Diagnostic significance of circulating immune complexes in patients with pulmonary tuberculosis

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Summary. A polyethylene glycol (PEG) precipitation method was used to examine sera of patients with active pulmonary tuberculosis (PT), leprosy and non-tuberculous pulmonary diseases and of healthy control subjects for immune complexes (ICs). *Mycobacterium tuberculosis* antigen 5 was detected in the ICs in 80% of patients with PT by the indirect (sandwich) enzyme-linked immunosorbent assay (ELISA). Detection of mycobacterial antigen in ICs has diagnostic potential as an adjunct in the laboratory diagnosis of PT, particularly when repeated bacteriological investigations for *M. tuberculosis* in clinical specimens are negative. Levels of ICs tend to decrease with the duration of anti-tuberculosis chemotherapy and their detection can also be used to assess the clinical response to therapy in patients with PT.

Introduction

Early diagnosis of patients with active pulmonary tuberculosis (PT) is essential for effective initiation of appropriate chemotherapy. Diagnosis of this disease depends upon the specific demonstration of *Mycobacterium tuberculosis* in clinical specimens. Bacteriological culture is both cumbersome and time consuming but detection of acid-fast bacilli in sputum smears is less sensitive.¹ For several years, enzyme-linked immunosorbent assays (ELISA) have been used to aid the diagnosis of tuberculosis.^{2–4} In these studies, *M. tuberculosis* antigen 5 has been used because this antigen is considered to be more specific than other mycobacterial antigens. Daniel and Anderson⁵ isolated this antigen from the unheated cell-free culture filtrate of *M. tuberculosis* strain H₃₇Ra by means of immunoabsorbent affinity chromatography and called it *M. tuberculosis* antigen 5 because it identified with this number in the nomenclature of mycobacterial antigens.⁶ By immuno-electrophoresis, this antigen gave a single precipitin arc with polyvalent rabbit antiserum to *M. tuberculosis*.⁷ Physicochemical studies have shown that antigen 5 is composed of amino acids rich in aspartic acid, suggesting that it is derived from the cytoplasm of *M. tuberculosis*. It does not contain muramic or diaminopimelic acids, which further suggests that antigen 5 is not derived from the cell wall of *M. tuberculosis*. Antigen 5 is stable at 70°C and, by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), it has been characterised as a single band of 35 Kda.⁵

The present study was undertaken (i) to isolate and characterise immune complexes (ICs) in sera of patients with PT, (ii) to evaluate the diagnostic relevance of detection of *M. tuberculosis* antigen 5 in the ICs by a sandwich ELISA and (iii) to correlate IC levels with clinical recovery during and after chemotherapy in patients with PT.

Materials and methods

Patients and serum samples

Sera from four groups of patients were examined. (A) Sera from 50 patients admitted to the Hospital for Tuberculosis and Chest Disease, Trivandrum, with a clinical diagnosis of PT made by the attending consultant physician and supported by radiological investigations. *M. tuberculosis* was demonstrated in sputum specimens from 15 patients. In the remaining 35 patients, repeated sputum examinations and cultures were negative for *M. tuberculosis* and atypical mycobacteria. At the time of completion of this study, all the patients had received continuous anti-tuberculosis chemotherapy for 1–6 months. Sera were collected also from 10 asymptomatic patients with PT who had received continuous therapy for 6–12 months. (B) Sera from 40 patients with non-tuberculous pulmonary diseases (bronchogenic carcinoma 16, chronic bronchitis 18, pneumococcal pneumonia 6) were collected. (C) Sera from 50 healthy voluntary blood donors attending the Blood Transfusion Services. (D) Sera from 10 patients with lepromatous and tuberculoid leprosy. None of the patients in clinical groups B, C and D showed either clinical or radiological evidence

of active PT. These patients were selected as controls for this study. Sera from tuberculous and control groups were coded and preserved at -70°C in sterile storage vials.

Antigen

M. tuberculosis antigen 5 was isolated from the unheated cell-free culture filtrates of *M. tuberculosis* strain H₃₇Ra by immuno-absorbent affinity chromatography⁵ with reference goat antiserum to *M. tuberculosis* antigen 5 (kindly supplied by Dr T. M. Daniel, Cleveland, OH, USA).

Antiserum to *M. tuberculosis* antigen 5

Adult male rabbits were used to raise the antiserum. A water-in-oil emulsion containing 1 ml (100 µg) of antigen 5 and 1 ml of *Bordetella pertussis* vaccine (Pasteur Institute, Kasuli, India) were mixed thoroughly in 1 ml of incomplete Freund's adjuvant. The material was injected intramuscularly and subcutaneously at multiple sites. The same procedure was repeated at 14, 21, 35 and 42 days after the primary immunisation. In Ouchterlony immunodiffusion plates, the antiserum demonstrated a single precipitin arc against both *M. tuberculosis* antigen 5 and a culture filtrate of *M. tuberculosis*. The antiserum was passed through a protein A-sepharose column⁸ and the immunoglobulin G (IgG) fraction was recovered. This IgG fraction was dialysed extensively and concentrated with an Amicon ultrafiltration unit (Amicon GmbH, Written, Germany). The protein content was estimated by Lowry's method⁹ and the IgG fraction was preserved at -70°C in sterile storage vials.

