Tuberculosis 106 (2017) 62–72

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

IMMUNOLOGICAL ASPECTS

Immunological characterization of latent tuberculosis infection in a low endemic country

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ARTICLE INFO

Article history:
Received 21 March 2017
Received in revised form
30 June 2017
Accepted 3 July 2017

Keywords:
Tuberculosis
Latent tuberculosis infection
Quantiferon
Tuberculin skin test
Lymphocyte stimulation

ABSTRACT

The diagnosis of a latent tuberculosis infection (LTBI) is based on detection of immunity against *Mycobacterium tuberculosis (Mtb)*. The tuberculin skin test (TST), the Quantiferon (QFT) and a prolonged lymphocyte stimulation test using either ESAT-6/CFP-10 (LST-EC) or PPD (LST-PPD) were evaluated in a cohort of 495 individuals, suspected to have LTBI, in a low endemic country. While the TST and LST-PPD were both positive in the majority (75%) of individuals, only one third responded in the LST-EC and in the QFT. The choice for LTBI treatment was significantly associated with ESAT6/CFP10 recognition, however the LST-EC detected considerably more individuals (21%) with immunity against *Mtb*, who might also be at risk for development of active TB, although none of them did during follow up. Follow-up for 2 years showed 7% conversions and 32% reversions for the QFT. The LST-EC showed higher conversion rates (~45%), although the percentage of individuals positive in the LST-EC did not change significantly within the follow-up period. LTBI treatment did not alter immune recognition of *Mtb* antigens. In conclusion, the sensitivity of tests for detection of cellular immunity to *Mtb* specific antigens depends on test methodology and may vary considerably over time in a low endemic region.

1. Introduction

The world-wide burden of tuberculosis (TB) is high and although the global TB incidence has been slowly declining for years, TB is still one of the most deadliest infectious diseases (global TB report 2016, www.who.int). Many TB cases result from reactivation in individuals with latent TB infection (LTBI) because migration and travelling across borders between countries and disease-burdened regions. An important part of TB control in low TB endemic areas is based on screening of individuals at risk for developing TB and provision of LTBI treatment to those infected [1]. The Netherlands is a low endemic country with a notification rate of less than 5 TB cases per 100,000 person-years (surveillance report 2016, euro.who.int). In contrast to active TB, LTBI notification is not mandatory in the Netherlands but since several decades confirmed LTBI cases are often registered by the TB department of Municipal Public Health Services (PHS). From 2005 till 2013 the annual number of registered cases with latent tuberculosis infections (LTBI) in the Netherlands dropped by 37%, of which approximately 75% percent received preventive LTBI treatment [2]. This drop was caused by the introduction of interferon-γ release assays (IGRA), such as the Quantiferon TB-Gold (QFT) and T-spot TB in the diagnostic algorithm of LTBI in 2010 [2].

TB screening is only offered to individuals at risk, such as contacts of TB patients, recent immigrants and travellers who have been in TB endemic countries for longer periods and health care workers. Immigrant screenings mostly aim at detection of TB disease, but LTBI infection may be a concomitant finding. The likelihood of latent infection and the risk of developing active TB are assessed per individual and form the basis for treatment [3]. A risk–profile is made based on the individual's infection related characteristics, chest X-ray findings and the tuberculin skin test (TST), but may also include assessment of the degree of exposure, likelihood of prior infection with *Mycobacterium tuberculosis (Mtb)*, BCG vaccination status, medical history and comorbidities. Between 2005 and 2010 IGRAs have been gradually implemented in the Dutch LTBI screening program [2] (guidelines at www.kncvtbc.org) and is currently advised for individuals with a

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http://dx.doi.org/10.1016/j.tube.2017.07.001
1472-9792/

positive TST result, a method which was found cost-effective [4].

The TST and QFT are immunological tests to assess whether an individual has developed cellular immunity to *Mtb*. In contrast to the TST, the QFT is based on recognition of mycobacterial antigens that are not present in *Mycobacterium bovis* Bacille Calmette-Guerin (BCG), the only currently available vaccine against TB and therefore considered to indicate infection with *Mtb* more specifically [5,6]. In the diagnosis of LTBI, QFT has a stronger correlation with exposure compared to TST for high risk groups in low endemic countries such as The Netherlands [7]. This can be explained largely by the fact that BCG vaccination or previous infection(s) with non-tuberculous mycobacteria (NTM) can give rise to false-positive TST results [8,9]. Indeed, in previous immunological studies in the Netherlands we have found that a considerable proportion of healthy Dutch Blood Bank donors do respond to PPD in *in vitro* laboratory tests such as 6 day lymphocyte stimulation tests (LST) [10,11]. The sensitivity of the TST and QFT is around 70 to 80% for patients diagnosed with active TB [12]. However, test results can vary considerably over time both due to technical and biological variation [13,14]. In serial testing spontaneous conversions and reversions were observed with the TST [15] and even more frequently with the QFT [16–20].

