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## Evaluation of the Humoral Immune Response in Pulmonary Tuberculosis Patients

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**Abstract:** The aim of this study was to evaluate the humoral immune response in the pulmonary tuberculosis patients. A total of 54 patients receiving treatment at the hospital and 41 healthy subjects were included in this study. Bactericidal activities of serum complements were assessed by the colony count method. The total serum immunoglobulin G (IgG) was measured by nephelometric method and complement components C3 and C4 were measured by immunoturbidometric methods. It was found that the patients had very high ESR ( $85.0 \text{ mm h}^{-1}$ ) and about 78% of them had BMI < 18.5. The bactericidal activity of complement from fresh serum of the patients was similar to that of the control group ( $p > 0.05$ ) but the heat-inactivated serum showed significantly high bactericidal activity ( $p < 0.001$ ). The IgG level was also significantly elevated than that of the controls ( $p < 0.001$ ). There was no difference in the levels of complement protein C3 between the patients and controls but C4 was significantly elevated in the patients ( $p < 0.01$ ). High IgG levels in the patients might have a relationship with enhanced killing of bacteria, which was observed in experiments with heat-inactivated serum. Elevated level of C4 indicated reduced utilization and thereby suppressed activity of the classical complement pathway. The findings suggest that the humoral immune response is altered in the pulmonary tuberculosis patients.

**Key words:** Pulmonary tuberculosis, bactericidal activity, C3, C4, IgG

### INTRODUCTION

Tuberculosis remains the most common infectious disease in the world, with an estimated one-third of the population infected and 2.5 million deaths annually (Haslett *et al.*, 2002). Two species of *Mycobacterium*, namely *M. tuberculosis* and *M. bovis* cause tuberculosis. The pathogenicity of *M. tuberculosis* is related to its ability to escape killing by macrophages and induce delayed type hypersensitivity (Quinn *et al.*, 1996). Alveolar resident macrophages are the primary cell type involved in the initial uptake of *M. tuberculosis*. After this first encounter, dendritic cells and monocyte-derived macrophages also take part in the phagocytic process (Henderson *et al.*, 1997; Thurnher *et al.*, 1997). Some individuals become reinfected with mycobacteria, reactivate dormant disease, or progress directly from the primary mycobacterial lesions into disseminated disease. Granulomas of secondary tuberculosis most often occur in the apex of the lungs but may be widely disseminated in the lungs. These granulomas, which fail to contain spread of the mycobacterial infection, are the major cause of tissue damage in tuberculosis and are a reflection of delayed type hypersensitivity (Samuelson, 2000).

Mycobacteria have many antigen-active components. The antigens are: polypeptide, which induces later cellular response and inhibits macrophage function and polysaccharide II- arabinomannan which induces suppressive T cell proliferation (Arend *et al.*, 1987; Tomoka *et al.*, 1990). Polysaccharide II consists of antigens (glucan, peptides, lipids-cord factor, sulphatides), which induce antibody synthesis and inhibits phagosome-lysosome fusion (Edwards and Kirkparile, 1986;

Tomoka *et al.*, 1990). Putative mechanisms involved in killing of *M. tuberculosis* within the phagolysosomes of activated macrophages include the production of Reactive Oxygen Intermediates (ROI) or Reactive Nitrogen Intermediates (RNI). *In vivo* it was found that mice lacking the cytosolic p47 (phox) gene that is essential for effective superoxide production by the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, suffer from increased early outgrowth of mycobacteria in experimental infection (Cooper *et al.*, 2000). Therefore, this supports a role for ROI in the killing of *M. tuberculosis*. *In vitro* human alveolar macrophages infected with *M. bovis* BCG display increased inducible nitric oxide synthase (iNOS) mRNA and inhibition of iNOS is followed by increased bacterial outgrowth (Nozaki *et al.*, 1997). In tuberculosis patients, alveolar macrophages show increased production of iNOS as well (Nicholson *et al.*, 1996).

