Evaluation of Cellular Immune Response against Purified Antigen 85 in Patients with Tuberculosis

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ABSTRACT

Background: Tuberculosis (TB) remains an important health problem throughout the world. Despite its significance in public health, mechanisms of protective immunity against *Mycobacyerium Tuberculosis* in humans have not yet been understood. **Objective:** To evaluate cell mediated immune response against purified Ag 85, PPD and Phytohemagglutinin (PHA) in patients with tuberculosis and healthy tuberculin positive and negative individuals. **Methods:** Thirty patients with tuberculosis and 60 healthy tuberculin skin test positive and negative volunteers were participated in this study. Cell mediated immunity was assessed by measuring [^{3}H]-thymidine uptake and detection of IFN-γ in the culture supernatant using commercial ELISA test. **Results:** In the present study, we showed that IFN- γ production and cell proliferation response to Ag 85 were significantly higher in tuberculin positive than tuberculin negative individuals ($P<0.01$). Among tuberculous patients, IFN-γ production and cell proliferative responses to Ag 85 was significantly lower in contrast to healthy tuberculin positive individuals ($P<0.01$). In addition, IFN- γ response in patients with cavitary tuberculosis was lower than patients without cavitation ($P<0.05$). **Conclusion:** Based on the higher cell mediated immune responses to Ag 85 in healthy tuberculin positive volunteers compared to patients (especially with advanced disease), purified Ag 85 can be used as a sensitive marker for analysis of immune responses in tuberculosis.

Keywords: Tuberculosis, *IFN- γ*, Cell Proliferation, Ag 85

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INTRODUCTION

Cell mediated immune response plays an essential role in tuberculose infection. T cells are known to have a crucial role in acquired resistance against mycobacterial infections (1). Healthy, tuberculin positive volunteers seem to have protective immunity with an enhancement of Th1 cell proliferation response and IFN- γ production, whereas magnitude of such responses is lower in patients with tuberculosis. In animal models, a protective immune response against *M. tuberculosis* depends on the emergence of $CD4^+$ T lymphocytes, which produce cytokines to activate macrophages to eliminate intracellular mycobacteria (2).

IFN-γ is an essential protective cytokine in mice (3,4). This cytokine peaks when protective immunity is maximally expressed and is produced by $CD4^+$ T lymphocytes, when these cells are in contact with previously infected macrophages (by live mycobacteria or primed with secreted mycobacterial antigens (Ags)) (3). Mice which fail to produce IFN-γ due to disruption of IFN-γ gene develop a fatal tuberculous infection upon intravenous or aerogenic challenge (4,5).

Accordingly much work has been focused on identification of mycobacterial antigens recognized by human T cells. Understanding mycobacterial antigenicity is important for evaluating immune response in patients with tuberculosis and healthy individuals. Studies in animal models have demonstrated that only live dividing mycobacteria efficiently induce protective immunity. This fundamental finding has been the basis for the hypothesis that proteins secreted from the multiplying and metabolizing bacilli at the early stage of infection are responsible for the recognition of infected host cells by protective T cells (6). Protein secretion from mycobacteria is thought to be important for eliciting of protective immune responses against TB.

Recently, experimental vaccines based on culture filtrate proteins have been demonstrated to induce high levels of protective immunity both in guinea pig and in mice (7,8). The Ag 85 complex (30-32 kDa Antigen) is a major fraction of the secreted proteins of *M.tuberculosis* and BCG culture filtrate which catalyses the transfer of mycolates via a mycolyltransferase exchange process, leading to the formation of Trehalose monomycolate (TMM) and Trehalose dimycolate (TDM), also known as cord factor (9,10). The Ag 85 complex induces a strong T-cell proliferative response and IFN-γ secretion in BCG–vaccinated mice and in most healthy individuals exposed to *M. tuberculosis* (6).

Therefore, Ag 85 can be considered as a specific Ag for analysis of the immune response to *M. tuberculosis* particularly for designing new TB vaccines.

In this study, we have analysed *in vitro* lymphoproliferative response and secretion against native Ag 85, Ag 85 purified from culture filtrate of BCG, PPD, polyclonal T in Iranian healthy volunteers and tuberculous patients, by using a whole blood assay.

