

ENZYME LINKED PROTEIN-A: AN ELISA FOR DETECTION OF IgG ANTIBODIES AGAINST *MYCOBACTERIUM TUBERCULOSIS* IN INTESTINAL TUBERCULOSIS

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Summary

Enzyme linked protein-A has been used to develop an enzyme-linked immunosorbant assay (ELISA) to detect circulating IgG antibodies to *Mycobacterium tuberculosis*. The specific binding of protein-A to IgG fractions through Fc receptors, makes the test more specific for detection of total IgG antibodies. The ELISA system has been used for detection of circulating antibodies to *M. tuberculosis* H₃₇Ra in 22 patients with histologically proven intestinal tuberculosis and 88 healthy controls, in addition to 7 diseased controls. The ELISA has been found to be a sensitive test as it was positive in all 22 patients with intestinal tuberculosis. Its specificity was 85 % in Indian controls and 97 % in Norwegian controls. The test is easy to perform and may be recommended for the serological diagnosis of intestinal tuberculosis.

Résumé

La protéine enzymatique A a été utilisée pour la mise au point d'un test immunosorbant lié aux enzymes (ELISA) permettant de déceler les anticorps circulants IgG vis-à-vis de *Mycobacterium tuberculosis*. La liaison spécifique entre la protéine A et des fractions d'IgG par les récepteurs Fc rend ce test plus spécifique pour le dépistage des anticorps IgG entiers. Le système ELISA a été utilisé pour déceler les anticorps circulants vis-à-vis de *M. tuberculosis* H₃₇Ra chez 22 malades porteurs d'une tuberculose intestinale confirmée par l'histologie, et 88 sujets témoins en bonne santé (22 Indiens et 66 Norvégiens) en plus de 7 sujets témoins malades. L'ELISA s'est avéré être un test sensible puisqu'il était positif chez les 22 sujets atteints de tuberculose intestinale. Sa spécificité a été de 84,6 % chez les sujets témoins indiens et de 97 % chez les sujets témoins norvégiens. Ce test est facile à réaliser et peut être recommandé pour le diagnostic sérologique de la tuberculose intestinale.

Resumen

Se ha utilizado la proteína A ligada a enzima para desarrollar una prueba de inmunoabsorbente ligado a enzima (ELISA) para detectar los anticuerpos circulantes IgG al *Mycobacterium tuberculosis*. La ligazón específica de la proteína A a fracciones de IgG por receptores Fc aumenta la especificidad de esta prueba para la detección de los anticuerpos IgG totales. Se utilizó el sistema ELISA para la detección de anticuerpos circulantes al *Mycobacterium tuberculosis* H₃₇ Ra en 22 pacientes con tuberculosis intestinal confirmada histológicamente y en 88 controles

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sanos (22 hindúes y 66 noruegos), además de 7 controles enfermos. La prueba ELISA demostró ser sensible, ya que fue positiva en los 22 pacientes con tuberculosis intestinal. Su especificidad fue de 84,6 % en los controles hindúes y de 97 % en los controles noruegos. La prueba es de fácil realización y puede ser recomendada para el diagnóstico serológico de la tuberculosis intestinal.

Introduction

The presence of circulating antibodies to *Mycobacterium tuberculosis* in the blood has been used as a diagnostic marker for tuberculosis [1–3]. A number of serological tests using different mycobacterial antigens have been used to distinguish acute, subacute and past tuberculous infections [4–6]. Soluble antigen fluorescent antibody (SAFA) test [7] has been shown to be specifically related to the presence of active intestinal tuberculosis. However, more emphasis is being laid on the diagnosis of tuberculosis by enzyme linked immunosorbant assay (ELISA) [8, 9] which has been found to be a simple and sensitive method for the immunodiagnosis of pulmonary tuberculosis [10, 12]. Most ELISA systems use anti-immunoglobulins as carrier for enzymes. These immunoglobulins may not be class specific. An enzyme linked protein-A ELISA system has recently been used for detection of circulating antibodies to amoebic antigen in patients with amoebic liver abscess [13]. Protein-A with its extraordinary affinity for Fc regions of IgG subclasses 1, 2 and 4 has ability to detect most of the IgG antibodies. In this report we have described an ELISA system for the detection of antibodies to *M. tuberculosis* in patients with intestinal tuberculosis, using enzyme linked protein-A.

