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Clinical Study

Evaluation of the Immune Response of Individuals Infected with *Mycobacterium tuberculosis* and Patients with Active Tuberculosis

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This study reports the association between *Mycobacterium tuberculosis* and the immune response to pulmonary tuberculosis (TB). Three groups were analyzed: (a) symptomatic patients with pulmonary tuberculosis (PTB), HIV-negative; (b) healthy individuals, tuberculin skin test reactive (TST^+); (c) asymptomatic individuals, TST nonreactive (TST^-). Groups B and C presented a negative bacilloscopic smear, normal chest radiographs, and negative HIV. The ELISA was used for IFN-γ, IL-10, TNF-α, and IgG quantification and lymph proliferative assay (LPA) to evaluate the cellular immune response. IgG and LPA increased in all study groups as well as IFN-γ and TNF-α, but IL-10 remained low in all study groups. There was an association between LPA and IFN-γ in group B. It was demonstrated an association between IgG and IL-10 and between IFN-γ and IL-10 in group A. There were direct and significant correlations between LPA and IgG, TNF-α and IFN-γ, IL-10 and IgG, and between IL-10 and IFN-γ, but an inverse relationship was observed between IFN-γ and LPA.

1. Introduction

Tuberculosis (TB) is caused mainly by *Mycobacterium tuberculosis*, an optional intracellular pathogen. Despite advances in the knowledge of TB, this disease remains a serious public health problem worldwide, especially in developing countries. However, despite the severity of this disease, the mechanism of protective immunity against *M. tuberculosis* in humans has not been fully elucidated. Numerous studies emphasize the important role of cellular immunity in the elimination of bacilli [1–3]. Resistance to mycobacterial infections is conferred by common immunological mechanisms mediated by $CD4^+$ T lymphocytes, involving cytokines that increase the microbicide activity of macrophages [4–6]. Studies in murine and human models allow differentiation between two subpopulations of $CD4^+$ T lymphocytes, denominated Th1 and Th2 that mediate the protection or the aggravation of the disease [7, 8]. This dichotomy between the existing protective or nonprotective immune responses is likely to be influenced by cytokine patterns produced by different subpopulations of lymphocytes that survive during the initial stages of the pathogens inside macrophages. IFN-γ acts as a powerful macrophage activator, increasing the molecular expression of the major histocompatibility complex (MHC) and potentialization in the cellular response, including the production of cytokines and nitric oxide as well as an increase in the cytolytic activity, with the fundamental role in the Th1 type [9, 10].

TNF-α also has a key role in the mycobacteria elimination process, acting in synergy with IFN-γ to stimulate the production of reactive oxygen and nitrogen intermediates, thereby mediating the tuberculostatic action of macrophages.

TNF-α stimulates immune cells to shift the focus of infection, promoting the formation of a well-organized structure (granuloma) capable of controlling the progression of the disease [10, 11]. The IL-10 cytokine produced by macrophages and T lymphocytes during infection with *M. tuberculosis* inhibits the production of pro inflammatory cytokines by antigen-presenting cells and blocking the activation of T cells by inhibiting the expression of class II MHC [6]. The IL-10 has an immunoregulatory function considering its ability to drive the response of T lymphocytes to a Th2 profile [12, 13]. The increased levels of IL-10 contribute to the inhibition of the IFN-γ production, compromising the microbicidal mechanisms of macrophages and the presentation of antigens. It also has a TNF-α opposite effect protecting against tissue damage by regulating inflammation and apoptosis [14]. The balance of the cytokines produced during the inflammatory response is a determinant key of the nature and effectiveness of the immune response. In addition to the importance of better understanding the immunological mechanisms that may contribute to healing, it becomes essential to investigate some cellular immunological mechanisms involved in TB and to evaluate a possible correlation between the disease and the activation of the immune system.