SUBJECTS AND METHODS

Patients and Controls. Whole blood was obtained from 30 patients in whom pulmonary TB was confirmed by clinical findings, radiography, and sputum smear. Patients with a history of HIV infection, corticosteroid treatment, and autoimmune diseases were excluded. All Patients were hospitalized at the Department of Tuberculosis at National Research Institute of Tuberculosis and Lung Diseases (Maseh

Danashvariy Hospital, Shahid Beheshti University of Medical Sciences, Tehran-Iran). Chest X-ray was obtained from each patient at the time of study, and patients were graded for the extent of TB infection according to the National Tuberculosis and Respiratory Association Criteria (11) and classified as either having active minimal TB, including patients with minimal lesions with slight to moderate density but without demonstrable cavitation, or patients with active advanced TB, including patients with marked cavitary lesions in the lungs. Blood samples were collected from patients either before or within two months after starting the antibiotic treatment. Control subjects were 30 healthy tuberculin-positive and 30 healthy tuberculin-negative volunteers. Skin indurance reaction of 10 mm or more after intradermal testing with 5 units of PPD (Razi Institute of Iran) was considered positive both in tuberculosis patients and in healthy volunteers. Ethics committees of Ahwaz University of Medical Sciences approved the research protocol.

Antigens. Purified Protein Derivative (PPD) was prepared at the Pasteur Institute of Brussels and Razi Institute, Iran. Ag85 was purified from culture filtrate of M. bovis (BCG) as described previously (12). In brief, culture filtrates of BCG were fractionated by sequential chromatography on phenyl sepharose, DEAE sephacel and Sephadex G75. The molecular weight of this protein complex was found by SDS-PAGE to be 30-32 KDa. Purification was confirmed by immunoblotting with a specific monoclonal antibody. Polyclonal T cell mitogen Phytohem- agglutinin (PHA) was purchased from Sigma (USA).

Whole Blood Assay, Lymphoproliferation and IFN-γ Assay. Heparinized whole blood was taken from the participants. After hematological analysis by cell counter, it was washed, plasma was removed and cells were diluted in RPMI-1640 medium with 10% FCS and adjusted to one million leukocytes /ml. Cell suspensions were distributed in 180-µl portions in round-bottomed 96 microwell plates to which 20 µl of PPD (10 µg/ml), Ag 85 (5µg/ml), PHA (4µg/ml) or RPMI (control) had been added. Cultures were incubated at 37^{o}C in a humidified Co_2 incubator for 5 days for PHA stimulated cultures and for 6 days for other cultures.

For the lymphocyte proliferation assay 5µCi/well tritiated thymidine ([^{3}H]TdR) with specific activity of 5 Ci/ Mmol was added to the cultures during the last 18h. Cells were then harvested by using a Cell Harvester system (Skatron Company, Finland). The radioactivity recovered from the filter was measured in a Wallac liquid scintillation counter. Results are expressed as mean CPM ± standard deviation (of quarter-plate cultures). Lymphocyte proliferation responses to antigens were considered positive when the values were more than 3 SD above mean values obtained from 30 healthy tuberculin-negative volunteers. For IFN-γ assay culture supernatant from four wells was pooled after 5 days (PHA) or 6 days (other antigens). IFN- γ activity was quantified by a sandwich ELISA. Coating antibody, biotinylated detection antibody and standard human recombinant IFN- γ were obtained from Pharmingen. Titers are expressed in mean picogram per milliliter.

IFN- γ production after antigen stimulation was considered positive at 3 SD values above the mean IFN- γ value obtained from 30 healthy tuberculin- negative volunteers.

Statistical Analysis. The student t-test was used to determine statistical significance between groups by using SPSS version 11 for windows.

RESULTS

Cellular Immune Response. Lymphocyte cell populations were stimulated *in vitro* with Ag 85 complex, PPD and PHA.
Cultures without stimulus were considered as negative control for cell proliferation. Proliferative responses were evaluated by tritiated thymidine uptake of cells in the well after 6 days of *in vitro* stimulation with Ag 85 and PPD and 5 days with PHA. While a statistically significant difference was observed between healthy tuberculin-positive and negative volunteers regarding the proliferative response to Ag 85 as well as to PPD ($P< 0.01$), no difference was recorded between the two groups of subjects in response to PHA and RPMI or in the cultures without stimulus (Figure 1). The proliferative lymphocyte responses to Ag85 complex for each group are shown in Figure 2. The cell proliferation response to Ag 85 complex is significantly different between tuberculin positive patients and tuberculin negative subjects ($P< 0.01$).

