Diagnostic Significance of Humoral Immune Responses to Recombinant Antigens of *Mycobacterium tuberculosis* in Patients with Pleural Tuberculosis

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Mycobacterium tuberculosis bacilli are seldom demonstrated in tuberculous pleural effusion (TPE) by conventional bacteriological methods. In this study, an indirect enzyme-linked immunosorbent assay (ELISA) was developed to detect IgG against four mycobacterial recombinant antigens (ESAT-6, PlcA, HspX and Tb8.4) in 69 pleural fluids of patients with TPE and 71 patients with malignant pleural effusion. To increase the sensitivity of the assay, a multi-antigen cocktail containing all the above antigens were also used. IgG positivity in ELISA for PlcA,

HspX, Tb8.4, ESAT-6 antigens and multi-antigen complex were 49.3, 60.8, 49.3, 53.6 and 75.4% respectively. Each one of the above four antigens and their multi-antigen cocktail were highly specific in distinguishing tuberculous and malignant pleural effusion. This new generation immunoassay will serve as a useful marker for the diagnosis of pleural tuberculosis patients in whom *M. tuberculosis* bacilli were not demonstrated by bacteriological methods. J. Clin. Lab. Anal. 24:283–288, 2010. © 2010 Wiley-Liss, Inc.

Key words: *Mycobacterium tuberculosis*; tuberculous pleural effusion; malignant pleural effusion; recombinant mycobacterial antigens; ELISA

INTRODUCTION

Pleural effusion is one of the common clinical manifestations in patients with pleural and pulmonary tuberculosis. Pleural effusion occurs as a result of the release of mycobacterial antigens into the pleural space from sub-pleural tuberculous lesions. In these patients, delayed hypersensitivity reactions mediated by CD4⁺ lymphocytes may also contribute to the development of pleural effusion (1). During the past decade, several imaging techniques have been introduced as an aide in the diagnosis of pleural tuberculosis (PTB). Despite these, precise etiological diagnosis of pleural effusion in many patients with PTB still remains a challenge. The radiological features of the thorax of patients with tuberculous and malignant pleural effusion often resemble each other and pose considerable diagnostic difficulty at the bed-side diagnosis and management. A distinction between tuberculous and malignant pleural effusion becomes extremely relevant because the treatment modalities differ vastly in these patients.

A confirmatory diagnosis of tuberculous pleural effusion (TPE) depends upon the demonstration of

Mycobacterium tuberculosis in pleural fluid specimens. Owing to the paucibacillary status, *M. tuberculosis* is seldom demonstrated in pleural fluid specimens by conventional bacteriological methods (2,3). As an alternative, several diagnostic parameters have been introduced and evaluated for TPE diagnosis. These include (a) histopathology in pleural biopsies (4), (b) nucleic acid amplification assays (3) and (c) estimation of biomarkers such as adenosine deaminase (ADA) (5), and gamma interferon (IFN- γ) (6). The sensitivities and specificities with these parameters are highly variable

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and hence restricted their routine application as a diagnostic tool in PTB patients (7).

In this study, an indirect enzyme-linked immunosorbent assay (ELISA) has been developed to measure IgG antibodies against four recombinant antigens of *M. tuberculosis* (ESAT-6, PlcA, HspX and Tb8.4) in the pleural fluid of patients with TPE. Sensitivity of the ELISA is appraised in PTB patients and specificity of the assay was evaluated in patients with malignant pleural effusion. Potential application of this diagnostic approach in patients with pleural effusion has been highlighted.