Precipitation of ICs

ICs in sera were precipitated with PEG 6000 (Sigma).¹⁰ Briefly, 0.8 ml of the serum sample was mixed with an equal amount of 0.2 M EDTA and centrifuged at 700 *g* for 20 min. To the supernate, 0.4 ml of PEG 12% w/v in 0.1 M sodium borate buffer, pH 8.4, was added and the mixture was incubated for 12 h at 4°C. ICs were collected by centrifugation at 5000 *g* for 30 min. The pellets were washed twice with PEG washing solution (PEG 2.5% w/v, 0.1 M NaCl, 0.025 M sodium borate, 0.08 M EDTA and 0.1 M Tris, pH 7.1) and resuspended in 0.15 M phosphate-buffered saline pH 7.4 (PBS). Usually, the PEG precipitate from 0.8 ml of serum was resuspended in 150 µl of PBS. The presence of IgG and complement (C3 component) in PEG precipitates were determined by the single radial immunodiffusion technique (Hoechst-Behring) with quantitation according to the protocol supplied by the manufacturer; the technique detected IgG and C3 at levels as low as 20 mg/100 g and 5 mg/100 g, respectively.

Detection of mycobacterial antibody in PEG precipitates

A non-competitive ELISA¹¹ was standardised in polyvinyl chloride microtitration ELISA plates (Dynatech Inc, Alexandria, VA, USA). The reaction temperature and assay volume were 21°C and 100 µl, respectively. The PEG precipitates of test sera were serially diluted (1 in 10–1 in 80) in 0.15 M PBS before use in the assay. Briefly, the microtitration plates were coated with *M. tuberculosis* antigen 5 (2 µg/well in carbonate buffer, pH 9.6 and the amount of antibody to antigen 5 in PEG precipitates was determined by the addition of anti-human IgG-alkaline phosphatase conjugate (Sigma) diluted 1 in 1000. P-Nitrophenyl phosphate 1 mg/ml in 1 M diethanolamine buffer, pH 9.8, was used as the substrate. The colour reaction was stopped after 30 min with 3 N NaOH. The absorbances in all samples were measured at 405 nm with an automated ELISA reader (Titertek-multiscan; Flow laboratories, USA). The assay on PEG precipitates of the tuberculous and the control groups was repeated on two different occasions and the inter-assay variation (coefficient of variation) was in the range 5–8%. The "cut-off" titre for the PEG precipitate was determined to be 80, on the basis that it gave the best discrimination between tuberculous patients and controls. At this "cut-off" titre, none of the PEG precipitates in the control group gave a positive colour reaction. Test sera with an ELISA value, at a dilution of 80, that was above the control mean + 2 SD, were considered to be positive.

Detection of *M. tuberculosis* antigen 5 in the PEG precipitates

The mycobacterial antigen was dissociated from the PEG precipitates with 4 M urea and the ELISA test was done on the dissociated samples. Inconsistent results were obtained with dissociated PEG precipitates and, as this could have been attributable to the presence of urea in the samples, the ELISA test was performed only on undissociated PEG precipitates for all patients. A sandwich method was standardised¹² in microtitration ELISA plates. The assay volume, reaction temperature and incubation time were 100 µl, 37°C and 3 h respectively. The plates were intermittently washed with 0.15 M PBS in Tween-20 (PBS-T) during the procedure. Briefly, the wells in microtitration plates were coated with rabbit IgG to *M. tuberculosis* antigen 5 (5 µg per well in carbonate buffer, pH 9.6) and the plates were quenched with bovine serum albumin 3% w/v. The PEG precipitates, diluted 1 in 80, from tuberculous and control groups were added in duplicate. The plates were incubated with anti-human IgG-alkaline phosphatase conjugate diluted 1 in 1000. The enzyme activity was assayed by the addition of nitrophenyl phosphate. The absorbance in every sample was recorded at 405 nm. The mean and SD of the absorbance at a dilution of 1 in

80 were calculated for the patients in the control group. A test was considered to be positive if the absorbance in a test sample was >2 SD above the mean value for the control. The assay was repeated on three occasions and with coded samples, to eliminate observer bias; the inter-assay variation (co-efficient of variation) was 6–8%.

