Comparative Study of Three Different Mycobacterial Antigens With a Novel Lipopolysaccharide Antigen for the Serodiagnosis of Tuberculosis

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Demonstration of *Mycobacterium tuberculosis* in a smear or culture is the most reliable method for diagnosing tuberculosis (TB). In the last 10 years, several enzyme-linked immunosorbent assays (ELISAs) based on mycobacterial antigens (such as antigen 60, 38 kDa antigen, and antigen Kp90) have been used for the rapid diagnosis of TB. In this study, we report the isolation of an immunodominant lipopolysaccharide (LPS) antigen from *M. tuberculosis* H₃₇Rv, which can be used for the serodiagnosis of TB. The LPS antigen was compared with three commercially available mycobacterium-specific antigens for the detection of TB. The antigens were evalu-

ated using serum samples obtained from 59 Indian patients (19 patients with active pulmonary TB, 20 with extrapulmonary TB, and 20 with nontuberculous pulmonary disease) and 20 healthy adults. Antigen 60 IgG (sensitivity 89%, specificity 97%) and LPS (sensitivity 84%, specificity 97%) were more sensitive and specific than 38 kDa antigen IgG (sensitivity 79%, specificity 97%) and Kp90 IgA (sensitivity 82%, specificity 40%). These results indicate that the LPS antigen can be used as a sensitive tool for the serodiagnosis of TB and could be utilized to develop an ELISA for the screening of patients for TB. J. Clin. Lab. Anal. 16:151–155, 2002. © 2002 Wiley-Liss, Inc.

Key words: tuberculosis; mycobacteria; serodiagnosis; lipopolysaccharide; ELISA

INTRODUCTION

Tuberculosis (TB) is a major health problem in both developing and developed countries. It is estimated that this disease causes 3 million deaths and 8 million new cases annually (1). Early diagnosis would help control the morbidity and infection from this disease. The diagnostic methods currently in use are the demonstration of tubercle bacilli in the sputum of infected patients, followed by the culture of *Mycobacterium tuberculosis* for confirmation. These methods require several months for completion and are not feasible in the countries where the disease is endemic. In some countries, tuberculin (purified protein derivative (PPD)) has been used for the diagnosis of TB (2,3). However, the test has a high rate of false-positive and false-negative cases associated with it. In the last few years, polymerase chain reaction (PCR)-based diagnosis of TB in sputum samples has also been demonstrated (4). However, this method also gives a high percentage of false-negative results. Alternatively, diagnostic techniques based on serology are easier to perform, are

cost-effective, and have a low frequency of false-positive and -negative results associated with them. The use of enzyme-linked immunosorbent assay (ELISA) to measure the humoral response generated against various antigenic components of tubercle bacillus has been described by a number of investigators (5,6). PPD antigen (2,3), a complex antigen from *M. tuberculosis* (7), sonicate of bacille calmette-Guerin (BCG) (8), and antigens 5 and 6 (9,10) have also been employed in the ELISA methods to improve both sensitivity and specificity, with varying success. At present, three kits—the andaelisa mycobacteria (A60 antigen complex); Pathozyme-Myco (Omega Diagnostics, Ltd., UK) (recombinant 38 kDa protein

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and a highly purified antigen of *M. tuberculosis*); and Kreatech Diagnostics (Madrid, Spain), an immuno-cross-reactive antigenic compound Kp90 from mycobacteria—are commercially available for the diagnosis of TB. However, the efficacy of all the commercially available kits to detect TB in Indian patients has not been reported. We have isolated a novel lipopolysaccharide (LPS) antigen from *M. tuberculosis* H₃₇Rv and attempted to develop a serodiagnostic test for TB. In the present study, we compared the LPS with commercially available kits for its use in TB detection in Indian patients.

