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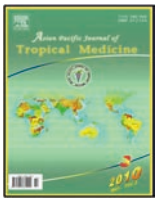

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Clinical and bacteriological correlates of whole blood interferon gamma (IFN-γ) in newly detected cases of pulmonary TB

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ABSTRACT

Objective: To determine the relationship of the capacity to produce interferon gamma (IFN-γ) in whole blood, bacteriological, hematological, radiographic and clinical presentations in new, HIV seronegative cases of pulmonary tuberculosis (TB). **Methods:** 80 cases and 50 control subjects aged 15 years onwards, representative of Kasturba Hospital and Nursing schools of Wardha district of Maharashtra state in India were examined for their health condition with standard methodology. **Results:** Among these TB patients, 73.8% were Quantiferon-TB gold (QFT) positive with IFN-γ concentration as 0.35 IU or more and there was none in healthy controls. The mean IFN-γ concentrations varied between 9.58 IU (50-59 yrs) and 2.58 IU (≥ 60 yrs), showing no trend. The differences in positivity and mean IFN-γ concentrations were statistically insignificant. Both the QFT positivity and IFN-γ concentrations were higher in normal lymphocyte percent as compared to below and above normal, but differences were not statistically significant. **Conclusions:** The IFN-γ concentrations are not correlated with any of the predictors of disease severity studied, the levels are significantly higher in observation group as compared to healthy group.

1. Introduction

Worldwide, there were 8.8 million new cases of active tuberculosis (TB) and an estimated 1.7 million deaths from TB in 2003[1]. India occupies only 2% of the land area and 15% of the total population but shares 30% of the TB burden. One person dies from TB in India every minute, more than 1 000, every day and 500 000 every year[2]. Sputum smear for acid fast bacilli (AFB) is the primary microbiologic method for diagnosis of TB. Culture for AFB requires more time, trained personal and laboratory facilities. Human immunodeficiency virus (HIV) co-infection can further complicate TB diagnosis with atypical clinical symptoms of pulmonary tuberculosis (PTB) and a higher likelihood of negative sputum smears for AFB[3]. TB is an important opportunistic disease among HIV infected persons world wide. In developing countries of Africa, Southeast Asia & Latin America an estimated 10 millions persons are co infected. The tuberculin skin test (TST) which is the standard for diagnosis of latent tuberculosis infection (LTBI), is the only test available for that purpose in resource-limited settings, and sometimes is used to aid in the diagnosis of active TB; however, its specificity is limited by cross reactivity with non tuberculous mycobacteria and bacille calmette-Guerin (BCG) vaccine strains of *M. bovis*, and its sensitivity can be affected by malnutrition and immuno-suppression[4].

Mycobacterium tuberculosis (*M. tuberculosis*) infection is chiefly controlled by activation of macrophages through type 1 cytokine production by T cells (Th1), and interferon gamma (IFN-γ) is central to this process[5]. Patients with defective IFN-γ receptor develop severe mycobacterial disease[6]. Several ex vivo studies have focused on the role of IFN-γ and its regulatory cytokines in active pulmonary TB (PTB) patients and they suggested that cytokine analysis

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in whole blood cultures may be able to discriminate between active TB infection and healthy controls[7]. Vankayalapati[8] shows that Th1 (helper T-cell 1) cytokines in sputum, serum and bronchoalveolar lavage fluid (BALF) correlate with disease activity during active TB of the lung. Advances in genomics and immunology have led to promising alternative- *in vitro* interferon gamma release assay (IGRA), based on the principal that T cells of individuals infected with *M. tuberculosis* release IFN-γ when they re-encounter TB specific antigens[9,10]. Newly discovered antigens that are specific to the *M. tuberculosis* complex are not produced by *M. bovis*. BCG vaccine strains offer the opportunity to develop IGRA with high sensitivity for TB and LTBI, and specificity for LTBI and potentially for TB as well[11].Three promising antigens for use in such assays are the 6-kDa early secreted antigenic target (ESAT-6), 10-kDa culture filtrate protein (CFP-10) , and the Rv2654 antigen (TB 7.7)[12]. These antigens, encoded by genes located within the region of difference 1 (RD1) segment of the *M. tuberculosis* genome, are more specific because they do not share with any of the BCG vaccine strains or certain species of non tuberculosis mycobacterium (NTM)[12].

