Levels of tumor necrosis factor-alpha (TNF-α) and Interferon gamma (IFN-γ) s During Tuberculosis treatment

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ABSTRACT

Cytokines play a major role in protection against Mycobacterium tuberculosis infection and regulate the immune responses at a cellular level. A T helper 1 (Th1) cytokine interferon gamma (IFN-γ) is one of the most important cytokines which activate the macrophages to produce tumor necrosis factor-alpha (TNF-α). At physiological levels, TNF-α has anti-microbial activities through the apoptotic effect. Excessive production of TNF-α have been implicated in immunopathogenesis of tuberculosis. The aim of the present study was to determine Th1 cytokine profile in patients with tuberculosis to identify immunological marker for follow up of the disease activity, and on the other hand, to study the outcome of anti-tuberculosis treatment. To examine this, blood samples were collected at several intervals before and during the treatment with anti-tuberculosis drugs. Levels of IFN-γ, TNF-α pre and during treatment using commercial available enzyme-linked immune-sorbet assay (ELISA). Data were analyzed using SPSS 20. Receiver Operating Characteristic (ROC) Curve analysis has been carried out to assess their discriminative power and to determine cut-off values. Analysis has been carried out further by calculating other measures of diagnostic test accuracy. The results showed that the levels of TNF-α were significantly increased in patients before and after the treatment than those in control (p=0.001). Levels for IFN-γ were not statistically different between patients and controls (p=0.351). A decrease in TNF-α levels dose not conflict with the finding that IFN-γ had highest level in patients under treatment as the difference between those and newly diagnosed patients was not statistically different. TNF-α can be used as a marker for TB severity, having that the area under the curve (AUC) for TNF-α is .824 (95% CI: .737 - .912). For the chosen cut-off level of 5pg/ml, it has a sensitivity of 85% and a lower specificity of 70%. TNF-α test is most beneficial where prevalence is very low or very high.

Keywords: Cytokines levels, TNF-α, IFN-γ, TB treatment
INTRODUCTION

The immune system has the capacity to inhibit the growth or kill mycobacteria in majority of cases immune cells for example Th1 and macrophages have the capacity to produce cytokines that capable killing of mycobacteria like interferon-γ (IFN-γ) and tumor necrosis factor alpha (TNF-α). These molecules serve both to attract other inflammatory effectors cells such as lymphocytes and activate them (Alberts et al., 1997).

IFN-γ is produced by activated CD4, CD8 T cells and is produced predominantly by natural killer (NK) and natural killer T cells (NKT) cells as part of the innate immune response. IFN-γ required for activation of macrophages by Th1 helper cells to eliminate mycobacterial infections. The activation of Th1 helper cells by macrophages is achieved by releasing of IL-1 and IL-12 and finally leads to granuloma formation. IFN-γ might also improve or augment antigen presentation, leading to recruitment of CD4+ T-lymphocytes and/or cytotoxic T-lymphocytes, which might participate in mycobacterial killing (Neil W., Schucer and Willam N. ROM 1998). IFN-γ also has the capacity to recruit more macrophages to the area of infection. Activated macrophages release a pro-inflammatory cytokine known as Tumor necrosis factor-alpha (TNF-α).

TNF-α is the principal mediator of the response to Gram-negative bacteria (Keertan Dhed et al., 2005). It activates inflammatory leucocytes to kill microbes. At physiological levels, TNF-α clearly plays an important but potentially complex role in the host response to M. tuberculosis, not only by synergizing with IFN-γ in activating macrophages but also by playing a role in the modulation of macrophages apoptosis and granuloma formation (Janis E. Wigginton and Denise Kirschner 2001) and thus TNF-α appears to be crucial for the infection control and elimination of mycobacteria (Dahir Ramos de Andrade et al., 2008; Levent Kart et al., 2003). During the early stages of TB there are elevated amount of TNF-α and this high level persists after the start of chemotherapy course and seems to play a critical role in controlling M. tuberculosis infection (Jacobs et al. 2007). Patients with latent TB infection rapidly progress to active disease when treated with TNF-α antagonists, such as Infliximab (MMWR Morb Mortal Rep. 2004). As such, patients should be thoroughly investigated for TB before commencing such therapy (BTS 2005).

