

Measurement of Peripheral Blood Mononuclear Cells Producing IFN-Gamma in Patients With Tuberculosis

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The type of immune response induced by tuberculosis (Th1 or Th2) and its correlation with the clinical outcome is unclear. We studied 13 patients with active tuberculosis (TBC). The peripheral blood mononuclear cells producing IFN-gamma (PBMC-IG) were measured by enzyme-linked immunospot (ELISPOT) technique. The control group had ten healthy individuals vaccinated against tuberculosis. We collected blood samples of each patient in two moments: a) in the hospital admission without treatment (TBC1); b) after seven to 20 days of treatment (TBC2). The comparison of the spots forming units of PBMC-IG between TBC group and controls showed that there was a significant difference between TBC1 and control group ($p < 0.001$) and between TBC2 and control group ($p < 0.005$), but there was no difference between TBC1 and TBC2 ($p > 0.05$). A positive correlation was found between PBMC-IG and hemoglobin value, as well as between PBMC-IG and weight loss. There was no correlation between PBMC-IG and other variables [age, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP)]. We conclude that tuberculosis activates Th1 immune response due to increase of PBMC producing IFN-gamma. There was no difference between the first sample (TBC1) and the second sample (TBC2) of PBMC-IG. This result can have occurred due to treatment influence, or can indicate that the immune response reaches a plateau. The positive correlation among PBMC-IG and both hemoglobin level and weight loss indicates that may exist a link between patient's clinical status and the immune response intensity.

Key-Words: Tuberculosis, Th1 immune response, ELISPOT.

The characterization of T cell responses by measurement of cytokine secretion by ELISPOT assay has become widely used, mainly in the vaccine production, cancer immunotherapy [1,2], and in the immune response evaluation against infectious agents [1,3,4].

The role of cytokines produced by Th1/Th2 lymphocytes is complex due to the action of TNF-alpha, IL-12, IFN-gamma (pro-inflammatory cytokines) and IL-4, IL-10 and TGF-beta (anti-inflammatory cytokines). Th1 lymphocytes produce pro-inflammatory cytokines while Th2 lymphocytes produce anti-inflammatory cytokines. Normally, the Th1 immune response is correlated with tissue injury. In this way, the clinical form of infectious diseases, as well as the cell death intensity will be determined by the predominant immune response, Th1 or Th2. In patients with tuberculosis (TBC) and PPD skin test positive the predominant immune response may be Th1, although there are few studies in this field.

The cellular immune response is essential for tuberculosis resistance, mainly through cytokines production such as IL-12, IL-18, IFN-gamma, IL-2 and TNF-alpha. The CD8+ T cells perform cytotoxic action mainly through major histocompatibility complex (MHC) class I molecules in association with CD1d molecules presenting glycolipids antigens to T lymphocyte cells [5]. Cytotoxic T lymphocyte

cells (CTLs) may have a protective role in TBC disease by several mechanisms: 1) Producing potent antibacterial cytokines such as IFN-gamma and TNF-alpha; 2) Presenting *M. tuberculosis* antigens associated to HLA-I complex; 3) Inducing apoptosis in infected cells [6,7].

The enzyme-linked immunosorbent spot (ELISPOT) technique has been used in a crescent form for quantification of peripheral blood mononuclear cells (PBMC) producing specific cytokines in patients with infectious diseases [2,8,9]. There are several advantages of the ELISPOT assay for use in field settings: it is relatively easy to perform, does not require expensive instrumentation, and has the potential for high throughput screening. Through ELISPOT technique, several authors reported that the quantification of peripheral blood mononuclear cells producing IFN-gamma (PBMC-IG) presented cytotoxicity in response to influenzae peptide [1,2,9]. Several studies have shown that ELISPOT is more sensitive for detection of cytokines than ELISA technique [10-12]. However, there are not studies about PBMC-IG in patients with tuberculosis.

The discovery of the predominance of a specific cytokine in the inflammatory microenvironment, as well as in the peripheral blood, may help to understand the evolution of an infectious agent in the organism. The CD8+ T cells perform cytotoxic action mainly through major histocompatibility complex (MHC) class I molecules in association with CD1d molecules presenting glycolipids antigens to T lymphocyte cells [5].