ESAT-6 and CFP-10 can elicit strong IFN-γ responses from T-cells of persons infected with MTB but not from T-cells of those vaccinated with BCG or at low risk of infection[11]. Although IGRAs are promising and offer logistic advantage, unresolved issues remain. One such is whether they can be used for diagnosis of active TB and for determining the disease severity. Effector response may be driven by antigen load, and there is evidence that reduction of the antigen load by treatment decreases T cell responses.

The sensitivity of IFN-γ in active PTB cases has been reported to be 75%-89% using TB specific antigens (ESAT-6, CFA-10 & TB7.7) and 96% using purified protein derivative (PPD)[13]. Several studies have reported significantly higher IFN-γ concentrations in serum[14] and broncho alveolar lavage fluid (BALF)[15] of active TB patients as compared to healthy controls.

Since IFN-γ production correlates strongly with ineffective immunity to TB, Sodhi *et al*[16] determined, if production of this cytokine varied in patients with different clinical manifestations of TB and observed that the radiographic extent of disease and the site of disease were the only independent predictors of IFN-γ production in HIV-negative and infected patients (P=0.001). It is concluded that reduced IFN-γ production by peripheral blood mononuclear cells (PBMCs) is a marker of severe TB in both HIV-negative and infected patients with TB. Inokuchi *et al*[17] in their attempt to determine if whole blood IFN-γ production IS correlated with the radiographic extent of PTB before treatment by using PPD and phytohemagglutinin (PHA) for stimulation, observed that PHA stimulated IFN-γ production was lower in patients than healthy volunteers (P<0.05) and inversely correlated with disease extent (P<0.01). Tuberculous PPD stimulated IFN-γ production, however, it did not correlate with disease extent. It is concluded that PPD-IFN-γ / PHA- IFN-γ may be useful for diagnosis of TB but not for evaluating the disease severity, and it is suggested that PHA-IFN-γ could be considered as a marker of disease severity. Tsiouris *et al*[18] suggested that TST combined with IGRA or with a single sputum smear may play a role in excluding the diagnosis of TB in some settings. IGRA with Quantiferon TB Gold (QFT-G) in tube kit (Cellestis Ltd Australia), which has been performed previously in estimating prevalence of LTBI among health care workers[19], study of IFN-γ responses among health care workers before and after LTBI preventive therapy and diagnosis of LTBI in children[20].

There has been practically no study from India that has studied the correlation of IFN-γ response following stimulation of PBMCs in whole blood using In-tube IGRA manufactured by Cellestis Ltd, Australia, with different parameters suggesting disease severity in PTB patients. Keeping in view the above, the present study had been undertaken (Ⅰ) to determine if the capacity to produce IFN-γ in whole blood is related to bacteriological, hematological, radiographic and clinical presentations in new, HIV seronegative cases of PTB; (Ⅱ) to confirm new cases of PTB by Zeihl Neelsen staining, culture on Lowenstein Jensen medium and X- ray chest; (Ⅲ) to determine HIV status of recruited patients using 4th generation enzyme-linked immunosorbent assay (ELISA) kit; (Ⅳ) to determine the disease severity in recruited patients through smear/culture grading and extent of disease by chest radiography; (Ⅴ) to determine total leukocyte count, absolute lymphocyte count and lymphocyte percent in recruited patients using automated coulter counter; (Ⅵ) to determine IFN-γ concentrations in whole blood among patients and controls using Quantiferon TB Gold (QFT) In tube commercial test kit; (Ⅶ) to correlate IFN-γ results and concentrations with smear, culture, symptoms suggestive of PTB, radiographic and hematological findings.

2. Materials and methods

The present work was performed in the Department of Microbiology, Mahatma Gandhi Institute of Medical Sciences, Sevagram, which is located in a rural Institute in Wardha district of Maharashtra state in Central India. The study was conducted from November 2004 to October 2005.

2.1. Study population

A total of 80 subjects attending the Kasturba Hospital attached to the Institute and fulfilling the following inclusion criteria:

Adult above 15 years of age; newly diagnosed case of active PTB (smear and/or culture positive and/or evidence of TB by chest X ray); new case having received treatment for no more than 4 weeks at the time of inclusion; HIV sero-negative.