The specific and nonspecific immune markers including *M. tuberculosis*-specific serum antibodies, the T-lymphocyte enzyme adenosine deaminase, the macrophage activation product neopterin, the mononuclear cell surface protein  $\beta_2$  microglobulin, soluble T-cell interleukin (IL)-2 receptors as well as soluble CD4 and CD8 receptors, macrophage and T-cell adhesion molecules and acute phase reactant proteins have all been proposed as indicators of disease activity in pulmonary and extrapulmonary TB (Chan *et al.*, 1991; Lai *et al.*, 1993; Ameglio *et al.*, 1994; Hosp *et al.*, 1997; Baumer *et al.*, 1998). The cytokines tumour necrosis factor (TNF- $\alpha$ ), IL-1, IL-6, interferon gamma (IFN- $\gamma$ ) and IL-12 and the chemokines IL-8, monocyte chemoattractant peptide-1 (MCP-1) and regulated on activation, normal T cell expressed and secreted (RANTES) have also been found to be elevated in the bronchoalveolar lavage fluid or serum of patients with active disease and have been proposed as markers of disease activity (Chensue *et al.*, 1986; Law *et al.*, 1996; Taha *et al.*, 1997; Bekker *et al.*, 1998; Juffermans *et al.*, 1998; Verbon *et al.*, 1999).

The complement system is an important non-specific mediator of immune responses. Detection and assessment of complement proteins show increased levels of C3 and C4 concentrations in active pulmonary tuberculosis (Van Schooten *et al.*, 1989; Bothamley, 1995). Measurement of serum immunoglobulins using different antigenic preparations has shown that IgM antibody levels have been so low that their reliable measurement has been difficult. IgA levels have generally paralleled IgG class, but also have tended to be low and more difficult to measure reliably. This feature suggests that the presence of IgM antibody to TB protein antigen might be characteristic of early disease. In view of the scarcity of data, this study has been undertaken to evaluate the humoral immune response and complement mediated bactericidal activity in patients with pulmonary tuberculosis.

## MATERIALS AND METHODS

### Study Subjects and Sample Collection

This study was conducted from November 2005 to March 2007. A total of 54 Pulmonary tuberculosis patients (39 males and 15 females) undergoing treatment at the National Institute of Diseases and Chest Hospital (NIDCH), Mohakhali, Dhaka, Bangladesh, were enrolled in this study. The duration of hospitalization of the patients varied from 0.1 to 6 months (mean:  $1.6 \pm 1.3$ , median: 1.0). In the hospital, the patients were treated with rifampicin, isoniazid, ethambutol and pyrazinamide. Forty-one healthy human subjects (21 males and 20 females) were included in this study as the control population. About 2-3 mL of peripheral blood was collected from the subjects with their full consent. The general information of the patients was recorded on preformed questionnaires. Serum was collected from each blood sample and preserved at  $-80^\circ\text{C}$  until further analyzed.

### Assay of Complement Mediated Bactericidal Activity

*Escherichia coli* DH5 $\alpha$  were allowed to grow in nutrient broth for 14 h at  $37^\circ\text{C}$  in an Orbital Shaker. Then the bacterial cells were harvested, washed twice using excess of Phosphate Buffer Saline

(PBS) and the optical density of the bacterial suspension was adjusted to 0.600 at 620 nm by using a Spectrophotometer. Immediately, an aliquot of 200  $\mu$ L of Bacterial Cell Suspension (BCS) were taken into separate tubes and mixed with 20  $\mu$ L of serum and incubated for 30 min at 37°C. At the end of incubation, the suspensions were serially diluted with PBS to 1:10,000. An aliquot of 20  $\mu$ L of this dilution was spread on each of 3 agar plates and incubated for 16 h at 37°C. The number of colonies formed was counted and the mean value for each serum was taken from the readings of three plates. For the negative control experiments, instead of using serum, 20  $\mu$ L of PBS was added to the BCS, incubated and then serially diluted with PBS to 1:50,000.

#### **Assay of Complement Inactivated Bactericidal Activity**

To determine non complement mediated bactericidal activity of each serum, complement proteins were inactivated by heat treatment at 56°C for 30 min in a water bath and then a 20  $\mu$ L aliquot was used to test for bactericidal activity. Both the negative control and inactivated serum treated bacterial preparations were serially diluted to 1:50,000. The rest of the procedure was the same as described before.

