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Detection of mannophosphoinositide antigens in sputum of tuberculosis patients by dot enzyme immunoassay

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Abstract. A simple and economical dot ELISA for the detection of mannophosphoinositide antigen in sputum samples of tuberculosis patients has been developed using affinity-purified antibodies. This test is able to detect free as well as bound antigen. Sputum samples from 94 patients suffering from tuberculosis and 30 non-tuberculosis patients were screened and an overall sensitivity and specificity of 89% and 93.3%, respectively, was obtained. The sensitivity of the test among the different groups of tuberculosis patients did not vary significantly.

Introduction

Over the past several years, attempts have been made to develop simple, sensitive and specific immunoassays for the diagnosis of tuberculosis. Various techniques like radioimmunoassays (RIA) and enzyme immunoassays (EIA) have been employed extensively to measure circulating antibodies to different mycobacterial antigens [1, 3, 4, 6, 8]. Irrespective of the type of antigen employed in EIA, no assay could detect more than 95% of the patients. Moreover, high antibody titres from normal healthy individuals from endemic areas as well as from convalescing patients, restrict their use for the routine serodiagnosis of tuberculosis.

In recent years, the demonstration of mycobacterial antigens in body fluids is gaining significance as it appears to be a more clinically useful approach. It has been successfully used for the detection of mycobacterial antigen in cerebrospinal fluids (CSF) obtained from patients suffering from tubercular meningitis [9, 10]. Similarly, Kadival and his associates [5] employed a RIA while Yanez et al. [11] have used an enzyme-linked immunosorbent assay (ELISA) to detect mycobacterial antigens in sputum of patients suffering from pulmonary tuberculosis. Both these techniques cannot usually be employed for conducting the epidemiological studies because of the high cost of reagents and the elaborate instrumentation

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required. An improvement in the ELISA test can be achieved by using a dot-ELISA which is rapid, easy and economical in comparison to other tests [2].

In the present study, we have developed a simple dot-ELISA test for the detection of mannophosphoinositide antigen in the sputum samples of tuberculosis patients using highly specific affinity-purified antibodies.

Materials and methods

Clinical samples

A total of 94 test sputum samples from clinically diagnosed and confirmed tuberculosis patients were collected. These patients were admitted to either the TB Hospital, Patiala or the TB Sanatorium, Dharampur and were on anti-tubercular chemotherapy. Along with the test samples a total of 30 control sputum samples were collected from confirmed non-tuberculous patients attending the chest Out-patients Department or were admitted to the Nehru Hospital attached to PGIMER, Chandigarh, suffering from diseases such as asthma, chronic bronchitis, pneumonia, hydropenumothorax and lung cancer. All the sputum samples (4–5 ml) were digested with a solution containing sodium hydroxide, sodium citrate and *N*-acetyl-L-CYSTEINE and concentrated to a minimum volume of 100 µl using routine methods for smear and culture. Cultures were performed on conventional Lowenstein Jensen medium. Residual sputa were then autoclaved and used for antigen detection.

Isolation of mannophosphoinositide antigen and antibodies

Antigen was isolated and purifed according to the method of Mehta and Khuller [6]. Hyperimmune serum was obtained from rabbits immunized with mannophosphoinositide-MBSA (Methylated Bovine Serum Albumin) complexes emulsified with incomplete Freund's adjuvant (IFA). The antigen was injected at weekly intervals, once into the footpad and twice intramuscularly.

A total of 10–12 mg of antigen was injected per animal. The total immunoglobulin fraction was obtained from the serum by precipitation with 40% ammonium sulphate.

Purification of mannophosphoinositide antibodies by affinity chromatography

Coupling of mannophosphoinositide antigen to Sepharose 4B. Mannophosphoinositide antigen was conjugated to Sepharose 4B by the method of Porath [7] with slight modifications. Five grams of washed sepharose 4B (Sigma, St. Louis, Mo.) was suspended in 5.0 ml 1.0M sodium carbonate buffer (pH 11.0) and was activated by slowly adding 1.0 ml divinyl sulphone (Sigma). The suspension was stirred continuously for 70 min at room temperature. The reaction was stopped by collecting the Sepharose 4B on a Buchner funnel and washing it extensively with distilled water. This was mixed with 9.5 mg mannophosphoinositide antigen (sonicated in 0.3 M sodium carbonate buffer, pH 10.0) and was put on a shaker at room temperature overnight. The conjugation was checked by measuring the phosphorous content of Sepharose 4B before and after the treatment with antigen suspension.

