

Interferon-gamma treatment kinetics among patients with active pulmonary tuberculosis

Olanisun Olufemi Adewole, Martin O. Ota¹, Greg E. Erhabor, Patrick Owiafe¹, Aliu Oladimeji, Daniel Obaseki

Department of Medicine, Obafemi Awolowo University and Teaching Hospitals, Ile Ife, Osun State, Nigeria, ¹Tuberculosis Immunology Unit, Medical Research Council Unit, Banjul, Gambia

ABSTRACT

Introduction: Interferon- γ (IFN- γ) is essential for defence against *Mycobacterium tuberculosis*; however, levels in patients with active tuberculosis (TB) and changes during treatment have not been documented in our tuberculosis patients in Nigeria, hence this study has been carried out. **Objective:** To determine variations, treatment kinetics, and predictive value of IFN- γ levels during treatment of active tuberculosis. **Design:** Patients with pulmonary tuberculosis were recruited and subsequently followed up for 3 months during treatment with anti-TB. Peripheral blood was collected for IFN- γ assays, C-reactive protein and others followed by a Mantoux test. IFN- γ levels produced by stimulation with TB antigens were determined by ELISA and repeated measurement of IFN- γ were done at 1 and 3 months of anti-TB therapy. Chi Associations and correlations between IFN- γ were determined. Regression analysis was done to determine association between serial IFN- γ and treatment outcome. **Results:** We recruited 47 patients with active tuberculosis with a mean age of 34.8 ± 3.6 years and M:F ratio of 1.12:1. Six (11%) were HIV positive. The mean level of IFN- γ induced by TB antigens was 629 ± 114.1 pg/ml, higher for HIV-negative PTB patients compared with HIV-positive PTB patients, 609.78 ± 723.9 pg/ml and 87.88 ± 130.0 pg/ml, respectively, P -value = 0.000. The mean level of IFN- γ induced by TB antigen increased significantly from 629 ± 114.1 pg/ml to 1023.46 ± 222.8 pg/ml, P -value = 0.03 and reduced to 272.3 ± 87.7 pg/ml by the third month on anti-TB drugs, P -value = 0.001. Negative correlation was observed between the mean of baseline and chest X-ray involvement, $P = 0.03$. There was no significant correlation between sputum smear grade with baseline and follow-up IFN- γ levels. Three-month IFN- γ level among cured patients were higher than those with treatment failure, regression analysis showed that it does not predict outcome. **Conclusion:** IFN- γ may be useful in early detection and monitoring response; however, large scale studies are needed.

Key words: Interferon-gamma, tuberculosis, treatment outcome

Address for correspondence:

Dr. Olanisun Olufemi Adewole,
Department of Medicine,
Obafemi Awolowo University/
Teaching Hospitals Complex,
Ile Ife, Nigeria.
E-mail: adewolef@yahoo.co.uk

INTRODUCTION

Despite advances in therapy and some progress made towards global targets of reducing Tuberculosis (TB) cases and deaths, the global burden of the disease still remains enormous. In 2011, there were an estimated 8.7 million new cases of TB (13% coinfecting with HIV) and 1.4 million people died from TB, including almost one million deaths

among HIV-negative individuals and 430,000 among people who were HIV-positive.¹ The high prevalence of TB in the world and especially in developing countries is attributed to a number of factors, including significant delays in diagnosis and treatment initiation.^{2,3} Diagnosis still depends largely on the conventional methods i.e., acid-fast staining of sputum which has a low sensitivity of 50-60% and Mycobacterial culture that usually requires 6-8 weeks to be interpretable.⁴⁻⁶

Resistance to Mycobacterial infections is mediated by macrophages, T-cells and their interaction, and is dependent on the interplay of cytokines produced by each cell.⁷ IFN- γ is essential for defence against *Mycobacterium tuberculosis* (*Mtb*) infection, as revealed by experimental studies using knockout mice and the unusually severe Mycobacterial infections in patients with defects in the IFN- γ or IL-12 signalling pathways.⁸⁻¹¹