MATERIALS AND METHODS

Study Population

Pleural fluid specimens (10 ml) were collected from 140 patients during thoracentesis, and they were simultaneously subjected to cytological, bacteriologic, and immunological investigations. The patients were admitted to the Hospital for Tuberculosis and Chest Diseases, Pulayanarkotta, Thiruvananthapuram, India ($n = 129$), and at the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, India ($n = 11$). There were 93 male and 47 female patients, and the age of the patients ranged from 20 to 82 years. Relevant clinical and radiologic features were recorded from the case records of these patients. The most common clinical symptoms were productive cough, breathlessness, chest pain, and pyrexia of more than 4 weeks duration. Constitutional symptoms such as loss of appetite, reduction in body weight, and general malaise were also recorded in majority of these patients. At the time of admission, 106 of 140 patients showed positive intradermal tuberculin test (> 12 mm) and elevated erythrocyte sedimentation rates (45–120 mm). The plain skiagrams of the thorax were suggestive of pleural effusion of varying degree, and the underlying lung lesions in the skiagrams were considerably masked because of the effusion. In 114 patients, the pleural effusion was unilateral, and 26 patients had bilateral effusions. Radiologic features of the thorax alone could not define the precise etiology of pleural effusion necessitating further analysis of pleural fluid in 140 patients.

Cytological features

Based on the cytological features in hematoxylin and eosin stained smears, pleural fluids could be broadly classified into two groups: (A) 69 pleural fluids were inflammatory in nature, predominantly composed of lymphocytes. Five among them showed an admixture of

macrophages, lymphocytes and mesothelial cells; (B) 71 cytopsin smears showed the presence of malignant cells.

Classification criteria

Based on the clinical and radiologic findings, along with cytological, biochemical and bacteriological investigations, we classified the patients into three groups: (a) 71 pleural fluid specimens showed the presence of malignant cells in the pleural fluid. Bacteriology studies in the pleural fluid did not demonstrate *M. tuberculosis*. Hence they were classified as malignant pleural effusions (disease control); (b) Bacteriological culture revealed the presence of *M. tuberculosis* in six pleural fluid specimens, and hence, they were regarded as “confirmed” patients with PTB; (c) The bacteriologic cultures in the 63 pleural fluid specimens did not grow *M. tuberculosis* or any other pyogenic bacteria. However, the radiologic and clinical features in these patients were suggestive of TPE, and these 63 patients received a course of ATT. The patients showed optimal clinical recovery. Based on the above, these 63 patients were classified as “probable” patients with PTB.

Cloning of *M. tuberculosis* Open Reading Frames into *Escherichia coli* Expression Vectors

Materials

E. coli BL21 (DE3) pLysS, pET-32a (+) and pET-28a (+) (Novagen, Madison, WI). *Nde* I, *Kpn* I, *Hind* III (NEB, MA, USA). pGEM-T Easy vector, Taq DNA polymerase, T4 DNA Ligase and Nickel–nitrilotriacetic acid (Ni-NTA) affinity agarose beads (Promega Corporation, Madison, WI). The DNA sequencing kit used was ABI PRISM Big Dye Terminator cycle sequencing Ready Reaction kit, Version 2.0 (PE Applied Biosystems, CA), Anti-HIS antibody and PCR gel elution kit (GE Healthcare Biosciences, NJ), Microtitre plate (Dynatech Laboratories, Alexandria, VA). Antibodies and substrate for ELISA were procured from Sigma Aldrich, MO. Primers were designed using Primer Premiere 5 (PREMIER Biosoft International, CA). All other chemicals were of molecular biological grade. Ultra-pure de-ionised water was used in this study.

Cloning of PlcA, HspX, Tb8.4 and ESAT-6 Genes

Genes coding for PlcA, HspX, Tb8.4 and ESAT-6 proteins were amplified from *M. tuberculosis* H₃₇Rv genomic DNA by PCR with specific forward and reverse primers that were designed with restriction sites (Table 1). The thermal cycling was done in a Bio-Rad iCycler (Bio-Rad Laboratories, CA). The temperature conditions employed were as follows: Initial denaturation at 95°C for 2 min, 35 cycles, each composed of