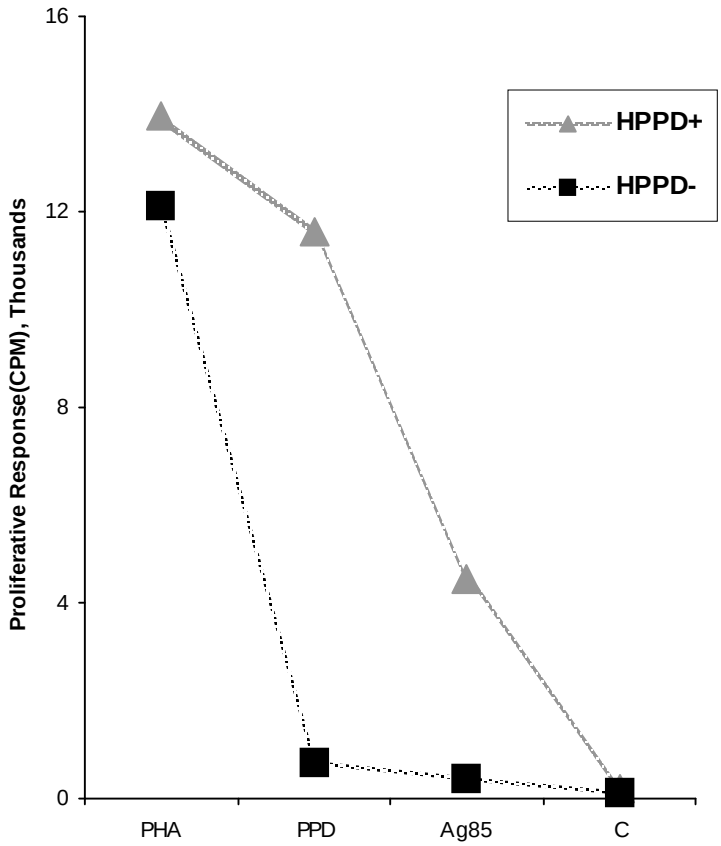

Figure 1. Proliferative responses to PPD, Ag 85, PHA, and C (RPMI without Ag) of cells derived from healthy tuberculin positive donors (HPPD+) and healthy tuberculin negative donors (HPPD–).