The aim of the present study was to evaluate the effect of treatment on the inflammatory process in patients with tuberculosis. To that end, we determined levels of the cytokines TNF-α, IFN-γ.

MATERIALS AND METHODS

Study design:

This a cross sectional, descriptive longitudinal study, conducted in Khartoum Hospital in the section of tuberculosis. It is one of tuberculosis Centre for treatment and follow up in Khartoum. It belongs to tuberculosis National Programme in Khartoum state.
Study population:

The study was conducted on 80 HIV negative tuberculosis patients who had a positive sputum for M. tuberculosis, matched with 57 healthy individuals in age and sex and ethnicity as controls. After the pre-treatment specimens were collected from patients received standard short-course chemotherapy.

Ethical Considerations:

Ethical approval for this study was obtained from the Institutional Review Board of Faculty of Medicine, Al Neelain University. The study protocol was scientifically reviewed by the ethical review board of AL Neelain University. The objective of the study was explained clearly to participating patients and controls. Written consents were obtained from participants.

Samples:

Venous blood samples (3-5 ml) were collected in plain vacutainers from patients before starting anti-tuberculosis treatment (ATT) and after 2,4,5 and 6 months of ATT. Blood samples were centrifuged at 5000 rpm for 10 minutes and then sera were collected and store at (−80) until use.

Cytokines Assays:

Sera from patients and control were screened for HIV using enzyme-linked immuno-sorbent assay Kit from Biorex and those whom were reactive for HIV were excluded from the study.

Cytokines levels in sera were assessed using commercial available ELISA Kits obtained from Komma biotech (Komma biotech INC., Seoul were used to determine IFN-γ, TNF-α levels. Results were reported as pg/milliliter (pg/ml). The detection range of the assay is 32-2000pg/ml for IFN-γ and 16-2000pg/ml for TNF-α.

Sandwich Enzyme Immuno-sorbent assay (ELISA) for the quantitative measurement of Human IFN-Gamma levels:

Briefly, 100 μl of each sample were added to each well in duplicate into 96 well plates pre-coated with antigen-affinity purified Rabbit anti-Human IFN Gamma along with controls. The plates were covered with the Plate Sealer and Incubated at room temperature for at least 2 hours. Washing was undertaken in 4 times by using washing buffer (1ml Tween-20 (50%) to 1L PBS). Then 100 μl of the reconstituted detection antibody (1 μg/ml) of biotinylated antigen-affinity purified anti- Human IFN Gamma were then added to each well. Plates were incubated at room temperature for 2 hours and then were washed 4 times. Then 100 μl of the diluted color development enzyme ((Streptavidin-HRP conjugate) (60 ul) (1/200 dilute)) were added per well, covered with the plate sealer provided and incubated for 30 minutes at room temperature (or 37°C for 30 minutes). Following washing 4 times, 100 μl of color development solution were added to each well (A mixture of 1 volume of color development reagent A (TMB solution) and 2 volumes of color development reagent B (Substrate (H₂O₂) Solution). Then plates were incubated at room temperature for a proper color development for (3-15 minutes) and to stop the color reaction, 100 μl of the stop solution (2M H₂SO₄) were added to each well. Plates were read at 450 nm wavelength.
Sandwich Enzyme Immuno-sorbent assay (ELISA) for the quantitative measurement of Human TNF-alpha levels:

