ORIGINAL ARTICLE

Isolation and Evaluation of Diagnostic Value of Two Major Secreted Proteins of *Mycobacterium Tuberculosis*

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ABSTRACT

Two secreted antigens of *Mycobacterium tuberculosis*, namely the antigen 85 complex (30/31) and 38kDa antigens, were purified from the whole culture filtrate by using two dimensional preparative electrophoresis and anion exchange chromatography, respectively. Individual components of the antigen 85 complex namely, antigen 85A, 85B and 85C, were separated using hydrophobic interaction chromatography. The humoral antibody activity to these antigens in sputum positive cases of active pulmonary tuberculosis and normal healthy volunteers was determined by enzyme linked immunosorbent assay (ELISA) and immunoblot. Recombinant 38kDa and antigen 6 were used as reference antigens for the assay. None of the healthy volunteers reacted with the 38kDa antigen, while 52% of the TB sera reacted with it. Of the three components of the antigen 85 complex, 85B gave the highest positivity of 40 per cent. The results of combination of 38kDa with antigen 6 offered better results with 76% positivity.

Key words : *Antigen 85 complex, ELISA, Immunoblot, M. tuberculosis; 38kDa, Preparative electrophoresis, Hydrophobic interaction chromatography.*

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INTRODUCTION

Tuberculosis is one of the most common infectious diseases in the world. The global incidence of tuberculosis was estimated to be 7.3 million cases approximately in 1995, causing three million deaths per year, more than that from any other single infectious disease[1]. Since effective chemotherapy is available for treatment, rapid and early diagnostic methods are the most essential tools to control the spread of the disease. A definite diagnosis of pulmonary tuberculosis can be made by the demonstration of the tubercle bacillus in clinical material by microscopy or by culture techniques. A direct smear is easy to perform, but is found to be relatively insensitive. Since *M. tuberculosis* is a slow-growing organism, conventional culture techniques are time consuming and expensive. Attempts have been made to improve the sensitivity and speed of the detection of tubercle bacilli, or components thereof, by techniques like radiometric determination of bacterial growth, gas chromatography/mass spectroscopy and DNA hybridization. All these have met with problems of either specificity or cost.

Despite an enormous amount of research done so far, there are no reliable serodiagnostic

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tests available for pulmonary or extrapulmonary tuberculosis, which have got routine clinical application. Therefore, a need exists for development of specific, sensitive, simple to perform, cost effective, rapid diagnostic tests. Earlier attempts with crude extract antigens such as PPD, whole culture filtrate (CF) sonicate etc, in ELISA had the limitation of lack of sensitivity and/or specificity. A major goal in immunologic studies of mycobacterial infections is identification of species-specific antigens and determination of occurrence and significance of corresponding immune responses.

The aim of the present study was to purify the individual antigens such as the 38 kDa antigen which is species specific and antigen (Ag) 85 complex (30/31kDa) which is abundantly secreted into whole CF of *M. tuberculosis* H37Rv, and to develop a simple and sensitive enzyme linked immunosorbent assay (ELISA) test for measurement of antibody levels in sera of patients with pulmonary tuberculosis. We also attempted to evaluate the most favourable antigen combination, using two polar categories of tuberculous patients and healthy control subjects.

MATERIALS AND METHODS

Study Population

Sera were obtained from the two polar categories (1) Sputum smear and culture positive cases of active pulmonary tuberculosis, pre-treatment (TB) (N=25); and (2) Normal healthy volunteers (NHS) (N=25). Sera from patients and normal healthy volunteers for this study were included from a sera bank collected for a larger study. The subjects had been interrogated for the symptoms of tuberculosis, duration of symptoms if any, history of contact with cases and history of treatment, and checked for the BCG scar. The other investigations done were tuberculin tests, chest x-ray read by two independent investigators, and two sputum smear and culture examinations. Sera were aliquoted and stored at -70° C till the time of use.

