Enzyme Immunoassay to Detect Antituberculous Glycolipid Antigen (Anti-TBGL Antigen) Antibodies in Serum for Diagnosis of Tuberculosis

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We report the development of an EIA specific for antituberculosis antibody in human serum for the clinical evaluation of tuberculosis. We developed a TLC immunostaining method to detect specific antigens for antibodies in the serum of patients with tuberculosis. The detected specific antigens, TDM and specific glycolipid fraction, were individually purified from *M. tuberculosis* H37Rv by column chromatography. The two purified fractions were mixed and the mixture, termed TBGL antigen, was applied to an enzyme immunoassay suitable for the measurement of

antituberculosis antibodies in serum. This EIA meets all the requirements of routine clinical assay in terms of sensitivity (detection limit : 0.125 U/ml), reproducibility (total CV : 3.3–6.0%), accuracy (recovery: 96–105%), simplicity and rapidity (<2.5 h). Clinical validation of the assay was confirmed by the measurement of the anti-tuberculosis antibody in the serum of normal subjects and patients with pulmonary tuberculosis. The EIA tested in this study showed a high serodiagnostic discriminating power (90% sensitivity and 98% specificity). J. Clin. Lab. Anal. 11:140–145, 1997. © 1997 Wiley-Liss, Inc.

Key words: *M.tuberculosis*; pulmonary tuberculosis; serodiagnosis; cord factor; ELISA

INTRODUCTION

Mycobacterial infections have long been known to be important in human beings and other animals. There is also current interest in mycobacteriosis associated with the acquired immunodeficiency syndrome (1). The diagnosis of pulmonary tuberculosis especially is of clinical importance and the establishment of a simple and rapid method to diagnose mycobacterial infection is expected. The diagnosis of mycobacterial infections is still based on finding bacilli in the sputum of patients either by smear-staining such as Ziehl-Neelsen or by isolation in cultures of sputum. However, such conventional methods are insufficient for clinical needs, i.e., the detection rate is low in the smear test and isolation by culture requires 4–6 weeks. Serodiagnosis is useful for the diagnosis of infectious disease, but has not been successful for tuberculosis. The enzyme immunoassay (EIA) provide a rapid and simple means for the measurement of antibodies against pathogenic organisms in serum. We previously developed an EIA method in which TDM (trehalose-6,6'-dimycolate) purified by TLC (thin-layer chromatography) was used as the specific antigen (2). This EIA method is useful for the rapid serodiagnosis

of mycobacterial infections, but there are some problems when applying it for widespread clinical use. For instance, it is difficult to obtain a supply of the antigen with constant quality and quantity by the TLC method, serum samples have to be diluted 1/160, which is a time-consuming job, and some reagents have to be prepared for every use, and to obtain reproducible and accurate results, the assay condition has to be rigorously controlled.

In this report, we developed a TLC immunostaining method to detect specific glycolipid antigens for antibodies in the serum of patients with tuberculosis, and the specific antigens were purified by a column chromatography method and also applied to EIA. The EIA, suitable for the measurement of the antituberculosis antibody in serum, which met the requirements of routine clinical assay, was developed using the purified antigens.

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MATERIALS AND METHODS

Chemicals

Reagents were obtained from the following sources; TMBZ (3,3',5,5'-tetramethylbenzidine) solution (TMBBlue, TSI Co., Milford, MA), BSA (bovine serum albumin, Miles, Kankakee, IL); HRP (horseradish peroxidase, Toyobo Co., Osaka, Japan), antihuman IgG rabbit IgG (Dako Japan Co., Kyoto, Japan), N-succinimidyl-6-maleimidohehexanoate (Dojindo Laboratories, Kumamoto, Japan), thimerosal (Sigma Chemical Co., St. Louis, MO), fetal calf serum (GIBCO, Grand Island, NY). All other reagents were of analytical grade.