MATERIALS AND METHODS

Study Population

Serum samples were obtained from 79 adult subjects, including 59 patients with TB (19 patients with active pulmonary TB, 20 patients with extrapulmonary TB, and 20 with nontuberculous pulmonary disease) and 20 healthy adults. The samples were obtained from the Gulabi Bagh Chest Clinic, Rajan Babu TB Hospital, and the All India Institute of Medical Sciences. The selection of patients with active pulmonary TB was based on the presence of acid fast bacilli (AFB) in the sputum, and clinical and radiographic response to antituberculosis treatment (ATT). Of the 19 patients with pulmonary TB, nine were sputum-positive. The remaining 10 patients were diagnosed to have sputum smear negative pulmonary TB on the basis of constitutional features, location of infiltrates in the upper lobes on chest radiographs, and good clinical and radiographic response to antitubercular drugs. Disseminated TB was diagnosed on the basis of histopathological and/or microbiological evidence of TB from two noncontiguous sites. Miliary TB was diagnosed according to criteria described previously (11). Lymph node TB was diagnosed by the demonstration of *M. tuberculosis* in the smear and/or culture of lymph node aspirates or biopsy specimen. The healthy control group comprised of 20 adults free from any disease. Another group included 20 patients with pulmonary problems other than TB, such as bronchial asthma, chronic obstructive airways disease, and bronchogenic carcinoma.

Isolation of LPS Antigen

M. tuberculosis H₃₇Rv was obtained from the New Delhi Tuberculosis Center and grown in modified Youmans and Karlson's medium as a surface culture (12). Cells were killed with acetone and dried for further use. To isolate the antigen, dried cells were resuspended in 50 mM Tris-HCl and 10 mM EDTA, pH 8.0 (buffer A) and lysed. The sonicated sample was deproteinized using Tris pH 8.0 equilibrated phenol and centrifuged at 14,000 rpm for 15 min at 4°C. Cold ethanol (99%) was added to the aqueous layer and the resultant precipitate was recovered by centrifugation, dissolved in 7.5 mM Tris-HCl and 10 mM EDTA, pH 7.4 (buffer B). The samples were then applied to the anion exchange column (DE-52;

Whatman, Biosystems, UK) pre-equilibrated with the same buffer. After washing with the same buffer, the sample was eluted with a linear gradient of 0–1.2 M NaCl. Different fractions obtained were tested for the antigenicity in an ELISA-based assay.

ELISA

Antigen 60 IgG measurement

The diagnostic kits were obtained from Anda Biologicals (Strasbourg, France). The antigen used in this kit was prepared from the cytoplasm of *Mycobacterium bovis* (BCG). In the procedure recommended by the manufacturer, 100 µl of diluted sera (1:100) were put into microtiter wells and incubated for 60 min at 37°C. The unbound components were removed by washing with a buffer solution. The wells were subsequently incubated with 100 µl of peroxidase-labeled antihuman gammaglobulin (IgG) conjugate at 37°C for 30 min. After the incubation period and washing, peroxidase substrate tetramethyl-benzidine (TMB) containing hydrogen peroxidase was added to each well and incubated for 15 min in the dark at 37°C. The reaction was stopped by addition of 100 µl of H₂SO₄ (0.5 N). The optical density (OD) was measured at 450 nm in a ELISA reader. Six standards of serum samples with varying reactivity to antigen (0, 1, 2, 4, 8, and 16 sero units) were provided for determining the reactivity of unknown serum samples to antigen.

Antigen 38 kDa IgG measurement

The serologic test of 38 kDa IgG (Omega Diagnostics Limited, Alloa, Scotland, UK) consisted of microtiter wells coated with 38 kDa antigen isolated from *M. tuberculosis*. The procedure employed was similar to that described above for antigen 60, except that the sera were diluted by a factor of 1:50 rather than 1:100. The cut-off level of the OD readings in this kit was determined by dividing the OD value of the low positive control by 1.5 (as recommended by the manufacturer). The antibody index derived from the ratio of the unknown sera to the cut-off level permits comparison of the samples by different measurements.

Antigen Kp90 IgA measurements

Diagnostic kits were obtained from Kreatech Diagnostics. Serum IgG antibody to a mycobacterial Kp90 ImCRAC (immuno-cross-reactive antigenic compound) was measured. The procedures were similar to those described for antigen 60, except that the sera were diluted by a factor of 1:500 and the second incubation time was 1 hr rather than 30 min at room temperature. The cut-off OD value was derived from the mean of the OD values of three cut-off controls (provided by the manufacturer). The ratio of the OD obtained from unknown sera to the cut-off OD value level provided the basis for predicting the presence or absence of the disease.