The exclusion criteria were as following:

Refusal to participate; unstable cardio-respiratory condition; past H/O active TB or those who have received treatment for more than one month; pregnant women; HIV seropositive.

The control group included 50 healthy students of MGIMS & Kasturba Nursing School. All patients and controls were

enrolled after obtaining written informed consent. Where ever necessary this was done in local language. The study was started after obtaining the approval of the Institutes ethical committee.

All patients were subjected to following studies:

2.2. Sputum smear examination by ZN method

Three sputum specimens were examined [spot/overnight/spot as per revised national tuberculosis control programme (RNTCP) criteria]. Grading of smears was done as TRC manual[21].

2.3. Sputum culture

All sputum samples were inoculated on two LJ slopes and incubated in a CO_2 incubator at 37 ℃ for a maximum of 8 weeks before declaring them negative. All slopes were examined weekly and the growth graded as TRC manual and the isolate identified by standard methods[21].

2.4. X-ray chest

This was done for all patients and examined by two independent consultants blinded to laboratory results. The presence or absence of cavitations was noted and the extent of disease was assessed as follows:

2.4.1 Minimal disease

The total extent of disease did not involve more than an area of lung equivalent to that above the second chondrosternal junction and the spine of fourth thoracic vertebra.

2.4.2. Moderately advanced disease

Dense and confluent lesions did not exceed an area of one third of the volume of one lung; non confluent lesions of slight or moderate density did not exceed the area of one lung.

2.4.3. Far advanced disease

More extensive than moderately advanced disease.

2.5. HIV status

This was determined by 4th generation ELISA kit, Vironostika (BioMerieux), which is capable of detecting HIV-1, HIV-2 antibodies and p24 antigen. The test was performed as the directions of the manufacturer.

2.6. Haemotological examination

Total lymphocyte count (TCL) and lymphocyte percent were determined for all patients using coulter counter.

2.7. IFN-γ estimation

The main kit was QuantiFERON®-TB Gold KIT (Cellestis Limited, Australia). The test was performed in following stages.

2.7.1. Collection of blood, incubation & harvest of plasma

Two mL blood was collected and 1 mL each was added to the two tubes up to the 1 mL mark: Nil Control (adjusts for background) and TB Antigen tube provided with the kit. Both tubes were shaken vigorously and incubated immediately upright at 37 ℃ for 24 hrs.

Following the incubation, the tubes were centrifuged at 2 200 rpm for 5-10 minutes to harvest plasma. Movement of gel plugs to separate plasma from cells indicated that centrifugation was successful. Plasma (300-500 μL) was pipetted out and transferred to separate 1mL sterile micro tube racks in a 96 well format (two for each patient). All plasma samples were stored at 2-4 ℃ till evaluated (4 weeks maximum).

2.7.2. Preparation of reagents

All reagents were prepared as kit instructions All plasma samples and reagents, except for Conjugate 100 ×Concentrate, were brought to room temperature (22±5) ℃ before use. At least 60 minutes were allowed for equilibration. One ELISA plate for 44 samples was used at one time. The standards (S1 to S4) and conjugate were reconstituted as kit instructions. The lay out of samples and controls was as Table 1. Prior to assay plasma samples were mixed. 50 μL of freshly prepared diluted conjugate was added to all wells using a multichannel pipette. Following this 50 μL of plasma was added to test 50 μL of the four standards in duplicate in respective wells as per layout (Table 1).

The conjugate and plasma samples/standards were thoroughly mixed using microplate shaker (Waveform=20, amplitude=6, time=1 minute). Plate was covered with a lid and incubated at room temperature (22±5) ℃ for (120±5) minutes. Wells were washed with 300-400 μL of working strength wash buffer forsicycles at room temperature with a fully automated ELISA washer. After the last washing plates were tapped face down on an absorbent wipe to remove residual wash buffer. 100 μL of enzyme substrate solution was added to each well and mixed thoroughly on the shaker. Plate was covered with a lid and incubated at room temperature for 30 minutes, starting from the time substrate was added to the first well. 50 μL of the enzyme stop solution was added to each well in the same sequence and same speed as was the substrate added, following completion of incubation. The stop solution was gently mixed. Optical density was read within 5 minutes of terminating the reaction using 450 nm filter, with a reference filter between 620 nm and 650 nm. Optical density (OD) values were used to calculate results. OD values were directly fed from the ELISA reader to the computer and the QFT-G in tube analysis software was used to analyze raw data and to calculate result. Interpretation of results shown in Table 2.