TABLE 1. List of Primers Used for Amplifying Genes from *M. tuberculosis*

<i>esat-6</i> (Rv3875) forward
5'-GGAATTCCATATGACAGAGCAGCAGTGAATTC-3'
<i>esat-6</i> (Rv3875) reverse
5'-CCCAAGCTTGGGCTATGCGAACATCCCAGTGACG-3'
<i>plcA</i> (Rv2351c) forward
5'-GGGGTACCCCGATGTCACGTCGAGAGTTTTTG-3'
<i>plcA</i> (Rv2351c) reverse
5'-CCCAAGCTTGGGTCAGCTGCACAGCCCGC-3'
<i>hspX</i> (Rv2031c) forward
5'-GGGGTACCCCGATGGCCACCACCCTTCCCTTC-3'
<i>hspX</i> (Rv2031c) reverse
5'-CCCAAGCTTGGGTCAGTTGGTGGACCGGATCTG-3'
<i>tb8.4</i> (Rv1174c) forward
5'-GGGGTACCCCGATGAGGCTGTCGTTGACCGCA-3'
<i>tb8.4</i> (Rv1174c) reverse
5'-CCCAAGCTTGGGTAAATAGTTGTCAGGAGC-3'

denaturation at 95°C for 30 sec, annealing at 72°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 1 min. The reaction mixture consisted of 1 × PCR buffer (pH 8), 1.5 mM MgCl₂, dNTPs (100 μM each), 10 pmol of each primer, sterile ddH₂O, 2.5 Units of Taq polymerase and 1 μl of target DNA. PCR products were gel eluted. The amplified PCR products were cloned in pGEM-T Easy vector at the TA cloning site. The correct orientation and sequence was checked by DNA sequencing performed on ABI 310 Automated DNA sequencer (Applied Bio-System, Perkin Elmer) using ABI PRISM Big Dye Terminator cycle sequencing Ready Reaction kit. To reduce the size of the resultant recombinant protein, a “thioredoxin tag” coding sequence from pET-32a (expression vector) was removed with *Nde*I restriction and the plasmid was re-ligated. Genes, *plcA*, *hspX* and *tb8.4* genes were further sub-cloned into pET-32a prokaryotic expression vector using *Kpn*I and *Hind*III while *esat-6* was sub-cloned into pET-28a prokaryotic expression vector using *Nde*I and *Hind*III digestion.

Expression and Purification of Proteins

The vectors containing the genes were transformed into competent cells of *E. coli* expression host BL21(DE3)pLysS. A single transformed colony was inoculated into LB broth containing 60 μg/ml ampicillin for *plcA*, *hspX* and *tb8.4* expression. Kanamycin (25 μg/ml) was used to culture cells containing *esat-6*/pET-28a vector. The cultures were grown overnight at 37°C. About 2 ml overnight culture was inoculated into 100 ml of LB medium containing appropriate antibiotic and grown at 37°C for 2 hr. Cells on reaching the logarithmic phase (*A*₆₀₀ of 0.6) were induced with 1 mM IPTG and grown further for 4 hr at 28°C. After induction, cells

were harvested, and pellet was resuspended in phosphate-buffered saline (PBS) (pH 7.4). Cell pellet was lysed by ultrasonication and the proteins were found to be present as inclusion bodies. HspX was found in the soluble fraction. As such, recombinant antigens were purified from cell extracts to near-homogeneity by Ni-NTA affinity method. HspX was purified under native conditions but as the yield was less, it was purified in denaturing conditions as well, while other three proteins were purified under denaturing conditions. Protein purification was monitored by 280 nm absorbance. Expression of proteins was confirmed with SDS-PAGE and further with anti-His antibody reactivity through western blotting. The solution of denatured protein was dialyzed against 500 ml of freshly made 6, 4, 2, 1, 0.5, and 0 M urea, respectively, with 5 mM Tris (pH 7.4) to remove urea and other salts. With each concentration, the protein was dialyzed 12 hr at 4°C. Protein was concentrated by ultra-filtration through 10.0-kDa cut-off Amicon-GMBH, Germany.

Protein Concentration Assay

Bradford method (8) was used to assay protein concentration. Bovine serum albumin (BSA) was used as a standard.

