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Detection of Lipoarabinomannan as a Diagnostic Test for Tuberculosis

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A coagglutination technique was established for the detection of lipoarabinomannan of *Mycobacterium tuberculosis* in human serum samples and evaluated for its utility in the diagnosis of tuberculosis at the Instituto Nacional de Enfermedades Respiratorias in Mexico City. The test had a sensitivity of 88% in patients with sputum-smear-positive active pulmonary tuberculosis. The sensitivity in patients with active pulmonary tuberculosis negative for acid-fast bacilli in sputum was 67%. Less favorable results were obtained for patients with AIDS and tuberculosis, with a sensitivity of 57%. The specificity in control patients with lung diseases different from tuberculosis and in healthy subjects was 100%. The positive predictive value was 100%, and the negative predictive value for patients with sputum-positive active pulmonary tuberculosis was 97%. The results of this study suggest that the detection of lipoarabinomannan is an accurate test for the diagnosis of pulmonary tuberculosis.

Tuberculosis is still a major worldwide problem, with a prevalence of 30,000,000 active cases, an incidence of 10,000,000 new cases each year, and 3,000,000 deaths annually (5); even developed countries have experienced an increase in the prevalence of tuberculosis as a consequence of the AIDS epidemic (8). The diagnosis of tuberculosis depends on the identification of *Mycobacterium tuberculosis* in clinical samples by preparing acid-fast smears, followed by culture. Direct sputum smears have a sensitivity in pulmonary tuberculosis of 22 to 43% (6, 23). Culture is more sensitive, but the results take weeks and facilities for culture are limited in most of the countries in which the disease is endemic. There are two major approaches to rapid noncultural diagnosis of tuberculosis. One is the detection of mycobacterial antigens in clinical samples, which has been shown to be useful for diagnosis of tuberculous meningitis (11, 30); the other is the use of nucleic acid probes. Polymerase chain reaction technology may be an ideal method for identification of mycobacterial DNA in clinical samples (4, 15). However, at the moment it is a very expensive technology and has technical problems, such as contamination, that require resolution prior to general application in laboratories.

An important indirect diagnostic method is enzyme-linked immunosorbent assay (ELISA) serology, which has been widely explored (12). Several antigens have been used, and the sensitivity for diagnosis has ranged from 70 to 85%.

Recently, we evaluated two different antigens by using ELISA for the serological diagnosis of tuberculosis in our population. We used an *M. tuberculosis* antigen of 30,000 Da; the test had a sensitivity of 70% in patients with pulmonary tuberculosis and a specificity of 100% (29). We also tested lipoarabinomannan (LAM) as an antigen for the ELISA; the test had a sensitivity of 80% in patients with pulmonary tuberculosis and a specificity of 91.5% (28). Since most of the patients with tuberculosis produce antibodies against LAM, we hypothesized that LAM is present in the serum of patients with tuberculosis and that the detection of antigenemia might be useful for diagnosis of tuberculosis. In the present study, we showed that most patients with pulmonary tuberculosis have LAM antigenemia.

MATERIALS AND METHODS

Study groups. Serum samples were obtained from patients seen at the Instituto Nacional de Enfermedades Respiratorias, a national reference hospital for patients with pulmonary diseases in Mexico City. All serum samples were code labelled and kept at −20°C until tested. The classification scheme and characteristics of patients in this study are described in Table 1. All diagnoses of tuberculosis were confirmed by identification of *M. tuberculosis* by culture according to standard methodology (27).

Production of antisera. Antisera to LAM were raised in New Zealand White rabbits by the injection of sonicated *M. tuberculosis*. The sonicate was prepared by growing *M. tuberculosis* H37Rv in 100 ml of Proskauer-Beck-Youmans (PBY) medium; the bacteria were harvested when the bacterial mass covered the surface of the PBY medium (4 to 6 weeks). The bacteria were killed with 10% phenol and sonicated by using 80 cycles per s for 30 min, after which the material was centrifuged at 3,000 rpm for 10 min and the supernatant was dialyzed against phosphate-buffered saline (PBS) overnight. Five rabbits were immunized with this material by using 3 mg of protein per ml intramuscularly once a week for 7 weeks, followed by administration of 1 mg/ml once a week for 9 weeks. After 13 weeks of this immunization regimen, titers of 1:32,000 (by ELISA) against purified LAM were obtained. LAM was a gift from Patrick Brennan, Department of Microbiology, Colorado State University, Ft. Collins. This material was purified by anion-exchange and gel-filtration chromatography in detergent as described by Hunter et al. (16).

