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The Study of Th1/Th2 Cytokine Profiles (IL-10, IL-12, IL-4, and IFN γ) in PBMCs of Patients with Multidrug Resistant Tuberculosis and Newly Diagnosed Drug Responsive Cases

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ABSTRACT

Background: Multi-drug resistant tuberculosis (MDR-TB), which is a worldwide clinical problem, is associated with high morbidity and mortality, as well as long-term survival of infected immunocompetent patients. In this study, the PPD-induced production of IL-12, IL-10, IFN γ , and IL-4 in peripheral blood mononuclear cells (PBMC) from patients with MDR-TB were investigated and compared with cytokine production capabilities in newly diagnosed, treated cases.

Materials and Methods: This study investigated the profiles of IFN- γ , IL-12, IL-10, and IL-4 in response to a purified protein derivative (PPD) in peripheral blood mononuclear cells (PBMC) from 15 HIV negative patients with multidrug-resistant tuberculosis (MDR-TB), 11 newly diagnosed, treated cases and compared those with 10 healthy negative tuberculin reactors as controls.

Results: ELISA results showed that the following stimulation with PPD, IFN γ production was significantly increased, whereas IL-10 was significantly reduced in MDR-TB patients compared with PPD negative controls. Production of IL-12 in MDR-TB patients showed elevation, induced by PPD stimulation of their PBMCs. However, MDR-TB patients were similar to healthy negative tuberculin controls in their IL-12 production and there was no statistically significant difference between them. IL-4 was detected to be in very low levels in three groups.

Conclusion: In this study MDR-TB patients have no dysregulation in IL-12 or IL-10 production during *Mycobacterium tuberculosis* infection, and profiles are prone to Th1 cytokines. (Tanaffos 2004; 3(10): 25-31)

Keywords: Th1/Th2 cytokine profiles, Tuberculosis, MDR-TB

INTRODUCTION

Multi-drug resistant tuberculosis (MDR-TB), which is a worldwide clinical problem, is associated

with high morbidity and mortality, in spite of long-term survival of infected immunocompetent patients. It is reported to be a frequent clinical infection and might be prevalent in as much as 70% of infected cases (1).

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Immunologic resistance and susceptibility to intracellular pathogens are mediated by CD₄⁺ T cells with specific patterns of cytokine secretion. Th1 cells that produce gamma interferon confer resistance to infection with Mycobacteria (2). In HIV-negative patients with MDR-TB, CD₄ lymphocytopenia appears to correlate with the severity of disease and low IL-2/IFN γ production in response to Mycobacterium tuberculosis and PPD (3). IL-12 is known to play important roles in anti-TB cell mediated immunity (4) and is the major cytokine for directing primary Th1 differentiation in CD₄⁺ T cells in vitro and in vivo (5). Active pulmonary TB is associated with enhanced production and activity of immunosuppressive molecules such as IL-10 and TGF- β 1 (6). These cytokines have many overlapping biological effects, including T-cell suppression, macrophage deactivation, modulation of pro-inflammatory cytokines, and interference with antigen-presenting cell function (7, 8).

In this study, the PPD-induced production of IL-12, IL-10, IFN γ , and IL-4 in peripheral blood mononuclear cells (PBMC) from patients with MDR-TB were investigated and compared with cytokine production capabilities in newly diagnosed, treated cases. We found that IL-10 production was significantly down-regulated after PPD stimulation by PBMC from MDR-TB patients, whereas IFN γ production was greatly increased compared with production in controls. In addition PPD-induced IL-12 production was higher in MDR-TB patients than PPD negative controls.

MATERIALS AND METHODS

Subjects

Patients and healthy volunteers consented to participate in this study. Whole blood was obtained from 15 HIV-negative patients with culture-proven

TB and nonresponders (for 6 months to at least 3 drug regimen received), and 11 newly diagnosed tuberculosis patients at Massih Daneshvari Hospital, National Research Institute of Tuberculosis and Lung Disease (NRITLD). MDR-TB patients were at various stages in their clinical course and had different treatment duration; minimally all patients showed resistance to rifampicin and isoniazid. All patients, except 3, had been infected for less than 3 years. The patients age range was from 16 to 70 years, with a mean age of 32.36 \pm 14.72 years. In ten healthy volunteers no skin reaction after an intradermal tuberculin test using 5 units PPD was observed.

