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Utility of B-cell epitopes based peptides of RD1 and RD2 antigens for immunodiagnosis of pulmonary tuberculosis

Bela Goyal [a], Krishan Kumar [b], Dheeraj Gupta [c], Ritesh Agarwal [c], Romica Latawa [a], Javaid Ahmad Sheikh [a], Indu Verma [a,*]

[a] Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Sector-12, Chandigarh, India
[b] ESI Baddi, India
[c] Department of Pulmonary Medicine, Postgraduate Institute of Medical Education and Research, Sector-12, Chandigarh, India

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ABSTRACT

Tuberculosis (TB) continues to be a major health problem due to lack of accurate, rapid, and cost-effective diagnostic tests. Serodiagnostic tests incorporating highly specific region of difference (RD) antigens (early secretory antigenic target 6 [ESAT-6], culture filtrate protein 10 [CFP-10], culture filtrate protein 21 [CFP-21], and mycobacterial protein from species tuberculosis 64 [MPT-64]) have recently been shown to be promising for specific diagnosis of TB in our lab. However, only few studies have reported the use of synthetic peptides of RD antigens, and none has used them to differentiate TB from sarcoidosis, a close mimic of smear-negative pulmonary TB (PTB) with entirely different management. The present study was conducted with an aim to study the utility of B-cell epitopes based peptides of RD1 (ESAT-6, CFP-10) and RD2 (CFP-21, MPT-64) antigens for immunodiagnosis of PTB for which sputum smear-positive PTB patients, sputum smear-negative PTB patients, sarcoidosis patients, and healthy controls (n = 24/group) were recruited. Bioinformatic software Bcepred was used to predict linear B-cell epitopes, using physico-chemical properties on a non-redundant dataset. Seven peptides as representative B-cell epitopes of ESAT-6, CFP-10, CFP-21, and MPT-64 were evaluated as targets of the antibody responses in TB patients and controls by enzyme-linked immunosorbent assay (ELISA). The current study showed sensitivity with individual peptides ranging from 37.5% to 83.3% for smear positive, 25% to 58.3% for smear negative as compared to 4.16% to 20.8% for sarcoidosis. Four out of 7 peptides that showed higher reactivity with TB patients and better discrimination from sarcoidosis patients representing ESAT-6, CFP-10, CFP-21, and MPT-64 were selected for multiepitope ELISA. The combination of peptides yielded 83.3% sensitivity for smear positive, 62.5% for smear negative, and only 4.16% for sarcoidosis. The specificity, however, for all the peptides/combination was 100%. Combination of peptides has proven to be better than individual peptides as per the latest criteria of the World Health Organization according to which a test that can replace smear microscopy with sensitivity of >90% for smear-positive patients and >65% for smear-negative TB patients with a specificity >95%, and thus, the present study suggests that a test based on combination of peptides selected from mycobacterial RD1 and RD2 antigens could be important for promoting an early diagnosis and management of otherwise difficult to diagnose smear-negative PTB patients. Moreover, it can also be used to discriminate sarcoidosis from PTB, thus preventing the misdiagnosis and mismanagement.

1. Introduction

Current diagnosis of tuberculosis (TB) relies on clinical interpretation, sputum microscopy, microbiology, and radiography. However, these diagnostic tools suffer limitations of speed, specificity, and sensitivity (Katoch, 2004). The development of new diagnostic tests for the rapid and specific diagnosis of sputum smear-positive TB and the improvement of the differential diagnosis of sputum smear-negative TB are the 2 priorities for TB diagnosis. In particular, the detection of TB by serological methods has been a subject of great interest, with regard to patients who are unable to produce adequate sputum or sputum smear-negative or suspected of having extra-pulmonary TB. Moreover, such a test would be rapid, easy to perform, and user friendly and could be implemented easily in the conditions found in developing countries. Recently, the World Health Organization (WHO) report, released at the end of December 2010, WHO Strategic and Technical Advisory Group (STAG-TB) for TB has issued the negative recommendations for the use of currently available commercial serodiagnostic kits for the diagnosis of active TB (www.who.int/tb/advisory_bodies/stag_tb_report_2010.pdf). However, WHO guidelines do not jeopardize future research and new antigen

* Corresponding author. Tel.: +911722755280.
E-mail address: induvermabio@gmail.com (I. Verma).

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and biomarker discovery programmes that would guide and inform the development of point-of-care tests (Morris, 2011), thus opening new horizons for developing assays using novel antigens.

Comparative genomic studies have helped in revealing some regions of the genome known as region of difference (RD) that is present in *Mycobacterium tuberculosis* complex but absent in *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) substrains and several non-TB mycobacteria (NTM), including *Mycobacterium avium* (Gordon et al., 1999; Behr et al., 1999). Recently, our lab has shown that by using combination of both antibody and antigen detection assays based on combination of two RD1 antigens, i.e., early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), and two RD 2 antigens, i.e., mycobacterial protein from species tuberculosis 64 (MPT-64) and culture filtrate protein 21 (CFP-21), lead to detection of ~65% smear-negative pulmonary TB (PTB) cases with 100% specificity. Moreover, this combination was not recognised by the serum samples of sarcoidosis patients (Kalra et al., 2010).