#### **Calculation of Bactericidal Activity**

Complement mediated bactericidal activity was calculated as described elsewhere (Islam *et al.*, 2006) using the following formula. For the negative control, if the mean colony-forming unit (cfu) on the plate was  $N_c$ , then one mL of the original bacterial cell suspension contained  $N_c \times 50 \times 50,000$  (dilution factor) cfu. For the test serum, if the mean cfu on the plate was  $N_s$ , then 1 mL of the bacterial cell suspension treated with serum complement contained  $N_s \times 50 \times 10,000$  (dilution factor) cfu. Therefore,

$$\text{Bactericidal activity (\%)} = \frac{(N_c \times 50 \times 50,000) - (N_s \times 50 \times 10,000)}{N_c \times 50 \times 50,000} \times 100$$

The bactericidal activity (%) of the inactivated serum for the mean cfu,  $N_i$ , was

$$\frac{(N_c \times 50 \times 50,000) - (N_i \times 50 \times 50,000)}{N_c \times 50 \times 50,000} \times 100$$

#### **Determination of Serum C3, C4 and IgG**

Quantitative estimates of serum complement components C3 and C4 were performed using a TURBOX plus Analyzer, Orion Diagnostica, Finland. The method was based on the principle of immunoprecipitation reaction of a specific antibody with its antigen. The light scattering caused by antigen-antibody complexes was measured after incubation. The intensity of the scattered light was directly proportional to the concentration of the tested complement protein present in the serum sample. IgG was measured by immunonephelometry using DADE Behring reagents (USA) and an autoanalyzer. The results were expressed in  $\text{g L}^{-1}$ .

#### **Statistical Analysis**

Data analyses were carried out using the Statistical Package for Social Sciences (version 11.5 for Windows, SPSS Inc., Chicago, USA). Independent t-test was done for the comparison of two groups (control and patients). The results were considered significant when  $p$  was  $\leq 0.05$ .

## RESULTS

### Baseline Characteristics of the Study Subjects

The baseline characteristics of the study subjects were determined from the preformed questionnaires. The mean age of the pulmonary tuberculosis patients was  $37.5 \pm 17.9$  years (range: 15-80 years), BMI was  $16.2 \pm 3.2$  (male  $16.2 \pm 2.7$ ; female:  $15.8 \pm 4.2$ ) and about 78% of the patients had BMI < 18.5. The mean ESR was 82.4 and 87.0 mm h<sup>-1</sup> for the male and female patients, respectively. In case of the control subjects, the age varied from 20-35 years, mean BMI was  $21.3 \pm 1.6$  and only 5% had BMI < 18.5. The mean ESR for the male controls was 8.4 mm h<sup>-1</sup> while that for the females was 17.8 mm h<sup>-1</sup>.

### Complement Mediated Bactericidal Activity

It was found that in the negative control experiments (n = 51) the numbers of *Escherichia coli* DH5 $\alpha$  colonies grown on agar plates after treating the bacterial cell suspensions with PBS varied from  $303-790 \times 10^6$  cfu mL<sup>-1</sup>. On the other hand, the numbers of colonies formed after treating the BCS with serum from control healthy subjects (n = 41) varied from  $0.16-10 \times 10^6$  cfu mL<sup>-1</sup>. After treating the BCS with serum from pulmonary tuberculosis patients (n = 54) the number of colonies varied from  $0.17-42.5 \times 10^6$  cfu mL<sup>-1</sup>. Compared to the negative control experiments, both types of sera exhibited highly significant bactericidal activities (p < 0.001). Further, the bactericidal activity of serum complements from the pulmonary tuberculosis patients varied from 93.6 to almost 100% compared to about 98.8 to almost 100% in the control healthy subjects. The complement mediated bactericidal activity of the pulmonary tuberculosis patients was not significantly different from that of the control group (p = 0.071).

### Effect of Serum Inactivation on Bactericidal Activity

The bactericidal activity of the complement inactivated serum (BACIS) from the control subjects varied from 12.9-33.1% whereas that from the pulmonary tuberculosis patients varied from 23.0-74.3%. The results are shown in Fig. 1a and b. The BACIS from the pulmonary tuberculosis patients was found to be significantly higher than that of the control subjects (p < 0.001).