Purification of mannophosphoinositide antibodies. Immunoglobulins which were initially precipitated by 40% ammonium sulphate were subjected to antigen-based affinity chromatography. Total immunoglobulins were repeatedly passed through a 1.0-ml (bed volume) column of the Sepharose 4B coupled to the antigen. Unbound molecules were removed by extensive washing with phosphate-buffered saline (PBS). Bound molecules were eluted with 1.0 M ammonium hydroxide and the pH was quickly adjusted to 7.4–8.0 with 1.0 M HCl. The eluted material was dialyzed against normal saline overnight and concentrated, if necessary, by dialysis against polyethylene glycol (PEG, mol. wt. 20,000).

Dot-ELISA for mycobacterial mannophosphoinositide antigen

Dot-ELISA was performed on simple nitrocellulose (NC) paper strips obtained from Advanced Microdevices Pvt., Ambala, India. Autoclaved sputum samples were used for spotting onto the NC paper. A total of 10 µl of the material was spotted in 1-µl aliquots. Non-specific sites were blocked with 3.0% BSA in PBS (0.01 M, pH 7.2) for 90 min at 37°C. The NC paper was then washed extensively with PBS and incubated with affinity-purified antibodies to mannophosphoinositides at a concentration of 100 µg protein/ml for 2 h at 37°C. The NC paper was again washed extensively with PBS and incubated with 1:1,000 diluted horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Sigma) for 90 min at 37°C. Finally, after extensive washings with PBS, the colour was developed with diaminobenzidine tetrahydrochloride (Sigma) in the presence of 0.05% hydrogen peroxide. The NC paper was rinsed with water, dried overnight and recorded visually or photographed.

Nature of the antigen in sputum

To check whether the antigen detected in sputum samples is dueto the whole bacteria or free antigen, some of the sputum samples which were found to be positive by dot-ELISA were subjected to filtration through a bacterial filter (millipore, pore size 0.45 µm) to remove whole bacteria. The filtrate was then checked for the presence of free antigen.

Results

The mannophosphoinositide antibodies obtained from hyperimmunized rabbits formed sharp precipitin lines with homologous antigen as seen by counter current immunoelectrophoresis (CCIEP). Affinity-purified anti-mannoside antibodies were found to be highly specific and did not cross react with other related components such as phosphatidylinositol, phosphatidylethanolamine, cardiolipin and phosphatidylserine.

Preliminary standardizations were carried out to develop dot-ELISA using free (purified mannophosphoinositides sonicated in normal saline) as well as bound (whole bacterial cells) antigen. This test could detect as little as 10 ng of free antigen and 3×10^2 organisms (*M. tuberculosis* $H_{37}Ra$ and *M. tuberculosis* $H_{37}Rv$). The positive samples appeared as distinct brown spots (Fig. 1) against the white background of the NC paper, which could be monitored visually, whereas the controls did not give any colour. The intensity of the colour varied depending upon the concentration of the antigen; however, the colour intensity was not taken into account when the results were recorded.

The results obtained from the 94 sputum samples of confirmed cases of tuberculosis are presented in Table 1. This table also shows the classification of all the patients on the basis of acid-fast staining and culture reports. The percent positivities obtained with dot-ELISA for mannophosphoinositide detection for various groups are as follows: smear-positive culture-positive (SPCP), 92%; smear-positive culture-negative (SPCN), 83%; smear-negative culture-positive (SNCP), 100%; and smear-negative culture-negative (SNCN), 94%. The overall sensitivity of the test was 89%. To check the specificity of the test (Table 2), sputum samples obtained from 30 non-tuberculous patients suffering from other diseases were processed similarly. Only two patients out of a total of 30 gave positive test.