Briefly, 100 μl of each sample were added to each well in duplicate into 96 well plates pre-coated with Antigen-affinity purified Rabbit anti-Human TNF-alpha along with controls. The plates were covered with the Plate Sealer and Incubated at room temperature for at least 2 hours. Washing was undertaken in 4 times by using washing buffer (1ml Tween-20 (50%) to 1L PBS). Then 100 μl of the reconstituted detection antibody (1 μg/ml) of Biotinylated antigen-affinity purified Rabbit anti-Human TNF-alpha were then added to each well. Plates were incubated at room temperature for 2 hours and then were washed 4 times. Then 100 μl of the diluted color development enzyme ((Streptavidin-HRP conjugate (60 ul)) (1/200 dilute)) were added per well, plates were covered with the plate sealer provided and incubated for 30 minutes at room temperature (or 37°C for 30 minutes). Following washing 4 times, 100 μl of color development solution were added to each well (A mixture of 1 volume of color development reagent A (TMB solution) and 2 volumes of color development reagent B (Substrate (H2O2) Solution)). Then plates were incubated at room temperature for a proper color development for (3-15 minutes) and to stop the color reaction, 100 μl of the stop solution (2M H2SO4) were added to each well. Plates were read at 450 nm wavelength.

Statistical analysis:

All statistical analyses were performed using IBM SPSS Statistics (version 20) except for the analysis of diagnostic test accuracy (ROC curve and other performance measures) which was performed using the biomedical Stats Direct Statistical Software v2.7.9 (7/9/2012). Graphs, however, were all created using SPSS. The conventional 5% level of significance was used for all statistical tests.

RESULTS

To assess the potential of Interferon-Gamma (IFN-γ) and Tumor necrosis factor-alpha (TNF-α) as markers for Tuberculosis (TB) during treatment, data were collected on subjects drawn from three groups. The groups were: (1) newly diagnosed cases of TB (the New cases group); (2) patients undergoing or already completed treatment of TB (the Under treatment group); and (3) apparently healthy individuals as controls (the Controls group). Each subject underwent one or more of the tests and the serum cytokines level determined by each test was recorded together with the subject’s age and sex. The number of subjects in each group differed with regard to the tests.

1. Interferon-Gamma

Interferon-gamma levels were measured in 40 subjects in the three groups. The mean age of the subjects was 32.2 (CI: 29.5 to 37) years. Table 1.1 below shows the characteristics of the three groups.
Table 1.1: Characteristics of the three groups of subjects tested for IFN-γ

<table>
<thead>
<tr>
<th>Group</th>
<th>Number Of Subjects*</th>
<th>Age (years)</th>
<th>Interferon-Gamma (pg/ml)</th>
<th>Percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (95% CI)</td>
<td>Minimum</td>
<td>Maximum</td>
</tr>
<tr>
<td>Controls</td>
<td>9 (5/4)</td>
<td>28.3 (22.4 to 34.3)</td>
<td>8</td>
<td>31.5</td>
</tr>
<tr>
<td>New cases</td>
<td>14 (9/5)</td>
<td>36.7 (28.8 to 44.6)</td>
<td>5</td>
<td>275</td>
</tr>
<tr>
<td>Under Treatment</td>
<td>17 (10/7)</td>
<td>33.2 (27.3 to 39.1)</td>
<td>5</td>
<td>450</td>
</tr>
<tr>
<td>All groups</td>
<td>40 (24/16)</td>
<td>32.2 (29.5 to 37)</td>
<td>5</td>
<td>450</td>
</tr>
</tbody>
</table>

* (Male/Female)

As can be seen by table 1.1, IFN-γ levels were measured in nine subjects in Controls group. Their mean age was 28.3 (CI: 22.4 to 34.3) years and their IFN-γ levels had a minimum of 8 pg/ml, a maximum of 31.5 pg/ml and a median of 16 pg/ml. The 25th and 75th percentiles indicate that the middle 50% of the levels were between 16 and 19 pg/ml.

In the New cases group, the median level of IFN-γ was about twice as much as that of the controls group (31.5 compared to 16 pg/ml). The 25th and the 75th percentiles were 16 and 31.5 pg/ml respectively. The interquartile range (the difference between 75th and the 25th percentile) was about 5 times as wide as that of controls. Although the Under treatment group showed the maximum level of IFN-γ (450 pg/ml), its median level was equal to that of the controls group and its interquartile range was three times as narrow. The median level of the three groups pooled together was 16 pg/ml. The 25th and 75th Percentiles were 15 and 31.5 pg/ml respectively.