### Levels of Complement Component C3

Of the total 54 pulmonary tuberculosis patients, 45 (83%) had serum complement component C3 level that was within the normal range (0.9-2.1 g L<sup>-1</sup>) whereas 7 (13%) had elevated level and only 2 (4%) had lower level. On the other hand, out of the total 41 control subjects, 32 (78%) had normal

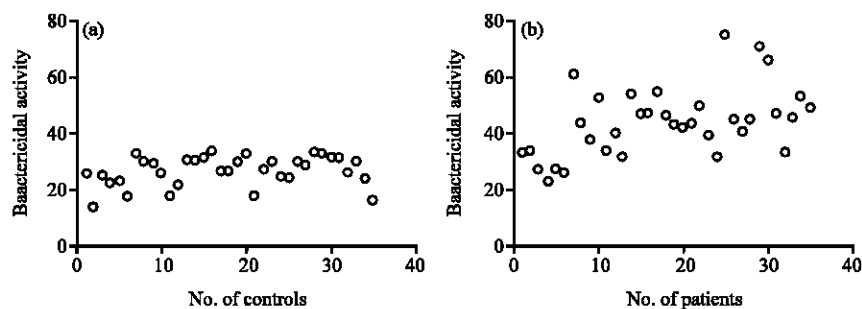


Fig. 1: Percentage bactericidal activity on *Escherichia coli* DH5 $\alpha$  cells by heat inactivated serum from (a) control subjects and (b) pulmonary tuberculosis patients. Each point represents the bactericidal activity of one individual expressed as the mean of three separate experiments

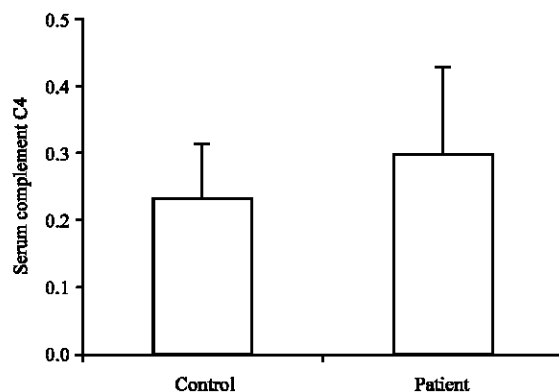


Fig. 2: Levels of serum complement C4 in the control subjects and pulmonary tuberculosis patients. The mean C4 level in the patients ( $0.30 \pm 0.13 \text{ g L}^{-1}$ ) was significantly higher ( $p < 0.01$ ) than that in the controls ( $0.24 \pm 0.08 \text{ g L}^{-1}$ ). The values are expressed in Mean  $\pm$  SD

level, 8 (20%) had elevated level and only 1(2%) had lower level of serum complement component C3. The mean level of C3 in the pulmonary tuberculosis patients was  $1.73 \pm 0.43 \text{ g L}^{-1}$  and that in the healthy control subjects was  $1.72 \pm 0.50 \text{ g L}^{-1}$ .

#### Levels of Complement Component C4

Evaluation of the levels of serum complement component C4 in the pulmonary tuberculosis patients showed 44 (81%) had C4 level within the normal range ( $0.1-0.4 \text{ g L}^{-1}$ ) while the remaining 10 (19%) had elevated levels. In contrast, of the total 41 control subjects, 40 (98%) had normal C4 level and only 1 (2%) had lower level. The whole population of patients had the mean value of C4 level of  $0.3 \pm 0.13 \text{ g L}^{-1}$  while that in the healthy control subjects was  $0.24 \pm 0.08 \text{ g L}^{-1}$ . Statistical analysis showed the complement component C4 level in the pulmonary tuberculosis patients was significantly higher than that in the control subjects ( $p < 0.01$ ) (Fig. 2).

#### Levels of Serum IgG

The levels of serum IgG in the pulmonary tuberculosis patients were found to vary from  $10.2-25.9 \text{ g L}^{-1}$  and those in the control subjects varied from  $8.2-16.4 \text{ g L}^{-1}$  (normal range:  $7.0-16.0 \text{ g L}^{-1}$ ). The patients had the mean value of total serum IgG level of  $18.5 \pm 4.3 \text{ g L}^{-1}$  compared to  $11.4 \pm 2.0 \text{ g L}^{-1}$  in the healthy control subjects. Statistical analysis showed that the total IgG level in the pulmonary tuberculosis patients was significantly higher than that in the control subjects ( $p < 0.001$ ) (Fig. 3).

