DIAGNOSTIC POTENTIAL OF FRACTIONATED *MYCOBACTERIUM TUBERCULOSIS* H₃₇R₆ EXCRETORY-SECRETORY (EST-DE₁) ANTIGEN IN PULMONARY TUBERCULOSIS

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ABSTRACT

Tuberculosis is emerging as a major public health problem in developing and developed world. Early and precise diagnosis is of prime importance in successful control of infection. Indirect ELISA with penicillinase as marker was developed using purified *M. tuberculosis* excretory-secretory (EST-DE₁) antigen for detecting IgG antibodies in pulmonary tuberculosis. The assay System gave a overall sensitivity of 82% for both smear positive and smear negative pulmonary tuberculosis cases with a specificity of 84%. The positive and negative predictive values were 75% and 88% respectively. Further studies with EST-DE₁ antigen revealed that, it contains two of the active antigen fractions of Mtb EST antigen i.e. Mtb EST-4 (56-68 KDa) and Mtb EST-6 (37-45 KDa), as demonstrated by inhibition ELISA. Reactivity with monoclonal antibodies HGT 3a showed the presence of 38 KDa molecule in EST-DE₁ antigen.

KEY WORDS: ELISA, EST-DE₁ antigen, Penicillinase, HGT 3a.

INTRODUCTION

Tuberculosis is long known to be a major cause of morbidity and mortality throughout the world, with one third (1700 million) of the worlds population infected and 3 million deaths annually (1). In India alone, there are 12-13 million individuals suffering from tuberculosis with 2.5 million new cases added up annually and the mortality rate exceeding 0.5 million per year (2). Operational difficulties in control programmes, coexistence with HIV infection, and emergence of MDR-TB have contributed to the increased incidence of the disease (1). WHO in April 1993 declared tuberculosis as "a global emergency" as the disease is out of control in many parts of the world (3).

The incidence of pulmonary tuberculosis is more than extra pulmonary form of the disease (4). while the sputum smear microscopy lacks the sensitivity, the culture method is time consuming with its utility

restricted to open cases of tuberculosis. These limitations make it imperative that more sensitive and specific serologic tests be developed for tuberculosis. Since the first description of Enzyme Linked Immunosorbent Assay (ELISA) for tuberculosis by Nassau et al (5), the assay has been widely tried in serodiagnosis, using various mycobacterial preparations ranging from crude extracts to purified antigens.

With the ELISA systems developed for diagnosis of tuberculosis, the sensitivity of 56% to 94% and specificity of 86% to 100% have been reported depending on the type of antigens used by different laboratories (6,7). Over the years attempts have been made to isolate species specific antigens but most of the purified antigens are found to share epitopes that are common to many bacteria (8).

In the recent years, the proteins that are secreted actively by mycobacteria in the in vitro cultures have drawn special attention (9). These proteins are equivalent to those released *in vivo*, and are found to be potential targets for specific protective immunity at the early stage of infection (10,11). Moreover, the secreted proteins are highly immunodominant, and measurement of humoral responses directed against them gives an indication of active infection (12,13,14).

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In earlier studies from our laboratory we have reported the potential of Mtb ES antigen in the diagnosis of pulmonary and extrapulmonary tuberculosis (15,16). In the present communication we have evaluated the diagnostic potential of fractionated EST-DE₁ antigen in pulmonary tuberculosis and recognized the antigenic molecules in it by Inhibition ELISA and monoclonal antibody studies.

MATERIALS AND METHODS

Human sera

Human sera belonging to different groups viz. pulmonary tuberculosis, disease control and healthy control were collected from the patients attending Kasturba Hospital, Sevagram and from the healthy volunteers among the staff of the Department. Of the 45 pulmonary tuberculosis cases, 35 were positive for AFB smear sample collected before initiation of anti-tuberculosis therapy and remaining 10 cases were negative for AFB smear, but were diagnosed clinically, radiologically and on positive therapeutic response to antitubercular therapy. In the disease control groups 38 sera samples were from the patients with the diseases simulating pulmonary tuberculosis which include, bronchial asthma (6), bronchiectasis (3), chronic obstructive airway disease (8), pyrexia of unknown origin (9), pleural effusion (6), lung abscess (3) and leprosy (3). Another 35 sera samples collected from healthy individuals served as healthy control group.