Bacteria

M. tuberculosis H37Rv were grown on middlebrook medium at 37°C. Grown cells were harvested after 3 weeks and were autoclaved at 121°C for 15 min and then centrifuged at $1500 \times G$ for 15 min. Heat-killed, packed bacteria of *M. tuberculosis* H37Rv were collected and then stored at -80°C until use.

Buffer

The regularly used buffers were 10 mM sodium phosphate buffer (pH 7.4) containing 0.1% (w/v) BSA and 140 mM NaCl; dilution buffer, 10 mM sodium phosphate buffer (pH 7.4) containing 0.05% (v/v) Tween 20 and 140 mM NaCl; washing buffer, 10 mM sodium phosphate buffer (pH 7.4) containing 140 mM NaCl; PBS, 50 mM sodium citrate buffer (pH 5.0) containing 0.015% H_2O_2 ; staining buffer.

Clinical Samples

Serum samples from patients of 45 men (ages 20–82) and 16 women (ages 20–85) with tuberculosis were collected from the Toneyama National Hospital. The diagnosis of patients with tuberculosis were established by the following findings: (1) chest X-ray lesion severity, (2) smear-positive sputum for acid-fast bacilli or culture-positive sputum for *M. tuberculosis*.

Serum samples of normal subjects were obtained from 28 men (ages 24–38) and 33 women (ages 20–29) of laboratory workers of Kyowa Medex. All normal subjects were healthy and were known not to suffer from tuberculosis.

Antigen Purification

Heat-killed, packed bacteria of Mycobacterium were suspended in chloroform-methanol (2:1, v/v) and disrupted by ultrasonication. The suspension was shaken with water (5:1, v/v) and allowed to stand. The resulting chloroform layer was recovered and evaporated, and then the residue was resuspended in a small volume of chloroform-methanol (2:1, v/v).

The antigens were purified from the extract of bacteria by column chromatography (8 cm $\times$ 60 cm) with silica gel (Wakogel C-200, Wako Pure Chemical Industries, Osaka,

Japan). Purification of TDM from the extract of bacteria by the TLC method was performed as previously reported (2). The purified antigens were dried in a desiccator under vacuum and were weighed.

Thin-Layer Chromatography Analysis

The samples were separated by TLC on a silica gel plate (HPTLC silica gel 60, E. Merck, Darmstadt, Germany) with chloroform-methanol-acetone acetic acid (90:10:6:1, v/v), or chloroform-methanol-0.2% $CaCl_2$ /water (65:35:7, v/v) as solvent, or two-dimensional TLC on a silica gel plate. First solvent system was chloroform-methanol-acetone-acetic acid (90:10:6:1, v/v), and the second solvent system was chloroform-methanol-water (90:10:1, v/v). The separated bands were made visible by primulin, orcinol- H_2SO_4 spray and heating at 135°C for 5 min.

TLC-Immunostaining

After separation of the antigens on the silica plates, the plates were incubated with 0.4% (w/v) polyisobutyl-methacrylic acid/chloroform-n-hexane (1:5, v/v) for 30 sec at room temperature and allowed to dry. Sample serum diluted 1/100 with dilution buffer was put on the plates, and the plates were then incubated overnight at 4°C under moisturized conditions. After incubation, the plates were washed five times with washing buffer and incubated with HRP labeled antihuman immunoglobulin at 4°C overnight. After washing five times with washing buffer, MCDP (3 μ g/ml in staining buffer) was put on the plates, and the plates were incubated for 30 min at room temperature in the dark. To stop the enzyme reaction, the plates were washed with water and the stained bands were observed.

Anti-human IgG Rabbit Fab'-HRP Conjugate

Antihuman IgG rabbit F(ab')₂ was prepared by digestion of antihuman IgG (gamma chains) rabbit IgG with pepsin by the method of Ishikawa (3). Antihuman IgG rabbit Fab' was prepared by reduction of F(ab')₂ with 2-mercaptoethylamine and was conjugated to HRP using N-succinimidyl 6-maleimidohehexanoate according to a previously described method (4). The produced conjugate was diluted to 10 ng/ml with 0.1 M phosphate buffer (pH 7.0) containing 0.1% BSA and 0.002% thimerosal, divided into 11 each in a brown polystyrene tube, and stored at 4°C until use. Under this storage condition, the conjugate was stable for at least 1 year and usable without preparation.

