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Analysis of humoral responses to proteins encoded by region of difference 1 of *Mycobacterium tuberculosis* in sarcoidosis in a high tuberculosis prevalence country

Sir,

Sarcoidosis is a granulomatous disorder of unknown aetiology characterized by the presence of noncaseating granulomas in multiple organs[1]. Mycobacterial nucleic acids have been demonstrated in sarcoidosis lesions[2], and we have reported 50 per cent prevalence of mycobacterial DNA in sarcoidosis samples using PCR for 65 kDa protein gene[3]. Genes located on the region of difference 1 (RD1) of *Mycobacterium tuberculosis* such as the 6-kDa early secreted antigenic target (ESAT-6) and the 10-kDa culture filtrate protein (CFP-10) are not shared by BCG strains and most non-tuberculous mycobacteria, and these antigens are specific indicators of *M. tuberculosis* complex infection[4]. We hypothesized that if mycobacteria are aetiologically linked to sarcoidosis, the humoral responses against ESAT-6 and CFP-10 should be demonstrable in the serum samples of sarcoidosis patients.

Newly diagnosed glucocorticoid-naïve cases of pulmonary sarcoidosis [18 patients (8 females); mean (95% CI) age, 34.9 (31.9-35.9) yr] defined on the following criteria were included consecutively in this study conducted at the department of Pulmonary Medicine, Postgraduate Institute of Medical Education and Research, Chandigarh, during January - March 2008. *(i)* Clinico-radiological presentation consistent with sarcoidosis; *(ii)* demonstration of non-caseating granulomas on bronchoscopic lung biopsy; *(iii)* absence of other known causes of granulomatous lung disease such as mycobacteria or fungal diseases on histopathology; and, *(iv)* response to steroid therapy[5]. All patients underwent clinical evaluation and investigations, including serum calcium, chest radiograph and high-resolution computed tomography (HRCT) scan of the chest, and tuberculin skin testing (TST) with one tuberculin unit (TU) purified protein derivative (PPD). The subjects were followed up for twelve months after the diagnosis. Patients with pulmonary TB [n=10 (4 females), mean (95% CI) age, 31.4 (22.4-40.4) yr] with clinico-radiological evidence of PTB and sputum smear positive for acid-fast bacilli (AFB), were also included. Patients with any immunodeficiency state were excluded from the study. All patients underwent TST with 1 TU PPD, and were followed up for six months after the diagnosis. The controls were healthy volunteers [20 subjects (7 females), mean (95% CI) age, 31 (26.3-31.7) yr] defined as asymptomatic individuals without any diagnosed illness and were normal on detailed physical examination. The controls were recruited from the healthy attendants accompanying the patients attending the outpatient departments. Hospital employees and direct family members of the study patients were not recruited as controls. All of them underwent routine physical examination, chest radiography, HIV serology, and TST with one TU PPD, and were followed up for twelve months after inclusion for any evidence of active TB. A written informed consent was taken from all patients and controls, and the study was approved by the Institute's Ethics Committee.

Venous blood (5 ml) was collected from patients and controls into tubes without anticoagulant (Becton Dickinson, USA) and serum was separated. Microtiter ELISA plates (Immulon 2HB, DYNAX, USA) were prepared by coating ESAT-6 and CFP-10 antigens [5 µg/ml, 50 µl/well) dissolved in coating buffer (1.59 g sodium bicarbonate, 2.93 g sodium carbonate, *p*H 9.6 in 1 liter phosphate buffer saline (PBS)]. The ELISA plates were incubated at 4^{o} C overnight to enable binding to wells. The plates were then washed with 0.05 per cent PBS with tween-20 (PBST) three times and blocked with 200 µl of blocking buffer [1% bovine serum albumin (BSA) in PBST for 2 h]. The plates were washed again with 250 µl of 0.05 per cent PBST three times. Fifty µl of 1:50 diluted serum (diluted in

0.1x 1% BSA-PBST) was then added and the plates were again incubated at 37° C for 90 min. The plates were washed six times with 300 µl/well of washing buffer (0.05% PBST). Fifty µl per well of protein-A alkaline phosphatase conjugate (1:2000) and anti-human IgA-alkaline phosphatase (1:1000) diluted in 0.1x BSA-PBST were added and the plates were again incubated at 37° C for one hour. The plates were washed eight times with 300 µl/well of 0.05M Tris. Next, the Substrate (Invitrogen, ELISA amplification system, USA), 50 µl/well was added and incubated for 12 min followed by addition of 50 µl/well of amplifier. The plates were then read at 490 nm. The mean optical density (OD) plus three standard deviations obtained with serum samples from healthy controls was used as the cut-off. The ELISA was performed thrice for every protein for each patient, and two positives out of the three ELISAs performed were taken as positive for every serum.