Antigens

Preparation of H37Rv Culture Filtrate Antigen

M. tuberculosis H37Rv was grown in Sauton's liquid medium for six weeks as a surface pellicle. Bacilli were removed by centrifugation and the culture supernatant was filtered through Seitz filter. The proteins in the culture filtrate were precipitated with 90% ammonium sulphate saturation. The final precipitate was dissolved and dialyzed against phosphate buffered saline (PBS), 0.1M, pH 7.2, extensively. The H37Rv CF antigen was aliquoted and stored at - 70 ºC in the presence of sodium azide and the protease inhibitor phenylmethyl sulfonyl fluoride (PMSF).

Purification of 38kDa Antigen

For the purpose of isolation and purification of 38kDa from *M. tuberculosis* H37Rv CF, two-dimensional preparative electrophoresis was used. The CF proteins were separated into 20 fractions based on their iso-electric point (Rotophor, BioRad, USA). The fractions rich in 38kDa were pooled together and separated on preparative SDS-PAGE (Prepcell, BioRad, USA). Every 10th fraction eluted out of the gel was analyzed on analytical SDS-PAGE, to select the region where 38kDa eluted.

Purification of Ag 85 Complex

Culture filtrate (CF) was separated into multiple fractions by passing through a pre-packed anion exchange column of QAE sepharuse. Tris-HC1 0.05M, pH 8.1-buffer was used as the starting buffer and 0.05 M Tris-HC1, pH 8.1 with 1.0M sodium chloride was used as the elution buffer. The run was carried in the high performance liquid chromatography (HPLC) system (Millenium v2.00, Waters, USA). The eluted fractions were analyzed in SDS-PAGE and the Ag 85 complex containing fractions was pooled and further purification of the A, B and C components from the complex was done by passing it through the hydrophobic interaction chromatography (HIC) column, namely the phenyl sepharose HP colomn. Three different buffers used during the run were as follows: *Buffer* A consisting of 0.01 M NaH_2ISO_4,

pH 6.8; Buffer B was 0.01M tris glycine pH 8 and *Buffer* C, 0.01 M tris glycine with 50% ethylene glycol, pH 8.9. The run conditions were such that the column was subjected to an isocratic flow of *Buffer* A for 10 min (flow rate lml/min) followed by the injection of the sample at a rate of 0.5 ml/min. Then an isocratic flow of *Buffer* B for 10 min at a rate of 1 ml/min was used for washing the unbound material. A linear gradient with *Buffer* C (from 0-100%) for 30 min with a flow of 1 ml/min resulted in the elution of individual components.

Other Antigens

Recombinant 38kDa (r38kDa) antigen of *M. tuberculosis* (Batch No. MTB 38–2) was obtained from the WHO Recombinant Antigen Bank. Ag6 and recombinant 85C were kind gifts from Prof. Thomas Daniel, Cleveland, USA and Prof. Patrick Brennan, Fort Collins, USA, respectively.

Analytical SDS-PAGE

The CF and fractions were analyzed by SDS-PAGE. The run was performed in slab gels by the method of Laemmli[2]. A 12.5% acrylamide-bis-acrylamide gel with 0.4% SDS in 0.375M Tris HC1, pH 8.8 was used to separate the antigens. The stacking gel was 4% acrylamide-bis-acrylamide with 0.4% SDS in 0.125M Tris HC1, pH 6.8. The gel thickness was 1.5 mm. The samples (usually 50 µg of Ag/cm length of the gel) were applied under reducing conditions by boiling the samples in sample buffer for five minutes at 96 °C. Gels were run in a vertical slab gel apparatus (Bio-Rad, USA).

After electrophoresis, the gels were stained with 0.2% Coomassie Brilliant Blue stain for one hour at room temperature with shaking and destained with successive changes of destainer consisting of 45% methanol and 10% acetic acid till the background became clear.

Immunoblotting

Resolved antigens were electrophoretically transferred to nitro-cellulose paper (NCP) (0.45 µg pore size) by the method of Towbin *et a1*[3], using a transblot apparatus (Bio-Rad Laboratories, California). The gels were soaked in transfer buffer (0.025M Tris, pH 8.3 containing 0.192M glycine and 20% methanol) for 30 min and transfer was carried out at 0.65 A for one hour. Reactive sites of the NCP were blocked by incubating with 5% skimmed milk powder in PBS with 0.3% (PBST). After washing, the strips were incubated overnight at 4 ºC with 1/100 dilution of tuberculous (TB) or normal sera. Antibodies were detected with peroxidase conjugated goat anti human immunoglobulins in a dilution of 1:4000 (Jackson Laboratories, USA). After repeated washes, the strips were developed using diaminobenzidine (DAB) as the substrate.

