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A Pilot Study of Correlations between an Ifn-Gamma Elispot Assay and the Tuberculin Skin Test

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Abstract

The global resurgence of Tuberculosis (TB), supported by HIV pandemic and *Mycobacterium tuberculosis* multi-drug resistance strains, underscores needs for new diagnostic measures. Here, persons with suspected TB infection were enrolled to compare the *in vitro* specific T cell-immune responses, using in-house IFNγ ELISPOT assay and Tuberculin Skin Test (TST). The TST is subject to variations, since PPD contains mixture several antigens also present in other mycobacteria. To avoid this drawback, *M. tuberculosis* proteins hsp65, Ag85A and Ag85B were used in PBMC cultures. No significant correlation was identified between the tests to detect potential associations, but the TST tended to show better sensitivity (83.3%) to detect confirmed TB infection. However, the Ag85A tended for higher specificity (83.4%). Only one individual showed nil results. Conversely, 2 suspected TB patients showed high reactivity to both Ag85, plus a strength TST (> 16 mm). Not surprisingly, there was a very significant agreement when the 2 Ag85 isoforms were compared. Larger studies are warranted to confirm those findings.

Keywords: Tuberculosis; ELISPOT; Antigen 85

Introduction

Tuberculosis (TB) is a major cause of illness and death worldwide. The rising of global incidence of TB is blamed for that burden mainly in developing countries [1]. The importance of cell-mediated immunity in the protective response against *Mycobacterium tuberculosis* is well established. At diagnosis, Tuberculin Skin Test (TST) is subject to substantial variations and other limitations, and false-positive results are common. Lately, peripheral blood-derived IFNγ responses to *M. tuberculosis*-RD1 specific antigens have been investigated for the management of TB. The results suggest that those assays may have advantages over the TST, in terms of higher specificity, better correlation with exposure to *M. tuberculosis*, and less cross-reactivity due to TB vaccination and other mycobacterial infections [2]. However, no other *M. tuberculosis* specific antigen has been tested in those platforms so far.

Essential requirements for the immune response in TB are the antigens recognized by the effective either CD4 or CD8 T lymphocytes. For several years, important efforts have been concentrated to identify candidate molecules for inclusion in a novel vaccine/booster against and immunodiagnostic for TB [3]. Evaluations of the T cell function in response to recombinant, soluble antigens have been performed in an endemic TB area. In previous studies, our data have shown that the IFNγ response within the TB patient group was enhanced in comparison to that of the controls, and that the CD69 and CD25 *in vitro* expression on T cells in response to Antigen 85B (Ag85B) and Ferritin was highest after 24 and 72 h, respectively [4,5].

In this study, we have extended those characterizations of specific *in vitro* cellular immune responses in terms of IFNγ production by Enzyme Linked Immunospot (ELISPOT) assay, comparing those data with Tuberculin Skin Test (TST) performed during a shelter screening [6]. Accordingly, selected comparisons among TST and *M. tuberculosis* recombinant proteins hsp65, Ag85A and Ag85B were made in primary Peripheral Blood Mononuclear Cell (PBMC) cultures purified within 24 hours of obtaining the specimens of 15 suspected TB patients. Demographic data, eligibility and exclusion criteria for those individuals are described elsewhere [7]. TST in duration was performed after intradermal placement of 5 tuberculin units of PPD (Statens Serum Institute, Copenhagen, Denmark). The IFN-γ ELISPOT was assayed as recommended by the T.SPOT-TB® manufacturer (Oxford Immunotech Inc. Oxford, UK) and slightly modified replacing the original panels A and B, by adding 5.0 μg/ml of either *M. tuberculosis* hsp65 or Ag85A or Ag85B (Kindly provided by Drs. T. Ottenhoff, LUMC, The Netherlands, and E. Sampaio, FIOCRUZ, Brazil). After developed and by using an AID plate reader (Germany), a reactive sample was scored if > 6 spots were formed. For a statistical analysis, the software Instat 3 was used to estimate the coefficient of correlation.

At screening, **the frequencies of** IFN-γ **ELISPOT responses** at the single-cell level already subtracted from the background and expressed as number of Spot Forming Colony (SFC) for the 3 *M. tuberculosis* recombinant antigens tested in the suspected TB patients are showed in the Figure 1. As internal controls, positive responses to PHA, and negative ones to empty plasmidial vector, were detected in all individuals (data not shown). A total of 8 out of 15 individuals assayed responded to TST, defined as the standard > 10 mm in duration cut off. On the other hand, only one individual (# 5297) did not show any SFC to all *M. tuberculosis* antigens used, neither to TST. However, 2 suspected TB patients (# 5267 and # 5375) showed a high reactivity (> 29 SFC) to both Ag85, plus a positive and strength TST (20 mm and 16 mm, respectively). Overall, the 53% of TST reactivity was more frequent than for the IFN-γ **ELISPOT**: 27% for