All relevant patient related information was recorded.

2.8. Statistical analysis

This was done using SPSS 10.0 version for Windows and Smart viewers software. Means were compared using the

Students't' test. Proportions, after converting into numbers, were compared using the Chi-square test and the Fisher's exact test, as appropriate. $P<0.05$ was considered as significant.

3. Results

Among the TB patients, 73.7% were QFT positive with IFN-γ concentration as 0.35 IU or more, and there was none in healthy controls. The IFN-γ concentrations were significantly higher in patients as compared to the control group ($P=0.015$). The age of the patients included in the study ranged between 18 to 80 years, with the mean age being 37.49 years. As per the kit criteria, both positive and negative test results were seen in all the age groups studied. The positive results varied between 92.3% (< 20 yrs) to 60.0% (30-39 yrs) and negative between 7.7% (<20 yrs) to 40.0% (30-39 yrs). The mean IFN-γ concentrations varied between 9.58 IU (50-59 yrs) and 2.58 IU (≥60 yrs), showing no trend. The positivity (81.0%) and mean IFN-γ concentrations (7.11 IU) were higher in the females with insignificant difference (Table 3).

Table 4 showed the correlation with clinical features. More patients with fever showed positive results (74.7%) and had higher mean IFN-γ concentrations (6.82 IU) with insignificant difference. Patients without cough showed higher positivity (100.0%) but lower mean IFN-γ concentrations (5.64 IU). However, those without cough were small in number. Haemoptysis is an important presentation of advanced disease. However, both positivity (75.0%) and mean IFN-γ concentrations (7.24 IU) were higher in those without haemoptysis with insignificant difference. Loss of weight was seen in 66 subjects. Those loss of weight patients showed higher positivity (74.2%) and lower mean IFN-γ concentrations (6.49 IU).

Table 5 showed the correlation with bacteriological status, including smear and culture. The higher the smear and/or culture grading the higher the severity of the disease, hence higher positivity and IFN-γ concentrations are expected in them. On correlation of smear with IFN-γ, it was observed that maximum positivity was seen in those with 2+ smear (92.9%) and there was no upward trend in positivity with increase in number of bacilli/mL of sputum, as those with 1-9 bacilli in their sputum showed higher positivity (76.9%) than those with 3+ smears (69.2%). It was interesting to note that the mean IFN-γ concentration was the maximum in smear negative cases (15.16 IU). None of the differences were significant. As seen in the smear correlation, maximum positivity was seen in patients with 2+ culture report (81.8%) and there was no upward trend as expected, either in the positivity or mean IFN-γ concentrations with increase in culture grading. The lowest mean IFN-γ concentrations were recorded in patients with 3+ culture report.

The degree of damage to the lungs is determined through chest radiography. The greater the damage, the higher the extent of disease. Though, the mean IFN-γ concentrations were higher in those with cavity, more patients without cavity showed positive QFT results. None of these differences were statistically significant.

On correlating the various grades of disease severity, though positivity was higher in those with moderate disease, mean IFN-γ concentrations were found to be higher in those with minimal disease. However, none of these differences were significant (Table 6).

Table 7 showed the correlation of hematological findings with IFN-γ. On the basis of TLC counts patients were divided into three groups: normal counts (4 000-11 000 cells/μL of blood), below normal (<4 000 cells/μL) and above normal (>11 000 cells/μL). Though the positivity was the maximum in those having TLC>11 000, the mean IFN-γ concentrations were the highest in those with TLC<4 000. None of the observations were significant. Both the percent positivity (68.4%) and mean IFN-γ concentrations (5.25 IU) were lower in those showing higher than normal absolute lymphocyte count (>4 500) than those with lower count (78.6% & 8.03 IU, respectively), but the differences were not significant. Both the QFT positivity and IFN-γ concentrations were found to be higher in those having normal lymphocyte percent as compared to those below and above normal, but then these differences were not statistically significant.

