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Rapid Screening Test for Tuberculosis Using a 38-kDa Antigen From *Mycobacterium tuberculosis*

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A screening test for the diagnosis of tuberculosis by immunodot (IDt) is described, using an antigen of *Mycobacterium tuberculosis,* namely, a 38-kDa glycoprotein which has shown great specificity in previous serologic analyses. The test was used to examine 28 sera from patients with lung tuberculosis. Of these, 85% were positive by micro-ELISA and by the IDt test herein described. Control sera from healthy subjects (n=20) gave negative results for ELISA and for IDt, which indicates that the screening test is highly specific. The test is easy to handle and requires no equipment and is therefore particularly useful for field studies. J. Clin. Lab. Anal. 12:126–129, 1998. © 1998 Wiley-Liss, Inc.

Key words: immunodot; ELISA; diagnosis; acid-fast bacilli; lung infection; serology

INTRODUCTION

Tuberculosis is a worldwide health problem and possibly one of the most common causes of death by an infectious agent especially in developing countries (1). It also accounts for a large proportion of deaths in HIV-coinfected AIDS patients (2). For this reason, diagnosis of the *Mycobacterium tuberculosis* infection is of great importance for public health. The diagnosis of lung tuberculosis is based on the demonstration of the bacillus in sputum smears or cultures (3). The presence of the tuberculosis bacillus at sites other than the lung has prompted the search for reliable serological methods which can additionally be useful for epidemiological studies. At present, the available screening method used to detect infection is the PPD skin test. This test has limited specificity and sensibility, especially with previous sensitization to environmental mycobacteria (4). Recently, a protein of 38-kDa specific to the *M. tuberculosis* complex was reported (5) which has also proved to be a potent immunogen (4). Furthermore, evidence has been set forth of its immunologic activity using a recombinant antigen, which suggests that it should be considered for use in skin testing and serological tests for the detection of tuberculosis (6). Given the prevalence of tuberculosis in developing countries, infection screening and epidemiological studies are difficult when laboratory facilities are not readily available. The present communication describes the evaluation of a rapid screening test for tuberculosis by immunodot (IDt) based on the detection (which does not require equipment) of antibodies against the 38-kDa antigen.

MATERIALS AND METHODS

Supernatants From *M. tuberculosis* Cultures

M. tuberculosis strain H37Rv was grown in the synthetic medium of Proskawer and Beck, modified by Youmans (7). After 4–6 weeks of culture, the medium was filtered and 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) was added. Protein was estimated with Folin phenol reagent (8).

Sera from 28 adults (14 males, 14 females; age range: 21–80 yrs.) with pulmonary tuberculosis were obtained from the Hospital Miguel Oraá, Guanare, Venezuela, and from the Centro Universitario de Ciencias Exactas e Ingenierías, Universidad de Guadalajara, Jalisco, México. Diagnosis was established by smear and/or culture of sputum. Information about BCG vaccination, extent of the disease, length of treatment, if any, and tuberculin reactivity were not obtained.

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Control sera were obtained from 20 healthy residents of the same countries.

SDS-PAGE Electrophoresis and Immunoblot

A discontinuous buffer system (9) was used with a 3% stacking gel and a 10% separating gel. Gels were run under nonreducing conditions. For immunoblot, gels were mounted in a transfer apparatus (Idea Scientific Co., Corvallis, OR) and transferred to nitrocellulose paper sheets (Bio-Rad Laboratories, Richmond, CA) by the method of Towbin et al. (10). The 38-kDa protein was identified by means of monoclonal antibodies and revealed with a peroxidase-labelled goat-antimouse IgG antiserum (Cappel Laboratories, Cochranville, PA), as previously described [11].

Purification of the 38-kDa Antigen

Isolation of the immunodominant 38-kDa antigen identified by immunoblot was carried out with minor modifications [11]. Briefly, ammonium sulfate was added to the supernatants of culture filtrates (0.5 g/ml). The precipitate was dialyzed and its pH adjusted to 4.0. The supernatant of this precipitation was adjusted to pH 8.0 and sufficient alcohol added to give a 30% alcohol concentration. The supernatant was brought to pH 4.0. Finally, to the obtained supernatant, alcohol was added to give a 70% concentration. The various precipitates and the last supernatant were analyzed by SDS-PAGE and a preparative gel of the fraction containing the 38-kDa antigen was done. The desired band was located by staining the gel with 4 M sodium acetate. Thereafter, a horizontal strip containing the antigen band was excised and treated with a solution (30 ml) containing 6 M urea, 50 mM phosphate buffer, pH 7, and 20 mM EDTA at 37°C for 18 h. Afterwards, the extract obtained was filtered, dialyzed against water, and finally liophylized (12).