Reagents

Complete medium consisting of RPMI 1640 (Sigma Chemical Co., with L. glutamine and without bicarbonate sodium) supplemented with 10% heat inactivated fetal bovine serum (Sigma Chemical Co.), penicillin (100 U/ml)/ streptomycin (100 μ g/ml) (LIFE Technologies. Gibco BRL.) was used for cell stimulation. PHA was purchased from Sigma Chemical Company, and PPD was a gift from Dr. Hedayati of Razi Vaccine and Serum Institute, Hesarak, Iran. Lymphocyte separation medium was purchased from Bahar Aphshan Corp.

Preparation of Cells

Heparinized venous blood was drawn from subjects into sterile blood collection tubes, and PBMCs were isolated by using the density gradient centrifugation method on Ficoll-Hypaque.

Stimulation of PBMCs

PBMCs were suspended at a density of 1 \times 10⁶ viable cell/ml in complete medium. A total of 2 \times 10⁵ cells/200 μ l were added to flat bottom, 96-well plates (Griner) and stimulated with PPD (12.5 μ g/ml) and phytohemagglutinin (PHA 16 μ g/ml) as a positive control for all reactivity and cells without stimulation

as negative control and incubated at 37°C in a 5% CO₂ humidified air atmosphere for either lymphocyte proliferation assay or supernatant fluid collection to measurement of cytokine production.

Lymphocyte proliferation assay

PBMCs (2×10^5 cells/200 μ l) were placed in each well of a flat bottom plate. They were stimulated with PPD and PHA; also, incubated for 72h 37°C in a 5% CO₂ humidified air atmosphere 1 μ Ci [³H]. Thymidine (TRK 686. UK.) was added in the final 18h. The cells were harvested on filter paper using a cell harvester (L.K.B. Wallac), and the incorporated radioactivity was measured in a liquid scintillation counter (Wallac 1409). The results were expressed as the mean counts per minute of triplicate wells for each donor. The stimulation index (SI) was calculated using this value and counts per min obtained in unstimulated wells.

Supernatant fluids were collected after 48h to measure the production of IL-10 and IL-12, after 72h for IL-4, and after 120h for IFN γ production. All supernatants were stored at -70°C until used. The frozen supernatant fluids were thawed at room temperature, and cytokine levels were measured by commercial immunoassay kits. IFN γ level was assessed using Biosource IFN γ ELISA kit with a minimum detection limit of ? 1IU/ml. IL-4 was assessed by Biosource IL-4 ELISA kit with a MDL of ? 12pg/ml. and also IL-10 with a lowest limit of detection around 11pg/ml. IL-12 was determined using Bender Med system and the lower limit of detection for this assay was 31.25 pg/ml.

Statistical method

The results are presented as the mean \pm S.D. Statistical analysis was performed using nonparametric ANOVA, Kruskal-Wallis test. Analysis and results were considered significantly different at $p < 0.05$.

RESULTS

Lymphoproliferative responses to PPD antigen were similar in PPD-stimulated PBMCs from MDR-TB patients to cells from PPD negative controls (mean 392.7 ± 432.8 versus control 157.9 ± 144.5 versus newly diagnosed patients 304.3 ± 295.7 , $p < 0.05$). Lymphoproliferative responses to PHA antigen had no significant difference in three groups (mean MDR-TB 2054 ± 1904 versus control 734.4 ± 1020 versus new case 1536 ± 2647 , $p < 0.05$) (fig 1. based on SI, data has not shown).

Six MDR-TB patients did not show response to the PPD (SI $<$ 2.5). The majority of the healthy non reactors showed no stimulation (7 of 10, SI $<$ 2.5) in lymphocyte response to PPD. Stimulation of PBMC with PHA resulted in greater lymph proliferation (700-2700) than PPD in three groups tested. Lymphoproliferative responses to PHA antigen (mean MDR-TB 2054 ± 1904 versus control 734.4 ± 1020 versus newly diagnosed patients 1536 ± 2674 CPM, $p > 0.05$) had no significant difference in statistical results.

Individual data on IFN γ production by PBMC were obtained following 120h stimulation with PPD (Figure 2). The mean IFN γ concentration of MDR-TB patients were significantly higher than corresponding values in non-reactor controls (mean 5.45 ± 3.44 versus 1.59 ± 2.05 IU/ml, $p < 0.01$). Mean IFN γ production in newly diagnosed patients was 3.59 ± 3.06 IU/ml, ($p > 0.05$). Stimulation of PBMC with PHA resulted in the secretion of IFN γ (0.4 - 10.3 IU/ml), and the mean IFN γ production in response to this antigen was significantly different in MDR-TB patients compared with newly diagnosed patients and controls (mean 6.08 ± 3.13 versus 5.84 ± 2.97 versus 2.68 ± 1.5 IU/ml, respectively, $p < 0.01$), indicating that there is no absolute qualitative defect in IFN γ production in these patients.