Table 1.2: Multiple comparisons of IFN-γ (Adjusted p values reported)

<table>
<thead>
<tr>
<th>Max-t</th>
<th>Kruskal-Wallis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated</td>
<td></td>
</tr>
<tr>
<td>Group1 – Group2</td>
<td>mean p-value</td>
</tr>
<tr>
<td>New - Controls</td>
<td>54.1 a</td>
</tr>
</tbody>
</table>
2. Tumor Necrosis Factor-Alpha (TNF-α)

Tumor Necrosis Factor-Alpha levels were measured in 136 subjects in the three groups. The mean age of the subjects was 30.3 (CI: 28.3 to 32.3) years. Table 2.1 below shows the characteristics of the three groups.

Table 2.1: Characteristics of the three groups of subjects tested for TNF-α

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Subjects*</th>
<th>Age (years)</th>
<th>Tumor Necrosis Factor-Alpha (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (95% CI)</td>
<td>Minimum</td>
</tr>
<tr>
<td>Controls</td>
<td>57(25/32)</td>
<td>28.8(25.9 to31.7)</td>
<td>2</td>
</tr>
<tr>
<td>New cases</td>
<td>26 (19/7)</td>
<td>37.2 (31.0 to 43.4)</td>
<td>4</td>
</tr>
<tr>
<td>Under Treatment</td>
<td>53(31/22)</td>
<td>28.7 (26.0 to 31.4)</td>
<td>1.5</td>
</tr>
<tr>
<td>All groups</td>
<td>136 (75/61)</td>
<td>30.3(28.3 to 32.3)</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* (Male/Female)

From table 2.1, the new cases group had the highest median TNF-α level; two and a half times that of controls and about one and a half times that of the under treatment group. While the highest level of TNF-α was observed in the under treatment group (125 pg/ml) followed by that of the new cases group (80 pg/ml), the highest level in the controls group was only 28 pg/ml. The interquartile range was about almost the same in both the new cases group and the under treatment group (9 pg/ml). That was about three times as high as the interquartile range of controls.

Pairwise comparisons were performed to identify the pair(s) within which the distributions of TNF-α level differed. Results of the pairwise comparisons are shown in table 2.2 and figure 2.2 below.
Table 2.2: Pairwise comparisons of the three groups

<table>
<thead>
<tr>
<th>Group1 vs Group2</th>
<th>Adjusted¹ p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls vs Under treatment</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>Controls vs New cases</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>New cases vs Under Treatment</td>
<td>0.308</td>
</tr>
</tbody>
</table>

Each row tests the null hypothesis that Group1 and Group2 are the same as regards TNF-α levels.

¹ P-values adjusted for multiple comparisons.

* Significant at 0.05 level.

From the pairwise comparisons, it was found that significant differences existed within two of the three pairs, namely Controls vs the under treatment group and Controls vs the New cases group (adjusted p-values < 0.001). In the last pair (New cases vs Under treatment), however, the null hypothesis that the distributions of TNF-α level were the same in both groups could not be rejected at the 5% level (adjusted p-value = 0.308).

Diagnostic accuracy of TNF-α test

Receiver operating characteristic (ROC) curve analysis was performed to assess the discriminative power of TNF-α as a marker for TB in new cases and to obtain a decision threshold (cut-off level). Having obtained a good discriminatory power for the test and a cut-off level optimized for equally important sensitivity and specificity, other measures of diagnostic accuracy were calculated.

ROC Curve analysis of TNF-α

Data from only the New cases group and the Controls group were used for the ROC curve analysis. Figure 1 below, shows the ROC curve.
Figure 1: ROC curve of TNF-α

The diagonal line connects points of equal values of sensitivity (true positive fraction) and one minus specificity (false positive fraction). It is the line of no discrimination or alternatively, the line of chance performance (random guessing). The area under the diagonal line is 0.5.