2. Materials and Methods

2.1. Subjects Studied. Patients with pulmonary or extrapulmonary tuberculosis (PTB), asymptomatic individuals with positive tuberculin skin test (TST) positive was defined as $\geq$10 mm, being considered as a latent *M. tuberculosis* infection (LTBI) and asymptomatic subjects with negative TST were selected for the study. All the patients responded to a standardized questionnaire and were assessed for the epidemiological (history of contact), clinical and laboratorial aspects (smear and/or culture for mycobacterium, serology for human immunodeficiency virus (HIV), and chest X-rays), and vaccination with Bacillus Calmette-Guerin (BCG). These patients were treated at the TB clinic, Hospital das Clínicas, Federal University of Minas Gerais (HC/FM/UFMG) and the Oswaldo Cruz Health Center, Belo Horizonte, Minas Gerais, Brazil, from December 2001 to June 2005. This study was approved by the Ethics Committee of the UFMG. The TST (0.1 mL) with a purified protein derivative (PPD-RT 23 *Mycobacterium tuberculosis* (Mtb), two UT, Copenhagen, Denmark) was administered by the Mantoux technique in the left forearm (TST1, intradermally). The second TST2 was performed (right forearm) after one week to assess the booster effect (increase in induration with and increment of 6 mm and $\geq$10 mm). The reading was performed by observing the induration after 72 to 96 hours and was done by trained and certified health care workers (degree of intrareaders >95% and interreaders $\geq$80%). The area was measured with a ruler and the result in millimeters was given of the greatest induration measured transversely.

Bacteriology (bacilloscopic smear, culture identification, and sensitivity testing of mycobacteria) was performed according to the recommendations of the Ministry of Health, Brazil (MS, 2008). The HIV antibody test was performed by the Western blot reaction (HIV LIATEK III kit - Organon Teknika). The radiological (X-ray) alterations were classified as follows: (1) Patients with pulmonary tuberculosis (PTB): (a) noncavitary lesions, (b) cavitary lesions. (2) Individuals with LTBI without signs and symptoms of active TB: (a) normal, (b) small calcifications. The diagnosis of extrapulmonary TB patients was provided by the clinical, histopathological, and TST-positive signs and by evaluation after treatment. The control group consisted of healthy, TST-negative, and HIV-negative volunteers, without radiological signs suggestive of active TB, from the School of Medicine and the School of Pharmacy, UFMG, Brazil.

2.2. Definition of Groups. All the participants were HIV seronegative and were divided into Group A: 23 (twenty-three) asymptomatic patients with negative TST, Group B: 52 (fifty-two) patients with LTBI, and Group C: 96 (ninety-six) patients with TB before and after treatment.

2.3. Antigen. Bacillus Calmette-Guerin (BCG), from the Ataulpho de Paiva Foundation, Lot 9910178, was kindly provided by the Municipal Health Secretariat of Belo Horizonte, Minas Gerais, Brazil.

2.4. Lymphoproliferative Assay (LPA). To isolate the mononuclear cells from the peripheral blood mononuclear cells (PBMCs), the collected blood was applied to a Ficoll-Hypaque mixture at a ratio of 1 : 2 (Ficoll-Hypaque : blood) in sterile polystyrene tubes with conical bottoms (Falcon, Corning, USA). After centrifugation at 400 g for 45 minutes at 18°C, the PBMC ring that formed at the Ficoll-Hypaque, plasma interface was removed. The cells were washed three times with RPMI-1640, centrifuged for 200 g at 4°C for 10 min, and resuspended in 1.0 mL of RPMI-1640. The cell concentration was adjusted to 1.5×10^6 cells/mL after counting in a Neubauer hemocytometer chamber by diluting in RPMI-1640 supplemented with 5% human AB, Rh-negative serum. A 0.2-mL aliquot of the PBMC suspension was added to each well of a 96-well polystyrene plate for cell cultivation. Cultures were performed in triplicate, with and without stimulus with 10 μL of BCG (1 mg/mL). Stimulation with the PHA mitogen (phytohemagglutinin, Sigma Chemical Co., USA) at a final concentration of 0.5 mg/well was used as a positive control. The cultures were then incubated at 37°C in 95% humidity atmosphere and 5% CO_2 for 5 days. On the last day of culture, 25 mL of RPMI-1640 containing 0.2 mCi of ^{3}H-thymidine (specific activity 5.3 Ci/mM, Sigma Chemical Co., USA) was added to each well. After 16–18 hours, cells were isolated on glass fiber paper (model 943-AH-Whatman, USA) with the aid of an automatic cell collector (Titertek Cell Harvester, Flow Laboratories, USA). The papers containing the cells were dried and placed in scintillation vials (Wheaton, USA) containing 1 mL of scintillation solution. Radioactivity was determined in counts per minute (cpm) using a beta scintillation spectrometer (model 1209, Rackbeta, LKB). The LPA intensity of the PBMC was determined by calculating the stimulation index