4. Discussion

PTB is one of the most important infectious diseases in the world. Both, TB occurring in immuno-deficient patients and multi-drug TB have emerged as clinical problems. T cells play a central role in the human immune response to mycobacterial pathogens and the balance of T-cell cytokines produced in response to infection is believed to have a profound effect on clinical outcome. Although the specific cytokines that mediate immunologic resistance to mycobacteria in humans remains unidentified, IFN-γ appears to be the key cytokine involved, as increased susceptibility to mycobacterial infections has been reported in individuals with IFN-γ receptor deficiency or mutations in IFN-γ receptor 1[6]. The primary function of this cytokine is to activate the macrophage and enable this cell to carry out its mycobacterial effecter functions. To date, the immune response to *M. tuberculosis* has been assessed by skin testing using the purified protein derivative from *M. tuberculosis*; however, TST measures both effecter and memory T-cell responses as against new generation IFN-γ assays which measures mostly effecter T-cell responses[22]. Both TST and IFN-γ assays have been used for diagnosing MTB infection, however, whether the results of whole blood IFN-γ assays is correlated with disease severity is not clear at present.

In the present study the sensitivity of the QFT TB Gold test in HIV sero-negative active PTB cases was 73.7%, taking ≥ 0.35 IU/mL IFN-γ concentration in whole blood as positive, as per the kit, following stimulation with TB specific antigens. Tsiouris *et al*[18] have reported an overall sensitivity of 75% in all and 82% in new PTB patients. Clinical studies have estimated the sensitivity of QFT TB Gold to be 89% approximately, for active TB disease. In comparison to QFT TB Gold, which utilizes TB specific antigens for stimulation,

Table 1
Sample layout.

Row	1	2	3	4	5	6	7	8	9	10	11	12
A	1N	5N	9N	13N	17N	S1	S1	25N	29N	33N	37N	41N
B	1A	5A	9A	13A	17A	S2	S2	25A	29A	33A	37A	41A
C	2N	6N	10N	14N	18N	S3	S3	26N	30N	34N	38N	42N
D	2A	6A	10A	14A	18A	S4	S4	26A	30A	34A	38A	42A
E	3N	7N	11N	15N	19N	21N	23N	27N	31N	35N	39N	43N
F	3A	7A	11A	15A	19A	21A	23A	27A	31A	35A	39A	43A
G	4N	8N	12N	16N	20N	22N	24N	28N	32N	36N	40N	44N
H	4A	8A	12A	16A	20A	22A	24A	28A	32A	36A	40A	44A

Note: 1N: Sample 1 Nil control plasma; 1A: Sample 1 TB specific antigen plasma; S1, S2, S3, S4: Standard controls with IFN-γ concentration: 4IU, 1 IU, 0.25 IU and 0.1 IU, respectively.

Table 2
Interpretation of results.

NIL(IU/mL)	TB antigen minus NIL (IU/mL)	Result	Interpretation
< 8.0	< 0.35	Negative	*M.tuberculosis* infection NOT likely
	> 0.35 & < 25 % of Nil value		
	> 0.35 & > 25 % of Nil value	Positive[1]	*M.tuberculosis* infection likely
> 8.0[2]	Any	Indeterminate [3]	Results are indeterminate for TB antigen responsiveness

[1] Where *M. tuberculosis* infection is not suspected, initially positive results can be confirmed by retesting the original plasma samples in duplicate in the QuantiFERON®-TB Gold ELISA. If repeat testing of one or both replicates is positive, the individual should be considered test positive.

[2] In clinical studies, less than 0.25% of subjects had IFN-γ levels of > 8.0 IU/mL for the Nil control.

[3] Refer to Trouble shooting section of the packing insert for possible causes.

Table 3
QFT result and IFN-γ concentration in health, age and sex.