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Measurement of the IFN- γ response to *Mtb* antigens has proven useful in detecting *Mtb* infection, both latent infection and infection manifesting as overt disease.¹²⁻¹⁴ Recently, interferon-gamma release assays (IGRAs) have been introduced and found useful as immunodiagnostic tests for MTB infection. These tests are based on the induction of interferon gamma (IFN- γ) by MTB-specific antigens. There are two widely available commercial forms of IGRAs, QuantiFERON-TB Gold in-tube (QFT-IT) (Cellestis Ltd, Victoria, Australia) and T-SPOT.TB (Oxford Immunotec, Oxfordshire, UK). Both contain a combination of MTB-specific antigens, early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10). TB 7.7 is an additional MTB-specific antigen present especially in QFT-IT. These proteins are present in *Mtb* but absent from Bacillus Calmette-Guérin (BCG) vaccine strains and most non-tuberculous mycobacteria.¹⁵ Both tests have shown promising results in the detection of latent TB infection.¹⁶

The dynamics of IFN- γ in tuberculosis have been extensively documented. Elevated level of unstimulated IFN- γ was found during active TB when compared with healthy controls, declining during and after treatment.¹⁷

Winkler *et al.*, in another study in Central Africa Republic demonstrated an increased IFN- γ level in PPD supernatant of patients with active TB.¹⁸ In Gabon, Nemeth *et al.*, demonstrated no difference in the level of IFN- γ stimulated by PPD of patients and controls.¹⁹ In a recent study, stimulation of peripheral mononuclear cells with TB-specific antigens was found to be associated with significant level of IFN- γ among culture-confirmed TB cases than control.²⁰ Knowledge and usefulness of peripheral blood level of TB antigen-induced IFN- γ is limited in our environment.

In this study we use supernatant from QuantiFERON-TB Gold in-tube to assess the IFN- γ levels in individuals with active TB and treatment kinetic of IFN- γ and relating IFN- γ level with clinical signs and symptoms in patients during a 3-month period. We also aim to determine the predictive value of IFN- γ for treatment outcome in patients with active TB.

MATERIALS AND METHODS

Participants and data collection

We prospectively enrolled consented individuals aged 18 years and above with active TB presenting at the Chest Clinic of Obafemi Awolowo University Teaching Hospitals Complex, OAUTHC, Ile Ife. The OAUTHC Ethics and Research Committee approved the study. Patients were evaluated according to the National TB and Leprosy Control Program (NTLCP) guideline. Only symptomatic patients with sputum smear-positive for acid-fast bacilli by Ziehl-Neelsen stain

and had not been commenced on anti-TB drugs were recruited.

After giving informed written consent, each patient at enrolment was asked to complete a questionnaire about his or her demographics, previous history of TB, smoking status and other factors. Clinical examination was done and presence and size of BCG scar (in millimetres) were noted. Chest radiograph was obtained and peripheral blood was collected and used for the QFT-IT assay, HIV testing while tuberculin skin test (TST) was performed. The chest radiographs were read by radiologists who were blinded to the status of the subjects.

Patients were then commenced on anti-TB therapy according to the NTLCP and evaluated at 1 and 3 months of treatment. At each time points, peripheral blood were collected and used for the QFT-IT assay and sputum samples collected for smear and culture. Outcome at the end of treatment was determined from the follow-up card. Outcome measures include treatment failure where sputum smear remains positive by the fifth month of treatment and cure when smear becomes negatives.

Whole blood was stimulated by TB-specific antigens: ESAT 1, CFP10 and TB 7.7 contained in a tube and another tube without any antigen. The tubes were obtained from Cellestis Ltd, Victoria, Australia. One millilitre of blood was collected into each tube and were mixed gently incubated for 24 hours. Sera were harvested and were stored frozen until further analysis.

Measurement of IFN- γ by ELISA

Thawed sera were used for IFN- γ concentrations measurement using an ELISA technique according to the Grand Challenge Protocol. It is a 3-day protocol after stimulation. Briefly, on first day, the plates are coated with assay cytokine, diluted with purified IFN- γ mAb and incubated overnight at 4°C. On the second day, test samples and standards were added to the wells and 150 μ l of blocking solution were added to each well and plates were incubated for 2 hours at room temperature. Fifty microlitres of standards and controls were added to appropriate wells. The standard curve was prepared by serial dilution from 4000 to 31 pg/ml. The plates were incubated overnight at 4°C. On the third day, the plates were washed four times and 100 μ l of detection antibody added to each well and incubated at room temperature. One hundred microlitres of Avidin peroxidase were added per well followed by another incubation at room temperature for 30 minutes, the plates were washed and 100 μ l OPD Fast solution were added to all wells and develop in the dark for 45 min. Fifty microlitres of H₂SO₄ were added to each well. The plates were read at 490 nm within 45 mins.

