Jpn. J. Med. Sci. Biol., 50, 1 - 7, 1997.

DETECTION OF ANTIBODIES AGAINST CELL WALL-ASSOCIATED ANTIGENS OF *MYCOBACTERIUM TUBERCULOSIS* (SIHV) BY AN ENZYME LINKED IMMUNOSORBENT ASSAY

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(Received July 18, 1996. Accepted January 10, 1997)

SUMMARY: We have used the cell wall-associated proteins of *Mycobacterium tuberculosis* (SIHV strain) as antigens in the serodiagnosis of pulmonary tuberculosis. Sera from 19 relapsed and 46 newly diagnosed cases of pulmonary tuberculosis and 21 healthy individuals were tested against the cell wall-associated proteins of *M. tuberculosis* (SIHV) by an ELISA technique. The results showed 92% and 100% positive titers in new and relapsed cases of tuberculosis, respectively. Control sera analyzed exhibited a negativity of 90%. Cell wall-associated proteins of *M. tuberculosis* were found to be useful in serodiagnosis of pulmonary tuberculosis in clear distinction from healthy subjects.

INTRODUCTION

Tuberculosis, caused by infection of *Mycobacterium tuberculosis*, is still a world health problem. According to the WHO estimation, there are 8 million new cases and 3 million deaths resulting from tuberculosis every year (1). Diagnosis of tuberculosis by the conventional culture method is time consuming and the diagnostic test has low specificity and sensitivity. Early diagnosis of tuberculosis would be of immense help in the treatment of tuberculosis. Therefore, a serological method, particularly one based on an enzyme linked immunosorbent assay (ELISA) for the measurement of antibodies to mycobacterial antigens seem to be

an appropriate approach to this problem. In the earlier attempts there were some differences in the ELISA methodology (2-5) and the major differences were in the antigens used to detect antibodies in patients and control sera. The main type of antigen preparations studied were purified antigens, including proteins and lipids and more commonly antigen mixtures adopted from skin test preparations. Serodiagnosis of pulmonary tuberculosis by ELISA with purified and non-purified antigens have been reported (6-8). Particular attention has been given to proteins associated with the mycobacterial cell wall since it is potentially important in protective immunity and in immunopathological events that occur during mycobacterial infection (9,10).

In the present study, we isolated the cell wall-associated proteins of South Indian strain of *Mycobacterium tuberculosis* (SIHV) and used them as antigens to detect antibodies in newly diagnosed and relapsed cases of tuberculosis.

MATERIALS AND METHODS

Patients: Sera from 65 individuals with pulmonary tuberculosis were collected from the outpatient department of Sri Ramachandra Medical College, Madras. Diagnosis was established by smear and/or culture of sputum. Among the 65 individuals with tuberculosis, 19 were relapsed infection patients and 46 new cases. Control sera were obtained randomly from 21 healthy individuals. All the sera were complement inactivated by heating at 58 C for 20 min and stored at -70 C.

Preparation of antigens: *M. tuberculosis* SIHV obtained from Tuberculosis Research Center, Madras, was grown in Middlebrook 7H9 medium at 37 C for 4 weeks and harvested for the preparation of cell wall-associated proteins as described before with minor modifications (11). Cells were extracted twice with $\text{CHCl}_2/\text{CH}_3\text{OH}$ (2:1) at 60 C to remove lipids. Delipidated cells were rehydrated in 10 ml dd H_2O before the sonication. Cells were then washed thrice with phosphate buffered saline (PBS; 10 mM sodium phosphate, 0.14 M NaCl, pH 7.2), re-suspended in Tris-buffered saline (110 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 4 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated for 15 min in 2.5 min bursts on ice. The sonic extract was layered on a discontinuous sucrose gradient composed of 1.5 ml of each 60, 45, and 30% sucrose containing 4 mM PMSF and then centrifuged at $100,000 \times g$ for 2 hr at 4 C. The fraction containing cell wall-associated proteins was collected from the interphase between 30 and 45% sucrose and washed thrice with PBS. After washing thrice with PBS,

the isolated cell walls were extracted with 2% SDS at 60 C to remove the soluble proteins.