The area under the curve (AUC) for TNF-α was found to be 0.824 (CI: 0.737 to 0.912), which is significantly different from 0.5 (p-value < 0.001). AUC is the average probability that the test will produce a value for a randomly chosen TB subject that is greater than the value for a randomly chosen TB-free subject. This probability can also be interpreted as the average sensitivity for all specificities or the average specificity for all sensitivities.

The 95% CI for the AUC of TNF-α test indicates that it has a moderate to high accuracy and can therefore be used as a marker for TB.

The optimum cut-off level, assuming equal weights (importance) for sensitivity and specificity, was 5 pg/ml. Subjects were classified according to the cut-off level; subjects with TNF-α levels ≥ 5 pg/ml were classified as test positives, while subjects with TNF-α levels < 5 pg/ml were classified as test negatives. The classification result is shown in table 2.3 below.
Table 2.3: TNF-α test results of 27 new TB cases and 56 TB-free controls

<table>
<thead>
<tr>
<th>Test Result</th>
<th>Disease Status</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TB +ve (New cases)</td>
<td>TB -ve (Controls)</td>
</tr>
<tr>
<td>TNF-α +ve</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>TNF-α -ve</td>
<td>4</td>
<td>39</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>56</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In order to achieve the balance between the inflammatory and the protective immune response, the productions of the anti-inflammatory cytokines like IL-4, IL-10 and TGFβ in response to M. tuberculosis antigens down regulate the immune response and limit tissue injury by inhibiting excessive inflammatory response. The opposing of this action will be achieved by increasing the interferon-γ and decreasing TNF-α. May determined the outcome of tuberculosis infection (Sadhna S. and Midula B 2001). However, TNF-α is involved both in the disease physiopathology and in the protective immune response. Its role is complex and it is act in synergism with IFN-γ inducing the formation of oxygen and nitrogen intermediate, but on the other hand it is involved in the destruction of pulmonary tissue (Moura EP. et al., 2004).

Several studies have demonstrated the importance of cytokines as markers of tuberculosis activity or of response to the specific treatment i.e. when treatment is effective, there is Th1 response recovery, with subsequent bacillus containment. There is evidence to suggest that TNF-α is necessary at the beginning of the inflammatory process in order to limit the multiplication of mycobacteria(Olobo JO. et al., 2001; Portales-Pérez DP. et al., 2002). Other studies demonstrated that high TNF-α initial levels in TB patients decreased significantly during the treatment, while the inflammatory process decreased at the same time (Sahiratmadja E. et al., 2007).

High level TNF-α from PBMCS in patients with active pulmonary tuberculosis was demonstrated, this agrees with our study that TNF-α was elevated in level, however, a significant difference was observed in the level of TNF-α after the administration of TB treatment (Moura P. et al., 2004), accordingly, this could be predicative marker indicate the activity of the disease. Even though small decrement had been occured during and after the completion of anti- tuberculosis therapy. This proves that TNF-α has a crucial role in the pathophysiology and in the protective
immunity against mycobacterium tuberculosis infection.

In the present study, TNF-α level in the new cases group was two and a half times that of controls and about one and a half times that of the under treatment group i.e., lower in control group than in patients before treatment and after treatment. However, the elevation in TNF-α levels in patients under treatment could be due to spontaneous or induced apoptosis of mononuclear cells and found to be increased among PBMC from patients with newly diagnosed TB, compared with that of healthy control subjects (Hirsch CS. et al., 1999). Increasing levels of TNF-α could be explained on one hand by that, the balance between the pro-inflammatory and anti-inflammatory activities persisted during the treatment until T6, when the patients evolved to Th2 profile, with normalization of IFN-γ levels, likely to protect from the effects of the Th1 profile pro-inflammatory activity and ensure appropriate cicatrization, with development of fibrosis (Eliana Peresi 2008). In addition to that, high levels of TNF-α in the current study may indicate that the enrolled patients may have necrotic granuloma with high number of with high number of TNF-α producing cells. Furthermore, a virulent strains of mycobacterium tuberculosis bacterium have been found to be more potent inducers of TNF-α-dependent apoptosis than their virulent counterparts (Keane J 2000), and apoptosis in response to a virulent strains can be enhanced by addition of TNF-α while that in response to virulent mycobacterium tuberculosis bacterium cannot (Keane J 1997).