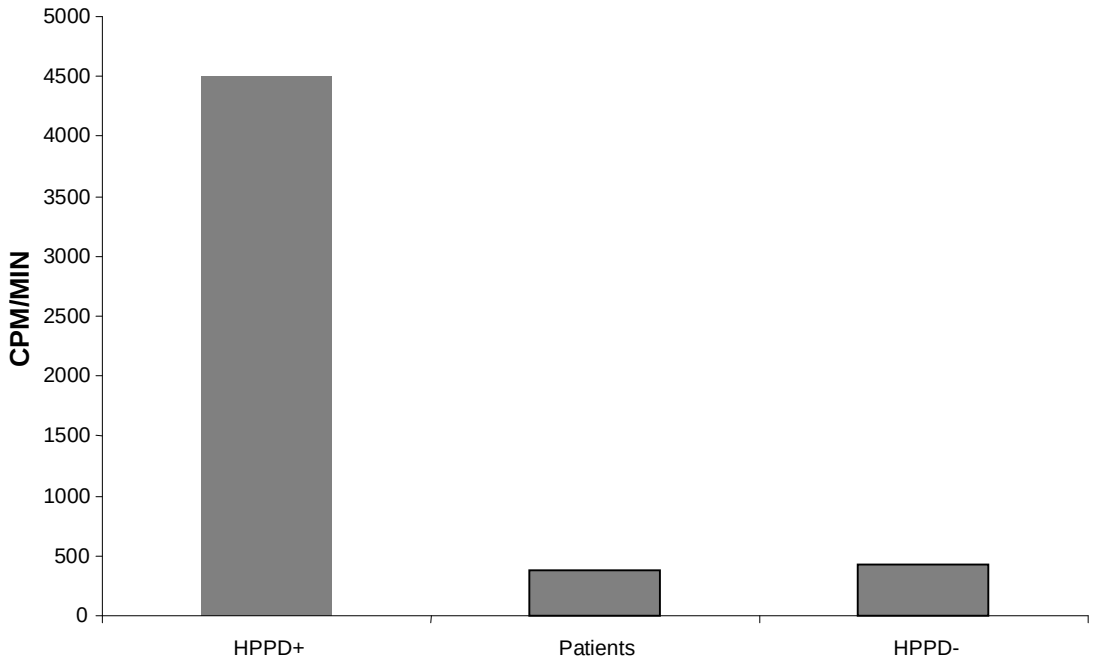

Figure 2. Mean Cell proliferative response to Ag 85 of cells derived from healthy tuberculin positive donors (HPPD+), patients, and healthy tuberculin negative donors (HPPD–).

Production of IFN-γ in response to Ag85 complex and PPD was determined by ELISA. The results for IFN-γ production are shown in table1. The mean concentration of IFN- γ in supernatants of Ag85 complex stimulated whole blood assay from TB patients was significantly lower than that of healthy tuberculin-positive subjects ($P<0.01$). Moreover, TB patients were divided according to clinical status into patients with active minimal tuberculosis and patients with active advanced tuberculosis. The average IFN-γ. production was lower in patients with extensive disease whereas patients with minimal disease demonstrated a markedly higher reactivity.

Table 1. IFN- γ production in responses to Ag85 and PPD

Groups (n)	Mean IFN gamma (Pg/ml) ±SD	
	PPD	Ag85 complex
Pulmonary TB (30)	944±892	247±234
Minimal (12)	1497±1004	390 ±298
Extensive (18)	482 ±412	128 ±68
Tuberculin Positive (30)	3544 ±2729	1227±1184
Tuberculin Negative (30)	240 ±168	129±85

The mean ± three SD values of the tuberculin–negative subjects were used as the cut-off level for IFN- γ production. Results in tuberculin–positive healthy controls and TB patients were analysed.

In tuberculin–positive group, IFN- γ responses to Ag 85 were found in 26 out of 30 subjects (86.6%). Among the tuberculosis patients, 6 out of 30 subjects (20%) had a positive IFN- γ response to Ag 85, all showing minimal disease clinically (Figure 3).