Group		*n*	Result[*n*(%)]		Mean concentration (IU)
			Positive	Negative	
Health*	Healthy controls	50	Nil	50(100.0)	23.55
	Patients	80	59(73.7)	21(26.3)	6.71
Age(yrs)	<20	13	12(92.3)	1(7.7)	7.19
	20–29	22	17(77.3)	5(22.7)	8.08
	30–39	10	6(60.0)	4(40.0)	9.37
	40–49	9	6(66.7)	3(33.3)	7.74
	50–59	5	4(80.0)	1(20.0)	9.58
	⩾60	21	14(66.7)	7(33.3)	2.58
	Total	80	59(73.7)	21(26.3)	6.71
Sex	Male	59	42(71.2)	17(28.8)	6.57
	Female	21	17(81.0)	4(19.0)	7.12
	Total	80	59(73.7)	21(26.3)	6.71

*: Significant at 1% level.

Table 4
QFT result and IFN-γ concentration by clinical features.

Parameters		*n*	Result[*n*(%)]		Mean concentration (IU)
			Positive	Negative	
Fever	Yes	75	56(74.7)	19(25.3)	6.82
	No	5	3(60.0)	2(40.0)	5.05
	Total	80	59(73.7)	21(26.3)	6.71
Cough	Yes	77	56(72.7)	21(27.3)	6.75
	No	3	3(100.0)	–	5.64
	Total	80	59(73.7)	21(26.3)	6.71
Haemoptysis	Yes	16	11(68.8)	5(31.3)	4.58
	No	64	48(75.0)	16(25.0)	7.24
	Total	80	59(73.7)	21(26.3)	6.71
Weight loss	Yes	66	49(74.2)	17(25.8)	6.49
	No	14	10(71.4)	4(28.6)	7.73
	Total	80	59(73.7)	21(26.3)	6.71

Table 5
QFT result and IFN-γ concentration by sputum smear and culture.

Group		*n*	Result[*n*(%)]		Mean concentration (IU)
			Positive	Negative	
Sputum smear	Negative	2	1(50.0)	1(50.0)	15.17
	1–9 bacilli	13	10(76.9)	3(23.1)	6.55
	1+	38	26(68.4)	12(31.6)	6.59
	2+	14	13(92.9)	1(7.1)	9.23
	3+	13	9(69.2)	4(30.8)	3.21
	Total	80	59(73.7)	21(26.3)	6.71
Culture	Negative	7	4(57.1)	3(42.9)	4.94
	< 20 colonies	7	4(57.1)	3(42.9)	7.47
	1+	29	21(72.4)	8(27.6)	6.37
	2+	22	18(81.8)	4(18.2)	9.53
	3+	15	12(80.0)	3(20.0)	3.69
	Total	80	59(73.7)	21(26.2)	6.71

Table 6
QFT result and IFN-γ concentration by Chest X ray positivity and grading.

Group		*n*	Result[*n*(%)]		Mean concentration (IU)
			Positive	Negative	
X-ray positivity	Not suggestive of TB	4	2(50.0)	2(50.0)	6.13
	Suggestive of TB	39	26(66.7)	13(33.3)	5.87
	Suggestive of TB + cavity	37	21(56.8)	16(43.2)	7.82
	Total	80	49(73.7)	31(26.2)	6.71
X-ray grading	Negative	4	2(50.0)	2(50.0)	9.71
	MD	8	5(62.5)	3(37.5)	13.95
	Md. D	23	18(78.3)	5(21.7)	7.13
	Ad. D	45	34(75.6)	11(24.4)	5.67
	Total	80	59(73.7)	21(26.3)	6.71

M: Minimal, Md: Moderate, Ad:Advance, D:Disease, C: Cavity.

Table 7
QFT result and IFN-γ concentration by TLC, absolute lymphocyte count and lymphocyte percentage.

