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Evaluation of Interferon-Gamma, Interleukin 6 and Interleukin 10 in Tuberculosis Patients in Umuahia

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Abstract

Tuberculosis has been a great threat to mankind and has been defying treatments. Tuberculosis presents with a lot of changes in the system of the patients. This study was done to determine the levels of interferon-gamma, interleukins of the tuberculosis patients in TB directly observed treatment-short course (TB DOTS) center of FMC, Umuahia. A total of 150 subjects aged 18-60 years were enlisted for this study. The subjects were grouped into: Group A (50 control subjects) and Group B (100 TB Subjects). About 4.5 ml of blood were collected into plain tubes for assay of interferon gamma and interleukins (6 and 10). The cytokines were measured using MELNI ELISA Kits. The results were expressed as mean ± SD. The data was analyzed with the statistical package for social science (SPSS) version 20 using ANOVA the level of significance set at P<0.05. There was a significant decrease (P<0.05) with increasing months of treatment for TB group (48.94 ± 3.73 pg/ml, 46.64 ± 1.71 pg/ml, 38.76 ± 1.48 pg/ml, 26.77 ± 1.27 pg/ml) and TB-HIV (54.04 ± 3.54 pg/ml, 37.58 ± 1.99 pg/ml, 28.27 ± 1.35 pg/ml, 18.81 ± 1.38 pg/ml) respectively. The results of interleukin 6 (IL-6) in TB group on baseline (15.92 ± 2.43 pg/ml) were significantly higher (P<0.05) than TB on 2 months, 4 months and 6 months treatment (14.10 ± 1.97 pg/ml, 11.14 ± 1.31 pg/ml, 8.66 ± 1.12 pg/ml) but no significant difference (P>0.05) in TB group on 6 months treatment (8.66 ± 1.12 pg/ml) compared to the control group (7.98 ± 0.22 pg/ml).There was significant decrease (P<0.05) in IL-10 with increasing months of treatment (2 months, 4 months, 6 months on treatment) for TB subgroups based on the duration of treatment (21.06 ± 2.84 pg/ml, 18.93 ± 1.34 pg/ml, 15.08 ± 1.12 pg/ml, 10.62 ± 1.19 pg/ml). The results showed significant decrease in IFN-γ, IL-6 and IL-10 with increase in duration of treatment of the TB patients. The study shows that IFN-γ, IL-6 and IL-10 are some of the biomarkers in the pathogenesis of TB The cytokines can be used as prognostic and diagnostic markers as their levels decreased with increased duration of treatment of the patients.

Keywords: Interferon-gamma; Interleukins 6; Interleukin 10; Tuberculosis patients; Umuahia

Introduction

Tuberculosis (TB) is a chronic bacterial disease caused by *Mycobacterium tuberculosis* (MTB) complex which commonly affects the lungs (pulmonary tuberculosis (PTB) but can affect other sites as well (extra-pulmonary TB (EPTB) as opined by Thumamo et al. [1]. *Mycobacterium tuberculosis*, the bacterium that causes human TB disease, is an old enemy. Historically; tuberculosis (TB) has a lineage that could be traced to the earliest history of mankind having been in existence for 150,000-200,000 years ago. It is believed that TB first made its ravaging presence felt in Europe and later got to US, Africa and Asia through voyagers and settlers [2]. *Mycobacterium tuberculosis* is an acid fast facultative intracellular rod-shaped bacterium. It is non-motile, obligate aerobe with stretched generation time and prefers specially to localize in macrophages [3,4].

Tuberculosis (TB) is a global public health problem and is the second leading causes of death. All inclusive, the disease takes a life every 20 seconds [5,6]. Although much progress has been made with regards to the control measures, the World Health Organization (WHO) estimated that 9 million people developed tuberculosis in 2013 and that 1.5 million deceased, including 360,000 people who were infected with HIV [7]. Tuberculosis is a major public health problem in Nigeria with an estimated prevalence of 616 cases per 100,000. Nigeria ranks first in Africa, and fourth among the 22 high TB burden countries in the world, and no fewer than 460,000 cases of TB are reported annually in Nigeria [8,9]. Ita and Udofia [10] reported the prevalence rate of 38.5% TB in Ikot Ekpene and 17.6% in Itu Local Government Area of Akwa Ibom State; they reported that male subjects had a higher incidence rate of TB (35.6%) compared to 29.6% in female. Similarly, Nwanta et al. [11] reported an overall prevalence rate of 37.9% TB in Enugu State, Nigeria.