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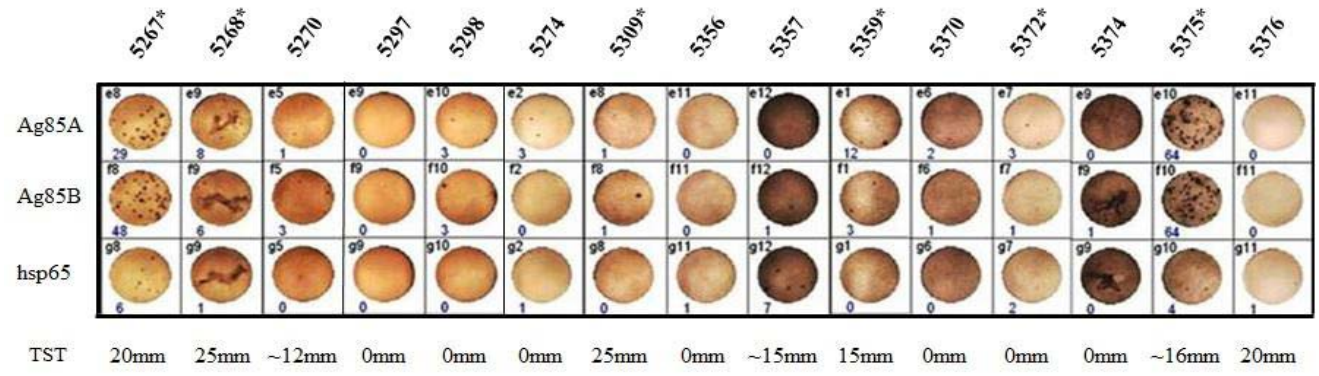

Figure 1: IFNγ production evaluated by a modified, in-house ELISPOT in primary PBMC cultures employing *Mycobacterium tuberculosis* specific Antigen 85A, Antigen 85B and heat-shock protein (hsp) 65 in fifteen suspected TB patients. Positive *in vitro* IFNγ responses were scored when ≥ 6 spots were formed after subtracted from the background. Antigens used at final concentration of 5μg/ml. The Tuberculin Skin Test (TST) indurations (5 units of PPD) were recorded in parallel, and scored as positive when ≥ 10 mm. *denotes confirmed TB infection detected by RD1 antigens.

Ag85A, 20% for Ag85B and 13% for hsp65. It should be emphasized that 6 individuals were confirmed as TB infected ones, as detected by RD1 antigens performed in parallel (data not shown). When we used this certified immunodiagnostic tool (T.SPOT-TB®) to sort out the positive group, the results are the following: 83.3% of TST reactivity compared to 66.7% for Ag85A, 50.0% for Ag85B and 16.7% for hsp65 by IFN-γ **ELISPOT**. However, the specificity changed the ranking as 83.4% of the IFN-γ **ELISPOT employing** the Ag85A was higher than 75.0% for both TST and Ag85B, but lower than for hsp65 (52.8%) IFN-γ **ELISPOT**. Based on that, and also t**o compare the magnitude of** IFN-γ **ELISPOT responses with the TST in duration size, correlation studies were preliminary performed.** It's worth of note that due to limited number of individuals no correlation was found when the 2 methods were compared, regardless the antigens employed or criterion. Not surprisingly, there was a very significant agreement ($p < 0.004$) in the mean number of SFC when the 2 Ag85 isoforms were compared.

New strategies for specific TB diagnosis and prevention of *M. tuberculosis* transmission are mandatory. The most obvious limitation of the current study was the small sample size. Even though, the results of this pilot study show that our in-house and modified IFN-γ **ELISPOT** assay, which uses a different set of *M. tuberculosis* antigens from the original and commercial kit, seems to be less accurate and sensitive in identifying subjects suspected to be infected with TB than the TST. On the other hand, the IFN-γ **ELISPOT employing** the Ag85A tended for a better specificity in detect confirmed TB infection when compared to the TST. Furthermore, on the basis of IFN-γ **ELISPOT** responses, we have not been able to define suitable *M. tuberculosis* antigens that correlate with TST other than the 2 in *M. tuberculosis* RD1. Arend and colleagues [8] confirmed that two commercial IFN-γ **release assays employing specific** R**D1 antigens** correlated with exposure parameters to TB, whereas the TST did not. A possible lack of sensitivity of these assays with a 10mm or larger TST cut off warrants further investigation.

The members of the Ag85 family are found in all mycobacteria, and sequence comparison indicates that the Ag85 gene family arose by duplication of an ancestral gene, before the emergence of the actually known mycobacterial species [9]. Based on that, we were not surprised that the only significant agreement was found between Ag85A and Ag85B, regardless the study size. Indeed, the three members of this mycolil-transferases family have shown higher immune reactivity [10].

Possible explanations for the low sensitivity of the *in vitro* assays rely on the concentration of the antigen employed, although a dose-response curve was previously generated and then corroborated latter [4,5]. The definition of positive or negative responses to ELISPOT assays is still empirical, even if a general consent on how to consider positive results exists in the literature [11]. Thus, we decided for maintain the cut off value as suggested by the T.SPOT-TB® manufacturer. Would this hamper the final result? Earlier, an ELISPOT cut-off for RD1 proteins was set at 4-8 SFC, with little benefit from the final results, and another for PPD skin test at 9-13mm was more rational when comparing with that ELISPOT data [12]. In contrast, Diel et al. [13] used a 10mm in duration cut-off for the TST among occasional contacts, and showed strong agreement between TST and ELISPOT in non BCG-vaccinated persons.

In conclusion, we primarily rule out the possibility to have the *M. tuberculosis* hsp65 recombinant protein included in an IFN-γ **ELISPOT assay in order to screen for suspected TB individuals. In contrast,** the potential of this *in vitro* **assay** employing the Ag85A deserves more attention. Further investigation among a larger population is warranted.

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