(SI) obtained by dividing the average cpm of three wells stimulated with antigen or mitogen by the mean cpm of three wells containing nonstimulated cells. Stimulation indexes greater than or equal to three were considered to be representative of the LPA values.

2.5. Cytokine Levels. The PBMC were grown in duplicate at a concentration of 1.5×10^6 cells/mL in 24-well plates in the presence of 50 μL of BCG (1 mg/mL) and in the absence of antigen to test the cytokine doses (IFN-γ, IL-10, and TNF-α). The supernatants were collected 72 hours after the antigen stimulus, in accordance with the standardization performed in preliminary studies. The concentrations of cytokines were determined by Enzyme-Linked ImmunoSorbent Assay (ELISA) using commercial kits (Quantikine Human Immunoassay, R & D, USA). The cutoff value for IFN-γ was 378, that for TNF-α was 36.3, and that for IL-10 was 126.2. The sensitivity of the commercial Quantikine kit was 8 pg/mL for IFN-γ, 4.4 pg/mL for TNF-α, and 3.9 pg/mL for IL-10.

2.6. Immunoenzymatic Assay for IgG. Polystyrene plates (Hemobag, Brazil) were sensitized with 2 μg/100 mL/well of BCG. To each well was added 100 μL of plasma (diluted 1 : 50) obtained from the groups analyzed. After washing, 100 μL of anti-human IgG conjugate (Sigma Chemical Co., USA) was added to each well. The reaction was developed by adding *o*-phenylenediamine (OPD) and H_2O_2 as substrates. The absorbances were determined with the aid of an automatic microplate reader fitted with a 492 nm filter (BioRad model 550). Immunoenzymatic reactions were performed with 20 volunteers presenting TST-negative plasma to determine the cutoff point, which was 214 for IgG.

2.7. Statistical Analysis

Method. Initially, a descriptive analysis of the variables used in the study was performed. For nominal categorical variables (e.g., gender), frequency distribution tables were employed. For numeric variables (such as age), measurements of a central tendency (mean, median) and variability (minimum, maximum, and standard deviation), the analyses were stratified by group, that is, independent analyses were performed for the negative TST and positive TST groups and for patients with tuberculosis.

Crossings. The Pearson Chi-square test, suitable for comparison of proportions, or the Fisher's test, for samples with small frequencies, was used at the crossings between categorical variables. The nonparametric Kruskal-Wallis test was employed to discriminate the differences between the three groups when numerical variables were considered. A nonparametric test was utilized because of the asymmetric nature of the variables tested. In the group with tuberculosis, the results of the tests before and after treatment were compared using the nonparametric Wilcoxon test.

Comparison between the Three Groups. The Chi-square test or the nonparametric Kruskal-Wallis test, suitable for comparing three or more groups, was used to compare between the three groups (negative TST, positive TST, and TB patients). A 5% level of significance was considered in all the tests, and the SPSS 12.0 software was used.

3. Results

3.1. Characteristics of the Samples Studied. In Group A, 52.2% of the patients were female. The mean age was 42 years (25 to 60 years). Of the individual patients, 60.9% had been vaccinated with BCG and 43.5% had contact with carriers of TB. The levels of IgG were high in 35.3% of the cases and the SI was high in 55% (median = 3.1). The levels of IFN-γ and TNF-α were high in 25% and 57.1%, respectively, but the levels of IL-10 were negative in 100% of the cases. The median for the IgG levels in this group was 146. None of the variables analyzed showed any statistically significant association with the LPA results.