Preparation of coagglutination reagents. Coagglutination reagents were prepared by using *Staphylococcus aureus* Cowan I by the method of Edward and Larson (14). Complete coagglutination reagents were prepared by mixing 500 μl of 10% (vol/vol) *S. aureus* and 200 μl of purified immunoglobulin G (IgG) antibody (1.6 mg/ml) to LAM purified by

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TABLE 1. Classification scheme and characteristics of patients

Group	No.	Description
(I) Pulmonary tuberculosis	50	Patients with active pulmonary tuberculosis with positive acid-fast smear and culture of *M. tuberculosis* from sputum.
(II) Pulmonary tuberculosis, negative smear	9	Patients with active pulmonary tuberculosis with negative acid-fast smear and positive culture of *M. tuberculosis* from bronchial washing obtained by bronchoscopy.
(III) Pulmonary tuberculosis and AIDS	21	Patients with active pulmonary tuberculosis and AIDS. Positive culture of *M. tuberculosis*.
(IV) Other pulmonary disease	63	Patients with established diagnosis of pulmonary disease other than tuberculosis. Tuberculosis was excluded in these patients by clinical, radiological, and microbiological evaluation.
(V) Healthy controls	63	Sera were obtained from a blood bank from persons presumed to be healthy.

protein A affinity chromatography. The bacteria and the IgG were incubated for 2 h at room temperature, washed three times with PBS, and resuspended in 1 ml of PBS with 1% bovine serum albumin (Sigma, St. Louis, Mo.). This suspension represented the final coagglutination reagent. As a control, another coagglutination reagent was prepared by using IgG purified from the serum of healthy rabbits.

Treatment of serum samples for concentration of LAM and reduction of nonspecific reactions. The serum samples from patients with tuberculosis and from healthy controls were pretreated by the method described by Doskeland and Berdal (13). Briefly, 100 μl of serum was mixed with 80 μl of EDTA (0.1 M), and this mixture was boiled for 3 min and then centrifuged for 8 min at 10,000 × *g*. The supernatant was precipitated with ethanol at −20°C and centrifuged again at 10,000 × *g* for 8 min. The sediment was suspended in 150 μl of PBS, and this suspension was boiled for 3 min and then centrifuged at 10,000 × *g* for 8 min. The supernatants were precipitated with ethanol a second time as described above and centrifuged at 3,000 × *g* for 10 min at 4°C, and the sediment was resuspended in 100 μl of PBS. This last material was used for agglutination reaction.

Agglutination and test interpretation. For the agglutination test, serial twofold dilutions of the treated sera from 1:2 through 1:16 were prepared in PBS-albumin, and 10 μl of each dilution was mixed with 10 μl of the coagglutination reagent and 50 μl of PBS-albumin in a 15-mm well of an agglutination test slide with 12 wells. On each slide, purified LAM (100, 50, and 25 ng/ml in PBS–1% albumin) was included as a positive control. A negative PBS-albumin control was also included to detect nonspecific agglutination. Each treated serum sample was tested with the anti-LAM reagent and normal immunoglobulin reagent to detect nonspecific agglutination. The agglutination reaction was read at the end of 15 min of rotation (100 rpm) at room temperature.

In the control reaction, 100 ng of LAM yielded a coarse clump of agglutination, 50 ng yielded visible clumps, 25 ng yielded cloudy granularity, and PBS-albumin yielded an unagglutinated clear suspension. A test was considered positive when the agglutination was comparable to the agglutination induced by 50 ng of LAM.

RESULTS

The results of the coagglutination test for all of the serum samples tested are given in Table 2. In the group of patients with pulmonary tuberculosis (group I), 49 of 50 serum samples (98%) yielded agglutination equal to or greater than a 1:2 dilution, and 44 (88%) yielded agglutination equal to or greater than a 1:4 dilution. In the patients with pulmonary tuberculosis and negative sputum smears (group II), 8 of 9 sera (89%) yielded agglutination at a dilution equal to or greater than 1:2, and 6 (66%) yielded agglutination equal to or greater than 1:4. In tuberculous patients with AIDS (group III), agglutination was detected in 19 of 21 sera (90%) with an agglutination titer equal to or greater than 1:2; 12 (57%) yielded agglutination at a dilution equal to or greater than 1:4. In the group of patients with lung diseases different from tuberculosis (group IV), 4 of 63 sera (6%) yielded agglutination at a dilution of 1:2; no serum yielded agglutination at a dilution equal to or greater than 1:4. The diagnoses of the patients of this last group with agglutination titers of 1:2 were nocardiosis, paracoccidioidomycosis, bronchiectasia, and lung fibrosis. In 63 healthy controls (group V), no antigenemia was detected.

After reviewing these results, as well as Fig. 1, we decided to consider a serum sample positive for the purpose of calculating test sensitivity and specificity (25) only if the serum sample yielded agglutination at a dilution equal to or greater than 1:4. Test sensitivities for patients of groups I to III and test specificities for patients of groups IV and V are shown in Table 3. Positive and negative predictive values were calculated by using a prior probability of 0.189 for pulmonary tuberculosis, which reflects the prevalence of tuberculosis among patients admitted to the Instituto Nacional de Enfermedades Respiratorias in 1988 (17).