In this study we quantified the PBMC-IG in patients with active tuberculosis employing the ELISPOT technique. Our results indicate that tuberculosis activates the Th1 immune response, and also that there is a positive correlation between PBMC-IG and some clinical and laboratory variables.

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Material and Methods

Patients Selection

Thirteen patients with active tuberculosis were selected from Infectious Diseases Clinic and Internal Medicine Clinic of the Clinics Hospital, School of Medicine, University of São Paulo. The control group had ten healthy individuals vaccinated against tuberculosis. Informed consent was obtained from each patient and the protocol for the research project was approved by the ethics committee of the institution.

We excluded patients with HIV test (ELISA) positive, and patients with immunodeficient diseases. Sera of patients and control group were obtained under sterile conditions and disposed in tubes containing sodium heparin (0.1 mg/mL). The maximum volume of the sample collected from each patient was 20 mL. We collected blood samples of each patient in two moments: TBC1 = in admission without treatment; TBC2 = after seven to 20 days under treatment.

Peripheral Blood Mononuclear Cells Isolation

The PBMC isolation followed the protocol based in other studies [8,9] as follows: PBMC were isolated by centrifugation gradient with Ficoll-Paque Plus (Amersham). The cells were washed with saline solution and resuspended in Iscove's B medium (Sigma) with 4 mM of l-glutamine (v/v), 100 U/L of penicillin, 100 U/L of streptomycin, enriched with 10% of human AB serum pool (Cultilab), and stored in fetal bovine serum containing DMSO 10% in liquid nitrogen. The freezing technique was efficient, producing similar replies to fresh cells [13]. The isolated PBMC were incubated overnight in Iscove's medium containing fetal bovine serum (10%) before ELISPOT assay.

The cells were counted in Neubauer chamber and disposed in a concentration of 1×10^4 cells/mL in Iscove's medium, containing fetal bovine serum (10%).

ELISPOT Assay

We added 100 mL of each sample (with 5×10^4 cells) of ten controls and 13 tuberculosis patients in a 96-well PVDF plate coated with a specific IFN-gamma antibody. The plate was incubated at 37°C in a CO₂ incubator for 48 h. The contents of the plate were discarded and the plate was thoroughly washed with 300 µL/well of 1x wash buffer, taking care to not damage the membrane. This procedure was repeated for a total of six washes. After washing, the plate was turned upside down and blotted onto paper towels. We added 50 µL of IFN-gamma-detection antibody to each well. The plate was covered and incubated for 1 h at room temperature. After this, it was washed six times again. We added 100 µL of diluted streptavidin-AP conjugate to each well. The plate was covered and incubated for one hour at room temperature, and it was washed six times again. Then, we added 100 µL of NBT/BCIP substrate solution to each well. The color reaction was developed at room temperature, in the dark, for 3-15 minutes. The contents of the plate were discarded and it was rinsed with ultra pure water to

stop the reaction. The plate was turned upside down, allowing the membrane to completely dry prior to analysis. The spot-forming units (SFU) had been counted by using a dissection microscope (Quimis).

Statistical Analysis

The variables were tested about Gaussian distribution through Kolmogorov-Smirnov test (K-S) and Shapiro-Wilks test, and they were classified as parametric or nonparametric. The values were expressed as mean and standard deviation for parametric variables. In this group, we realized Student's t test unpaired for two groups comparison, and ANOVA test followed by Newman-Keuls post-test for comparison of more than two groups. Otherwise, the values were expressed as median and sample variability is reported as 25% and 75% percentiles for nonparametric variables. In this group we utilized the Kruskal-Wallis multiple comparisons test with Muller-Dunn post-test when comparing two or more unpaired groups and the unpaired Mann-Whitney test was used to compare two distinct groups.

The Pearson's correlation was made when indicated. An alpha risk equal or lower than 5% ($p \leq 0.05$) was considered statistically significant.