Mycobacterium tuberculosis H₃₇ Ra excretory-secretory (Mtb ES) Antigen

Excretory-secretory (Mtb ES) antigen was isolated from the 7 days old *in vitro* cultures of the *Mycobacterium tuberculosis* H₃₇ Ra strain in synthetic Sauton medium as described by Lodam et al (15).

TCA precipitation and DEAE fractionation

The trichloroacetic acid (TCA) soluble fraction of Mtb ES antigen (Mtb EST) was prepared by adding TCA to it to a final concentration of 6% as described by Lodam et al (15). The Mtb EST antigen was further fractionated on DEAE cellulose (diethylaminoethyl, Whatman DE 52) by anion exchange column chromatography following the procedure described by Cheirmaraj et al. (16). Four mg of Mtb EST antigen

was applied onto DEAE cellulose column (1.2 x 20cm) and eluted at a flow rate of 10 ml/hr with 0.05M sodium phosphate buffer (pH 7.2). After collecting the unbound fraction (EST-DE₁), the bound proteins were further eluted using an ionic gradient with 1M sodium chloride. The eluted fractions were pooled, concentrated and labelled as EST-DE₂ (Fig. 1).

Fig. 1: Fractionation of Mtb EST antigen into two components (EST-DE₁ and EST-DE₂) by anion exchange chromatography on a DEAE cellulose column (for details see text).

Mtb EST-4 and Mtb EST-6 antigens

The SDS-PAGE fractions of Mtb EST antigen i.e. Mtb EST-4 and Mtb EST-6 antigens were prepared as described earlier (15).

Anti Mtb EST antigen antibodies

Antibodies specific to Mtb EST antigen from hyperimmune goat serum were isolated by affinity chromatography as described earlier (15).

Monoclonal antibody HGT 3a

Monoclonal antibody HGT3a specific to *Mycobacterium tuberculosis* 38 KDa antigen was a kind gift from Dr. G.V. Kadival, Radiation Medicine Centre, Parel, Mumbai.

Inhibition ELISA

To demonstrate the presence of antigenic molecules in EST-DE₁ antigen an Inhibition ELISA was carried out using affinity purified anti Mtb EST antigen antibodies as described by Malhotra et al (17) with few modifications. In brief, anti Mtb EST antigen antibodies were conjugated with enzyme penicillinase (Sigma Chemical Co., USA) following single step method of Avrameas (18) using glutaraldehyde. The substrate consisted of 150 mg soluble starch (Sigma Chemical Co., USA) in 27.5 ml of 0.25M sodium phosphate buffer (SPB) pH 7.2, and added with 10.64 mg penicillin 'V' and 100 µl of 0.8M iodine in 3.2M potassium iodide. Wells in the polyvinylchloride (PVC) microtitre plates (Dynatech, USA) were sensitized with EST-DE₁ and EST-DE₂ antigens (5 µg/50 µl/well) in 0.06M carbonate buffer (pH 9.6) and further blocked with 1% BSA (100 µl/well) in the same buffer at 37°C for 1 hr. The wells were washed with 0.01M phosphate buffered saline containing 0.05% Tween 20 (PBS/T). The Mtb EST-4 and Mtb EST-6 antigen fractions (100 µg/ml and further 10 fold serial dilutions) were preincubated separately with the optimally diluted anti-Mtb EST antigen antibody penicillinase conjugate in PBS/T at 37°C for 15 minutes and then added into the wells (50 µl/well) for further incubation. Followed by final washing, the wells were incubated with starch-iodine-penicillin 'V' substrate. The wells in which the decolourization of blue coloured substrate was delayed by at least 5 min as compared to the control wells (without Mtb EST-4 and Mtb EST-6 antigen fractions) were considered to be positive. The highest antigen dilution showing positive reaction was taken as the optimal inhibitory antigen concentration.

