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Research on the occurrence of *Mycobacterium tuberculosis* antigens in the circulating immune complexes, isolated from serum of patients with tuberculosis

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Tuberculosis is one of the most dangerous infectious diseases and has among the highest mortality rates of all infectious diseases. There are 9 million cases of active tuberculosis reported annually; however, an estimated one-third of the world's population is infected with *Mycobacterium tuberculosis* and remains asymptomatic. Despite the great progress in its diagnosis and treatment, tuberculosis is still a serious health and social problem. The contact between the immune system and *Mycobacterium tuberculosis* initiates cell-specific (Th1) and humoral-specific (Th2) responses. Many studies about the presence of antituberculotic antibodies in the serum have produced inconsistent results because of a high proportion of false-positive or false-negative results. The purpose of this study was to confirm whether circulating immune complexes (CIC) isolated from the serum of patients with tuberculosis are accompanied by antigenic proteins typical of *Mycobacterium tuberculosis*.

Material/Methods: We assayed serum samples from 42 patients with tuberculosis. The control group consisted of the sera samples taken from 45 healthy subjects. The immunochemical analysis of dissociated immune complexes using the dot blot method demonstrated positive reaction on the presence of *Mycobacterium tuberculosis* antigens in all patients with tuberculosis.

Results: All patients with tuberculosis demonstrated a high serum concentration of CIC protein. The mean serum concentration of CIC protein was significantly higher in patients than in controls: 0.081 g/l in the control group and 0.211 g/l in the tuberculosis patients.

Conclusions: The analysis of CIC suggests that it may be a helpful test for patients with tuberculosis because of its quickness, simplicity of the idea, and limited invasiveness.

Key words: *Mycobacterium tuberculosis* • serum • circulating immune complexes • antigen

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Background

Tuberculosis is one of the most dangerous infectious diseases and has among the highest mortality rates of all infectious diseases. Despite the great progress in its diagnosis and treatment, tuberculosis is still a serious health and social problem [1–4]. WHO estimates that, in 2011, there were 8.7 million new TB cases worldwide, equivalent to 125 cases per 100,000 population, and 1.4 million people died of TB, of whom 0.5 million were women. There were 0.9 million cases occurring among people living with HIV. There were also an additional 0.43 million HIV-associated deaths. By the end of 2011, XDR-TB had been reported by 84 countries; on average, 9.0% of MDR-TB cases had XDR-TB. These estimates imply that in 2011, among notified patients with pulmonary TB, there were around 310,000 MDR-TB cases and ~25,000 XDR-TB cases [5]. A quick and reliable test would increase the chance of effective treatment.

The contact between the immune system and *Mycobacterium tuberculosis* initiates cell-specific (Th1) and humoral-specific (Th2) responses [4–10]. Many authors suggest that the dominance of the humoral-specific response is related to the progression of the disease [1,8]. Many studies of the presence of antituberculotic antibodies in the serum have produced inconsistent results because of a high proportion of false-positive results [6,11–14]. Some authors note the high levels of circulating immune complexes (CIC) in the serum of patients with tuberculosis [7,10,14,15]. The initial work on this issue appeared in the 1980s [16]. It is possible that part of the CIC fraction contains protein antigens secreted and exfoliated by *Mycobacterium*, and that the presence of these antigens is related to the occurrence of a pathogenic strain of the bacteria. The purpose of this study was to confirm whether immune complexes isolated from the serum of patients with tuberculosis are accompanied by antigenic proteins typical of *Mycobacterium tuberculosis*.

Material and Methods

Study subjects

We analyzed the sera from 42 patients with culture-proven patients with pulmonary tuberculosis (PTB). Tuberculosis can be diagnosed only if *Mycobacterium tuberculosis* bacteria are found in a clinical specimen taken from the patient. The other types of tests may strongly suggest tuberculosis as the diagnosis, but they cannot confirm it. The complete medical evaluation for tuberculosis (TB) must include a medical history, a physical examination, a chest X-ray, and a microbiological examination (of sputum or some other appropriate sample). It may also include a tuberculin skin test, other scans and X-rays, and a surgical biopsy. Tuberculosis is diagnosed if the patient has a positive culture for *M. tuberculosis*. The average age of the patients was 51.0±12.2 years (range, 21–85 years). The study population characteristics are presented in Tables 1 and 2. Sera were also obtained from 45 healthy subjects, whose average age was 44.1±12.8 years (range, 27–67 years), who served as the control group.

Table 1. Demographic characteristics of study population.