Enzyme–Linked Immunosorbent Assay (ELISA)

ELISA was carried out to estimate the IgG antibody titre against the 38kDa antigen and individual components of the Ag 85 complex of *M. tuberculosis* H37Rv in sera from patients and normal controls. Polystyrene ELISA plates (Nunc Maxisorp, flat bottom) were coated with 100 µl of purified 38kDa antigen or Ag 85A, B, C, in a concentration of 1 µg/ml in carbonate buffer (pH 9.6). The plates were incubated with the antigen overnight at 4 °C.

The plates were washed four times with PBS, containing, 0.1% Tween20 (PBST) by the ELISA washer (Organon Teknika, Austria). The non-specific sites in the wells were blocked with 1% bovine serum albumin (BSA) for one hour at 37 °C. After four washes with PBST, the plates were incubated with 1:l00 dilution of the sera from patients and normals. The plates were washed after one hour incubation at 37 ºC and incubated with anti human IgG-peroxidase (Bangalore Genei, India) conjugate at a dilution of 1:l000 in PBST containing 1% BSA. At the end of one hour of incubation at 37 ºC and washing colour was developed by the addition of 100 µ1 of the substrate, O-phenylene diamine (OPD) (Sigma Chemical Company, USA) to each well. After arresting the reaction with 50 µl of 8N H_2SV_4, the optical density reading was taken in the Spectromax ELISA (Spectromax 250, Molecular devices, UK.) reader at 490nm wavelength.

RESULTS

The 38kDa antigen was purified to homogeneity using 2-D preparative electrophoresis and individual components of the Ag 85 complex were separated using HIC, from *M. tuberculosis* H37Rv CF SDS-PAGE picture of the separated components, along with other reference antigens is shown in figure 1.

Sera from 20 patients with active pulmonary tuberculosis and 20 normal subjects were tested for qualitative analysis by immunoblot. The pattern developed is shown in figure 2. Sixteen out of 20 TB sera recognized the 30/31kDa, while 5/20 control sera also recognized the doublet. Recognition of 38kDa was shown by 15/20 TB sera. Combination of the result did not increase the positivity among the TB cases.

The titres of IgG antibodies in the above sera (TB=25; Control=25) were determined for their reactivity to the individual components of the Ag 85 complex and to the 38kDa antigen. In addition to the antigens purified in our laboratory, refercnce antigens from other sources were also included, such as r38kDa, rAg85C and Ag6 (30kDa). At a serum dilution of 1:100, the O.Ds were compared and mean ± 2 SD of the control O.Ds was considered as the cut-off value. Using this cut-off, the number of antibody positives and negatives with these antigens was calculated and is presented in table 1.