ELISA for LPS

Polystyrene 96-well ELISA plates (Dynatech, Greiner, Germany) were coated with the LPS antigen at a concentration of 1 µg/ml. All additions were made in 100 µl volumes. Sera were added at a dilution of 1:400. Antihuman IgG-peroxidase conjugate (a gift from Dr. J.K.Batra, NII, Delhi) was used as the secondary antibody, and the color was developed using o-phenylenediamine as substrate. The absorbance, A_{490} , of each well was then recorded on an ELISA Reader (Molecular Devices, Inc., CA). The ELISA ratio was calculated by dividing the A_{490} of each serum sample by that of the control negative serum. The selection of 1 µg of antigen per ml and of a serum dilution of 1:400 was standardized from preliminary experiments. This concentration of the antigen and dilution of the sera were considered optimal for the assay. It was also noticed that the measurement of IgM antibodies against LPS antigen resulted in a high rate of false-positive results. Therefore, IgG levels against LPS antigen were measured in all the serum samples.

ELISAs using LPS antigen and all of the kits were performed simultaneously. Assays with kits were performed according to the manufacturer's instructions and to previous reports (13–15).

Data Analysis

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 patients correctly identified by the test, and the specificity is the proportion of normal subjects correctly identified as normal by the test. In the test with A60 antigen and 38 kDa antigen, sero-units/ml of serum samples were calculated by plotting the values obtained with standards as recommended by the manufacturer. In the assays with Kp90 antigen, the ratio was calculated using values obtained from test sera and cut-off control. In the assays with LPS antigen, the ELISA ratio was calculated by dividing the absorbance obtained with each serum by the absorbance of the control negative pool serum. On the basis of assays with known tuberculous patients (187) and healthy controls (159), the ELISA ratio was observed to be 3.12 and 1.25, respectively. The former was significantly higher ($P < 0.01$) than the latter. This ratio was consistently higher in tuberculous patients. The cut-off point for the ratio was calculated to be 2.015. This cut-off point was found to have $13 \pm 2.5\%$ and $1 \pm 0.9\%$ chances of false negativity and false positivity, respectively. Thus the sensitivity of the antigen in the standardization assay was 84%, and the specificity was 97%.

RESULTS

Serum samples were obtained from 79 adult subjects, including 20 healthy controls and 59 patients (19 patients with active TB, 20 with extrapulmonary TB, and 20 with diseases

TABLE 1. Serological test with sera obtained from various groups using mycobacterial antigen

Patients groups	Sputum acid fast stain		Extra-pulmonary tuberculosis	Non-tuberculosis diseases	Healthy controls
	+ve	-ve			
Anda 60					
IgG	8/9	9/10	18/20	0/20	1/20
IgM	8/9	10/10	14/20	4/20	3/20
IgA	8/9	8/10	10/20	0/20	3/20
Pathozymemyco					
IgG	9/9	7/10	15/20	0/20	1/20
IgM	6/9	6/10	7/20	9/20	9/20
IgA	7/9	7/10	7/20	0/20	2/20
Kreatech					
IgA	9/9	8/10	15/20	14/20	10/20
LPS					
IgG	7/9	10/10	16/20	0/20	1/20

Numerator is the number of samples detected by the antigen and denominator is number of subjects in the groups.

other than TB). Patients were grouped based upon disease status as shown in Table 1. Sera obtained from all the individuals were evaluated using commercially available diagnostic kits and LPS. All the assays were performed as specified by the manufacturers.

ELISA With Antigen 60

The sensitivity and specificity obtained with antigen 60 are shown in Table 2. The sensitivities with three kits based on antigen 60 (IgG, IgM, and IgA) were 89%, 82%, and 66%, respectively. The specificities with antigen 60 (IgG, IgM, and IgA) were 97%, 82%, and 92%, respectively. These results indicate that the antigen 60 IgG has the highest sensitivity and specificity.