Tuberculin skin test

The TST was performed after blood sampling by trained personnel following standard procedures. For this test, 0.1 mL (2 TU) purified protein derivate (RT23; Statens Serum Institute, Copenhagen, Denmark) was injected intradermally into the volar surface of the left forearm. Transverse induration at the TST site was determined by palpation method and measured in mm after 48-72 hour by the study clinician. An induration of ≥ 10 mm was considered as a positive test.

Statistical analysis

Initial data exploration was done and presented using descriptive statistics. Means of continuous variables were determined and compared among groups using Student t-test, while Chi-square test was used to test for association between dichotomous variable and the study groups. Correlation test was performed to find out association between values of IFN and other continuous parameters. Logistic regression was done to determine predictive value of IFN- γ . Level of significance was set at ≤ 0.05 . Data were analysed using SPSS version 17.

RESULTS

Characteristics of the study population

Forty-seven TB cases including 25 (53%) males and 22 (47%) females with a mean age of 34.8 ± 3.6 years were recruited [Table 1]. Seven (13.1%) and four (8.5%) of them had previous history of TB, respectively. Six (11%) of the TB cases were HIV positive. The mean (SD) BMI was 18.11 ± 3.4 kg/m². The mean diameters of TST induration and BCG scar were 18.11 ± 6.8 mm and 0.87 ± 3.0 , respectively, while the mean values for ESR and C-RP 72.2 ± 6.7 mm/hr and 8.65 ± 3.5 , respectively.

Table 1 also shows the baseline level of IFN- γ in the stimulated tubes. To calculate the actual level of induced

IFN- γ , the value obtained from the unstimulated tube was subtracted from the level obtained in each of the stimulated tubes. The mean level of IFN- γ in the unstimulated tubes was 545.1 ± 1258.8 pg/ml. The mean level of IFN- γ induced by TB antigens was 284.8 ± 1191.4 pg/ml, while the level of IFN- γ induced by mitogen was 890.0 ± 2418.9 pg/ml. This difference was statistically significant, P -value = 0.014.

Serial changes in IFN- γ level during treatment

Figure 1 shows the treatment kinetics of IFN- γ following treatment with anti-TB drugs.

As shown, the mean level of IFN- γ induced by TB antigen increased from 506.8 ± 652.4 pg/ml before therapy to 865.9 ± 1538.0 pg/ml at 1 month of anti-TB drugs, P -value = 0.3 and reduced to 111.6 ± 956.7 pg/ml by the third month of treatment, P -value = 0.004. However, the greatest drop in the level of IFN- γ occurred between the first and third month of treatment, P -value = 0.002. Patients who are HIV positive had a lower IFN- γ compared with those who are HIV seronegative, 87.88 ± 130.0 pg/ml compared with 609.78 ± 723.9 pg/ml, this was statistically significant, P -value = 0.00.

Correlations between baseline IFN- γ

Table 2 shows the correlations between pre-treatment baseline IFN- γ induced by TB antigens and some clinical and laboratory parameters. As shown, IFN- γ levels is positively correlated with age, diameter of Mantoux skin induration, mean lymphocyte count and mean ESR, P -values > 0.05 each. Weak negative correlations were obtained between baseline IFN- γ levels and mean CRP, P -value = 0.5, sputum smear grade, P -value = 0.9 and number of zones affected on CXR, P value = 0.04. BMI was also positively correlated with IFN- γ , but the association was not significant.