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA assay was performed as previously described (11) with minor modifications. Each well in a polyvinylchloride microtiter plate was coated with the 38-kDa antigen (0.5 μg/50 μl) in carbonate/bicarbonate buffer, pH 9.6 at 37°C for 5 h. The plates were then washed three times in phosphate-buffered saline (PBS), pH 7.4 with 0.1% Tween 20 (Tw) (Sigma) and 100 μl of 1% bovine serum albumin (BSA) diluted in PBS was added to the wells; they were then incubated for 30 min at 37°C to minimize nonspecific reactivity. The plates were again washed three times with PBS-Tw, and 50 μl of the test sera diluted 1:1000 were then added to the wells and incubated for 1 h at 37°C. Subsequent to a three times wash in PBS-Tw, 50 μl/well peroxidase-conjugated affinity-purified goat anti-human IgG (Fc specific) (Sigma) at 1:1,600 dilution in PBS-Tw-BSA was added and incubated for 1 h at 37°C. After another set of washes, 50 μl of 0.1 M phosphate-citrate buffer, pH 5.0, containing 10 mg *O*-phenylenediamine dihydrochloride (Sigma) and 10 μl of 30% H_2O_2 per 25 ml, was added and incubated for 30 min at room temperature. The enzymatic reaction was stopped by the addition of 50 μl of 2.5 N HCl. The wells were scanned with an STL 210 ELISA reader (Kontron, S.A., Madrid, Spain). Optical densities (OD) were read at 492 nm after 15 min.

IDt Assay

The IDt was performed by dotting 2 μl of the 38-kDa *M. tuberculosis* antigen preparation (2 μg) onto nitrocellulose strips and allowing to dry for 30 min. The strips were then soaked in PBS with 0.3% Tw for 30 min and stored dry until use. For each assay, strips were placed in 2 ml of a 1:1,000 dilution of each test serum for 10 min, washed 3 times with PBS-Tw and incubated with 2 ml of a 1:1,000 dilution of peroxidase-conjugated affinity-purified goat anti-human IgG (Fc specific) (Sigma) for 10 min. After washing as above, the strips were incubated with 3.3´ diaminobenzidine tetrahydrochloride (Sigma) upon addition of 30 μl of 30% H_2O_2 in 10 ml of 0.1 M Tris buffer, pH 8.3. Finally, the reaction was stopped by washing the strips with distilled water not more than 10 min later. All incubations were carried out at room temperature, mixing by hand periodically. In the laboratory a rack tray on a rocking platform can be used.

RESULTS

The studies carried out with the 28 sera of patients with pulmonary tuberculosis showed the presence of antibodies to the 38-kDa antigen from *M. tuberculosis*, as determined by the micro-ELISA test. In patients, mean OD ± SD was 0.656 ± 0.54, and in controls 0.135 ± 0.030. The antibody titers in the sera of patients with pulmonary tuberculosis differed significantly ($P < 0.0001$) from those of sera obtained from healthy control individuals (Fig. 1). In order to estimate the sensitivity (fraction of control sera below threshold), three SD above the mean OD of the control sera was chosen as threshold value. The cut-off value for healthy individuals at the 1:1,000 dilution tested was calculated to be 0.225. At this dilution, 24 out of the 28 serum samples exhibited OD readings above the cut-off value. On the other hand, none of the serum samples from the control group exhibited OD readings above the cut-off value. Thus, the sensitivity of the ELISA was 0.85 and the specificity 1.0.

The IDt test performed in the 28 sera of tuberculous patients was positive in 24 out of the 28 sera assayed (Fig. 2). Only individuals with an ELISA OD value above the calculated cut-off value gave a positive reaction with the 38-kDa antigen. On the other hand, the 20 sera obtained from healthy individuals were negative by the IDt assay. Therefore, comparison of the IDt results with the standard ELISA assay showed good correlation (Fig. 1).