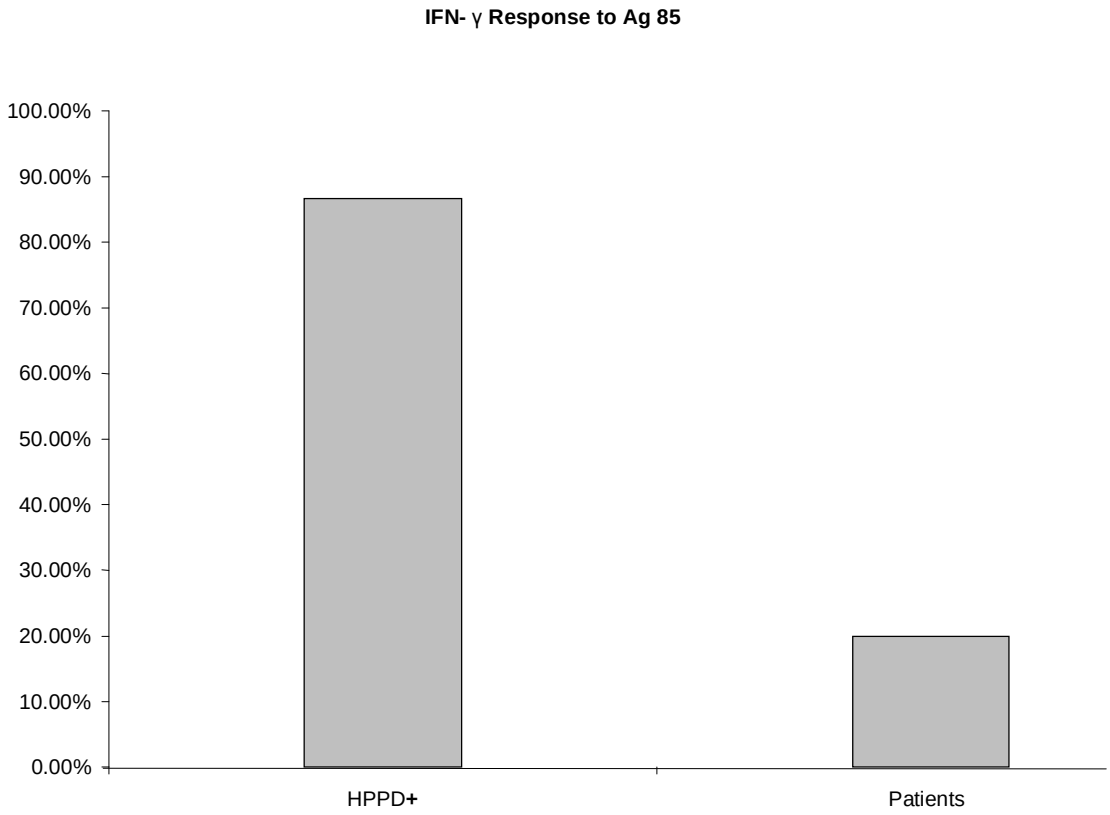

Figure 3. IFN- γ response to Ag 85. Bar represent the percentage of healthy subjects with tuberculin positive test (HPPD+) and patients who had a IFN- γ positive response to Ag 85.

DISCUSSION

This study focused on the immune response to Ag85 by whole blood assay of patients with tuberculosis and healthy subjects. There are some studies that compare the immune response to *M. tuberculosis* in healthy tuberculin positive subjects and infected patients (13-15). The objectives of these studies are to characterize the difference between protective and non-protective human immune response to tuberculosis, and to identify the involved antigen in the development of protective response.

In this study, we showed that 86% of healthy tuberculin-positive individuals had high lymphoproliferative and IFN-γ production in response to Ag 85. This high rate of responsiveness suggests the possibility of using Ag 85 as a subunit vaccine against TB (16,17). The only TB vaccine currently available for humans is a live attenuated strain of *M. bovis* (BCG). The protective efficacy of the BCG vaccine remains controversial and was reported to range from 0% to 80% (18,19). The search for purified mycobacterial antigens useful for vaccine production has been extensive (20).

In animal models, secreted antigens of mycobacteria induce a protective immune response (3). Ag 85 complex is a major secretary protein of *M. tuberculosis*. In mice, this Ag induces the production of IFN-γ (21), and in guinea pigs Ag85 confers protection against TB (22). Epitope mapping of the Ag85 has elucidated distinct peptides of this protein that stimulate human T cells, suggesting specific sites that may induce protection (23). Also Huygen et al. demonstrated that a DNA vaccine encoding Ag 85 complex induced Th1 response (tumor necrosis factor (TNF), IFN-γ, and IL-2),

cytotoxic activity against purified native Ag 85, and protection of mice against challenge with BCG (24). Along with the results of the present study, Huygen's results indicated that Ag85 complex could induce a strong T-cell proliferation response and IFN-γ production in healthy individuals exposed to *M. tuberculosis* and may be of use as a predictive *in vitro* correlate of protection in areas of TB endemicity (24). However, T cell response to Ag 85 complex was about two to three fold lower than to PPD, probably because PPD is a mixture of antigens from Mycobcterium (25).

In conclusion, our results showed decreased proliferative responses and production of IFN-γ to Ag 85 complex in TB patients, especially in patients with advanced disease. These results are consistent with most human studies performed by other investigators (13, 14, 26). The decreased production of IFN-γ in patients might be related to the lack of production of IL-12 at the onset of infection, which is known to be essential for the generation of Th1 subset (27,28). Other factors, such as malnutrition, weakened immune response, and also host genetic factors may be involved. In addition, strong cell mediated immune responses to Ag 85 in healthy tuberculin positive volunteers and weak response in patients, especially with advance disease, indicates that purified Ag 85 can be used as a sensitive marker for the analysis of immune response in tuberculosis.

ACKNOWLEDGEMENT

This work was supported by a grant from Ahwaz University of Medical Sciences.

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