Group		*n*	Result[*n*(%)]		Mean concentration (IU)
			Positive	Negative	
TLC groups(cells/μL)	<4 000	11	7(63.6)	4(36.4)	10.31
	4 000–11 000	34	23(67.6)	11(32.4)	4.32
	>11 000	35	29(82.9)	6(17.1)	7.90
	Total	80	59(73.7)	21(26.3)	6.71
	Significant level		$P = 0.255$		$P = 0.108$
Absolute lymphocyte count (μL)	900–4 500	42	33(78.6)	9(21.4)	8.03
	>4 500	38	26(68.4)	12(31.6)	5.25
	Total	80	59(73.7)	21(26.3)	6.71
	Significant level		$P = 0.303$		$P = 0.186$
Lymphocyte(%)	<20	31	21(67.7)	10(32.3)	5.24
	20–45	29	24(82.8)	5(17.2)	7.71
	>45	20	14(70.0)	6(30.0)	7.54
	Total	80	59(73.7)	21(26.3)	6.71
	Significant level		$P = 0.379$		$P = 0.054$

sensitivity & specificity of whole blood gamma interferon released following stimulation with PPD has been reported to be 96% and 98% for the diagnosis of TB[13].

In the present study IFN-γ concentrations were significantly higher in patients with disease than healthy controls, as report of Vankayalapati *et al*[8]. Tsao *et al*[15] demonstrated high levels in BALF as compared to serum of active PMTB and they advocate IFN-γ estimation in BALF rather than serum in such cases, as PTB is a local disease and IFN-γ is secreted by local immune cells. Moreover, they observed that T-lymphocytes from BALF spontaneously secreted significantly greater quantities of IFN-γ as compared to those in peripheral blood. Some workers have determined a defect in peripheral blood lymphocytes from tubercular samples for IFN-γ production after stimulation with mitogens or antigens[21]. Together with reasons for depressed PPD induced IFN-γ production in PBMC of new TB patients (both HIV infected and no infected) in comparison to healthy controls viz: genetic defect in IFN-γ production / response, compartmentalization of antigen responsive cells to sites of active disease, active and selective depletion of circulating MTB responsive T cells during TB or increase in spontaneous and MTB induced programmed cell death among PBMC, it could also possibly explain the reason for low IFN-γ production or negative QFT result in our study. Inokuchi *et al*[17] observed phyto-hemagglutin (PHA) induced IFN-γ production to be significantly low in patients but no such difference with PPD induced IFN-γ.

All our patients were HIV sero-negative, however, in absence of TB HIV infection alone reduces IFN-γ production in response to antigens or mitogens. Tsiouris *et al*[18] observed significantly lower IFN-γ concentrations in HIV infected patients than in HIV negative patients with culture positive TB (P=0.033).

Though the patients in age group <20 yrs in the present study showed highest positivity (92.3%), no significant change in sensitivity was observed with advance in age. Quantiferon TB Gold appears to function well in persons of all ages in report of Mori *et al* [23] on 110 culture confirmed TB patients stratified by age. A significant decline in test sensitivity was observed by them for the Mantoux test in contrast to QFT TB Gold, suggesting measurement of *in vitro* IFN-γ responses appropriate in the elderly. IFN-γ concentrations were the lowest in age group ⩾60 in our study, however these concentrations were not significant compared to other age groups, as reported by Sodhi *et al* [16] and Tsiouris *et al*[18].

MTB induced IFN-γ production was correlated with extent of disease, which was assessed either by the signs and symptoms of PTB, bacteriological grading (both smear and culture), radiographic extent of disease and hematological parameters viz. TLC, absolute lymphocyte count and lymphocyte percent.

With respect to signs & symptoms, QFT positivity and IFN-γ concentrations were more in patients with fever and weight loss while it was low in those with cough and haemoptysis, however, the differences were not significant when compared to those without them, suggesting that there was no correlation of IFN-γ response with signs and symptoms. Fever, weight loss, cough and haemoptysis are usually associated with more intensive disease, hence a higher IFN-γ response is expected in patients presenting with them. Tsao *et al*[15] reported significantly higher IFN-γ levels in active TB patients with fever or loss of body weight, but they were observed in BALF and not serum.