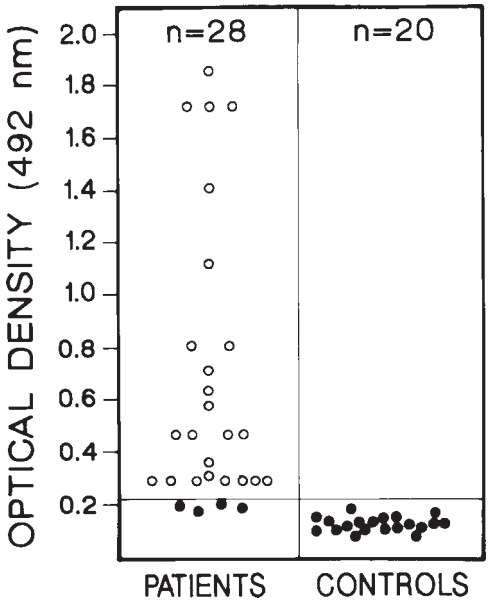

Fig. 1. Serum antibody titers against the 38-kDa protein from *M. tuberculosis* by ELISA in pulmonary tuberculosis patients and in healthy control individuals. The horizontal line represents the cut-off value of 3 SD above the mean OD of control sera (0.229). Closed circles indicate a negative IDt reaction.

DISCUSSION

The present report describes a simple and rapid technique of IDt for screening of infections by *M. tuberculosis*, using a species-specific antigen of 38-kDa. The IDt produced results similar to those obtained by ELISA. Negative results of tuberculosis patient sera observed with both techniques are very likely due to lack of antibody response in affected individuals, rather than to false negative reactions. This has also been observed by other authors when determining antibodies in patients with the disease (13,14). Noteworthy is the fact that no positive reactions were obtained with sera from healthy subjects.

The bacteriologic diagnosis of lung tuberculosis fails in 25% of the cases (15) partly due to patients with incipient disease or to children who are generally poor sputum producers. Besides, in samples from pulmonary origin, it is frequent to isolate atypical mycobacteria of saprophytic character. The development of a reliable immunodiagnostic test for tuberculosis has been impeded by cross-reactions with other microorganisms (16–18) and by the sensitization of the general population due to resolved exposure to *M. tuberculosis* or BCG vaccination. Among the mycobacterial molecules, the 38-kDa protein antigen has attracted much interest since it shows high immunogenicity at the B-cell (11) and T-cell (4) levels. At present, the 38-kDa antigen has proved to be an antigen with very high potential for the serological diagnosis of tuberculosis (4, 11, 19) since it is highly specific in recognizing patients with lung tuberculosis and does not react with normal sera (11).

In summary, we have developed an IDt test for the screening of tuberculosis using a 38-kDa antigen from *M. tuberculosis* that shows high specificity and sensibility and permits the screening of a large number of samples in a short period with no need of equipment.

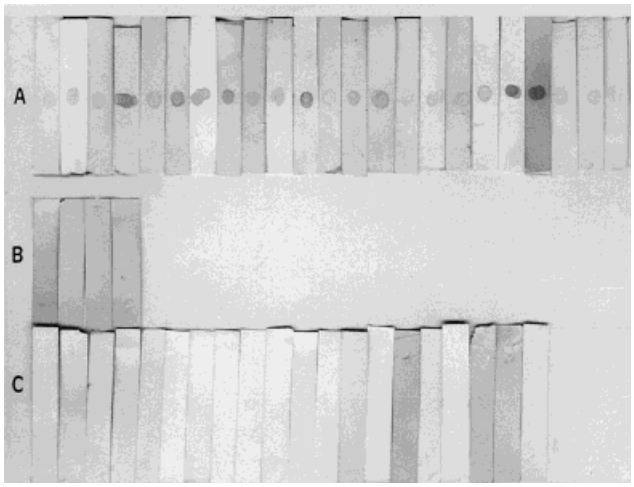

Fig. 2. Determination of the presence or absence of antibodies to the 38-kDa protein from *M. tuberculosis* by the IDt assay in pulmonary tuberculosis patients (**A** and **B**) and in healthy control individuals (**C**).

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