The sample size for the study was calculated (StatsDirect 2.7.2, *www.statsdirect.com*) assuming a positivity rate of mycobacterial antibodies being 95 per cent in the TB arm vs. 45 per cent in the sarcoidosis group[6,7]. With this calculation, 12 patients were required in each group to detect these differences [confidence level (1-α) 95%, power level (1-β) 80%][8].

Difference between categorical variables was assessed using Fisher's exact test. Difference between continuous variables was analyzed using Kruskal-Wallis test.

The baseline characteristics of the study population were similar in the three groups with respect to age and gender. Four patients with sarcoidosis had inappropriately received anti-tuberculous therapy in the past. Nine patients with pulmonary TB were TST positive while none with sarcoidosis was TST positive. The cut-off values for OD at 490 nm for PPD negative control subjects were 0.219, 0.197, 0.182 and 0.395, 0.312, 0.318 for ESAT-6 and CFP-10, respectively (Fig.). If both PPD negative and PPD positive control subjects were included, the cut-off values were 0.214, 0.211, 0.219 and 0.351, 0.299, 0.323 for ESAT-6 and CFP-10, respectively. None of the PPD positive controls were positive for either ESAT-6 or CFP-10 antibodies by the PPD negative cut-off. The overall prevalence of antibodies against any antigen was eight out of 18 (44.4%) in sarcoidosis and nine out of 10 (90%) in PTB if both PPD +/- were used as controls, whereas 11 of 18 (61.1%) and nine of 10 (90%) with sarcoidosis and PTB, respectively showed overall reactivity to either of the antigens if only PPD- subjects were used as controls.

The humoral response in sarcoidosis against RD1 antigens of *M. tuberculosis* has not been extensively studied despite numerous studies of T cell responses to RD1 antigens[6,7,9-11]. Demonstration of humoral responses to specific mycobacterial proteins in patients with TB and sarcoidosis could help in elucidating the relationship between these two granulomatous pathologies. Song *et al*[12] found anti-mycobacterial katG IgG in the serum of 48 per cent sarcoidosis patients versus none of healthy PPD negative controls. Dubaniewicz *et al*[13] demonstrated a humoral response to *M. tuberculosis* heat shock protein 70 (hsp70) in 12 of 37 patients with sarcoidosis and six of 29 TB subjects versus none in the 18 controls. However, B cell responses to specific mycobacterial proteins have not been studied, and none has been published from high TB prevalence countries[14]. The detection of antibodies against specific mycobacterial proteins would suggest that these antigens are the target of adaptive immune response in sarcoidosis, providing evidence of a mycobacterial aetiology in a subset of patients with sarcoidosis. We found similar frequency of circulating antibodies to RD1 antigens in patients with sarcoidosis and PTB. Moreover, none of the PPD positive controls were positive for either ESAT-6 or CFP-10 antibodies by the PPD negative cut-off suggesting a possible role of mycobacteria in the pathogenesis of sarcoidosis. Sarcoidosis patients may develop tuberculosis or both diseases may co-exist[15], however, it seems unlikely in our patients because all patients were treated with glucocorticoids and followed up for one year.

Our study had certain limitations. The major limitation was the small number of study subjects although the sample size was calculated *a priori*. Another limitation was that only B cell responses were evaluated in patients with sarcoidosis and PTB. It would have been interesting to note the T cell responses and mycobacterial DNA amplification in these patients. Other limitation was the choice of control group. We chose a healthy control group not related to the patients. Inclusion of control group from family members would have reduced bias due to environmental, economic and social conditions, thus leading to a better method group to allow direct comparison for the disease. The strength of the study included the fact that all blood samples were drawn from patients before any specific therapy was started (glucocorticoids in sarcoidosis