Table 3 shows the comparisons between mean values of IFN- γ at baseline, 1 and 3 months of anti-TB medications and treatment outcomes. By the second month of anti-TB

Table 1: Characteristics of study subjects

Characters	Patients <i>n</i> = 47 (%)
Male	25 (53%)
Past History of TB	7 (15%)
History of smoking	4 (8.5%)
HIV Positivity	6 (11%)
BCG scar	60 (95%)
Mean Age, yrs \pm SD	34.8 \pm 3.6
Mean BMI, kg/m ² \pm SD	18.11 \pm 3.4
Mean TST induration \pm SD	18.11 \pm 6.8
Mean ESR, mm/hr \pm SD	72.2 \pm 6.7
Mean C-RP \pm SD	8.65 \pm 3.5
Mean diameter of BCG scar	0.87 \pm 3.0
TB antigen induced IFN- γ , pg/ml	506.8 \pm 652.4
HIV positive	87.88 \pm 130.0
HIV negative	609.78 \pm 723.9

* P -value < 0.05; SD – Standard deviation; BMI – Body Mass Index; BCG – Bacillus Calmette Guerin; TST – Tuberculin Skin test; ESR – Erythrocytes sedimentation rate, C-RP – C-reactive protein; IFN- γ – Interferon gamma

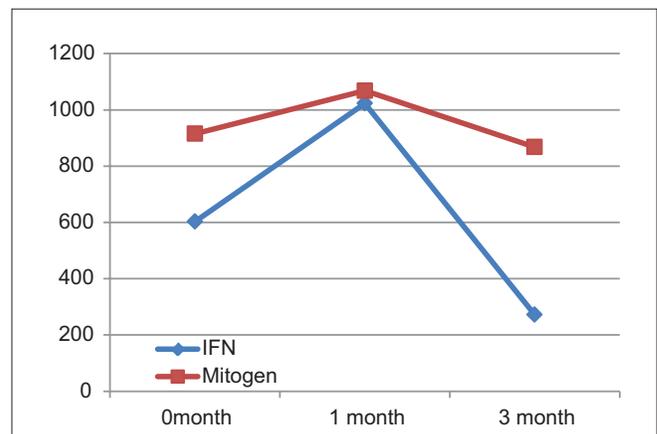


Figure 1: Treatment kinetics of IFN- γ following treatment with anti TB drugs

Table 2: Correlations between baseline Interferon-gamma and some parameters

	Age	Mantoux Size	Lymphocyte Count	Mean ESR	Sputum Smear Grade	Mean CRP	Number of Zones on CXR	Mean BMI
Pearson Correlation	-0.05	0.003	0.073	-0.01	-0.007	-0.33	-0.136	0.08
P-values	0.6	0.987	0.711	0.952	0.9	0.34	0.04	0.6

Table 3: Comparisons between mean levels of IFN at 0, 1 and 3 months and treatment outcome in patients

Parameters	2-Month Outcome			7-Month Outcome		
	ETF N=4	Smear conversion N=36	P-values	Treatment failure N=2	Cured N=38	P-values
0 month	196.81 (340.9)	589.08 (721.5)	0.1	-7.34 (10.4)	369.08 (494.5)	0.01
1 month	253.4 (2459.5)	848.6 (1460.286)	0.09	4.8 (7.8)	829.69 (980.4)	0.01
3 month	50.32 (42.108)	110.5 (969.0)	0.7	148 (104.4)	102.32 (1167.6)	0.8

medications, four patients were still smear-positive while two patients died within 1 month and five patients were transferred out. The remaining 36 became smear converted. As shown, the mean baseline value for IFN- γ was higher among those who had become smear converted at 2 month and those that were declared cured at 7 months, *P*-values were 0.1 and 0.01, respectively. The mean levels of IFN- γ at 1 month was also higher among those that became smear positive compared with those were not, 848.6 (1460.286), versus 253.4 \pm 2459.5 pg/ml, *P*-value = 0.09. The same trend was noticed among those who are cured compared with those who had treatment failure, 829.69 \pm 980.4 versus 4.8 \pm (7.8) pg/ml, *P*-value = 0.01.

Third-month value of IFN- γ was lower among who became cured compared with the value obtained among those who were not by the seventh month of treatment, 102.32 \pm 1167.6 versus 148 \pm 104.4 pg/ml, *P*-value = 0.8.

Table 4 shows the result of regression analysis for IFN- γ and treatment outcome.

At the end of treatment two patients were still smear positive, five were transferred out while two died within 1 month of enrolment, while the remaining were cured. As shown baseline IFN- γ and serial measurement did not predict treatment outcome, *P*-values greater than 0.05 in each case.