	Frequency (N=42)	Percentage
Sex		
Female	16	38.10
Male	26	61.90
Residence		
Urban	32	76.19
Rural	10	23.81
Marital stasus		
Separated/divorced/widowed	17	40.48
Married/cohabitating	25	59.52
Education		
Primary	18	42.86
Secondary	22	52.38
Above secondary	2	4.76
Smoking habits		
Smokers	30	71.43
Nonsmokers	12	28.57
Alcohol use		
Alcoholics	12	28.57
Non alcoholics	30	71.43

Circulating immune complexes assay by the PEG test

Each serum sample (2 ml) was diluted in 2 ml of 7% PEG-6000 in borate buffer (0.1 M, pH 8.4). The samples were incubated at 4°C for 24 h and centrifuged at 15,000 *g* for 30 min at 4°C. The supernatant was decanted, and the precipitate was washed with 3.5% PEG-6000 in borate buffer, suspended in 2 ml of 0.1 M NaOH, and incubated at 25°C for 30 min. The optical density was estimated at 280 nm on a spectrophotometer (0.1 optical density unit was read as 0.07 g/l of CIC protein). The results were considered positive when the optical density (OD) value was >0.130 based on the value of 0.112±0.018 OD of healthy men reported in our earlier publication [17].

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Table 2. Clinical characteristics of study population.

	Frequency (N=42)	Percentage
Symptoms		
Present	40	95.24
Absent	2	4.76
Tb new/retreatment		
New TB	32	76.19
TB retreatment	10	23.81
Radiological presentation		
Right Lung	13	30.95
Left Lung	10	23.81
Bilaterally	19	45.24
Cavitation on chest X-ray		
Yes	22	52.38
No	20	47.62

Circulating immune complexes isolation

A serum sample (0.5 ml) from each patient was mixed with 0.5 ml borate buffer (0.1 M, pH 8.4) and 1 ml of 7% PEG in borate buffer, and incubated for 24 h at 4°C. The precipitate was washed twice with 3.5% PEG in borate buffer, centrifuged at 15,000 g for 20 min at 4°C, and resuspended in 0.5 ml of solution for dissociation [17].

Circulating immune complexes dissociation

The identification of *Mycobacterium tuberculosis* antigens was preceded by the dissociation of immune complexes. To expose the antigenic determinants, 2-mercaptoethanol was used to cut the sulfide bridges in the hinge regions of the immunoglobulins. CIC samples were diluted in dissociation buffer (Tris-HCl, pH 6.8; 5% 2-mercaptoethanol, 6% sodium dodecyl sulfate) and applied to nitrocellulose filters.

Research on the occurrence of *Mycobacterium tuberculosis* antigens in CIC

Antigens of *Mycobacterium tuberculosis* were identified by dot blot analysis on nitrocellulose filters. The mouse monoclonal antibody to *Mycobacterium tuberculosis* (Vector Laboratories, catalogue number VP-M660) was used as the first antibody. This antibody reacts with the most common forms of mycobacterial species associated with human disease, including *Mycobacterium tuberculosis* [18]. Alkaline phosphatase-conjugated polyclonal rabbit anti-mouse immunoglobulin

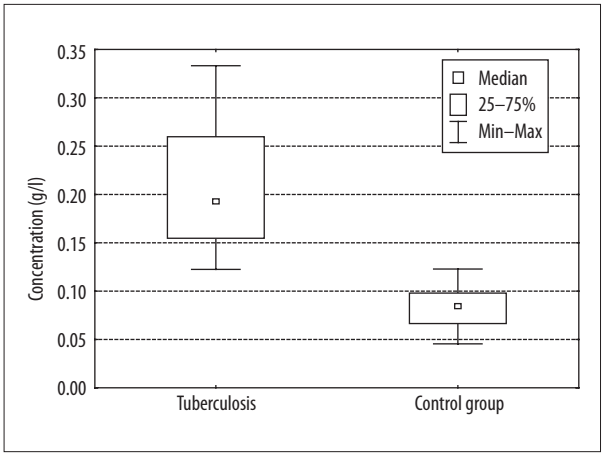

Figure 1. The concentration of CIC in the serum of patients with tuberculosis (n=42) and persons considered healthy (n=45).

(DakoCytomationDanmark A/S, catalogue number D0314) was used as the second antibody. A BCIP/NBT Alkaline Phosphatase Substrate Kit IV was used to visualize the results (Vector Laboratories, catalogue number SK-5400) [18, 19].

Statistical analysis

Data were sorted, initial calculations were performed, and figures were drawn using Office 2010 (Microsoft Corp., Seattle, WA). Data were analyzed with the non-parametric Wilcoxon-Mann-Whitney test and were assessed using the STATISTICA data analysis software system (version 10, StatSoft, Inc; www.statsoft.com). Selected results are significant from value $p<0.05$.

The study protocol was accepted by the Ethics Committee of Ludwik Rydygier, Medical University in Bydgoszcz, in June 2004 (KB 385/2004) and a written informed consent was obtained from each participant.

Results

Circulating immune complexes concentration in serum estimated with the PEG test

All patients with tuberculosis demonstrated a high serum concentration of CIC protein (Figure 1). The mean serum concentration of CIC protein was significantly higher in patients than in controls – 0.081 g/l in the control group and 0.211 g/l in the tuberculosis patients (Table 3).