Estimation of antigen-specific IgG antibody levels in pleural effusion samples

For the detection of IgG antibodies against the four recombinant antigens (ESAT-6, *PlcA*, HspX and Tb8.4), an ELISA in pleural fluid was standardized. Briefly, round-bottom microtitre plates were initially coated with respective purified recombinant mycobacterial protein (500 pg/well) for 2 hr at room temperature (RT), following which the wells of the microtitre plate were quenched with 1% BSA in PBS. Subsequently, the pleural fluid samples from disease control and tuberculosis groups were serially diluted in 1% BSA in PBS and 100 μl (1:5,000 dilution in BSA/PBS) was added to each well and incubated overnight at 4°C. The plates were then washed thoroughly and incubated at RT for 2 hr with 100 μl of (1:1,000) anti-human IgG-alkaline phosphatase conjugate. The color reaction was developed by the addition of a substrate containing para-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer) and the plates were incubated for 30 min at RT. About 3 N sodium hydroxide (25 μl) was added to the wells to stop the reaction and the absorbance was read at 405 nm using a microplate reader (Bio-Tek Instruments, Mumbai, India).

Whenever ELISA was performed in a batch of ten specimens, a pleural fluid from a culture-proven patient with PTB was used as a positive control. Inter-observer variation, reproducibility of assay and batch-to-batch

variation of recombinant antigens were checked by using two different batches of antigen in the same pleural fluid specimen at two different occasions. Mean absorbance of disease control group plus 2 standard deviations was used to determine the "cut-off" to score a pleural fluid positive for tuberculosis etiology.

ELISA was also performed in all pleural fluid specimens using a cocktail of all the four recombinant antigens in equal concentration (200 pg each). Coating concentration was standardized based on initial experiments. The technical procedures adopted were identical as described.

Statistics

The diagnostic value of the ELISA was evaluated in terms of sensitivity and specificity. The sensitivity of a diagnostic test was interpreted as the proportion of TB patients correctly identified by the test as having tuberculosis; the specificity is the proportion of normal or disease control subjects correctly identified as not having tuberculosis by the test.

RESULTS

The mean absorbance and standard deviation in tuberculous and malignant pleural effusion with individual recombinant mycobacterial antigens and their multi-antigen cocktail is given in Table 2. The "cut-off" value separating the PTB from the diseased control group by ELISA was determined using the following formula: mean absorbance of disease controls + 2 SDs. A test was found positive when the absorbance is greater than 0.513, 0.604, 0.506, 0.521 and 0.649 for PlcA, HspX, Tb8.4, ESAT-6 and multi-antigen complex respectively. Based on this criterion, IgG positivity for PlcA, HspX, Tb8.4, ESAT-6 antigens and multi-antigen complex were 49.3, 60.8, 49.3, 53.6 and 75.4% respectively. Antibody titre in culture-positive pleural fluids was significantly higher than culture-negative patients with PTB ($P < 0.05$). Sensitivity of the assay using multi-antigen complex was higher than individual antigens. The differences between the multi-antigen complex and PlcA, Tb8.4, HspX and ESAT-6 were

significant ($P < 0.01$). All the four antigens and multi-antigen gave positive results in three pleural fluids with malignant pleural effusion.

DISCUSSION

Immunoassays to detect the presence of mycobacterial antigen as well as antibody response to *M. tuberculosis* have been developed as reliable markers for the diagnosis of TPE (9,10). Exudates in pleural effusion in PTB patients are usually rich in lymphocytes. These lymphocytes are composed of both T and B lymphocytes. T-lymphocyte population are more predominant than B lymphocytes in tuberculosis. Several studies have highlighted the T-lymphocyte responses to mycobacterial antigens in patients with TPE. These include the release of pro-inflammatory cytokines like IFN- γ (11). On the other hand, B lymphocytes produce antibodies against mycobacterial antigens. Immunoassays to detect the presence of antibodies in pleural fluid against purified protein derivative (12), tuberculous glycolipid antigen (13) and lipoarabinomannan (14) in patients with TPE have been documented. Recently, Kaisermann et al. detected IgA antibodies against MPT-64 and MT-10.3 recombinant mycobacterial antigens in pleural fluids (15). Although these assays were sensitive, false-positive results were encountered in patients with non-TPE. In an effort to enhance the specificity of ELISA, an attempt was made to select a mycobacterial antigen that would give positive results only in patients with TPE. Hence, we evaluated the role of four mycobacterial antigens (PlcA, HspX, Tb8.4 and ESAT-6) in PTB diagnosis.