Materials and methods

The serological studies for antibodies to *M. tuberculosis* by the technique of enzyme linked protein-A ELISA were carried out on sera from 22 patients with intestinal tuberculosis, 7 patients with other intestinal disease, 22 healthy control subjects from India and 66 healthy control subjects from Norway.

Criteria for selection of subjects

Intestinal tuberculosis: The diagnosis of tuberculosis was made on the basis of positive bacteriology and/or histopathology. The tissue was obtained either through surgery or colonoscopy [14]. All patients were adults and had had vaccinations for BCG long ago.

Healthy controls: (Indian). This group included age and sex matched healthy subjects of different socioeconomic status. Most of them were employees of the All India Institute of Medical Sciences. There was no recent or past history of tuberculosis in these subjects and all had had vaccinations for BCG long ago.

Diseased controls: Seven suspected subjects did not prove to have intestinal tuberculosis and were grouped as diseased controls. This group included patients with sprue (1), lymphoma (2), gastric carcinoma (2), Budd-Chiari syndrome (1) and Crohn's disease (1).

Healthy controls from Norway: This group included male and female students and healthy blood donors of different socioeconomic status from the University of Bergen. The samples were received through courtesy of Claus Ola Solberg and Bjerne Bjorvatn, University of Bergen. There was no recent or past history of tuberculosis among these subjects.

Table I. Number of samples positive for antibodies to *M. tuberculosis* in different groups.

Group	No. tested	No. positive	% Positivity
Intestinal tuberculosis	22	22	100.0
Normal healthy controls	22	3	13.6
Normal healthy controls from Norway	66	2	3.0
Diseased controls	7	3	42.9

Assay

All sera were stored at -20°C until tested in dilutions of 1:50 and further in two fold dilutions in positive samples for antibodies to *M. tuberculosis*. The ELISA test was performed as detailed below. After activation with 0.25 % glutaraldehyde for 1 h, the 96 wells 'Nunc' Polyvinyl microtitre plates were precoated with 50 μl of *M. tuberculosis* H₃₇Ra antigen in a concentration of 0.1 $\mu\text{g}/\text{ml}$ in carbonate buffer 0.1 M, pH 9.5 for overnight at room temperature, usually 18–20 hours. After washing with phosphate buffer saline (PBS) containing 0.05 % tween (PBS-T), post coating was carried out with 200 μl of 0.5 % gelatin in PBS-T for 1 h at 37°C . The samples to be tested for antibodies to *M. tuberculosis* and the positive and negative controls were diluted 1:50 in PBS-T and 50 μl added to the wells on plate and the plates incubated for 90 min at 37°C . The plates were washed and incubated for 90 min at 37°C with 100 μl of 1:2000 dilution of the protein-A-HRPO conjugate in PBS-T containing 20 % fetal calf sera (FCS). 100 μl of the freshly prepared substrate (orthophenylene diamine (OPD) 4 mg/10 ml of citrate phosphate buffer pH 5.0, made by mixing 98.6 parts of 0.1 M citric acid and 101.4 parts of 0.2 M disodium orthophosphate and containing final concentration of 0.006 % hydrogen peroxide) was added to each well in the dark at room temperature and after 5–30 min, the reaction was stopped by addition of 100 μl of 4 M sulphuric acid. The optical density (OD) was measured at 492 nm in a Dynatec micro-ELISA mini reader.

The test was considered positive with OD of 0.214 or above. This cut off value was obtained by taking 2 S.D. (2×0.028) above the mean value (0.158) in healthy subjects.

Statistics: The test of proportion was applied to test for the difference between the groups.