Results

The data of patients' clinical status were: 9/13 had weight loss ranging between three and 26 Kg; 8/13 had fever; 8/13 had cough without sputum; 6/13 had night sweats; 2/13 had cough with sputum. The diagnostic imaging tests showed: 10/13 had chest X-ray abnormal, of which 1/13 developed pulmonary cavitations, and 3/13 had signs of lymph nodes necrosis. The tuberculosis group had four women and nine men, and the control group had seven women and three men.

The tuberculosis patients had the following clinical forms: 9 = pulmonary, 1 = pleural, 1 = lymphatic + splenic, and 2 = lymphatic. In relation to the clinical therapy, 10/13 did not have received anti-tuberculosis drugs at the time of blood sampling, and 3/13 were using therapeutic scheme with isoniazid, rifampin and pyrazinamide for less than one week. The PPD skin test was positive in 4/13. The C-reactive protein was high in 6/13. The acid fast bacilli in sputum were positive in 11/13.

SFU counting of PBMC-IG of control group is shown in Table 1, and the SFU counting of tuberculosis group is shown in Table 2 (Figure 1). The numbers are concerning to spots number by 1×10^4 PBMC. The comparison of the spots counting between PBMC-IG and the control group are shown in Table 3. There was a significant difference of PBMC-IG counting between TBC1 (first sample) and control group ($p < 0.001$) and between TBC2 (second sample) and control group ($p < 0.05$), but there was no difference between TBC1 and TBC2 ($p > 0.05$).

The values (mean and standard deviation) of TBC1 and TBC2, age, weight loss, hemoglobin, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) are shown in Table 4. A positive correlation was found between

Table 1. Count of PBMC-IG spots of control group

Patients	Count of PBMC-IG
1	6
2	2
3	1
4	1
5	3
6	2
7	0
8	2
9	1
10	0

Table 2. Count of PBMC-IG of tuberculosis patients

Patients	TBC1*	TBC2
1	5	0
2	26	10
3	23	9
4	16	21
5	27	25
6	21	18
7	3	-
8	23	-
9	30	24
10	45	-
11	11	12
12	12	21
13	65	13

Note: * = first sample; ** = second sample.

Table 3. Comparison of PBMC-IG counts among TBC1, TBC2 and control group in patients with tuberculosis

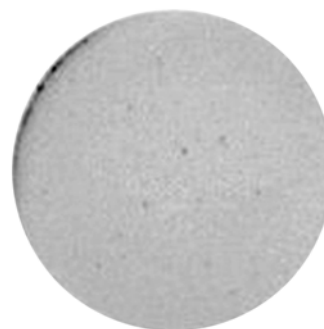
	Control	TBC1	TBC2
Mean	1.80	23.62**	15.30 **
Standard-Deviation	1.75	16.73	7.89
Standard-Error	0.55	4.64	2.50

Note: We used the ANOVA one-way test. ** p = 0.0004. Statistical difference among groups (Newman-Keuls post-test): Control versus TBC1 = p < 0.001; Control versus TBC2 = p < 0.05; TBC2 versus TBC1 = p > 0.05 (not significant).

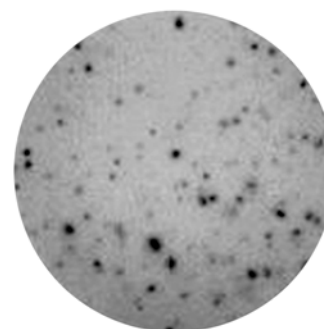
Table 4. Values of mean and standard-deviation of PBMC-IG counts for TBC1, TBC2, clinical and laboratory variables in patients with tuberculosis

	Patients (N)	Mean	Standard-deviation
TBC1	13	23.62	16.731
TBC2	10	15.30	7.889
Age	13	40.54	16.184
Weight loss	8	8.13	7.936
Hemoglobin	12	12.833	1.8763
ESR	11	46.64	36.694
CRP	7	78.537	65.4619

Figure 1. Comparison of spots obtained in control and TBC patient groups.



CONTROL GROUP
(Control number 7)



TBC GROUP
(Patient number 9)

PBMC-IG and hemoglobin value (Pearson correlation = 0.714; p = 0.031; n = 9), as well as between PBMC-IG and weight loss (Pearson correlation = 0.702; p = 0.05; n = 8) degree. There was no correlation between PBMC-IG and other variables.