In group B, 69.2% of the patients were female and the mean age was 39.1 years (20–64 years). In this group, 71.4% of the patients had been vaccinated with BCG, 13.6% had been revaccinated, and 59.1% had been in contact with individuals infected with TB. The X-ray presented small calcifications in 57.5% of the cases, but without disease signs. The IgG levels were high in 30.6% of the cases (median 147), and a positive SI occurred in 48.6% of the cases, although the median was 2.8%. The levels of IFN-γ, IL-10, and TNF-α were high in 23.1% (median 141), 50% (median 123), and 80% (median 1168) of the cases, respectively. There was a statistically significant association between the results of the SI and the IFN-γ ($P = 0.027$) (Table 1).

In group C, 45.8% were female. The mean age was 33.9 years (15–66 years). In patients group, 94 had PTB and two had extrapulmonary TB (eye and skin). In 93 patients (96.6%), the bacteriology was positive. The patient with ocular TB presented a probable diagnosis after epidemiological and clinical evaluation (history of contact) and exclusion of other causes of eye disease such as toxoplasmosis, syphilis, collagen diseases, sarcoidosis, and positive TST. The diagnosis of the patient with skin TB was made by clinical, epidemiological, histopathological (the skin biopsy showed caseous necrosis), and positive TST examination. The TB patients presented ocular uveitis, negative serology for toxoplasmosis and syphilis, no signs or symptoms of sarcoidosis or collagen disease, history of contact with TB, a positive TST, no improvement with the use of topical, and systemic corticosteroids. All the PTB and extrapulmonary TB patients were considered as cured at the end of treatment and had no recurrence of the disease by the end of the study. In 59.2% of the cases, the X-rays presented cavitations. The IgG levels before treatment were altered in 82% of the cases and 81.1% presented high SI levels. The levels of IFN-γ, TNF-α, and IL-10 were high in 47.6%, 66%, and 100% of the cases, respectively. There was a statistically significant difference between the measurements of IL-10 ($P = 0.030$) and TNF-α ($P = 0.006$) in groups A, B, and C (Table 1). The medians

Table 1: Comparison of the results of the immunological tests by group.

Immunological tests	Group A	Group B	Group C	*P* value
IgG				
≤214 (cut off)	11 (64.7%)	25 (69.4%)	11 (18.0%)	<0.001*
>214	6 (35.3%)	11 (30.6%)	50 (82.0%)	
Median	146	147	685.1	
LPA (SI)				
≤3 (cut off)	9 (45.0%)	19 (51.4%)	7 (18.9%)	0.011*
>3	11 (55.0%)	18 (48.6%)	30 (81.1%)	
Median	3.1	2.8	6.5	
IFN-γ				
≤378 (cut off)	3 (75.0%)	10 (76.9%)	22 (52.4%)	0.268*
>378	1 (25.0%)	3 (23.1%)	20 (47.6%)	
Median	174.8	141	2045	
IL-10				
≤126.2 (cut off)	4 (100.0%)	4 (50.0%)	16 (34.0%)	0.030**
>126.2	0 (0.0%)	4 (50.0%)	31 (66.0%)	
Median	0	123	250	
TNF-α				
≤36.3 (cut off)	3 (42.9%)	2 (20.0%)	0 (0.0%)	0.006**
>36.3	4 (57.1%)	8 (80.0%)	24 (100.0%)	
Median	370	1168	1038.5	

* Chi-squared test, ** Fisher's test.