Results

The results of enzyme linked protein-A ELISA test are presented in Table I and Figure 1. Sixty four of the 66 healthy controls from Norway were negative at dilution of 1:50, while two were positive at this dilution. Among the 22 healthy Indian controls, three showed the presence of antibodies to *M. tuberculosis* at a dilution of 1:50 or above while the rest were negative at 1:50 dilution. However, all the patients with intestinal tuberculosis were positive at dilution of 1:50 or above. Among seven subjects of the diseased control group, ELISA test was positive for *M. tuberculosis* in three, one each with carcinoma of stomach, Budd-Chiari syndrome and Crohn's disease.

Discussion

Among the various techniques used to detect antibody response to mycobacteria in tuberculosis, the use of ELISA technique to measure antibodies to *M. tuberculosis* has

provided increased sensitivity and specificity [2, 11]. The protein used as carriers for enzyme has been anti-immunoglobulin, which may not be monospecific. A stable protein, protein-A from *Staphylococcus aureus*, which is a single polypeptide of molecular weight 42 000, has been found to label easily with HRPO [18] and has strong affinity for Fc regions of IgG. In the present study, HRPO-conjugated protein-A has been used to detect IgG antibodies to *M. tuberculosis*.

Enzyme linked protein-A test was positive for antibodies to *M. tuberculosis* in 1:50 titre and above in 3 of the 22 normal healthy controls but in the remaining 19 subjects, the titre was less than 1:50 or the test was completely negative. However, in patients with intestinal tuberculosis, the antibodies to *M. tuberculosis* were found to be positive in 100% of the patients in a dilution of 1:50 or above. If we consider 1:50 dilution as the cut off point for a positive test, the sensitivity (percentage of positive tests among patients) of the ELISA test would be 100% and specificity (percentage of negative tests among controls) would be 85 % for the diagnosis of patients having antibodies to *M. tuberculosis*. However, the specificity of the test was raised to 97 % where only two of the 66 healthy controls from students and blood donors from Norway were positive for antibodies to *M. tuberculosis*. This group served as reference in comparison to our own normal healthy controls where 75 % of them were exposed to *Mycobacterium tuberculosis* bacilli as revealed by the tuberculin test [19]. However, the difference between two healthy control groups was not significant.

In three of seven diseased controls ELISA was positive for *M. tuberculosis* antibodies although there was no histological evidence of tuberculosis. They included one patient each with carcinoma of the stomach, Crohn's disease and the Budd Chiari syndrome. As *Mycobacterium tuberculosis* is responsible for a number of other clinical conditions such as pulmonary tuberculosis, genitourinary tuberculosis, bone and joint tuberculosis, tuberculous meningitis, tuberculosis of the brain etc. and its antibodies are detected for long periods, the positivity of antibodies to *M. tuberculosis* in non-tuberculous intestinal diseases may be explained by the presence of latent, inactive or subclinical infections [20]. The difference between intestinal tuberculosis group and diseased control group was significant ($p < 0.01$).

The possibility of cross reactivity among antigens of mycobacteria, fungi, gram positive and gram negative infections [21] is not ruled out. The mycobacterial saline extract antigen used for ELISA test was found to be the most suitable antigen for SAFA test [22] in discriminating between control and tuberculous subjects, as the incidence of non-specific or natural antibodies in normal sera has been shown to vary [23] according to the preparation of test antigen and choice of immunological procedures. The present protein-A ELISA system detects all the IgG antibodies directed against this purified saline extract antigen and is sensitive enough to detect very small amounts of antibodies, thereby raising the chances of detecting normal healthy controls with subclinical infection or past infection due to *M. tuberculosis*, at the cost of specificity of the test.

For specific diagnosis of any clinical variety of tuberculous infection, clinical observations must be supported by positive serology or pathology or both. The present enzyme linked protein-A ELISA system is highly sensitive for sero-detection of IgG antibodies against *M. tuberculosis* and it may be used to help with the diagnosis of intestinal tuberculosis.

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