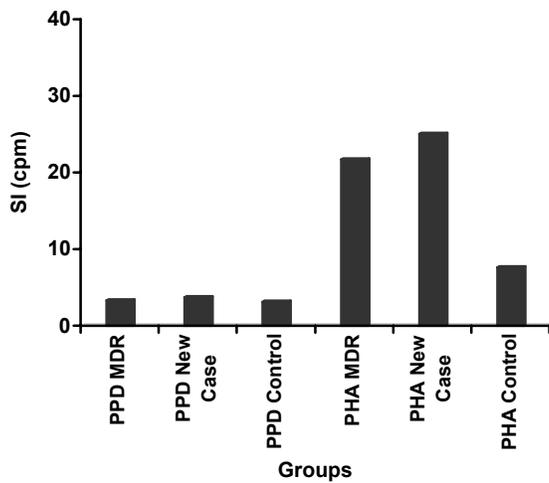


Figure 1. Lymphoproliferative responses to PHA antigen in three groups.

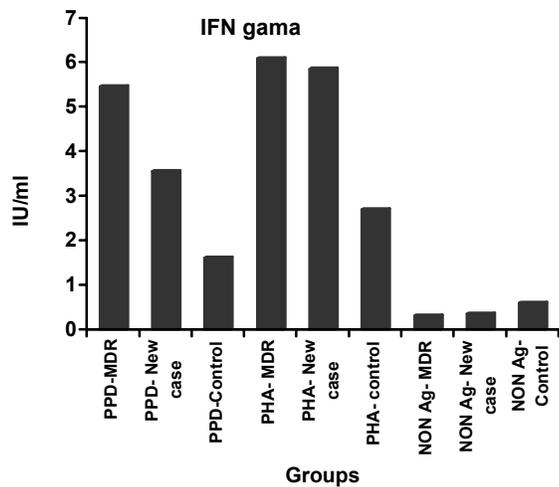


Figure 2. IFN γ production by PBMC following 120h stimulation with PPD.

As shown in Figure-3 there was no statistically significant difference between the two groups of patient and control, although the mean IL-12 production was higher in MDR-TB than in newly diagnosed patients and non-reactor controls(mean 126.06 ± 114.52 versus 87.03 ± 57.48 versus 38.49 ± 27.71 pg/ml, respectively, $p > 0.05$).

In addition, IL-12 production in response to PHA from PBMC in controls was higher than newly

diagnosed and MDR-TB patients (mean 235.54 ± 92.18 versus 210.25 ± 140.30 versus 171.07 ± 99.05 pg/ml, respectively, $p > 0.05$).

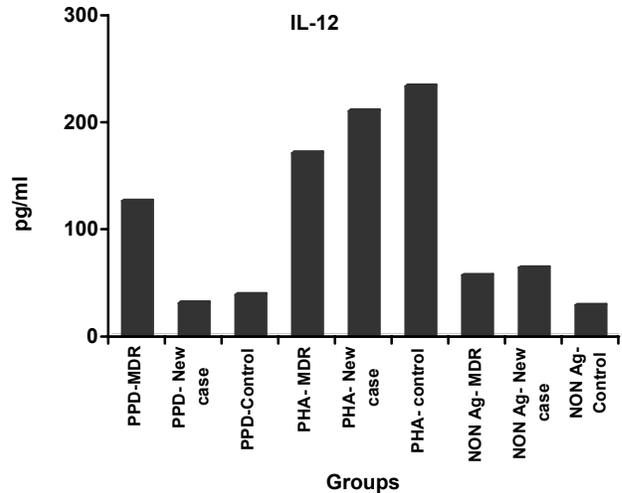


Figure 3. Various levels of IL-12 production in three groups.

Various levels of IL-10 was measured in the patients under study; this cytokine was produced significantly lower after stimulation with PPD in PBMC from MDR-TB patients comparing with controls (mean 92.70 ± 151.29 versus 140.10 ± 90.88 pg/ml, $p < 0.05$) (Figure 4).