IgG levels determined by ELISA in the plasma of healthy TST$^-$ individuals (Group A), of LTBI (Group B), and patients with active tuberculosis (Group C). Calculation of the stimulation index (SI) obtained after testing the lymph proliferative assay (LPA) after PBMC stimulation with BCG for 72 hours. Measurement of the cytokine levels (IFN-γ, TNF-α, and IL-10) obtained from the supernatant of a PBMC culture from individuals from Groups A, B, and C after stimulation for 72 hours with BCG. The median represents the absorbance obtained by the ELISA reaction for IgG levels in plasma and cytokines in supernatants of PBMC cultures stimulated with BCG. However, in the LPA, the median signifies the SI.

of these two cytokines were higher after treatment (Table 2). Significant direct correlations were observed for LPA (SI) with IgG, TNF-α with IFN-γ, IL-10 with IgG, and IFN-γ with IL-10 (Table 3). There was a significant inverse correlation between IFN-γ and LPA (SI). According to Table 1, there was a significant difference between the IgG, LPA, IL-10, and TNF-α group. The high IgG, LPA, IL-10, and TNFα values were more frequent in the group with tuberculosis.

Table 2: Descriptions of the continuous tests before and after the treatment of the tuberculosis group.

Treatment		IgG	IFN-γ	TNF-α	IL-10
Before	N	61	42	24	47
	Median	666.0	**204.5**	**1038.5**	250.0
After	N	20	35	28	41
	Median	431.0	**952.0**	**1361.0**	354.0
	P Value*	0.060	**<0.001**	**0.019**	0.210

This table shows a significant difference between the levels of IFN-γ and TNF-α before and after treatment (*P* value <0.05). The median values were higher after treatment.

4. Discussion

The cellular and humoral immune responses were determined in individuals with latent infection (TST positive) and TB patients and compared with those without TB (TST negative) to assess the immunological profile of the immune response. The TB was treated with rifampicin, isoniazid, and pyrazinamide for two months, followed by rifampicin and isoniazid for four months [15]. In this study, the groups were matched according to sex and age.

There was an immune response to BCG antigens in group A, but not as intense as that observed in group C. Although the patients were TST negative, an immunological memory was observed in all the parameters evaluated, probably as a result of the BCG vaccination or due to TB. This fact can be explained by rendering sensitive to Mycobacterium antigens and the TST response in the skin may not be evident since it already has been shown that not all individuals respond to the test (low sensitivity and specificity) [16, 17]. Wang et al. [18] demonstrated a reduction in the relative risk of obtaining a positive TST of 3.56 to 1.46 when the time between vaccination and the TST is less than or equal to 15 years or greater than 15 years, respectively [19]. The fact that certain individuals were not immune did not respond to TST, but had contact with TB, may be explained by physical and chemical barriers to the innate immunity that is important for the prevention of infection [20]. This defense mechanism must be efficient, since only 5% of the individuals who come into contact with the infection develop TB [15].

Table 3: Spearman's correlation between the markers of the whole group.

Immunological tests	Correlation	IgG	LPA (SI)	IFN-γ	TNF-α	IL-10
IgG	Correlation	1				
	P value	—				
LPA (BCG)	Correlation	0.133	1			
	P value	0.275	—			
IFN-γ	Correlation	**0.401**	**0.358**	1		
	P value	**0.002**	**0.027**	—		
TNF-α	Correlation	0.098	0.069	**0.502**	1	
	P value	0.564	0.706	**0.006**	—	
IL-10	Correlation	**0.425**	−0.253	**0.373**	0.315	1
	P value	**0.001**	0.143	**0.007**	0.079	—

There is a significant and direct correlation between TNF-α and IFN-γ; IL-10 and IgG; IL-10 and IFN-γ. There is a significant inverse correlation between IFN-γ and BCG.

A response to immunological parameters, similar to that of group A, was observed for group B. The cellular immune response was significant for the SI and IFN-γ results. This result might be caused by the *M. tuberculosis* antigen, since individuals in this group had a history of contact, positive TST, and X-rays showing minor changes suggestive of prior infection (suggestive Gohn complex), probably due to LTBI. This result corroborates to those of other authors [21]. TNF-α presented high levels compared to those of Group A. This fact suggests that the infection was controlled, since this cytokine has an important involvement in granuloma formation ($P < 0.05$). These data corroborate those reported by Burns et al. [11].