Studies have reported that the severity of TB is associated with depression in lymphocytes and CD_4^+ cell counts in HIV negative patients, a feature that could be due to either compartmentalization of antigen-responsive cells to sites of active MTB infection or active and selective depletion of circulating MTB responsive T cells during TB. This could result in unresponsiveness or poor response of the peripheral blood cells to produce IFN-γ, as both the number and function of lymphocytes can influence the amount of IFN-γ production[17]. In the present study, not only normal and above normal lymphocyte count, lymphocyte percent and leukocyte count were observed in the active TB patients, but also the IFN-γ concentrations did not correlate with the counts, rather the IFN-γ concentrations were more in those with low leukocyte and absolute lymphocyte counts as compared to those having normal or above normal counts. Sodhi *et al*[16] also did not observe any correlation between IFN-γ concentrations with either absolute lymphocyte count or lymphocyte percent. Inokuchi *et al*[17] observed significantly lower total lymphocytes in active TB patients as compared to controls and reported that though the PHA induced IFN-γ per lymphocyte remained significantly lower (P<0.05) in patients than healthy volunteers, tuberculous PPD induced IFN-γ was not different between the two groups.

Understanding correlation of IFN-γ response with TB would be perfect, provided it is studied in active TB cases confirmed by a definite marker. There could be no less a perfect marker as a positive smear and/or culture for this and in addition the severity of PTB can be assessed through bacterial burden (smear and/or culture grading). Keeping this in mind we tried to find if the severity of disease, as assessed through smear or culture had any correlation with either QFT results or IFN-γ concentrations. This study didn't show any such correlation. We could not come across any studies in the literature that have looked into this type of correlation. However, a study on estimation of sensitivity of IFN-γ in culture confirmed cases of TB has been done[23], which have shown the sensitivity of IFN-γ to be 89% against 66% by TST in such cases. Tsao *et al*[15] demonstrated highest IFN-γ levels in BALF of patients with higher bacterial load as assessed by smear and culture and have suggested that IFN-γ actually might be a marker of local inflammation and/or bacterial load in the lungs. Tsiouris *et al*[18] compared the sensitivity of QFT TB Gold kit, TST and sputum smear in diagnosis of culture confirmed cases of TB and found it to be 82%, 92% and 53% respectively in new PTB cases. Since they observed that the sensitivity of the TST combined with in tube IFN-γ estimation or sputum smear for AFB was high in PTB suspects (TST+QFT=96%, TST+ one sputum smear=93%), they are of the opinion that such combined testing strategy could be useful, in some settings, to exclude a diagnosis of PTB, considering the poor sensitivity of smear microscopy and specificity of TST. However, Inokuchi *et al*[17] did not find any correlation between PPD induced IFN-γ and sputum smear.

Sodhiratmadja *et al*[21] observed strong correlation of IFN-γ

(PBMCs stimulated by *M. tuberculosis*) with radiographic extent of disease, both by univariate (P=0.0004) and multivariate (P=0.001) analysis in PTB HIV sero negatives patients only. On comparison with different degrees of disease extent, estimated by X ray, IFN-γ levels were significantly low (P=0.0001) in far advanced than moderately advanced in report of Dlugovitzky *et al*[14]. PPD induced IFN-γ in whole blood was found to be significantly low in far advanced disease (P<0.01) by Inokuchi *et al* [17]. Our study showed the same result, though the differences were not statistically significant.

In the present study mean IFN-γ levels were observed to be higher in patients presenting with cavity as compared to those without it. This is in contrast to that reported by Sodhi *et al*[16]. However, the differences in both were not significant. Moreover, the later measured IFN-γ released by PBMC's following stimulation with killed MTB, while our study measured it in whole blood following stimulation with TB specific antigens.

Sodhi *et al* [16] suggested that reduced MTB induced IFN-γ production by PBMCs reflects disease severity. Tsao *et al*[15] regarded it as not only a marker of local inflammation but also bacterial load when measured in BALF. Inokuchi *et al*[17] found tuberculous PPD-IFN-γ / PHA-IFN-γ a useful marker for TB diagnosis and reduced PHA- IFN-γ a marker of disease severity, while Tsiouris find IGRA plus TST useful in excluding the diagnosis of TB.

Though the IFN-γ concentrations is not correlated with any of the predictors of disease severity studied, the levels were found to be significantly higher in diseased as compared to healthy subjects. Considering the exorbitant cost of the kit (Rs 50 000/ per kit-44 tests), the study could be performed on limited patients only. To evaluate the correlation between disease severity and IFN-γ released by cells, further studies in larger population not only in whole blood but also in those from local site of infection need to be done.

Conflict of interest statement

We declare that we have no conflict of interest.

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