## DISCUSSION

*Mycobacterium tuberculosis* is a potent human pathogen parasitizing macrophages. It has been shown that inhibition of phagosome-lysosome fusion is critical for *M. tuberculosis* persistence in human populations (Vergne *et al.*, 2004). Historical data indicate that protein-energy malnutrition plays an independent role in the susceptibility to tuberculosis (TB). Study showed among Finnish elderly men, those with a normal BMI ( $>23.0$ ) had a lower risk of the subsequent development of TB than those with a low BMI while the relative risk reduction associated with obesity (BMI  $>27.0$ ) was even greater (Hemila *et al.*, 1999). In mice, protein deficiency profoundly impairs the pulmonary

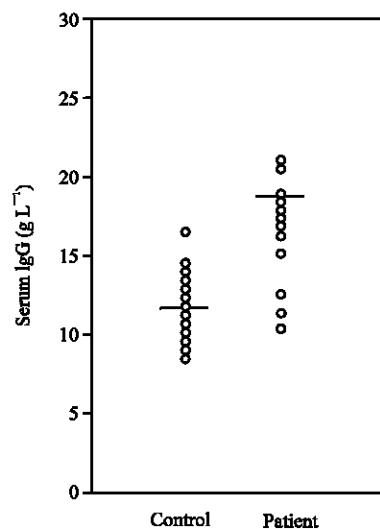


Fig. 3: Levels of total serum IgG in the control subjects and pulmonary tuberculosis patients. The mean IgG level in the patients ( $18.5 \pm 4.3 \text{ g L}^{-1}$ ) was significantly higher ( $p < 0.001$ ) than that in the controls ( $11.4 \pm 2.0 \text{ g L}^{-1}$ ). Each point represents the concentration of IgG in one individual

production of TNF and of nitric oxide (NO) and increases the lethality of infection (Chan *et al.*, 1996). Protein deficiency heavily affected macrophage function, their TNF- $\alpha$  and IFN- $\gamma$  production and led to increased production of transforming growth factor  $\beta$  (TGF- $\beta$ ) after infection with virulent *M. tuberculosis*. An association between malnutrition and a non-protective T<sub>H</sub>2 cytokine pattern was found in children with TB (Hanekom *et al.*, 1999). The effect of TB on the nutritional state has been reviewed elsewhere (Macallan, 1999; Miller *et al.*, 2000).

In the present study, about 78% of the total patient had BMI lower than 18.5 compared to only 5% of the control subjects. Thus a relation between TB and low BMI was also found in the present study. Malnutrition among TB patients might be the underlying cause of low BMI. From previous studies it was found that infection could rapidly lead to nutritional stress and weight loss, thereby worsening nutritional status and immunologic function (Chandra, 1991). The ESR may be elevated in the presence of infectious diseases, other inflammatory or destructive processes, collagen vascular disease or malignancy (Sox and Liang, 1986; Saadeh, 1998). In the present study, the ESR values of both the male and female pulmonary tuberculosis patients were significantly higher than the control males and females. This observation suggested that, inflammation of the lung might be a cause of higher ESR value among the pulmonary tuberculosis patients.

Complement proteins play an important role in the innate immune system. *Escherichia coli* DH5 $\alpha$  used in this study was a nonpathogenic Gram-negative bacterium and therefore, no appreciable levels of antibody should be present in the sera of normal healthy subjects against this organism. Therefore, killing of bacteria would be solely due to serum complement. We found that the complement-mediated bactericidal activity in the patients was similar to that in the control group. The concentration of C3 in the patients was similar to that in the controls while C4 was significantly higher in the pulmonary tuberculosis patients. Previous workers have found normal levels of complement protein C3 in the pulmonary tuberculosis patients (Ganguly *et al.*, 1977). C3 took part both in the alternative and classical pathways of complement activation, suggesting a significant role of this

protein in exhibiting bactericidal activity by the alternative pathway only in which C4 did not take part. Thus, our finding of elevated levels of complement component C4 in the patients indicated suppressed activity of the classical pathway.

We found that the complement inactivated bactericidal activity of the heat-treated patient sera was significantly higher than that of the control group. The drugs rifampicin, isoniazid, ethambutol and pyrazinamide might have been responsible for high bactericidal activity (Chopra and Brennan, 1998), which had been resistant to heat treatment. We found the patients had significantly elevated levels of total IgG than that of the controls. Other researchers have observed increased levels of IgG and IgA against mycobacterial glycoprotein in the pulmonary tuberculosis patients (Kato *et al.*, 1987). About the elevated levels of IgG, we suggest that since the pulmonary tuberculosis patients suffer from different types of bacterial infections, their sera might contain wide range of IgG antibodies against these organisms. In addition to the effects of antimicrobial drugs, these IgG antibodies might have killed the bacterial cells in a nonspecific manner while the serum complements had been destroyed by heat inactivation. In conclusion, the humoral immune response is altered in pulmonary tuberculosis patients.

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