Standard

Five patients' serum were pooled for the primary standard in the enzyme immunoassay. These patients were bacteriologically confirmed to have mycobacteriosis by use of both smear staining and cultivation of sputum, and in all the pa-

tients' serum, the existence of specific antibody was recognized by TLC immunostaining. The concentration of specific antibody in this pooled serum was arbitrarily determined to be 64 U/ml for convenience. A secondary standard was prepared from pooled high titer patients' serum, and the antibody concentration was determined by EIA with the primary standard. The secondary standard was diluted to 64 U/ml with FCS containing 0.05% NaN₃, and each practical calibrator for the enzyme immunoassay was prepared from the 64 U/ml secondary standard by a serial dilution with FCS containing 0.05% NaN₃, divided into 0.5 ml each in a brown stoppered polystyrene tube, and stored at 4°C until use. Under this storage condition, the calibrator was stable for at least 1 year.

Antigen-Coated Plate

Polystyrene 96-well microtiter plates (Polysorp immunoplates, NUNC, Roskilde, Denmark) were sensitized by coating them with a 25 µl/well of the antigen solution (10 µg/ml in n-hexane), placed at room temperature, and allowed to dry. The sensitized plates were sealed in an aluminum coated pack with a drying agent and stored at 4°C until use. Under this storage condition, the sensitized plates were stable for at least 1 year.

Enzyme Immunoassay

The stored plates were washed three times with PBS and patted dry. All samples, serum or calibrator, were diluted 1/41 with dilution buffer, and 100 µl of the diluted samples were added to each well and the plates were incubated for 60 min at room temperature. After washing five times with washing buffer, antihuman IgG rabbit Fab'-HRP was added to each well and the plates were incubated for 60 min at room temperature. The plates were washed three times with washing buffer and 100 µl of TMBZ solution was added to each well, and then the plates were incubated for 15 min at room temperature. To stop the enzyme reaction, 100 µl of 1M H₂SO₄ was added and the absorbance at 450 nm was measured with a MTP-120 plate reader (Corona Electric Co., Tokyo, Japan).

RESULTS

Antigens

To supply the antigen with constant quality and quantity, TDM was purified from *M. tuberculosis* H37Rv using the column chromatography method. The extract of bacteria was applied to the column equilibrated with chloroform and then eluted with methanol. Fractions (100 ml) of the eluate were collected and all fractions were subjected to TLC-analysis. The fractions containing TDM were pooled, concentrated, applied to the column equilibrated with chloroform-methanol-water (65:25:3, v/v), and eluted with the same solvent. The fractions containing TDM were collected based on the TLC analysis, concentrated, applied to the column equilibrated

with chloroform-methanol (95:5, v/v), and eluted with the same solvent. The TDM fractions were collected based on the TLC analysis. In one run, 1.4 g of the TDM was obtained from 670 g (wet weight) of packed bacteria. The antigen obtained was shown to be homogeneous upon TLC and also two-dimensional TLC (Figure 1A). However, the antigen obtained by the TLC method was found to be contaminated by a trace amount of glycolipids upon analysis with two-dimensional TLC (Fig. 1B).