Results

The table records the incidence of ICs detected (antigen 5, IgG antibody and C3 component of complement) in the PEG precipitates of sera from patients with PT, leprosy, non-tuberculous pulmonary diseases and healthy controls. The ICs were detected, with different incidences, in the two PT and the leprosy groups, but not in the non-tuberculous or healthy control groups. Fig. 1 shows the ELISA data for antigen 5 in the various clinical categories. In the 35 culture-negative patients with PT, absorbances (OD) in the ELISA for antigen 5 were in the range 0.28–0.69 (mean 0.57 SD 0.15). The mean absorbance in 15 culture-positive patients with PT was 0.592 SD 0.11. In patients with non-tuberculous pulmonary diseases and healthy controls, the mean absorbances in the ELISA tests were 0.249 SD 0.13 and 0.202 SD 0.09 respectively. A test was considered to be positive for the presence of antigen 5 if the absorbance was >0.509 (the mean absorbance + 2 SD in the non-tuberculous group). By this criterion, with sera from 25 out of 35 culture-negative patients with PT and all the 15 culture-positive patients with PT, results were positive for antigen 5 in the ICs. By the same criterion, sera from two of 10 patients with leprosy were considered to be positive for the presence of antigen 5 in the ICs, but none of the sera from 40 non-PT and 50 healthy control patients gave a positive result.

Fig. 2 shows the ELISA data for anti-antigen 5 IgG antibody in ICs from patients with PT and leprosy and from non-tuberculous subjects. Anti-mycobacterial antibodies were present in ICs from all the 15

Table. Incidence of ICs in PEG precipitates of sera from patients in various clinical categories

Patient groups (n)	Percentage of patients with detectable ICs
Culture-positive, with pulmonary tuberculosis (15)	100 (15)*
Culture-negative, with pulmonary tuberculosis (35)	71 (25)
Leprosy (10)	40 (4)
Non-tuberculous pulmonary diseases (40)	0
Health controls (50)	0

* Figures in parentheses are number of samples that gave positive results.

Fig. 1. Detection of *M. tuberculosis* antigen 5 by indirect ELISA in the ICs from patients in various clinical categories. Each dot represents the absorbance value recorded for an individual patient. The horizontal lines represent the mean value for each group.

Fig. 2. Detection of IgG antibody to *M. tuberculosis* antigen 5 by ELISA in ICs of tuberculous, leprosy and non-tuberculous patients. Each dot represents the absorbance value recorded for an individual patient. The horizontal line represents the mean value for each group.

culture-positive patients with PT and absorbances in the ELISA were in the range 0.79–0.97 (mean 0.86 SD 0.24). In sera from 35 culture-negative patients with PT, the absorbances in the ELISA varied between 0.52 and 0.93 (mean 0.81 SD 0.22). The mean absorbance in samples from patients with non-tuberculous pulmonary diseases was 0.31 SD 0.21. Therefore, a test was considered to be positive when the absorbance value was >0.72 (mean absorbance + 2 SD in non-tuberculous subjects). By this criterion, sera from 25 out of 35 culture-negative patients with PT had anti-mycobacterial antibody in the ICs. The absorbance values for four of the patients with leprosy were >0.72 and were considered to be positive for anti-mycobacterial antibody in their ICs. Two of these had antigen 5 as a component of the ICs. Thus, both antigen 5 and anti-mycobacterial (antigen 5) antibody could be detected in ICs of 80% of patients with clinically diagnosed PT (including culture-positive and culture-negative cases).

For both types of ELISA test, the differences in the mean values from culture-positive and culture-negative patients were not statistically significant ($p > 0.05$). PEG precipitates from patients with non-tuberculous pulmonary diseases and healthy controls did not contain detectable amounts of either antigen 5 or the anti-mycobacterial antibody. PEG precipitates in 10 patients with PT who had received continuous

chemotherapy for more than 6 months showed neither antigen 5 nor IgG antibody in their ICs, whereas in patients who had received chemotherapy for less than 6 months, both factors were present in the ICs.

Discussion

The presence of ICs in patients with active pulmonary tuberculosis¹³⁻¹⁶ and their diagnostic significances^{12,17} have been emphasised in published reports. Bhattacharaya *et al.*¹⁷ have reported an ELISA for the determination of ICs in tuberculous patients. They have stated that the antibody present in ICs was predominantly IgG, and was specific for *M. tuberculosis* antigen. In their study, although an immuno-dot assay was used to detect antigen in ICs, information about defined antigenic components of *M. tuberculosis* in the ICs was not presented. Moreover, only a small number of tuberculous subjects was studied and patients with non-tuberculous pulmonary diseases were not included among the control group. Mehta and Khuller¹² have developed an ELISA test to detect phosphatidylinositolmannoside, a glycolipid mycobacterial antigen in ICs of patients with PT. Their assay had a sensitivity of 88% and 66%, respectively, in sputum-smear-positive and smear-negative patients with PT. However, in that study, patients with non-tuberculous pulmonary diseases were not included. Because the specificity of ELISA should be evaluated in non-tuberculous subjects, patients with non-tuberculous pulmonary diseases were selected as controls in the present study.

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We thank the Director, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum-695 011, for his kind permission to publish this article. We are indebted to Dr T.M. Daniel, Department of Medicine, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA, for the kind supply of reference *M. tuberculosis* antigen 5.