DISCUSSION

This study reports the changes over a 3-month time period in the IFN- γ levels of active TB patients during treatment with anti-TB medication and relate this to outcome. Baseline IFN- γ induced by TB antigens rose to the highest level by 1 month of treatment and fell to the lowest level by the 3 month of treatment. Baseline IFN- γ correlated negatively with the number of zones affected on the CXR. Patients who are HIV positive had a significantly lower level of IFN- γ compared with HIV-negative patients. In this study, IFN- γ levels at baseline and 1 month was higher among

patients who were cured. However, IFN- γ levels does not predict outcome.

The increase in the level of IFN- γ during anti-TB treatment has also been demonstrated in other studies carried out in patients with TB.^{21,22} The initial rise in IFN- γ and cytokine storm, in the first month of treatment may be related to stimulation of lymphocytes by antigens released after the death of Mycobacterial caused by chemotherapy, causing a reversion of the antigenic status or by increase in peripheral T lymphocytes antigen released from the lungs. This was different from a study from South Africa where over a 42-day study period, plasma levels of IFN- γ was observed to decrease while the levels of TNF increased.²³

The reduction observed by the third month of treatment may be indicative of the effectiveness of the anti-TB therapy eliminating further source of antigen stimulation.

This reduction occurred well after the patients had become sputum smear negative. IFN- γ stimulation has been noted to persist during and even after treatment.^{21,22} We use TB-specific antigen to stimulate whole blood unlike the use of PPD used these studies.^{21,22,24,25} However, findings by Lee *et al.*,²⁶ corroborated our study and Mattos *et al.*,²⁴ whose findings reveals that IFN- γ stimulated by MTB antigens was significantly lower at the end of chemotherapy. This may be indicative of the usefulness of IFN- γ as a marker of bacterial clearance.

We found a negative correlation between IFN- γ level and severity of active TB measured by the number of zones affected on the CXR. Relationship between the productions of some cytokines by peripheral blood mononuclear cells and the severity of the disease had previously been noted.²⁵ The relationship we found in our study was similar to that reported by Dlugovitzky *et al.*²⁷ We observed a non-significant negative correlation between IFN- γ and sputum smear grading. Taken together, it may indicate that severe disease is associated with lower level of IFN- γ and vice versa. This is supported by the sequestration of lymphocytes which are attracted and present in large number in the lung and pleural space.²⁸⁻³⁰ Because these cells migrate to the lung

Table 4: Regression analysis for IFN- γ and treatment outcome

Model	Standardized Coefficients Beta	Sig
Baseline IFN- γ	0.05	0.8
IFN- γ at 1 month	-0.5	0.2
IFN- γ at 3 month	0.04	0.9

Dependent variable – Treatment outcome/cured or failed

and pleural tissue during active disease, therefore, they may be reduced, temporarily, in the peripheral blood. This phenomenon of recruitment of *Mycobacterium tuberculosis*-specific CD4+ T cells to the sites of active infection has also been observed in a recent study and has been proposed to have diagnostic usefulness.³¹ In a recent study by Zhang *et al.*, patients with severe or extensive lesions or cavity on chest radiographs had reduced IFN- γ level and CD4/CD8 cell.³² They also observed that cellular immune function significantly correlated with the extent and cavity formation of pulmonary lesions. Hence, possibility of its usefulness in early detection of active TB or monitoring of response to treatment may be explored.

IFN- γ level was significantly reduced in patients with HIV. HIV alters plasma and *M. tuberculosis*-induced cytokine production in patients with tuberculosis and this has been associated with reduced IFN- γ level in HIV patients coinfecting with TB.³³ This is consistent with our finding. Hodsdon WS, *et al.*,³⁴ also found in their study that IFN- γ responses to soluble mycobacterial antigen *in vitro* were reduced in peripheral blood, but increased in pleural fluid, of HIV-positive subjects.

Despite the significantly lower levels of IFN- γ at baseline and 1 month of treatment, it was not a predictor of outcome. This may partly be due to small size on both outcome groups especially among those that had treatment failure. In view of this a large-scale study is suggested which will minimise and eliminate this limitations. Such a study will also provide answers to important questions like the differential levels of IFN- γ in healthy individuals and patients and the level of IFN- γ that will constitute a high risk for adverse outcome.

Peripheral blood level of IFN- γ undergoes a rise and fall phenomenon during treatment. Hence, it may be a useful marker to monitor response to treatment and effectiveness of anti-TB medications. The diagnostic usefulness of IFN- γ may be further explored in individuals with mild radiographic involvement and in HIV positive subjects.

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