PlcA is a probable membrane-associated phospholipase C1 of *M. tuberculosis*, which is also referred to as Mtp40 antigen (16). PlcA activities seem to be restricted to pathogenic *Mycobacterium* subsp. Preliminary studies indicate that PlcA elicit high antibody titre in sera of pulmonary tuberculosis patients (17). ESAT-6 is the early-secreted antigenic target 6-kDa protein, specific for *M. tuberculosis* complex, but is reported to be absent from *M. bovis* BCG (18). ESAT-6 has been reported to elicit strong antibody responses and delayed type hypersensitivity skin reactions in guinea pigs (19). HspX (acr/16-kDa antigen/ α -crystallin) was reported to be more sensitive than ESAT-6, CFP10 and antigen 85 in terms of sensitivity in serodiagnosis for the diagnosis of pulmonary tuberculosis (20). Tb8.4 is an immunodominant T-cell antigen of *M. tuberculosis* and this antigen predominantly elicit cell-mediated immunological responses in human beings and animal models (21).

In this study an effort was made to evaluate the potential application of the above recombinant mycobacterial antigens for laboratory diagnosis of TPE. The sensitivity

TABLE 2. The Mean OD in Tuberculous and Malignant Pleural Effusions

Antigens	Control group (n = 71)	Tuberculosis test group (n = 69)
PlcA	0.247 $\pm$ 0.133	0.698 $\pm$ 0.14
HspX	0.36 $\pm$ 0.122	0.715 $\pm$ 0.187
Tb8.4	0.31 $\pm$ 0.098	0.667 $\pm$ 0.089
Esat-6	0.281 $\pm$ 0.12	0.703 $\pm$ 0.143
Multi-antigen cocktail	0.289 $\pm$ 0.18	0.848 $\pm$ 0.173

of ELISA using individual antigens was found to be low and ranged between 40 and 60%. To enhance the sensitivity of the assay, we used all the four antigens in a cocktail. An ELISA using this cocktail antigen gave a sensitivity of 75.4%. HspX gave higher titre than other three when assayed individually in active tuberculosis cases. Earlier studies reported that increased immune responses to HspX are observed in latent TB (22). But the results of this study confirm that antibodies to this antigen are predominantly found in individuals with active TB as reported (23). Although not highly sensitive individually, PlcA, Tb8.4 and ESAT-6 antigens contributed in increasing sensitivity in the multi-antigen complex, without affecting the specificity.

False-positive results were encountered in three pleural fluid specimens from patients with bronchial carcinoma. These three patients subsequently underwent surgery and the pneumoectomy specimens were subjected to histopathology. In these patients the histological factors were suggestive of squamous cell carcinoma. In addition, non-caseating granuloma suggestive of tuberculosis was also observed in the histological sections of lungs. Reactivation of tuberculous lesions is well known to occur in patients with bronchial carcinoma (24). Therefore, it is likely that IgG antibody to recombinant antigens detected by ELISA in these three patients with bronchial carcinoma could be due to the reactivation of tuberculous lesions. Hence IgG antibodies to *M. tuberculosis* in three patients with bronchial carcinoma were not regarded as false positive.

The IgG antibody response in pleural fluid specimens in PTB patients are specifically directed against the recombinant mycobacterial antigens and are produced by B-lymphocyte population in the pleural effusion (25) and not as a result of passive diffusion from serum to pleural fluid. Intrathecal synthesis of IgG against mycobacterial antigens is well documented in patients with tuberculous meningitis (26). To substantiate this observation in PTB, we are currently undertaking a prospective study to measure the antibody responses in both serum and pleural fluid of the same patient with PTB.

In conclusion, results of this study indicate that a multi-antigen complex composed of PlcA, HspX, Tb8.4 and ESAT-6 is a useful marker in making a diagnosis of PTB. The assay is reliable and reproducible. IgG detection using ELISA is a rapid and cost-effective method and hence we recommend the use of ELISA using this multi-antigen cocktail for the diagnosis in patients with PTB.

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