Interleukin 6 (IL-6) is a proinflammatory cytokine that regulates various physiological processes [12]. It plays a key role in the acute phase response and in the transition from acute to chronic inflammation [13]. Evidence has accrued to suggest that dysregulation of IL-6 production is a major contributor to the pathogenesis of chronic inflammatory diseases [12,14]. This study will find out the changes that may be associated to the

IL-6 levels in pulmonary tuberculosis patients. Interleukin 6 (IL-6) is known to exhibit multifactorial function. It will be important to determine the changes the co infection could cause to this cytokine.

Aim

The study was done to determine the levels of interferon-gamma, interleukin 6, interleukin 10 Umuahia, Abia State, Nigeria

Objectives

- To determine levels of interferon-gamma, IL-6, and IL-10 among TB patients.
- To compare the levels of interferon-gamma, IL-6 and IL-10 among TB based on duration of treatment.

Materials and Methods

Study area

This study was carried out at the directly observed treatment-short course tuberculosis (TB DOTS) center of Federal Medical Centre (FMC), Umuahia, located in South-Eastern Nigeria and serve patients of high, middle and lower socio-economic status and with Ibo as the dominant tribe.

Advocacy, mobilization, pre-survey contacts and ethical consideration.

With a letter of introduction from the Head of the Department of Medical Laboratory Science, Imo State University, Owerri, the secretary Health Research and Ethical Committee of the Federal Medical Centre (FMC), Umuahia was met with a well detailed research proposal and full ethical approval for the research was obtained.

Study population and enrolments

The subjects were grouped into:

(1) Group A: 50 control subjects.

(2) Group B: TB subjects (n=100) which was further grouped into: Group i: naïve TB subjects (n=25), Group ii: TB subjects on 2 months therapy (n=25), Group iii: TB subjects on 4 months therapy (n=25), Group iv: TB subjects on 6 months therapy (n=25)

Subjects criteria

Patients who presented at the TB DOTS Clinic confirmed for TB infection and were recruited for this study as test participants. Apparently healthy age and sex matched individuals were recruited as control.

Sampling technique

The participants were recruited by purposive sampling technique.

Inclusion criteria

Subjects of both genders aged 18-60 years positive for Mycobacterium tuberculosis were included in the study.

Exclusion criteria

The following subjects were excluded:

1. Pregnant women.
2. Diabetes mellitus patients.
3. Persons below 18 years and above 60 years.
4. Those that withheld their consents before or in the course of the study were excluded in the study.

Sample collection

About 4.5 ml of venous blood was collected from each subject and was dispensed into plain tubes. Serum was obtained after clotting by spinning at 3000 RPM for 10 minutes and was used for interferon-gamma, interleukin 6 and interleukin 10 determinations.

Three samples (consisting of one early morning sample and two spot samples) were collected in a wide mouth container from the subjects for Tb diagnosis.

The sample was analyzed in Links Laboratory, Owerri by Sandwich ELISA method for interferon-gamma, interleukins (6 and 10) and Ziehl Nelson and GeneXpert were done in Federal Medical Centre, Umuahia, Abia State.

Methods of Sample Analysis

Ziehl-Nelson technique by *Mycobacterium tuberculosis* diagnosis

Smear preparation: A piece of clean stick was used to transfer and spread sputum materials evenly covering an area of about 15-20 mm diameter on a glass slide. The smear was air dried and labeled.

Heat fixation: The slide with the smear uppermost was rapidly passed three times through the flame of a Bunsen burner and was allowed to cool.