ELISA With Antigen 38 kDa (Pathozyme-Myco)

The sensitivities with Pathozyme-Myco IgG, IgM, and IgA were 79%, 61%, and 52%, respectively. The specificities with

TABLE 2. Sensitivity and specificity of various kits: Anda 60, Pathozyme-myc (38 kDa), Kreatech (Kp90) and lipopolysaccharide antigen

Antigen	Sensitivity	Specificity
Anda (A60)		
IgG	89%	97%
IgM	82%	82%
IgA	66%	92%
Pathozyme-myc (38 kDa)		
IgG	79%	97%
IgM	61%	55%
IgA	52%	95%
Kreatech (Kp90)		
IgA	82%	40%
LPS		
IgG	84%	97%

the IgG-, IgM-, and IgA-based kits were 97%, 55%, and 95%, respectively. The results suggest that the sensitivity of the antigen was low in comparison to antigen 60.

ELISA With Antigen Kp90 (Kreatech)

The sensitivity and specificity with antigen Kp90 was 82% and 40%, respectively. The specificity of this kit was lowest among the three kits used in this study.

LPS Antigen

Using the ELISA cut-off value of 2.015, sera from patients suffering from diseases other than TB were evaluated. The ELISA ratio in all such disease categories was below 2.015. In the present study, using a cut-off value of 2.015, the LPS antigen showed 84% sensitivity and 97% specificity. The results suggest that the LPS antigen is highly specific for *M. tuberculosis* and is similar to the antigen 60 IgG assay system.

DISCUSSION

The identification of organisms using serological methods is very commonly used to detect several infectious diseases. The success of serological testing depends on the specificity and sensitivity of the antigen. For the serodiagnosis of TB, several antigens have been isolated and shown to be useful in the detection of the disease (5), and have been applied by commercially available kits. The antibody level against mycobacterial components in healthy controls will vary depending upon the prevalence of the disease in a specific area. Large numbers of people are exposed to *M. tuberculosis* in developing countries, including India. In view of this fact, it is important to evaluate the efficacy of the commercially available diagnostic kits for serodiagnosis of TB. Kits based on IgG, IgM, and IgA assays, and an LPS antigen isolated from *M. tuberculosis* were evaluated for detection of TB. The selection of the patients in each group was strict, and for most of them the diagnosis was confirmed by culture of mycobacteria and staining.

The sensitivities and specificities of the kits varied widely, with sensitivity ranging from 52% to 90%, and specificity ranging from 40% to 97%. In general, the sensitivity and specificity were higher in IgG-based detection (Table 2). However, both sensitivity and specificity were lower in IgM- and IgA-based assays. Among the three kits tested here, antigen 60 (IgG-based) showed 89% sensitivity and 97% specificity. Although the specificity of IgA-based assay with antigen 60 was higher (92%), the sensitivity was low (66%). In a previous study using antigen 60 (with combined IgG and IgA antibody titers) for the detection of TB in Indian patients, similar sensitivity (91.6%) and specificity (90%) were observed (13). The 38 kDa antigen kit showed very low sensitivities with all of the assays measuring IgG, IgM, and IgA titers, whereas

specificity was higher with the IgG- and IgA-based assays (Table 2). In contrast, antigen Kp90 showed very low specificity and moderate sensitivity (Table 2). The LPS antigen was comparable to antigen 60 in both sensitivity (84%) and specificity (97%). The results indicate that IgG-based assays with antigen 60 and LPS antigen are highly sensitive and specific for the diagnosis of TB. LPS is a major constituent of the outer face of the surface membrane of mycobacteria, and is highly antigenic. Lipoarabinomannan (LAM) is one of the constituents of LPS antigen, and is present in all members of the genus *Mycobacterium*. In an earlier report, highly purified LAM from *M. tuberculosis* showed a high degree of specificity (91%) and sensitivity (72%) for the detection of TB (16). In our study, the specificity and sensitivity was improved, possibly due to the presence of lipopolysaccharides containing other carbohydrate moieties in addition to LAM.

The ELISA test in combination with other clinical findings will be useful for obtaining a rapid and definite diagnosis—especially in the case of extrapulmonary TB, in which many diagnostic tests fail.

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