In the group of TB patients (group C), before treatment, an equilibrium in the levels of cytokines produced by the Th1 (IFN-γ) and Th2 (IL-10) lymphocyte subpopulations observed, since the patients were sick, but still able to maintain the stability between the disease and the immune response (Table 2). After treatment, the levels of IFN-γ were higher ($P < 0.01$), which means a more intense immune response, probably due to an antigenic discharge released into the bloodstream due to the treatment that eliminates the bacilli or perhaps to a reduction in the transit of T-lymphocytes antigen-specific to the lung. An increase in the lymph proliferative response of the PBMC from tuberculosis patients when compared to those of groups A and B was observed. In group C, 81.1% of the patients had positive stimulation indexes (SI > 3).

The increase in IFN-γ levels obtained after treatment reinforces its important role in the immune response against *M. tuberculosis*. IFN-γ is the main cytokine involved in the protective immune response against infection caused by mycobacteria. Its main function is the activation of macrophages, making them able to carry out their microbicide functions. It also acts by increasing the expression of molecules of the principal class I and II of major histocompatibility complex (MHC) and promotes the differentiation of lymphocytes to the Th1 subset [5, 22]. The protective immunity against *M. tuberculosis* is not completely understood but depends on a wide range of innate and adaptive immune mechanisms. T-cell-mediated immune responses are important in the host control of *M. tuberculosis* infection. The ability of $CD4^+$ T cells to produce IFN-γ which activates phagocytes to trap the intracellular pathogen is central to protection. This fact is evident from the increased risk of tuberculosis in individuals with deficiencies in their IFN-γ and IL-12 (which promotes Th1 cell differentiation) signaling pathways [9], from the association between $CD4^+$ T cell depletion, and elevated susceptibility to TB in HIV^- infected individuals [23]. Studies of people with gene mutations in receptors for IFN-γ proved that they had high susceptibility to infections caused by atypical mycobacteria. In this study, the average IFN-γ level observed in the PBMC supernatants culture from the patients with pulmonary TB was high before and increased significantly after the treatment. All the patients presented values of IFN-γ above the preestablished cutoff value (82 pg/mL). Several authors have shown that the pleural fluid of patients with TB contained a high number of IFN-γ-producing cells [24–26] and have suggested that these cells might migrate to the lung and pleural tissue during the active disease. Therefore, they might be reduced temporarily in the peripheral blood. This fact might also explain the lower levels of IFN-γ found before the use of anti-TB drugs in this study. However, to confirm this hypothesis, more invasive techniques may be used to collect pulmonary cells from infected individuals. The increase in the production rates of IFN-γ after the anti-TB treatment has also been demonstrated in other studies performed in patients with TB [1, 2].

The results from the present study corroborate to those reported by Wang et al. [18], who reported an increase in TNF-α levels in the supernatant of PBMC cultures from patients with PTB. The principal TNF-α-producing cells are activated macrophages, T lymphocytes, and dendrite cells [7, 27]. TNF-α acts in synergy with IFN-γ stimulating the production of reactive oxygen and nitrogen intermediates, and mediating the tuberculostatic action of macrophages [28]. No significant alteration was observed in the production of IL-10, a cytokine produced by macrophages and T lymphocytes during infection with *M. tuberculosis* in patients

before and after treatment, since there was no inhibition of production of proinflammatory cytokines by antigen-presenting cells [6]. Knowledge of the protective immunity against TB remains incomplete; a classical example is that the role of B cells remains undefined, even though these cells are found in substantial numbers in granulomas [29].

These results do not represent a populational study; therefore, they cannot be extrapolated to the general population. Other studies with a larger sample should be performed to better characterize the cellular immune response in TB in healthy individuals showing negative TST, in individuals with newly converted TST, but without clinical symptoms of the disease, in patients with active PTB, and in patients clinically cured of TB to better differentiate between infection and disease states so that a better assessment of patients for definitive diagnosis, new diagnostic methods, or the development of new vaccines can be achieved.

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