Indirect ELISA

Indirect ELISA for the detection of tuberculous IgG antibody was carried out as described earlier, using cellulose acetate membrane (CAM) fixed sticks as the solid support. Five µl of optimally diluted antigen (Mtb EST, or EST-DE₁ or EST-DE₂ (20 µg/ml) in 0.05M SPB, pH 7.2) was coated onto the CAM sticks and air dried. The unbound sites were saturated with 3% gelatin in the same buffer by incubating at 37°C for 2 hr. After washing with PBS/T 5 times, the sticks were incubated with 0.5 ml of optimally diluted serum samples in PBS/T at 37°C for 1 hr. After incubation the sticks were washed 5 times with PBS/T and incubated further for 30 min at 37°C with 0.5

ml of anti human IgG penicillinase conjugate. After final washing the sticks were incubated with freshly prepared starch-iodine-penicillin-V substrate at 37°C. Complete decolourization or decolourization with slight tinge of blue colour substrate denoted a positive reaction.

Reactivity with HGT 3a monoclonal antibody

The seroreactivity of EST-DE₁ antigen fraction with Moab HGT 3a was carried out by indirect ELISA as described by Reddy et al (19). Wells in the PVC microtitre plates were sensitized with EST-DE₁ antigen (10 µg/50 µl/well and serial 10 fold dilutions) in 0.06M carbonate buffer (pH 9.6) and further blocked with 1% BSA (100 µl/well) in the same buffer at 37°C for 1 hr. The wells were washed with PBS/T and incubated with optimally diluted Moab HGT 3a (50 µl/well) in PBS/T containing 0.05% BSA at 37°C for 1 hr. After incubation the wells were washed with PBS/T and incubated further with anti-mouse IgM

Fig. 2: Evaluation of Mtb EST, EST-DE₁ and EST-DE₂ antigens by indirect ELISA in detecting tuberculous IgG antibody in pulmonary tuberculosis, disease control and healthy control sera (10 sera samples in each group).

peroxidase conjugate (Cappel, USA) in PBS/T containing 0.05% BSA at 37°C for 1 hr. Following the final washing, the wells were incubated with O-phenylenediamine. The colour development was arrested by adding 2N HCl and the wells were then read in ELISA reader (Dynatech, USA) at 490 nm.

RESULTS

The elution profile of Mtb EST antigen on DEAE column chromatography is shown in Fig. 1. Comparative diagnostic efficacy of the 2 DEAE column chro-

matography fractions viz., EST-DE₁ and EST-DE₂ in comparison with whole Mtb EST antigen is shown in Fig. 2.

The analysis of reactivity of EST-DE₁ and EST-DE₂ antigens by inhibition ELISA showed that the Mfb EST-4 and Mfb EST-6 antigens inhibited the binding of EST-DE₁ antigen with anti Mtb EST antigen antibody at a concentration of 500 ng.

The distribution of the tuberculosis IgG antibody titres in different groups of human sera against

Fig. 3: Scattergram showing the tuberculous IgG antibody titres in different groups viz., (A) smear positive pulmonary tuberculosis, (B) smear negative pulmonary tuberculosis, (C) disease control and (D) healthy control by indirect ELISA using EST-DE₁ antigen. The geometric mean titre of each group (—) & the threshold antibody titre of 1:600 for positivity (---) are also shown.

EST-DE₁ antigen is shown in Fig. 3. At a serum dilution of 1:600, 89% of smear positive and 60% smear negative pulmonary tuberculosis sera and 16% of controls sera showed positive reaction for tuberculous IgG antibody. The geometric mean titres (GMT) of tuberculous IgG antibodies in smear positive (894) and smear negative (424) pulmonary tuberculosis cases were significantly higher than the GMT of antibodies in control groups (Disease control (231) and healthy control (182) respectively). EST-DE₁ antigen fraction showed reactivity upto a protein concentration of 100 ng/well with HGT 3a Moab in indirect ELISA.