Enzyme linked immunosorbent assay: The solution of antigen (30 µg/ml) was coated to polystyrene microtitration plates (Nunc, Roskilde, Denmark) by incubating at 37 C for 1 hr. The plates were then washed with PBS, pH 7.2, containing 0.05% Tween 20 (PBS/T) for 10 min. All sera were diluted to 1:100 in PBS/T containing 0.5% bovine serum albumin and 200 µl volumes of each were added to microwells in duplicate. After incubating at room temperature for 1 hr, the plates were again washed for 10 min with PBS/T and 200 µl of 1:1,000 diluted goat anti-human IgG-peroxidase conjugate was added to each well. The plate was incubated at 37 C for 1 hr and washed with five changes of PBS/T for 20 min. After thorough washing, orthophenylenediamine substrate (40 mg/ml) of phosphate citrate buffer, pH 5.0, containing 40 µl of 30% H₂O₂ was added in equal amounts of 200 µl to each well and the reaction was allowed to proceed for 25 min at room temperature. The reaction was terminated by adding 50 µl of 1 N H₂SO₄ to each well and the readings were taken on an ELISA reader (Dynatech, McLean, VA) at 492 nm. The results were expressed as "antibody activity". This was mean A₄₉₂ of each test serum multiplied by 100. Antibody activity >50 was taken as a positive result.

RESULTS

Figure one shows the antibody activities as measured by the ELISA method by using the cell wall-associated proteins of *M. tuberculosis*. All sera control subjects with an exception of two cases gave negative results showing a negative titer value in 90%. Of new 46 cases of pulmonary tuberculosis, 42 showed positive results with a titer value in 92%, whereas the sera from relapsed cases showed a positive titer in 100%. Among the positive sera, a low antibody titer value was observed in 15 new and three relapsed cases.

Fig. 1. Scatterogram showing distribution of antibody activities in healthy controls and new and relapsed pulmonary tuberculosis patients. Each point represents one patient serum. Antibody activity of >50 was taken as a positive titer.

Cut off value = control mean + 2 SD. Statistically significant difference between the values of control and patients was designated * $P < 0.001$.

DISCUSSION

Cell wall-associated proteins are of fundamental importance to the pathogenesis of bacterial infection. The unique adjuvant activity of mycobacteria has long been known as a component feature of cell walls (12,13). Furthermore, induction of protective immunity against *M. tuberculosis* can be accomplished by immunization with cell wall components (14,15). Significant advances have been made in biochemical characterization of some of the key components responsible for the immunogenicity of mycobacterial cell walls (11,16). It is generally accepted that infection with *M. tuberculosis* often induces a humoral response which can be detected and measured with mycobacterial antigens (17). In this context,

it is noteworthy to measure the antibody activities in tuberculosis patients against cell wall-associated antigens to find whether it could be used as a simple, fast and reliable means of diagnosis of *M. tuberculosis* infection in pulmonary tuberculosis patients. Our data show the use of cell wall-associated antigens of *M. tuberculosis* by ELISA with high titer value in identifying new and relapsed cases of pulmonary tuberculosis (92% and 100%, respectively) in clear distinction from the control sera. It is also interesting to note that a few of the control sera were found to be positive, probably because they were from an area where many types of mycobacterial infection are endemic and BCG vaccination is widespread.

In the present study, the low titer antibody activities and negative results observed among some of the tuberculosis patients are stated to be due to several reasons. According to the hypothesis (18) based on existence of an immunologic spectrum where at one extreme tuberculosis is localized to confined loci with well-developed cell-mediated immunity but little or no detectable antibody, and at the other end there is disseminated disease with poor cell-mediated immunity but with high antibody levels. Alternatively, it is also reported that chronic stimulation of antibody synthesis resulting in progressive production of low avidity antibody and formation of circulating immune complexes of the more reactive antibodies with the relapsed antigen resulting in a considerable reduction of antibody (5). Suspected cases of tuberculosis patients who have low antibody titers should therefore, be followed up and, if possible, investigated for circulating mycobacterial antigens. In conclusion, the ELISA test using cell wall-associated antigens used in the present study was found to be useful in the serodiagnosis of cases with pulmonary tuberculosis. However, further study is warranted to test the efficiency of the assay with a large number of tuberculosis patients and also with a great variety of serum specimens, especially from patients infected with other species of mycobacteria.

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