Ziehl-Nelson staining: The slide containing the smear was placed on a slide rack and the smear covered with carbol fuschin stain. The stain was heated until vapor just begins to rise. The heated stain was allowed to remain on the slide for 5 minutes. The stain was washed off with clean water and then covered with 3% v/v acid alcohol for 5 minutes or until smear is sufficiently decolorized, that is pale pink. The slide was washed off with clean water. The smear was covered with methylene blue stain for 2 minutes and then washed off with clean water. The back of the slide was wiped clean and placed in a drinking rack for the smear to air dry.

***Mycobacterium tuberculosis* diagnosis:** The smear was examined microscopically using the X100 oil immersion objective. Scanning of the smear was done systematically and

when any definite red bacillus is seen, it was reported as AFB positive.

GeneXpert method for detection of *Mycobacterium tuberculosis* and rifampicin resistance (GeneXpert MTB/RIF)

The assay consists of a single-use multi-chambered plastic cartridge pre-loaded with the liquid buffers and lyophilized reagent beads necessary for sample processing.

DNA extraction and hemi-nested real-time PCR: Sputum samples were treated with the sample reagent (containing NaOH and isopropanol). The sample reagent was added in the ratio of 2:1 to the sputum sample and the closed specimen container was manually agitated twice during 15 minutes of incubation at room temperature. About 2 ml of the treated sample was transferred into the cartridge, the cartridge was loaded into the Gene Xpert instrument and automatic step completed the remaining assay steps.

The assay cartridge also contained lyophilized *Bacillus globigii* spores which served as an internal sample processing step and the resulting *B. globogii* DNA was amplified during PCR step. The standard user interface indicates the presence or absence of Mycobacterium tuberculosis, the presence or absence or rifampicin resistance and semi quantitative estimate of *Mycobacterium tuberculosis* concentration (high, medium, low and very low). Assays that are negative for Mycobacterium tuberculosis and also negative for *B. globogii* internal control will be reported as invalid.

Human interferon-gamma (IFN-γ) ELISA kit by Melsin Medical Co Limited, catalogue number: EKHU-0162

Dilutions of standard was prepared to get a concentration of 240 ng/l, 160 ng/l, 80 ng/l, 40 ng/l and 20 ng/l. 50 µl of standards were pipette into the standard wells. 10 µ of test serum were added into each well. 40 µl of sample diluents was added to the sample well. Sample blank was included (to contain only chromogen solution A and B, stop solution). 50 µl of HRP-conjugate reagent was added to all wells except blank, covered with an adhesive strip and incubated for 30 minutes at 37°C. It was washed for 4 times. 50 µl of chromogen solution A and 50 µl of chromogen solution B was added to each well. They were mixed and incubated for 10 minutes at 37°C. 50 µl of stop solution was added to each well. Optical density of the samples was read in a microtiter plate reader at 450 nm wavelength within 15 minutes taking the blank well as zero concentration.

Calculation: A standard curve of optical density against concentration of standards was plotted and the concentration of the tests determined from there.

Interleukin 6 (IL-6) assay

Human Interleukin 6 commercial ELISA Kit by MELSIN Medical Co Limited was used. Catalogue Number: EKHU-0140:

Dilutions of standard was prepared to get a concentration of 240 ng/l, 160 ng/l, 80 ng/l, 40 ng/l and 20 ng/l. 50 µl of standards were pipette into the standard wells. 10 µ of test serum were added into each well. 40 µl of sample diluents was added to the sample well. Sample blank was included (to contain only chromogen solution A and B, stop solution). 50 µl of HRP-conjugate reagent was added to all wells except blank, covered with an adhesive strip and incubated for 30 minutes at 37°C. It was washed for 4 times. 50 µl of chromogen solution A and 50 µl of chromogen solution B was added to each well. They were mixed and incubated for 10 minutes at 37°C. 50 µl of stop solution was added to each well. Optical density of the samples was read in a microtiter plate reader at 450 nm wavelength within 15 minutes taking the blank well as zero concentration.

Calculation: A standard curve of optical density against concentration of standards was plotted and the concentration of the tests determined from there.