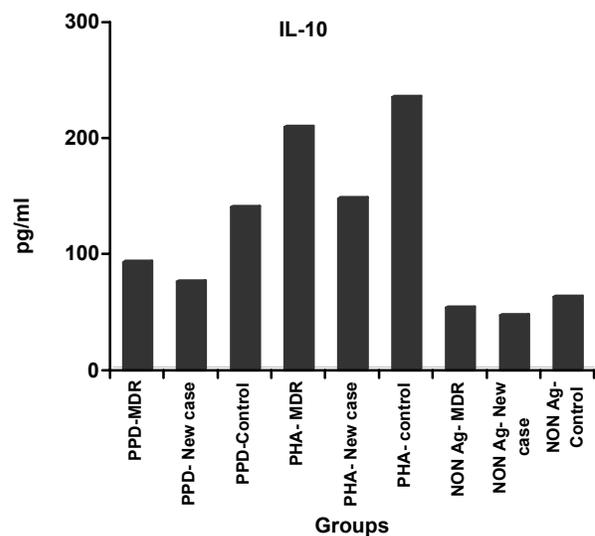


Figure 4. Various levels of IL-10 production in three groups.

Production of IL-10 in stimulation of PBMC with PHA was not statistically significant in three groups (mean in MDR-TB 209.4 ± 261.93 versus newly diagnosed patients 147.59 ± 168.77 versus controls 235.05 ± 109.63 pg/ml, $p > 0.05$).

IL-4 could not be detected above the lower limit of the ELISA method (>12 pg/ml) and all culture supernatants showed negligible levels.

DISCUSSION

MDR-TB emerged as a new clinical complication of mycobacterial infection worldwide and showed a progressive prevalence among HIV-negative patients. Recently immune deviation is considered as a major pathogenic cause for drug resistance in TB. Protective immunity to intracellular microbes predominantly relies on release of T cells cytokines, such as IL-12 and IFN γ which activate macrophages to kill intracellular mycobacteria (9).

In order to elucidate the possible concordance in Th1/Th2 cross-regulation, the cytokine profiles of Th1/Th2 cells are measured in PPD and PHA-stimulated PBMC in vitro. The mean concentration of IFN γ in supernatant of stimulated PBMC is reported to be significantly higher in MDR-TB patients. During mycobacterial infection the bidirectional interaction between macrophage and T cells resulted in macrophage activation. Macrophages that have phagocytosed microbes produce IL-12, stimulating undifferentiated naive CD $_4^+$ T cells to Th1 subset which produce IFN γ . In a recent study (3) IFN γ production of stimulated PBMC was inhibited in MDR-TB patients with absolute CD $_4^+$ counts less than $500/\text{cm}^3$ while in those patients with partially normal CD $_4^+$ T cells ($>500/\text{cm}^3$) interferon release was close to normal. Therefore, it is concluded that T cell proliferative response to PPD is most probably normal in those patients with normal quantitative level of CD $_4^+$ T cells. Mansoori et al. studied the efficacy of IFN α in the treatment of MDR-TB and

concluded that cytokines had at least a temporary effect on disease remission and could be used as adjunctive therapy (10). IL-12 is a potent induced substance by IFN γ of mononuclear phagocytes and is expected to rise in parallel with IFN γ . IL-12 P40 subunit is considered as a remarkable biological indicator of Th1 activity (11) and is probably closely related to IFN γ production which is not true for IL-12 dimeric molecule.

The prominent stimulation of Th1 causes down regulation of Th2 resulting in lowered production of IL-10. Several cytokines produced by Th2 cells including IL-4, IL-10, and IL-13, inhibit macrophage activation; thus, Th2 cells may serve to terminate Th1-mediated DTH reaction. IL-4 participates mainly in differentiation of Th2 cells from naive CD $_4^+$ precursor and stimulation of IgE production by B cells. IL-4 is a stable molecule and can not be easily detected by conventional immunoassays unless it is markedly stimulated through Th2 activation, which does not occur during mycobacterial infection.

The predominant Th1 activity reported in MDR-TB patients in this study is not compatible with the data reported by Lee and McDyer's studies (3,11), and it is suggested that the probable local respiratory immune cells may react with intracellular microbes differently. Measurement of local cytokine profile is strongly recommended that must be performed on the cellular infiltrate of broncho-alveolar lavage (BAL). Schwander et al. reported that the mononuclear cells infiltrate of BAL produced 24 times more interferon in response to PPD as compared with normal individuals (12).

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