The TDM purified by the column chromatography method was applied to enzyme immunoassay as an antigen for detection for the antituberculosis antibody. With TDM purified by the TLC method as the antigen, almost all the patients tested were detected (specificity 86%). In contrast, with TDM purified by the column chromatography method, some patients were not detected (see Fig. 6). For the detection of glycolipid antigens specific for antibodies in patients' serum, TDM purified by TLC was separated on silica plates and the plates were reacted with patients' serum and immunostained. Figure 2 shows the reaction of antibodies in various patients or normal subjects. Under this condition, the diversity of the antibody was found among patients and highly hydrophilic components were also reacted with the antibodies, indicating that only TDM purified by the column chromatography method was insufficient to detect the specific antibodies in almost all the patients with tuberculosis. Specific antigens, which are more hydrophilic than TDM and also reacted with patients' serum and did not react with normal serum, were purified by column chromatography. The extract of bacteria was applied to the column equilibrated with chloroform and was eluted with methanol. The fractions containing the desired antigens were pooled based on the TLC immunostaining, concentrated, applied to the column equilibrated with chloroform-methanol-water (65:25:3, v/v), and eluted with the same solvent. The desired fractions were collected based on the TLC immunostaining analysis. In one run, 0.6 g of the antigen was obtained from 670 g (wet weight) of packed bacteria. Purified components (Fig. 1C) were mixed with TDM to reconstruct the antigen purified by TLC and the mixture, termed TBGL antigen (Fig. 1D), was used as the specific antigens of the EIA.

Enzyme Immunoassay for Detection of Antibody Specific for Tuberculosis

An enzyme immunoassay for the detection of antibodies specific for tuberculosis was done using a low-binding, 96-well, flat-bottom microtiter plate as the solid phase, with TBGL antigen purified by column chromatography as the specific antigen, with antihuman IgG Fab'-HRP conjugate as the second antibody, and TMBZ solution as the enzyme substrate. Figure 3 is a typical calibration curve generated by plotting absorbance at 450 nm vs. the logarithm of the specific antibody concentration of each calibrator. Serum samples

Fig. 1. Detection of antigens directed against antibodies in human serum by TLC immunostaining analysis. TLC was performed with chloroform methanol-0.2% CaCl_2 /water (65:35:7, v/v) as solvent. lane 1~7: patients with

tuberculosis, lane N: normal subject. Indicated fractions were collected. In this TLC condition, TDM was fail to be stained because of its large amount and water-repellent character.

Fig. 2. Two dimensional TLC analysis of various antigens. **A**, TDM purified by column chromatography method. **B** TDM purified by TLC method. **C** Glucolipid antigens purified by column chromatography method. **D** TBGL antigen. TDM is indicated by the arrow.

serially diluted gave results close to linearity (Fig. 4), indicating that a quantitative assay was accomplished.

Detection Limit

The detection limit of specific antibodies in serum using the present assay was determined. Human serum containing

Fig. 3. A typical standard curve for the enzyme immunoassay of anti tuberculosis antibody. Each point represents the mean of two measurements.

Fig. 4. Detection limit of anti-tuberculosis antibody by the enzyme immunoassay. Each point represents the mean $\pm$ 2 SD of eight measurements.

8 U/ml specific antibodies was serially diluted and used for testing. A significant difference from 0 U/ml was confirmed by a Student t-test ($n = 5$, $P < 0.05$). Figure 5 shows that 0.125 U/ml specific antibodies could be detected by this assay, indicating that it is of sufficient sensitivity for detection of the antituberculosis antibody (see Fig. 6).

Fig. 5. Dilution curve for patients' serum.

Antigen TDM TDM TBGL TBGL
Method TLC Column Column Column

Fig. 6. Individual concentrations of antibodies against various antigens with EIA. Cutoff value is indicated by the dot line. (○); patients with tuberculosis, (●); normal subjects.

Assay Variation

Assay variation in the present immunoassay was examined using serum samples that contained the specific antibody at three levels (high, middle, low). Table 1 shows that the coefficients of variation for within- and between-assay were 3.3–4.6% and 3.5–6.0% respectively.

To determine the analytical recovery, a standard (64 U/ml) was mixed with the serum samples containing 2.5 U/ml and 8.7 U/ml specific antibodies (1:9). Recoveries of the specific antibodies ranged from 98.0 to 104.5% (2.5 U/ml, $n=3$) and from 96.4 to 104.9% (8.7 U/ml, $n=3$), respectively.