Human interleukin 10 (IL-10) assay by commercial ELISA kit by MELSIN Medical Co Limited, catalogue number: EKHU-0155

Dilutions of standard was prepared to get a concentration of 240 ng/l, 160 ng/l, 80 ng/l, 40 ng/l and 20 ng/l. 50 µl of standards were pipette into the standard wells. 10 µ of test serum were added into each well. 40 µl of sample diluents was added to the sample well. Sample blank was included (to contain only chromogen solution A and B, stop solution). 50 µl of HRP-conjugate reagent was added to all wells except blank, covered with an adhesive strip and incubated for 30 minutes at 37°C. It was washed for 4 times. 50 µl of chromogen solution A and 50 µl of chromogen solution B was added to each well. They were mixed and incubated for 10 minutes at 37°C. 50 µl of stop solution was added to each well. Optical density of the samples was read in a microtiter plate reader at 450 nm wavelength within 15 minutes taking the blank well as zero concentration.

Calculation: A standard curve of optical density against concentration of standards was plotted and the concentration of the tests determined from there.

Statistical Analysis

The results were expressed as mean ± standard deviation. The data were analyzed with the statistical package for social science (SPSS) version 20 using t-test, ANOVA and the level of significance was set at $P<0.05$.

Results

There was a significant decrease ($P<0.05$) with increasing months of treatment for TB group (48.94 ± 3.73 pg/ml, 46.64 ± 1.71 pg/ml, 38.76 ± 1.48 pg/ml, 26.77 ± 1.27 pg/ml) and TB-HIV (54.04 ± 3.54 pg/ml, 37.58 ± 1.99 pg/ml, 28.27 ± 1.35 pg/ml, 18.81 ± 1.38 pg/ml) respectively. The results also showed significant higher ($P<0.05$) level of IFN-γ of non-ART group (29.31 ± 1.44 pg/ml) than HIV ART group (18.49 ± 1.48 pg/ml).

The results of interleukin 6 (IL-6) in TB group on baseline (15.92 ± 2.43 pg/ml) were significantly higher ($P<0.05$) than TB on 2 months, 4 months and 6 months treatment (14.10 ± 1.97

pg/ml, 11.14 ± 1.31 pg/ml, 8.66 ± 1.12 pg/ml) but no significant difference (P>0.05) in TB group on 6 months treatment (8.66 ± 1.12 pg/ml) compared to the control group (7.98 ± 0.22 pg/ml). There was significant decrease (P<0.05) in IL-10 with increasing months of treatment (2 months, 4 months, 6 months on treatment) for TB subgroups based on the duration of treatment (21.06 ± 2.84 pg/ml, 18.93 ± 1.34 pg/ml, 15.08 ± 1.12 pg/ml, 10.62 ± 1.19 pg/ml) (**Tables 1 and 2**).

Table 1: Sex distribution of the subjects.

Group	Female	Male
TB	13 (8.67%)	12 (8.00%)
Baseline	13 (8.67%)	12 (8.00%)
2 Months on Treatment 25 (16.67%)	13 (8.67%)	12 (8.00%)
4 Months on Treatment 25 (16.67%)	13 (8.67%)	12 (8.00%)
6 Months on Treatment 25 (16.67%)	13 (8.67%)	12 (8.00%)
Total 100 (66.67%)	52 (34.67%)	48 (32.00%)
Control 50 (33.33%)	25 (16.67%)	25 (16.67%)
Grand Total 150 (100.00%)	77 (51.33%)	73 (48.67%)

Table 2: Mean values of interferon-gamma, interleukin 6, interleukin 10 of control, TB, HIV and TB-HIV groups based on duration of treatment.

Groups	IFN-γ (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml
Control	16.25 ± 0.87a	7.98 ± 0.22c	8.52 ± 0.62c
TB			
Baseline	48.94 ± 3.73a	15.92 ± 2.43c	21.06 ± 2.84b
2 months Therapy	18.93 ± 1.34b	46.64 ± 1.71a	14.10 ± 1.97c
4 months therapy	38.76 ± 1.48a	11.14 ± 1.31c	15.08 ± 1.12b
6 months therapy	26.77 ± 1.27a	8.66 ± 1.12c	10.62 ± 1.19b
a>b>c			

Discussion

Cytokines are important immunomodulating agents of immune system. Human immunodeficiency virus co infection has been suggested to alter blood cell populations and change Th1/Th2 balance [16], which affects the course of TB, clinical presentation, signs and symptoms [17], leading to misdiagnosis or delay in diagnosis of TB [18].

