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A new method of screening for latent tuberculosis infection: Results from army recruits in Beijing in 2014

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ABSTRACT

Latent tuberculosis infection (LTBI) lacks diagnostic gold method. Effective method is important to the control of tuberculosis. IFN-γ responses in 600 military recruits were tested by ELISA using whole blood incubation with latent protein Rv2029c, Rv2659c and recombinant protein CFP10-ESAT6 (rCE) respectively. They also received tuberculin skin test. Their BCG vaccination status was recorded. When 30.7% (184/600) of recruits gave higher IFN-γ responses (≥470 pg/mL) to rCE as LTBI, the rests as healthy control, the AUC of rRv2029c was 0.856 and rRv2659c was 0.827 for LTBI diagnosis. IFN-γ responses to rCE were higher in PPD-positive group (≥5 mm) than negative group (<5 mm) ($p < 0.05$), while for rRv2029c and rRv2659c were not ($p > 0.05$). IFN-γ responses induced by rRv2029c and rRv2659c were higher in the moderately-positive group (≥5, <15 mm) than the strongly-positive group (≥15 mm) ($p < 0.05$), while for rCE were not ($p > 0.05$). IFN-γ levels to three antigens were not related to BCG vaccination status ($p > 0.05$). Rv2659c and Rv2029c are good candidate antigens to complement the role of rCE for LTBI diagnosis, which provide a basis for developing cost-effective LTBI screening methods in the army.

1. Introduction

Latent *Mycobacterium tuberculosis* infection (LTBI) is composed of a diverse range of host states, from those who have completely cleared the *M. tuberculosis* to those who are incubating actively replicating *M. tuberculosis* in the absence of clinical symptoms [1–3]. Approximately 10% of healthy LTBI individuals have risk of developing active TB disease in their lifetime [4]. The enormous reservoir of LTBI embodies a major obstacle in achieving worldwide control of TB. But there is no gold standard for diagnosis of LTBI [5,6]. As a special population, new army recruits may be at high risk of TB infection because they will live and work in close contact environments [7]. Therefore, a cost-effective method for LTBI screening is important for large-scale screening.

The tuberculin skin test (TST) is often used for detecting *M. tuberculosis* infection in developing countries, as it has low cost and does not require complex laboratory operations. However, TST has several problems using purified protein derivative (PPD) as the antigenic stimulus, which contains more than 200 *M. tuberculosis* antigens that are also present in the vaccine *M. bovis* BCG and most environmental mycobacterial species. Hence, the TST has low specificity and cannot differentiate among the infection with *M. tuberculosis*, prior vaccination with BCG vaccine and infection with environmental mycobacteria [8,9]. The commercial in vitro interferon-γ (IFN-γ) release assays (IGRAs) are more specific than PPD skin tests because they only contain antigens that are specific to *M. tuberculosis*: 6 kDa early secreted antigenic target (ESAT6) and culture filtrate protein 10 (CFP-10) [10–12]. However, ESAT-6 and CFP-10 are secreted by the bacteria in the proliferative phase of infection and it is not clear if adding latency-associated antigens might help for detecting LTBI. Similarly, it is not known if high responses to ESAT-6 and CFP-10 might predict a higher risk of developing active TB. In either circumstance, diagnostic tests suitable for use in high TB prevalence countries might be improved.

We focused on 2 latency-associated proteins, Rv2659c and Rv2029c. The rv2659c encoded a kind of phiRv2 phage integrase, which located in the 11th region of difference (RD11) in *M. tuberculosis* genome [13]. Rv2659c was one of the starvation regulon expressed in vitro during 6-week-starved culture of *M. tuberculosis* [14]. The *rv2029c* encoded a kind of phosphofructokinase pfkB in *M. tuberculosis*, which was one of the dormancy (DosR) regulon expressed in vitro during hypoxia and low-dose nitric oxide stimulation [15]. Several researchers had demonstrated that the two antigens could be preferentially recognized by persons with LTBI, compared with persons with TB disease [16–18]. They elicited stronger T-cell immune responses in LTBI than in active TB patients

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in several countries and districts [16–18] and we have reported higher immune responses to these antigens in LTBI than in active TB in China [19]. Accordingly, this study aimed to evaluate the potential value of quantifying responses to latency-associated antigens Rv2029c and Rv2659c in addition to CFP10-ESAT6 using whole blood incubation and enzyme-linked immunosorbent assay (ELISA) in new recruits to the Chinese army.

2. Materials and methods

2.1. Study subjects

A total of 600 new recruits to Beijing garrison troops were selected as the participants in 2014. They received routine physical examinations, chest X-ray and PPD skin test. All tested showed normal physical and X-ray examination results and negative for HIV infection. They were all male and their ages ranged from 17 to 25 years old with an average age of 19.0 ± 1.0 years old. We recorded BCG vaccination status by looking for vaccination scars on the arms. During the study, if subjects appeared to have signs or symptoms of relevant disease, monitoring personnel would immediately investigate, register and report. The study was overseen by the Ethics Committee of PLA 309th hospital. Written informed consent was obtained from all participants.

2.2. PPD skin tests

0.1 mL of 5 IU tuberculin PPD was injected intradermally in the left forearm of participants (Mantoux technique). The diameters of both axes of skin induration were measured by the same certified doctor at 72 h after antigen injection. Subjects with skin induration of maximum diameter not less than 5 mm was taken as the positive group and a strongly positive group was defined when the induration was not less than 15 mm in diameter according to the standard proposed by Chinese anti-tuberculosis association [20].