Effect of Temperature and Incubation Time on Assay Results

The results of this EIA was not affected by the change in temperature (20–37°C) and the incubation time (30–120 min), indicating that the present EIA was suitable for clinical use under various assay conditions.

TABLE 1. Within- and Between-Assay Precision

	Within-assay			Between-assay		
n	9	9	9	8	8	8
Mean (U/ml)	6.3	11.3	21.1	6.3	11.2	20.8
CV(%)	4.6	3.3	3.5	6.0	3.8	3.5

Clinical Samples

Clinical validation of the present EIA was confirmed by the measurement of specific antibodies in the serum of normal subjects and patients with pulmonary tuberculosis (Fig. 6). The normal range for EIA was determined to be lower than two standard deviations above the mean of the normal subject values. The present cutoff value was set to 1.7 U/ml. For patients with active pulmonary tuberculosis, the EIA had a sensitivity of 90% and a specificity of 98%.

DISCUSSION

The goal of this study was to purify specific antigens for antituberculosis antibodies by column chromatography from bacterial glycolipids in order to supply them with constant quality and quantity and to develop a highly sensitive EIA for the measurement of the antituberculosis antibody in serum for the diagnosis of pulmonary tuberculosis. Serological diagnosis of mycobacterial infections has been done with various antigens such as protein, polypeptides, polysaccharides, phospholipids, and the components of the cell-wall skeleton (5–9). However, the specificity of the serodiagnosis with these antigens has not been highly satisfactory. An antibody against the cord factor (TDM) has been experimentally produced (10,11), but an antibody against the cord factor was not found in the serum of patients with tuberculosis. We previously reported an EIA method with the cord factor (TDM) purified by TLC as the antigen and that this EIA was useful for the rapid diagnosis of mycobacterial infections (2). The cord factor (TDM) is a cell-wall glycolipid widely found among *Mycobacterium*. In this study, to supply this antigen with constant quality and quantity, the TDM was purified from *M. tuberculosis* H37Rv by column chromatography, and the obtained antigen was shown to be homogeneous. However, with TDM purified by column chromatography, the sensitivity decreased by 68%. In order to reconstruct the TDM purified by TLC and to improve the sensitivity, the homogeneous TDM was mixed with glycolipids, which were more hydrophilic than TDM and also reacted with patients' serum and did not react with normal serum, and the resulting mixture, termed the TBGL antigen, was applied to the enzyme immunoassay as an antigen. We determined that in addition to TDM, the TBGL antigens included at least trehalose-6-mycolate, 2,3-diacyltrehalose, phenolic glycolipid, 2,3,6,6'-tetraacyltrehalose 2'-sulfate, and 2,3,6-triacyl-trehalose. These bands could be made visible by the immunological method but almost invisible by the chemical method on a TLC plate, indicating that the main antigen in the TBGL antigen was TDM.

The present EIA has several advantages: nonspecific binding was reduced due to the use of the Fab'-HRP conjugate,

thus giving high accuracy and reproducibility. The use of the Fab'-HRP conjugate also contributed toward simplifying the dilution procedure of the samples. The Fab'-HRP conjugate was prepared by the method by which highly reproducible results were simply obtained (3). The sensitivity is sufficient (detection limit of 0.125 U/ml) for measuring normal human serum (cutoff point of 1.7% U/ml). The total CV being between 3.3% and 6.0% shows that the precision of this assay is sufficient for measuring the specific antibody in serum. Accuracy, as determined by the recovery of added specific antibody, is high enough to measure serum samples.

The present EIA was easy to handle. This EIA requires only pretreatment of the samples, dilution to 1/41, and all the reagents were not needed for each preparation and were ready for use. The same results were obtained in various assay conditions due to standardization.

In conclusion, this EIA results in a very practical tool for detecting patients who have been exposed to pulmonary tuberculosis and have developed an immune response.

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