The lack of reliable biomarkers to indicate or predict the different clinical outcomes of *M. tuberculosis* infection has been given as a key reason for the failure of developing new diagnostic and prognostic tolls, drugs and vaccines against tuberculosis [19].

The results showed that the levels of IL-6 based on the duration of treatment showed significant decrease among TB subgroups on 2 months, 4 months and 6 months compared to TB on baseline and TB on 6 months treatment showed no significant difference. This shows that treatment of tuberculosis has a decreasing effect on IL-6 which is seriously implicated in the pathogenesis of tuberculosis as the major regulatory cytokine that regulates the major hormone, hepcidin that regulates the synthesis and release of iron which is a major mechanism of the mycobacterium tuberculosis survival means in the patients. The treatment outcome may not be affected by modifying the levels of IL-6 in HIV patients. Activated inflammation, as demonstrated by persistently higher IL-6 levels, may have profound and far-reaching clinical implications. It has been shown that IL-6 is a proinflammatory cytokine that controls different physiological processes [12]. Interleukin 6 plays a major role in the acute phase response and in the transition from acute to chronic inflammation [13]. Evidence has occurred to suggest that dysregulation of IL-6 production is a major contributor to the pathogenesis of chronic inflammatory and autoimmune diseases [20]. This can be a biomarker in the diagnosis and prognostic monitor of the progress of the disease as well as the cure of the disease. It shows that a longer duration will have a more pronounced decreasing effect on the patients to avert drug induced haemolytic anaemia. Some works have revealed that when tuberculosis infection occurs, a variety of pro and anti-inflammatory cytokines are produced at disease sites and then released into circulation [21,22].

Interleukin 10 (IL-10) is one of the most important anti-inflammatory cytokines reported to affect multiple cell types, including macrophages, monocytes, dendritic cells, CD4 T cells and CD8 T cells [23]. The dominant function of IL-10 is to down-regulate the immune response and limit tissue injury. However, the excessive production of this cytokine directly inhibits CD^{4+} T cells responses which may result in a failure to control the infection. Interleukin 10 is one of the most important anti-inflammatory cytokines reported to inhibit CD^{4+} T cell responses by inhibiting APC function of cells infected with mycobacteria [24]. The results showed that the levels of IL-10 were significantly higher in TB patients compared to healthy subjects (P<0.05). The findings are similar with previous studies that have shown higher levels of IL-10 in the active TB group than in the control group [25-27]. Interleukin 10 can be found in the serum, plasma and bronchoalveolar larvage fluid of active TB patients and may contribute to the energy and failure of lymphocytes to proliferate in response to TB) [28,29]. It is the balance between the inflammatory and protective immune response that determines the outcome of tuberculosis infection.

The study showed lower levels of Il-10 in the control compared to TB subgroups based on duration of treatment. It also implies that duration of treatment decreases the levels of interleukin 10 in the patients both in TB. The study revealed a statistically significant decreasing of IL-10 level during treatment. Another study has shown a consistent decrease in IL-10 levels in active TB patients at all time points of therapy, suggesting that patients who maintain high IL-10 levels at the end of treatment are exposed to TB recurrence [30]. Sahiratmadja et al. [31] also observed reduced IL-10 production

during TB therapy, suggesting that this cytokine may be a useful biomarker signature to assess the disease progression [31]. Interleukin 10 functions to limit the immune response to TB and may contribute to TB pathogenesis [32].

Conclusion

The study shows that interferon gamma, interleukin 6 and interleukin 10 are some of the biomarkers in the pathogenesis of TB infection. The cytokines can be used as prognostic and diagnostic markers as their levels decreased with increased duration of treatment of the patients. The levels of these cytokines should be included during the time of treating TB patients to ensure improvement of their live by monitoring the levels of interferon-gamma, interleukin 6 and interleukin 10 which affect the response of the patients to the treatment bused on the duration of the treatment.

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