Discussion

The results showed that ELISPOT method used here was efficient for measurement of PBMC-IG in patients with active tuberculosis. We verified that patients with active tuberculosis produce significantly higher PBMC-IG in relation to control group. These results suggest that tuberculosis activates Th1 immune response due to increase of PBMC-IG. IFN-gamma is a product mainly of natural killer cells (NK), activates T cells, and has an important role in host defense against mycobacteria. IFN-gamma modulates the macrophages activation, increases MHC class II molecules expression and antigen presentation, and also increases differentiation of lymphocyte populations [14]. This cytokine has been measured *ex vivo* in serum [15], bronchoalveolar lavage fluids [16] and pleural effusions of TBC patients [17]. Out of seven studies using ELISPOT, for found that TBC patients produce more IFN-gamma than controls [18-21], one found that they produce less [22], and two found no difference [23,24].

Therefore, in order to establish definitive conclusions about these discrepant results, more studies should be done.

In patients with active tuberculosis, the PBMC-IG count in second sample (TBC2) was lesser than PBMC-IG count in first sample (TBC1), but without statistical difference. TBC2 determination was made within seven to 20 days of the beginning of anti-tuberculosis treatment. In the same way, Bekker et al. [25] showed that the serum IFN-gamma levels decreased in patients with TBC after beginning of therapeutic. Hirsch et al. [26] reported that IFN-gamma production was lower at the TBC diagnosis, and this phenomenon remains for 12 months. The authors suggested that TBC can induce a lesser response of T lymphocytes during and after infection. We speculate that the stability of PBMC-IG values between TBC1 and TBC2 can have occurred due to treatment influence, and to the consequent decrease of the bacterial load in each case. Another possibility is that the immune response reaches a plateau in normal conditions, or it suffers inhibition for still unknown mechanism.

In the analysis of the correlation between PBMC-IG levels and clinical and laboratory variables, we found a positive correlation among PBMC-IG and hemoglobin and weight loss. This result indicates that may be a link between patient's clinical status and the immune response intensity. We may consider that greater weight loss indicates higher bacterial load. In relation to hemoglobin level, it is possible that a patient with higher hemoglobin is in better clinical condition, and its immune response will be better too. The correlations between PBMC-IG and clinical and laboratory variables may indicate that the immune response against tuberculosis are not "neck or nothing" type. It is possible that the immune response may be modulated by other factors, as patient's clinical status. We did not find a correlation between PBMC-IG and C-reactive protein, as well as between PBMC-IG and erythrocyte sedimentation rate. These two clinical tests may not have correlation with the true inflammatory response that is occurring.

The positive correlation between weight loss and PBMC-IG is coherent with other studies that had found correlation between serum TNF-alpha levels and weight loss. TNF-alpha and IFN-gamma take part in the Th1 immune response. Bossola et al. [27] showed that serum TNF-alpha concentrations were significantly higher in patients with cancer and severe weight loss when compared with patients having cancer and low weight loss. Cakir et al. [28] stated that serum TNF-alpha level and leptin might be responsible for the weight loss in pulmonary tuberculosis patients. Another study also correlated chronic administration of TNF-alpha with weight loss in rats [29].

Another existing knowledge indicates that both TNF-alpha and IFN-gamma participate in the granuloma formation and they also appear crucial to the formation of *M. tuberculosis*-constraining granuloma. Beyond this effect, IFN-gamma seems to exert a main role as endogen activator agent for murine macrophages and their antimycobacterial effects

[30,31]. Other study showed that IFN-gamma actuates synergistically with TNF-alpha for these antimycobacterial effects [32].

Our study suggests that true immunological tests as ELISPOT for PBMC-IG detection must be added to therapeutical arsenal for patients with TBC. The greater knowledge about immune response type in infectious diseases provides new perspectives for treatment of these diseases in a near future. The predominance of Th1 immune response induces a greater cellular death by necrosis or apoptosis, reaching both host and inflammatory cells. We reasoned that the inhibition of Th1 response for reduction of tissue injury might be considered in selected cases. We think that the modulation of the immune response besides of the treatment of infectious agents will be part of infectious diseases therapy.

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