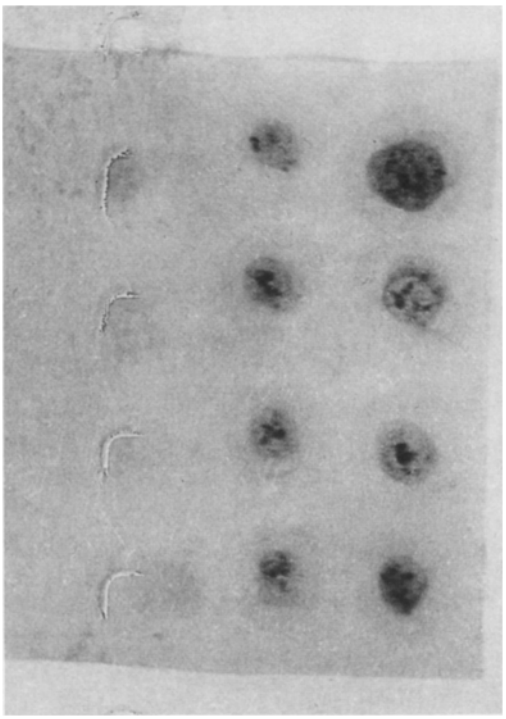

Fig. 1. Dot-enzyme-linked immunosorbent assay (ELISA) for detection of mannophosphoinositide antigen in sputum of samples of tuberculosis patients (positive samples appear as brown spots against the white background of nitrocellulose paper; intensity of the colour varies with the antigen concentration). The *first column* represents some of the negative sputum samples

Table 1. Dot-enzyme-linked immunosorbent assay for detection of mannophosphoinositide antigen in sputum samples of tuberculous patients

Group	No. of cases screened	No. of positive cases	% positives
SPCP	35	32	92
SPCN	36	30	83
SNCP	7	7	100
SNCN	16	15	94

SPCP, Smear-positive culture-positive; SPCN, smear-positive culture-negative; SNCP, smear-negative culture-positive; SNCN, smear-negative culture-negative

Table 2. Sensitivity and specificity of Dot-ELISA for detecting mannophosphoinositide antigen in the sputum samples

Sensitivity	Specificity
No. of positive tests/total no. of confirmed tuberculosis patients	No. of negative tests/total no. of non-tuberculous cases[a]
84/94 (89%)	28/30 93.3%)

[a] Sputa obtained from patients with either asthma, chronic bronchitis, pneumonia, hydropneumothorax or lung cancer

The sputum samples which were passed through bacterial filters also gave a positive reaction which indicates that the dot-ELISA is able to detect free as well as the bound form of the antigen.

Discussion

Demonstration of mycobacterial antigens in body fluids have always been thought to be a confirmatory test for the association of the disease with the host. In the present study, we have used affinity-purified antibodies to mannophosphoinositides to develop a sensitive and specific dot-ELISA test for the detection of antigen (mannophosphoinositides) in the sputum samples of tuberculous patients.

Our test yielded an overall sensitivity of 89% in confirmed cases of tuberculosis. The sensitivity of the test, however, did not vary significantly among different groups of tuberculosis patients being 92%, 83%, 100% and 94% for SPCP, SPCN, SNCP and SNCN groups, respectively. In earlier studies, Yanez et al. [11] demonstrated a positivity of 60% for SPCP, 55% for SPCN, 50% for SNCP and only 9% for SNCN groups. Similarly Kadival et al. [5] could detect significant amounts of antigen (58.5 ± 30.5 mg/ml) only in the SPCP group and the amount of antigen detected decreased from the SNCP (26.6 ± 19.7 mg/ml) to SNCN (1.4 ± 1.8 mg/ml) groups. In these studies, the % positivity as well as the antigen concentration seems to be directly correlated with the bacterial load indicating that these tests probably detect antigen which is bound to the whole bacteria. In the present investigation, the high positivity (93.7%) obtained especially with SNCN group is in contrast with earlier findings. This group necessarily represent those tuberculosis patients who are on anti-tubercular therapy, which renders them non-infectious (sputum and culture negative) after 4- to 6-week period. However, the breakdown products of the killed/dying bacteria can still be expected to be present in circulation. The high positivity of this group could be due to the detection of free antigen rather than the whole bacteria, which might be responsible for a positive dot-ELISA in other groups. The ability of this test to detect the free as well as the bound form of antigen makes it a uniformally useful test for different groups of tuberculous patients. The overall increase in sensitivity can also be partially due to the use of affinity-purified antibodies as compared to the polyclonal antibodies used in earlier studies [5, 11].

Along with the good sensitivity, a high specificity (93.3% was observed in this study. Two false-positive cases out of a total of 30 might be complicated by a secondary mycobacterial infection. Moreover, these samples do not represent true negative controls.

This assay method is also safe and without the risk of exposure to live bacterium since autoclaved sputum samples can be used. In addition, compared to RIA and EIA which have problems of high cost and reproducibility, this method is easy, economical and can be used for field trial studies involving large populations, especially in developing countries where tuberculosis is still a major health problem.

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