DISCUSSION

The humoral responses elicited by mycobacterial components have been of great significance in the immunodiagnosis of tuberculosis. In the recent years, proteins that are secreted by mycobacteria have gained importance due to the high host immune response against these proteins and further they are secreted by live bacilli thus indicator of active infection. There have been reports on the usefulness of culture filtrate antigens in the diagnosis of tuberculosis by ELISA method (5,6,20,21). In the earlier studies from our laboratory we have reported the potential of *Mycobacterium tuberculosis* H₃₇R_a excretory secretory (Mtb ES) antigen in the diagnosis of tuberculosis (15; 22, 23). In the present study, ELISA was developed to detect circulating antibodies against purified EST-DE₁ antigen of *Mycobacterium tuberculosis* H₃₇R_a in patients suffering from pulmonary tuberculosis and related diseases. The study was further extended to determine the active antigenic molecules present in the EST-DE₁ preparation.

In the earlier studies, we have reported that 2 of the Mtb EST antigen fractions viz., Mtb EST-4 (56-68 KDa) and Mtb EST-6 (37-45 KDa) have diagnostic potential. Further Mtb EST-6 antigen fraction was shown to contain two antigenic molecules of molecular mass 41 and 38 KDa reactive with pulmonary tuberculosis sera, as demonstrated by immunoblotting studies (15). As the yield of these proteins on SDS-PAGE fractionation was too low and the procedure being cumbersome, we attempted fractionation of Mtb EST antigen by anion exchange chromatography so that the proteins in ample amount could be obtained and thus could be helpful as routine screening reagents.

The unbound fraction EST-DE₁ was superior compared to bound protein fraction EST-DE₂ in detecting tuberculous IgG antibodies. Using EST-DE₁ antigen IgG antibodies could be detected in 80% of pulmonary tuberculosis and 10% of control cases, whereas 60% of pulmonary tuberculosis and 20% control cases showed positive reaction with EST-DE₂ antigen when limited number of sera (10 samples from each group viz., pulmonary tuberculosis, disease control and healthy control) were analysed by indirect penicillinase ELISA. In fact none of the pulmonary tuberculosis cases that showed antibodies against EST-DE₂ were missed when EST-DE₁ was used in ELISA. When these 2 fractions were analysed for the presence of Mtb EST-4 and/or Mtb EST-6 antigens by inhibition ELISA, it was observed that both the immunoreactive components were present in EST-DE₁ antigen only. Both the Mtb EST-4 and Mtb EST-6 antigen fractions effectively inhibited the binding of anti Mtb EST antigen antibody conjugate to EST-DE₁ antigen at a protein concentration of 10 µg/ml. Hence, EST-DE₁ antigen was explored for its diagnostic utility to see whether the presence of both the active antigen fractions Mtb EST-4 and Mtb EST-6 antigens adds any advantage over a single antigen.

At a cut off serum dilution of 1:600 for positivity the indirect ELISA using EST-DE₁ antigen gave a sensitivity of 89% for smear positive and 60% for smear negative pulmonary tuberculosis cases (overall sensitivity 82%). While the positive predictive value of the assay was 75% the negative predictive value was 88%. At a lower dilution of serum (1:300), the specificity gets decreased to 49% while at higher dilution, of serum (1:1200) the sensitivity of assay decreases to 42%. Though the assay with EST-DE₁ antigen (sensitivity 82% and specificity 84%) is not superior to that of Mtb EST-4 (sensitivity 81% and specificity 86%) and Mtb EST-6 (sensitivity 91% and specificity 88%). The EST-DE₁ antigen can be easily isolated in required amounts for field studies. The antibody assay with EST-DE₁ antigen showed a better sensitivity and comparable specificity to the assays reported by Nassau et al (5) and Benjamin et al (24) using culture filtrate antigen (sensitivity 56% and specificity 84-98%) and to the assays using antigen 5 and LAM (sensitivity 70-72% and specificity 89-91%) by Benjamin et al (25).

Among the secretory proteins of mycobacteria a 38 KDa molecule has been shown to be the only potential diagnostic reagent with high antibody re-

sponse directed against it and having high specificity for tuberculosis at epitope level (14,26,27,28). Further EST-DE, showed the presence of 38 KDa molecule as demonstrated by reactivity with Moab HGT 3a. From this study it can be stated that, the diagnostically useful EST-DE, antigen can be obtained by simple anion exchange chromatography, which would be helpful as routine screening agent for large number of samples.

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