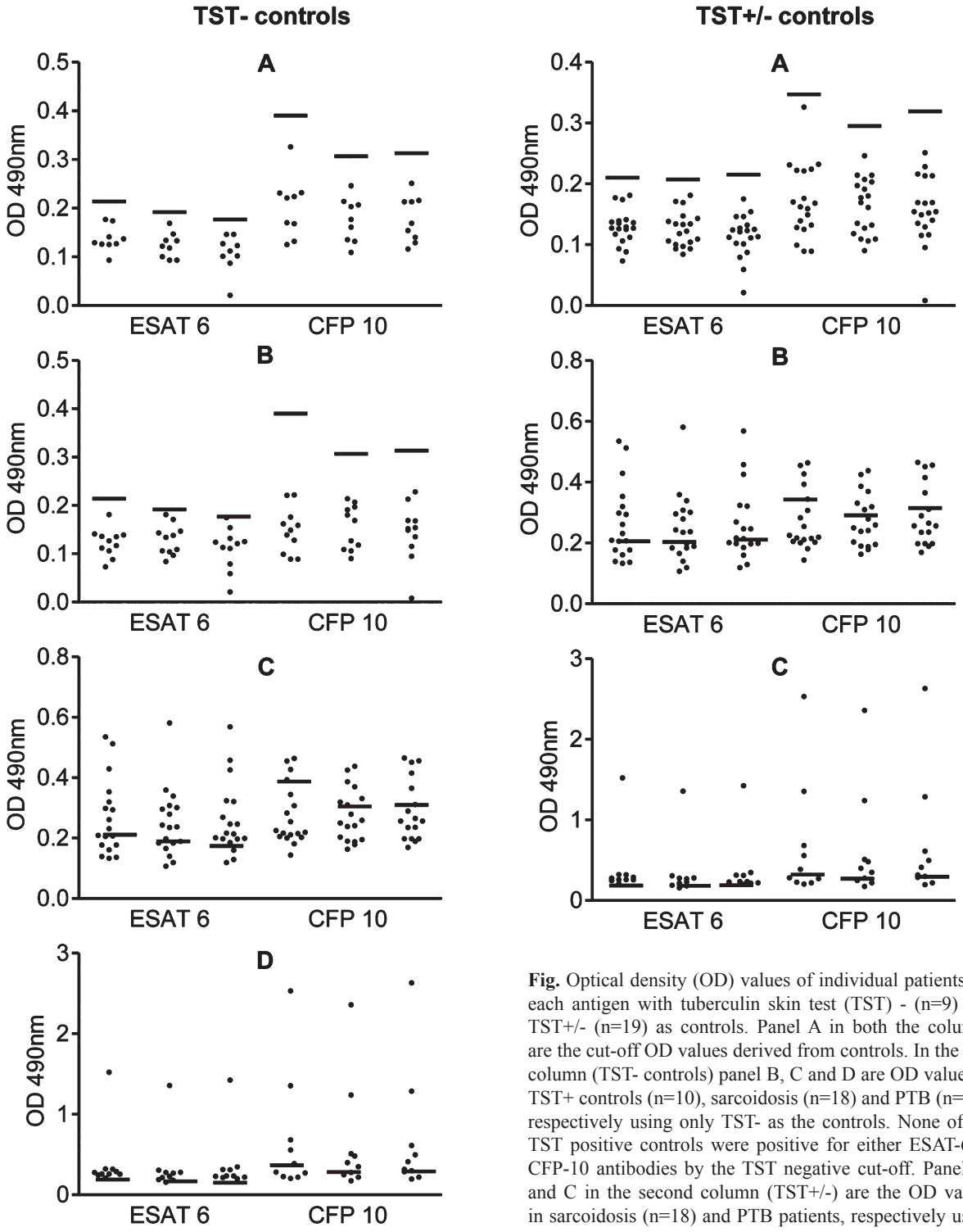

Fig. Optical density (OD) values of individual patients for each antigen with tuberculin skin test (TST) - (n=9) and TST+/- (n=19) as controls. Panel A in both the columns are the cut-off OD values derived from controls. In the first column (TST- controls) panel B, C and D are OD values in TST+ controls (n=10), sarcoidosis (n=18) and PTB (n=10), respectively using only TST- as the controls. None of the TST positive controls were positive for either ESAT-6 or CFP-10 antibodies by the TST negative cut-off. Panels B and C in the second column (TST+/-) are the OD values in sarcoidosis (n=18) and PTB patients, respectively using both TST-/+ as controls.

and anti-tuberculosis drugs in PTB). Finally, the study was based on patients selected from those attending the hospital and thus the results of study might not be applicable to general population.

In conclusion, patients with PTB and sarcoidosis showed evidence of seroreactivity to RD1 antigens. The positive results in patients with pulmonary sarcoidosis but not in PPD+ controls indicate a possible pathogenic role of mycobacterial antigens in sarcoidosis.

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