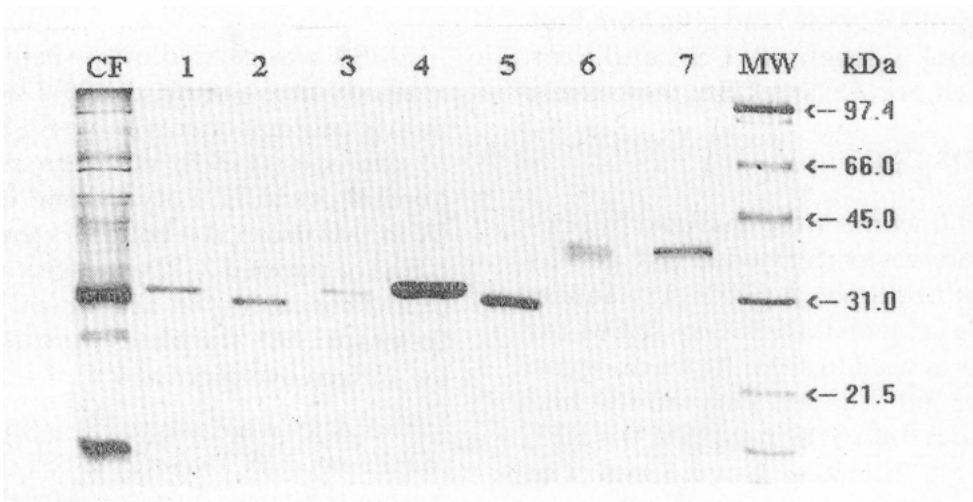

Figure 1. Coomassie blue stained ge1 showing the pattern of the purified antigens.
Lane : 1. Ag85A, 2. 85B, 3. 85C, 4. r85C, 5. Ag6, 6. 38kDa, 7.r38kDa. MW : Standard molecular weight, CF : Culture filtrate.

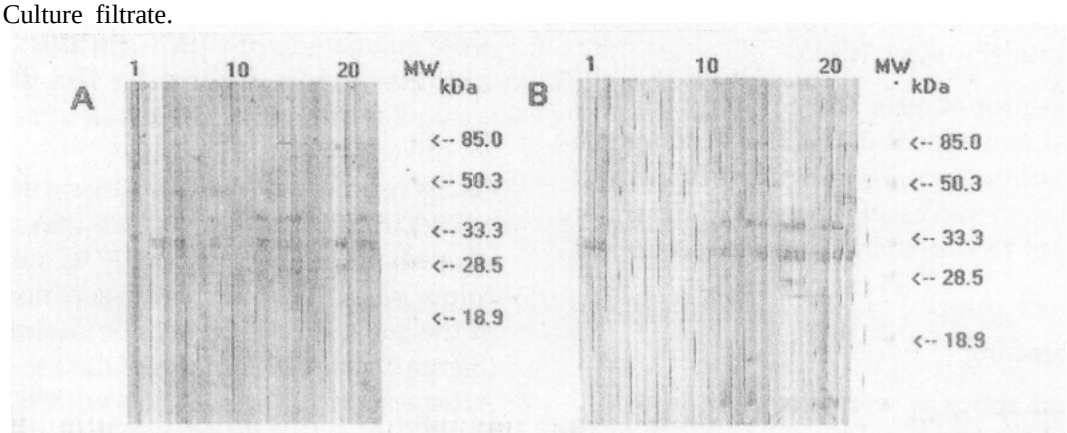

Figure 2. Immunoblot of culture filtrate antigen with normal and TB sera. Strips 1 to 10 and 11 to 20 of blots A and B correspond to the reactivity pattern of the control subjects and TB patients to the resolved CF antigens on the nitrocellulose membrane, respectively, MW : Standard molecular weight.

Table 1. *Positivity of ELISA with purified antigens*

Antigen	Positivity (%) (TB)	Negativity (%) (INHS)
H37RvCF	52	80
85A	28	95
85B	40	95
85C	12	95
r85C	32	95
38	52	100
r38	60	100
Ag6	60	100

The 38kDa (purified at TRC) reacted positively with 52% of the TB sera and 0% of the normal control sera. Recombinant 38kDa and Ag6, recognized 60% and 0% of the TB and normal control sera, respectively. Of the three components of Ag 85 complex, the 85 B detected more number of positives among the patients (40%). Lesser positivity was observed with the antigen 85A (28%), 85C (12%) and r85C (32%).

Different combinations of results obtained by ELISA with the 30kDa (85B), Ag6, 38kDa and r38kDa antigens were analyzed in order to increase the sensitivity of the assay while keeping the specificity high. The results of the combination are given in table 2. The combination of either the native 38kDa or recombinant 38kDa, with Ag6 was offered the maximum positivity of 76% among TB and negativity of 100% among the normal controls.

Table 2. *Positivity of ELlSA by combination of antigens*

Antigen	Positivity (%) (TB)	Negativity (%) (INHS)
85B & 38	64	95
Ag6 & 38	76	100
85B & r38	64	95
Ag6 & r38	76	100
38 & r38	76	100

DISCUSSION

Diagnostic tests, (serologic and delayed type skin tests) for tuberculosis are often complicated because of cross-reactive responses induced by subclinical infections with mycobacteria other than *M. tuberculosis.* It is of utmost importance that antigens used in ELISA are highly purified and species specific.