The current study demonstrates that, from the analysis of the diagnostic accuracy of TNF-α test, TNF-α can be used as marker for TB disease activity in new cases, having had a good discriminatory power (AUC = 0.824) at the chosen cut-off level of 5 pg/ml, it has a sensitivity of 85.2% and a lower specificity of 69.6%. In addition to that, the diagnostic odds ratio was greater than one (at least 3.6, with 95% confidence), indicating that the test has the ability to discriminate between TB and TB-free subjects and may be of use for the monitoring of treatment efficacy. This is supported by the finding by the current study that, negative TNF-α test results provide more clarification of the disease status of subjects as their likelihood of having TB approaches zero. A negative test result will at most clarify 79% of the diagnostic uncertainty. Positive results provide more clarification of the disease status of subjects as their likelihood of having TB approaches one.

IFN-γ contributes to protective immunity against M. tuberculosis by activating macrophages to more effectively eliminate these organisms. It is also observed that individuals with IFN-γ receptor deficiency have disseminated mycobacterial disease (Sadhu Sharma and Mridula Bose 2001). Studies looking at the severity of TB disease have shown that there is relationship between the production of some cytokines by PBMCs and the severity of the disease, observed that patients with the disease in a moderate stage presented significantly higher levels of IFN-γ compared to the levels presented by patients with the disease in the advance stage (Moura EP. et al., 2004). It had been found that plasma IFNγ levels were significantly higher in active TB cases than in controls normalized during treatment and correlated
with both TB disease activity and severity (Sahiratmadja E. et al., 2007).

The current study demonstrates that cytokine directing a Th1 response (IFN-γ) was not significantly elevated in serum of patients with TB during treatment with anti-TB drugs. This finding is in agreement with results presented by Hirsch and others (1999) that IFN-γ immunoreactivity in PPD-stimulated culture supernatants from HIV-uninfected patients remained depressed for at least one year after initiation of chemotherapy. Suggesting that the immunosuppression of TB is not only immediate and apparently dependent (at least in part) on immunosuppressive cytokines early during the course of Mycobacterium TB infection but is also long lasting, presumably relating to a primary abnormality in T-cell function. Conflicting results, reporting increased, unchanged or decreased cytokine production (Surcell H-M 1994; Lai CKW 1997), suggesting a relationship between low IFN-γ levels and lack of protection (Zhang M 1995). On the other hand, detection of high levels of IFN-γ in some studies may be due to selective concentration of Th1 cells at the site of disease in tuberculosis (Robinson, D. S 1994). This assumption was clearly demonstrated by measuring levels of IFN-γ in the supernatant of PBMC culture of patient with pulmonary tuberculosis before and after the treatment. It has been shown that the pleural fluid of tuberculosis patients contain a high number of IFN-γ producing cells and these cells may migrate to lung and pleural tissue during the active disease, and therefore they may be reduced temporarily, in the peripheral blood. The increased in the production capacity of IFN-γ after the anti-tuberculosis treatment was also demonstrated after the antigen released after death of mycobacteria caused by chemotherapy (Moura EP. et al., 2004).

In conclusion, serum levels of TNF-α in TB patients is useful in the evaluation of the disease activity of TB during therapy, not replacing clinical parameters of disease activity in TB, such as symptoms, chest X-rays, and culture and smear results, but used in addition to these conventional parameters. TNF-α can be used as marker for TB severity, having had a good discriminatory power (AUC = 0.824). Levels of both IFN-γ were not statistically significantly deferent between the three groups.

The current study recommends determination of treatment response phenotypes before the start of treatment, and reliable predictive models, using combinations of host markers would allow targeted interventions for patients at risk for slow treatment response to standard tuberculosis therapy. Further study with large sample size to study INF-γ in Sudanese TB patients should be conducted in future

REFERENCES


British Thoracic Society "BTS recommendations for assessing risk and for managing Mycobacterium tuberculosis infection and disease in patients due to start