2.3. Recombinant antigen preparation

Three antigens were produced and quality controlled by our laboratory as described previously [19,21–24]. Briefly, genes *rv2029c*, *rv2659c* were amplified by PCR and cloned into pET30a plasmid (Invitrogen, San Diego, CA, USA) and fusion gene *cfp10-esat6* were amplified and cloned into pET28a plasmid (Invitrogen, San Diego, CA, USA) respectively, both bacterial expression vectors containing an N-terminal histidine tag. The proteins were overexpressed in Escherichia coli BL21 (DE3) and purified, as described previously [19,21,22]. Purity and size were checked by gel electrophoresis. Residual endotoxin levels were determined with a Limulus Amebocyte Lysate (LAL) Assay (Associates of Cape Cod Inc., East Falmouth, MA, USA) and were found to be below 50 IU/mg recombinant protein. Recombinant antigens were freeze-dried and stored at −80 °C until testing in whole-blood assays was done.

2.4. Whole blood incubation

Whole blood was collected in heparinized syringes before the PPD skin test was done and transported to the laboratory within 2 h after collection. Blood was diluted in RPMI-1640 medium (Solarbio. Co. Ltd., Beijing, China) containing 10% fetal bovine serum (Sigma, final dilution 1:1), and incubated with a *M. tuberculosis* recombinant CFP10-ESAT6 protein (rCE, final concentration 20 μg/mL), recombinant Rv2029c protein (rRv2029c, final concentration 20 μg/mL), or recombinant Rv2659c protein (rRv2659c, final concentration 20 μg/mL), in 800 μL volumes in 24-well U-bottom plates (Costar, Washington, D.C., USA), for 24 h at 37 °C and 5% CO_2 in a humidified incubator. All antigens were prepared in our Laboratory. Blood incubated without antigen or with phytohaemagglutinin (PHA, final concentration 20 μg/mL) were used as negative and positive controls, respectively. After the incubation, 400 μL supernatant (SN) was carefully harvested and stored at −80 °C.

Table 1
Whole-blood IFN-γ release induced in 600 new recruits.

Levels of IFN-γ(pg/mL)	Cases (rate%) of 4 stimulators			
	CFP10-ESAT6	Rv2029c	Rv2659c	PHA
≥ 580	123 (20.5)	77 (12.9)	26 (4.3)	259 (43.6)
≥ 470, <580	61 (10.2)	83 (13.9)	23 (3.8)	81 (13.6)
≥ 300, <470	119 (19.8)	110 (18.4)	76 (12.7)	91 (15.3)
≥ 190, <300	122 (20.3)	99 (16.6)	105 (17.5)	71 (12.0)
≥85, <190	11 (19.3)	128 (21.4)	155 (25.9)	47 (7.9)
>0, <85	47 (7.8)	80 (13.4)	191 (31.9)	35 (5.9)
0	12 (2.0)	20 (3.4)	23 (3.8)	10 (1.7)
Total	600	597	599	594

2.5. IFN-γ measurement in supernatants

The IFN-γ level in culture supernatants was quantified by sandwich ELISA (BD OptEIA™ ELISA Sets). Wells of 96-well ELISA plates (Costar, Washington, D.C., USA) were coated with 100 μL per well of Capture Antibody diluted in coating buffer and incubated overnight at 4 °C. On the following day, the plates were washed 3 times with wash buffer and blocked with 200 μL of blocking buffer at room temperature (RT) for 1 h. Then the plates were washed 3 times. 100 μL of each standard or harvest supernatant was added into appropriate wells. Sealed plates were incubated for 2 h at RT then washed 5 times, 100 μL of Working Detector (Detection antibody + SAv-HRP reagent) per well was added and incubated for 1 h at RT then the plates were washed 7 times, 100 μL of Substrate Solution per well was added and the plates incubated for 30 min at RT in the dark. For the final detection step, 50 μL of 2 N H_2SO_4 per well was pipetted into plates and then optical density (OD) was determined at 450 nm on an ELISA plate reader. The standard curve was generated as a 7-point curve using serial dilutions of the IFN-γ standard; the point values were 600, 300, 150, 75, 37.5, 18.75 and 0 pg/mL.

2.6. Data management and statistical analysis

All data were recorded in Microsoft Office Excel file. Antigen-specific IFN-γ responses were calculated after subtracting the amount found in un-stimulated blood aliquot. Because the majority of the data did not show a normal distribution, they were expressed with the median and interquartile range (IQR). The data were grouped to 7° according to the IFN-γ value as follows: 0; >0 to <85; ≥85 to <190; ≥190 to <300; ≥300 to <470; ≥470 to <580; ≥580 pg/mL. The recruits who responded to rCE with IFN-γ values ≥ 470 pg/mL were taken as LTBI, which was based on the assumption that one-third of the population was LTBI worldwide and the observation that one-third of our recruits showed IFN-γ values ≥ 470 pg/mL [1]. The Kruskal-Wallis test was used to assess the difference among different groups. The statistical analyses were done using SPSS18.0; $P < 0.05$ was considered to be significant. Graphs were generated using basic Microsoft Excel software.

3. Results and discussion

3.1. IFN-γ levels induced by three recombinant proteins

IFN-γ release induced by *M. tuberculosis* rCE, rRv2029c, rRv2659c proteins and PHA are shown in Tables 1 and 2 and Fig. 1. The numbers and proportions of participants with responses at the different rank levels are shown in Table 1. PHA was used as the