Daniel and Anderson[4] were the first to report the species specific antigen 5 of *M. tuberculosis* which was synonymous with 38kDa antigen[5,6]. This could be purified using affinity chromatography[4,7,8], as well as other procedures, including gene cloning[9,10]. In the present study, two-dimensional preparative electrophoresis, based on the combination of isoelectric focusing (IEF) and preparatory electrophoresis allowed the complete purification of the 38kDa antigen from whole bacterial culture filtrate, using an entirely different approach, as compared to the previous isolations.

The other antigen used in this study was Ag 85 complex. This protein complex consists of at least three distinct, but related proteins designated as 85A, 85B and 85C[11]. It is an early antigen and is a major constituent of *M. tuberculosis* CF[12,13]. Thus, it can prove to be useful in early diagnosis. Ag 85 complex has also been found to possess species specific epitopes[14,15]. In the studies carried out so far, antigen 85 complex was obtained from CF by DEAE chromatography. Separations of individual components based on analytical IEF have also been reported[16]. In the present study, the Ag 85 complex was purified from *M. tuberculosis* CF by preparatory HPLC using DEAE column, separated into A, B and C components by HIC and evaluated by ELISA.

The 38kDa isolated by different methods has been used in diagnostic tests with varying outcome. As antigen 5, it has shown high specificity in all the studies conducted in different parts of the world (88%–98%), while sensitivity has varied from 49%-89%[17]. The MAb column purified 38kDa[18,19] and recombinant 38kDa (r38kDa)[20-24] widely vary in their sensitivity to detect smear-positive cases (51-89%).

Thus, it is obvious from the available literature that 38kDa is highly specific, while the sensitivity varies in different geographica areas. Also, the purificaion steps employed by various

laboratories might influence the folding of protein and thus the outcome of the assay. In our population, 38kDa purified in our laboratory has demonstrated 100% specificity, with 52% sensitivity, which are comparable with results obtained with r38kDa.

Of the three components of Ag 85 complex used in ELISA, the 85B detected more number of positives among the patients (40%). Lesser sensitivity was observed with the antigen 85A (28%), 85C(12%) and r85C(32%). Van Vooren *et al*[25] too using a semiquantitative western blot assay concluded that 85B is the most useful component of the antigen 85 complex for the diagnosis of active forms of tuberculosis.

The other forms of Ag 85 complex have been previously evaluated. Antigen 6 had a 94% sensitivity in patients treated for active bone and joint TB. The specificity was 100 per cent[26]. Sada and co-workers[27] obtained a sensitivity of 70% in patients and specificity of 100% in control subjects using a 30kDa native antigen of *M. tuberculosis.* Subsequently, 30kDa ELISA test was reduced to a simple dot assay on nitrocellulose paper strips but this resulted in loss of specificity[2S].

Recognition of 30 and 38kDa bands in immunoblot are also in line with the results of ELISA. Our observations of the immunoblot pattern matches those of Espitia and colleagues[9], who have reported that the 38kDa band reacted with antibodies of 80% of sera of patients with tuberculosis and with 15% of the healthy control sera. Van Vooren *et al*[16] have observed that the components reacted with 71% serum samples from tuberculous patients.

A combination of tests using several antigens has been recommended in view of the large individual variation in serological response[29,30]. Different combinations of results obtained by ELISA with the 30kDa (85B), Ag6,38kDa and r38kDa antigens were analyzed in order to increase the sensitivity of the assay, while keeping the specificity high. The combination of either the native 38kDa or r38kDa, with Ag6 offered the maximum favourable test characteristics - 76% positivity in TB and 100% negativity in normal controls.

In the present study, the two polar groups, namely confirmed cases and normal controls were evaluated. The number of samples used for ELISA was limited. However, as the purpose of the study is to compare seven antigenic preparations, the sample size could not be increased. Valid comparison among the antigens rather than the study groups could be obtained with these numbers. In view of the high specificity and marked sensitivity, the combination of 30 and 38kDa antigen is worth evaluating in smear negative pulmonary tuberculosis and other forms of tuberculosis where diagnosis is difficult. The 30kDa might prove especially useful in the early diagnosis, as it is one of the earliest to be secreted, during mycobacterial growth.

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