As compared to recombinant/native protein antigens based assays, diagnostic assays using the peptides are considered to be more advantageous particularly in resource-poor countries due to lower production costs and easier standardization protocols. There are few reports indicating the promising role of serodiagnostic assays based on the peptides of various mycobacterial antigens, e.g., Ag 85B, bfrB, PTRP (Shen et al., 2009; Sartain et al., 2006). However, in the case of RD antigens, studies are available where peptides of ESAT-6 and CFP-10 have been screened for Th1 response (Mustafa et al., 2008) and various commercially available interferon γ release assays such as QFT-G and T-Spot are based on the mixture of these peptides (Pai et al., 2004). On contrary, until today, only few scattered reports are available on the B-cell epitopes of various immunodominant RD antigens including ESAT-6 and MPT-64 (Harboe et al., 1998), and the role of peptides of RD antigens for the development of serodiagnostic assays particularly for the endemic countries is yet to be established.

Sarcoidosis, an idiopathic disease, has always been a cause of concern for clinicians due to its similar clinical and radiological presentation to TB. Due to their entirely different line of management, need for differentiating TB and sarcoidosis is immense. In this regard, RD antigens appear to be an attractive option owing to their specificity. The present study was, therefore, planned to develop a rapid enzyme-linked immunosorbent assay (ELISA)–based assay for the specific diagnosis of TB using synthetic peptides corresponding to B-cell epitopes of 4 well-characterised immunodominant RD1 and RD2 antigens (ESAT-6, CFP-10, MPT-64, CFP-21) individually and in combination.

2. Materials and methods

A case-control, comparative group study was conducted in study population. Serum specimens obtained from 96 subjects who provided informed consent were collected and stored at −80 °C until assay was performed and grouped into following 4 categories. Of these, 60 were males, 36 were females, and their age ranged from 17 to 73 years with mean age of 39.51 years.

2.1. Subjects

Sputum smear Acid fast bacilli-positive PTB (n = 24) subjects with clinical and radiological evidence of PTB were recruited from Directly Observed Treatment Short-Course centre of our institute. They had symptoms like fever, cough, dyspnoea, night sweats, loss of appetite, or loss of weight among clinical evidence (Table 1). Consolidations, nodules, hypodense region indicating Koch's complex or any opacity in lungs in chest x-ray were the most common radiological findings in TB patients. In view of low seroprevalence, a lack of significant risk in patient's history, HIV status of patients was not tested, and these patients were considered HIV negative (Dewan et al., 2010). Sputum smear Acid fast bacilli-negative PTB (n = 24) subjects were culture positive from sputum/brochoalveolar lavage with clinical or radiological evidence of PTB as described above.

Table 1
Clinical details of TB/sarcoidosis patients and healthy controls.

Clinical and radiological details	Smear-positive TB	Smear-negative TB	Sarcoidosis	Healthy controls
Mean age (y)	39 (19–60)	45.7 (17–73)	42.75 (24–61)	30.6 (21–57)
Male/female ratio	17:7	20:4	12:12	11:13
Dyspnoea	12	8	8	-
Cough	24	18	17	-
Chest pain	8	4	3	-
Fever	14	9	6	-
Hemoptysis	6	8	3	-
Expectoration	11	9	0	-
Loss of appetite	15	2	1	-
Loss of weight	3	1	1	-
Night sweats	1	13	2	-
Skin manifestations	-	-	3	-
Joint pain	-	-	1	-

Although specifically isolates were not further identified for *M. tuberculosis*/NTMs, however, considering that all the subjects recruited in this study were not having any history of immunocompromisation/HIV as well as their successful response to antitubercular therapy (ATT) confirms that these patients had TB. Sarcoidosis (n = 24) patients with clinical (pulmonary or extrapulmonary) features and radiological evidence suggestive of sarcoidosis or noncaseating granuloma on transbronchial biopsy and healthy controls (n = 24) were also included in this study. These samples were not tested for Purified protein derivative skin test; however, it has been reported that BCG-vaccinated subjects are more likely to give positive skin test (Wang et al., 2002). Blood samples were collected from all the subjects after obtaining written informed consent, and the study was also approved by the Institute Ethics Committee, PGIMER, Chandigarh.

2.2. Prediction of B-cell epitopes and synthetic peptides

Linear B-cell epitopes of RD1 (ESAT-6, CFP-10) and RD2 (CFP-21, MPT-64) proteins were predicted on the basis of bioinformatic software Bcepred program based on various physicochemical properties as described by Saha and Raghava (2007). The server is able to predict epitopes with 58.7% accuracy using hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity, and antigenic propensity. Biotinylated peptides with >90% purity, confirmed by Mas spectrometry (MS) and analytical High performance liquid chromatography by commercial source, were got synthesised having sequence corresponding to predicted epitopes.

2.3. Indirect ELISA

For detecting antibody to peptides of RD antigens in sera of TB/sarcoidosis patients and healthy controls, indirect ELISA was performed. Based on the principle of high affinity interaction between avidin and biotin, ELISA plates were overnight coated with 0.0625 mg/mL of avidin. Biotinylated peptides diluted to the concentration of 10 μg/mL in 3% Bovine serum albumin as blocking buffer were added. 100 μL of optimally diluted sera (1:20 in 0.1× blocking buffer) was then dispensed in plate. Antihuman IgG alkaline phosphatase conjugates (sigma P 7488) at the dilution of 1:2000 and antihuman IgA alkaline phosphatase (sigma A 3400) at the dilution 1:1000 were used as secondary antibodies (Shen et al., 2009). Each experiment was carried out in triplicates at different time points to ensure reproducibility, and the sample showing reactivity at least twice was taken as positive. Mean + 3 SD of Optical density of healthy control group for each assay was taken