Dot blot analysis

The immunochemical analysis of dissociated immune complexes from the serum of healthy persons did not show the

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Table 3. Table of sections descriptive statistics N=87.

Group	Average g/l	Weighted g/l	Standard deviation (SD) g/l	Q25 g/l	Median g/l	Q75 g/l
Tuberculosis	0.210950	42	0.066783	0.154700	0.192850	0.259700
Control group	0.081238	45	0.018160	0.066500	0.084500	0.098000
Total	0.143857	87	0.080902	0.082600	0.112700	0.191800

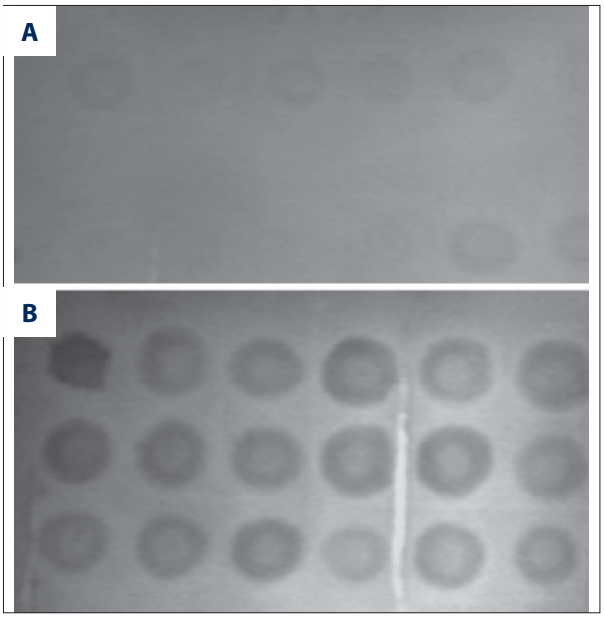

Figure 2. Analysis of dissociated immune complexes isolated from the serum of healthy persons (**A**) and patients with tuberculosis (**B**).

pattern of antigenic determinants typical of those exposed to *Mycobacterium* (Figure 2A). However, in the immune complexes isolated from the sera of infected persons, all samples showed positive reaction on the presence of *Mycobacterium tuberculosis* antigens (Figure 2B).

Discussion

The diagnosis of tuberculosis is a constant challenge. The diagnostic nature of the disease changes constantly and can take a treacherous and uncharacteristic course. The diagnosis of tuberculosis is based on microbiological methods augmented by genetic and molecular methods. The culture of *Mycobacteria* is a reliable diagnostic method, although it is time consuming [3,20]. There is a great need to develop a simple, cheap, and reliable diagnostic method. The value of serologic tests supporting the diagnosis of tuberculosis is limited by the high proportion of both false-positive and false-negative results [6,13].

The usefulness of serologic tests using specific antituberculotic antibodies in the diagnosis of tuberculosis has been assessed, but has produced divergent results [6,13].

Mycobacterium antigens stimulate the immune system towards cell-specific and humoral responses in the early stage of developing tuberculosis. This process is connected with the exfoliation of the surface antigens of the bacteria. The presence of exfoliated antigens can stimulate the immune system towards the less effective humoral response, which gives the bacteria a better chance of surviving [1,3,7,10,14]. At present, there is no effective serologic method to identify *Mycobacterium tuberculosis* at the early stage of the disease [1–3,6], although the exfoliated *Mycobacterium* antigens may be present in CIC in the early phases of the disease [8].

We found a characteristically high concentration of CIC in the serum of patients with the disease compared with the healthy controls (Table 3 and Figure 1). The immunochemical analysis of dissociated immune complexes using the dot blot method demonstrated a positive reaction on presence of *Mycobacterium tuberculosis* antigens in the group of all patients with tuberculosis. Until now, no studies have used this method to analyze CIC composition in the serum of patients with tuberculosis.

We used CIC dissociating buffer containing 2-mercaptoethanol. This compound has the ability to cut sulfide bridges that integrate heavy and light chains of the immunoglobulins. There is no information in the available literature on the use of CIC dissociation buffer containing 2-mercaptoethanol. The presence of this compound causes an exposure of the blocked antigenic determinants by removing the immunoglobulin-related immune complexes. This process allows specification of the identity of the antigen bound by specific monoclonal antibodies.

This method is quick and simple, and should be considered when diagnosing tuberculosis.

The method described for obtaining immunochemical information about the presence of *Mycobacterium* antigens in CIC may be helpful to work-up of methods relevant to Tb diagnostics.

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Conclusions

The positive reaction on antigens typical of *Mycobacterium tuberculosis* was shown in circulating immune complexes, isolated from the serum of patients with tuberculosis. Our results suggest that the analysis of CIC may be a helpful test for patients with tuberculosis because of its quickness, simplicity of the idea, and limited invasiveness.

Competing interests

The authors declare that they have no competing interests.

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