrimination between LTBI and active TB. Several studies used ESAT-6 and CFP-10 peptides in an in-house ELISPOT to investigate if LTBI can be detected. These studies showed that there was a strong correlation between a positive response in the ELISPOT and a gradient of exposure of healthy symptomless contacts of active TB patients, in contrast to the TST (65-69). Another study showed that there was strong recognition of ESAT-6 and CFP-10, measured as the concentration of IFN-γ produced, in those healthy household contacts who later developed active TB (70). Furthermore, a study in the Netherlands showed that responses to ESAT-6 and CFP-10 were more specific than the TST for LTBI, but the study also showed that these responses were also less sensitive (71). Together these data suggested that IGRA might be more accurate in identifying those LTBI individuals than the TST (64;72), but more studies were required also with regard to the sensitivity and specificity of both commercial IGRA for determination of LTBI. The advantage of both commercial IGRA in comparison with the TST is that no cross-reactions are expected to occur in BCG vaccinated individuals or individuals infected with most NTM. Besides this, only one patient visit is required, eliminating loss of patients who do not show up for reading of the test as is a not uncommon occurrence with the TST.

At the time of initiation of this PhD research project many questions were still unanswered with regard to the use of IGRA for the diagnosis of LTBI. E.g. the sensitivity and specificity of IGRA for detection of LTBI had not been studied in detail. A selection of these questions has been addressed in this thesis, which has focused on the use of IGRA for detection of LTBI in various clinical-epidemiological settings.

OUTLINE OF THE THESIS

The focus of this thesis is on the evaluation of the MTB specific IGRA as supplement to or replacement of the century old TST.

In the study described in **Chapter 2** we evaluated the use of the commercially available QuantiFERON-TB Gold in-tube (QFT-GIT) for the diagnosis of LTBI and

differentiation from NTM infections in personnel of the Royal Dutch Armed Forces after deployment to a region where TB is endemic (73).

Subsequently, we performed IGRA repeatedly during follow-up of subjects with a positive skin test or those with a positive IGRA result in association with a negative skin test, who were included in a large scale contact investigation carried out among clients of a supermarket after diagnosis of an employee with highly contagious TB. The study that was conducted at the time of the contact investigation and from which study subjects for the follow-up were recruited is summarized in **Chapter 3** (74). In **Chapter 4** the results are described of the follow-up of those individuals with a positive TST in the large scale contact investigation and who were either treated for LTBI or followed without treatment by the Municipal Health Authority (76). Individuals were asked to participate whether or not they received preventive treatment and both IGRA were repeated every 6 months for a 2 year period. **Chapter 5** focuses on those individuals detected during the large scale contact investigation as having a positive IGRA but a negative TST result (75). All assays were repeated one year later.

Another unknown aspect of the commercially available IGRA is whether or not they can predict who will develop active TB. To answer this question a study was initiated that included first ring, immigrant contacts of a smear-test positive TB patient. The participants did not receive preventive treatment as treatment of LTBI is not part of TB control policy among BCG vaccinated subjects who do not normally undergo TST in a contact investigation. For the purpose of this study, TST as well as both IGRA formats were performed and participants were followed during 2 years to detect newly developed TB cases. **Chapter 6** describes the base-line analysis of this study, including risk factors for a positive TST and IGRA result and in **Chapter 7** the results after 2 year follow up period are discussed.

The next two chapters address more technical questions about the assays. **Chapter 8** addresses agreement and disagreement between observers of different laboratories and automated readers with regard to the interpretation of the T-SPOT.*TB* assay results of data that were available from the study described in Chapters 5 and 6. **Chapter 9** describes the influence of the prozone effect in determining the exact interferon-γ concentration produced in the QuantiFERON-TB Gold in-tube when levels are in the high range.

IGRA both rely on the in vitro response of T cells towards the TB specific antigens ESAT-6 and CFP-10. In stead of using these antigens in in-vitro assays, another application could be to use these antigens as skin test reagents in order to replace the nonspecific and ill-defined PPD used in the standard TST. **Chapter 10** describes the results of a phase I clinical trial using recombinant dimer (rd) ESAT-6 in comparison to PPD as skin test reagent in healthy subjects and cured

TB patients (77). Previous research in our laboratory showed that some latently infected individuals are negative in the short term IGRA (20/24 hours) but are positive when cells are stimulated for 6 days in a lymphocyte stimulation assay (78). This encouraged us to investigate differences in cell populations producing interferon-gamma after 24 hours and 6 days of stimulation as well as comparing responses of former patients with active TB with the immune response of latently infected individuals who were IGRA negative but positive in a 6 day assay system. Results of this study are discussed in **Chapter 11**.
In **Chapter 12** results of the previous chapters will be summarized and discussed.

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