The QFT detects a cellular immune response against peptides of *Mtb* antigens ESAT-6, CFP-10 and TB7.7 by measuring IFN-γ release after stimulating whole blood *in vitro* for 16 to 24 h. Individuals with more remote LTBI infection may have low frequencies of circulating memory or effector T-cells and may therefore have a negative QFT test result [11]. Moreover, QFT responses may be established slowly and/or may be reduced by regulatory T-cells [21]. Hence, a prolonged stimulation with *Mtb* antigens could enhance assay sensitivity. Six day stimulation of peripheral blood mononuclear cells (PBMCs) with PPD or recombinant *Mtb* antigens has previously been applied to increase the sensitivity of detecting presumed memory T cell responses [22–24]. The prolonged lymphocyte stimulation test (LST, 6 days) was previously compared with two commercially available IGRA formats (QFT and T-spot TB) and with the TST [22]; the LST was in good agreement with the TST, while IGRAs were positive in only roughly half of the infected individuals, indicating that the LST was more sensitive for the detection of (remote) TB infection.

In the current study we describe the characteristics of a Dutch LTBI follow-up cohort (600 participants). Predominantly individuals with a positive TST (≥10 mm) were included and during 2 years of follow-up the kinetics of QFT and LST responses were measured, using both PPD and recombinant ESAT-6/CFP-10 fusion protein (EC) in the 6 day LST.

2. Methods

2.1. Data collection

The study was approved by the local Medical Ethics Committee of the Leiden University Medical Center (METC project nr: P07.048). All participants signed written informed consent forms. Between April 2007 and September 2013 individuals with a positive TST (≥10 mm) were referred to our research study nurse (CP) by a physician at the PHS or a physician at the Leiden University Medical Center (LUMC). Occasionally individuals with TST values below 10 mm were referred to the study by consulting physicians because of clinical indications for LTBI with a desire to characterise *in vitro* immune recognition. Amongst them were immigrants, health care workers and individuals who had been in close contact with active TB patients (Tables 1A and B). Individuals with an age between 18 and 65 years were included. Immunocompromised individuals and individuals with other diseases that may affect immune responses such as arthritis, hepatitis, Pfeiffer's disease, psoriasis and cancer were excluded. Individuals with clinical suspicion of AIDS were screened for HIV infection and excluded from immunological analysis when positive (n = 1). Based on the medical history, recent exposure to TB, complaints, TST result, chest X-rays findings, QFT result and/or detection of *Mtb* in sputum (Ziehl-Neelsen staining, Polymerase Chain Reaction or culture), TB/LTBI treatment or 2 years of X-ray follow-up was proposed, according to the national guidelines (www.kncvtbc.org). The chest X-ray was considered positive for abnormalities when pleural and/or lung abnormalities, compatible with active TB or old healed TB, were found. By venous access, 45–50 ml of whole blood was drawn at the time of inclusion and all participants were invited again at 3, 6, 12 and 24 months thereafter. At the start of this study the QFT was not yet commonly used in The Netherlands as this was introduced only gradually from 2005 [2], however the physicians of the PHS Leiden were informed in real-time regarding the QFT results obtained in this study and weighed these in the diagnostic process. The physicians were not informed about the LST results.

2.2. Immunological assays

The TST was performed by trained staff from LUMC or PHS by intradermal injection of 2 TU Tuberculin (RT23, SSI, Denmark). The induration was measured after 3 days, a TST result ≥10 mm was considered positive. TST administration and reading was performed at the PHS and the LUMC occupational health department. Reference for the study occurred after positive TST reading, blood samples for immunological assessment were taken at a median of 16 days (mean 46 days) post TST reading. QuantiFERON-TB Gold in tube test (QFT) (Qiagen, The Netherlands) was performed at inclusion and after 24 months of follow up. The QFT was performed according to the manufacturer's instructions, all at a single laboratory (Department of Infectious Diseases, LUMC). QFT results were considered positive if ≥ 0.35 IU/ml IFN-γ; QFT results are reported as obtained, however it is important to consider all values above 10 as 'high' and not compare the absolute values as this is outside the range of the standard curve. In addition, a standardized 6 day lymphocyte stimulation test (LST) was performed on freshly isolated peripheral blood mononuclear cells (PBMCs). PBMCs were isolated from heparinized blood by Ficoll separation (LUMC Pharmacy, The Netherlands) using LeucoSep tubes (Greiner Bio-one, The Netherlands). $0.5*10^6$ PBMCs were cultured in a 48 wells plate (Corning Costar, USA) in 0.6 ml AIM-V culture medium (Gibco, Life Technologies, The Netherlands) in the presence of medium alone, purified protein derivative from *Mtb* (PPD) (5 μg/ml, RT49, SSI, Denmark), *Mtb* ESAT-6/CFP-10 (EC) fusion protein (5 μg/ml, LUMC [25,26]) or phytohemagglutinin (PHA) (2 μg/ml, Oxoid Ltd, UK) as a positive control. Cells were incubated at 37 °C in a humidified incubator with 5% CO_2 for 6 days, after which supernatants were collected and IFN-γ levels were determined by ELISA according to the manufacturer's protocol (U-CyTech, The Netherlands). The final LST response to PPD (LST-PPD) and to EC antigens (LST-EC) was calculated by subtracting the value of the medium control from the value of the PPD or EC stimulation, respectively. Results were regarded valid if the medium (negative) control was <500 pg/ml and the PHA (positive) control was >1000 pg/ml. The maximum value of the standard curve was 10,000 pg/ml, results above the standard curve were expressed as ≥10,000 pg/ml.

2.3. Statistical analysis

Proportional analyses were performed using the Pearson's χ^2 test. The Fisher's exact test was chosen if there were, as expected, less than 5 individuals present in a sub-group. For comparison of