DISCUSSION

The detection of polysaccharides has been shown to be a useful test for the diagnosis of some bacterial infections, including those caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* (18, 31). It has been shown that patients with these infections have antigenemia, and it has been possible to detect it by using various agglutination tests, including coagglutination or latex

TABLE 2. Results of the detection of LAM by using a coagglutination test with sera, expressed as antigen titer

Group	No. of serum samples tested	No. of serum samples yielding agglutination at an antigen titer (reciprocal) of:				
		0	2	4	8	16
I	50	1	5	29	13	2
II	9	1	2	4	2	0
III	21	2	7	4	7	1
IV	63	59	4	0	0	0
V	63	63	0	0	0	0

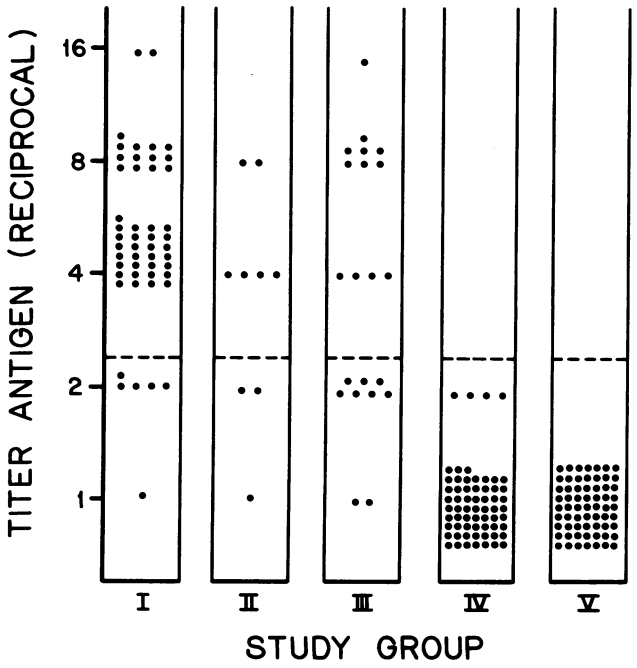

FIG. 1. Results of detection of LAM by using coagglutination in serum samples from patients with tuberculosis and from control groups. The results are expressed as the reciprocal of the agglutination titer for each serum sample. The broken line represents the cutoff value to consider a test positive for antigenemia.

agglutination (9, 19). These techniques are easy to perform and have been used as a routine test in some microbiology laboratories.

The present study showed that the detection of mycobacterial polysaccharide such as LAM in sera by using coagglutination can be used as a very accurate diagnostic test for tuberculosis. The simplicity of this technique makes it especially useful for laboratories in developing countries where the disease is endemic.

Agglutination techniques have been used for the detection of mycobacterial antigens in cerebrospinal fluid of patients with tuberculous meningitis (22) and, recently, in bronchial specimens of patients with pulmonary tuberculosis (7).

The presence of circulating antigen in sera from tuberculosis patients has been demonstrated before by Krambovitiz et al., who found, by using an ELISA for detection of mycobacterial antigens, antigenemia in 75% of the serum samples from patients with tuberculosis (21). In addition, mycobacterial antigens have been found as components of circulating immune complexes (1, 24), and it has been suggested that their presence could be a tool for diagnosis of tuberculosis (1). However, purification of immune complexes and treatment for the release of the antigen are cumbersome and therefore not applicable to routine clinical laboratories. Other studies (20) have shown evidence that other polysaccharides, including arabinogalactan, are circulating in the serum in tuberculosis and are important for the immune response against *M. tuberculosis*.

TABLE 3. Diagnostic test characteristics obtained by using coagglutination for detection of LAM in serum samples[a]

Group	Sensitivity or specificity[b] (%)	Positive or negative predictive value
I	88	0.976
II	67	0.93
III	57	0.898
IV	100	1.0
V	100	1.0

[a] Prior probability of tuberculosis, 0.189.
[b] Sensitivity values apply to group I, II, and III patients, with tuberculosis. Specificity values apply to control groups IV and V.

This is the first study in which LAM has been detected in serum from patients with tuberculosis. LAM is an important polysaccharide obtained from the wall of *M. tuberculosis*, and it has been calculated that 25 to 40% of the cell wall is composed of polysaccharide (3); therefore, it is not surprising that this antigen is released into the serum during infection. We suspect, because of the high level of antibody in patients with tuberculosis (28), that LAM travels as immune complexes in sera. In fact, the treatment regimen for serum described in this study is capable of dissociating immune complexes.

The sensitivity, specificity, and predictive values of the coagglutination test described in this paper are among the best reported for any test for the diagnosis of tuberculosis. In pulmonary tuberculosis, the sensitivity of the test is greater than that of a serological test with nonpurified antigens as well as one with purified antigens (12), and it is even greater than the sensitivities of those using monoclonal antibodies (2). This test is better than the gold standard technique, the acid-fast bacillus sputum smear, because it is capable of detecting patients with negative sputum results and positive culture results. These patients are not detected by any other known method, including serology (10).

We included in our study patients with AIDS and tuberculosis, for whom there is a need to develop noninvasive methods of diagnosis. The sensitivity of the coagglutination test is lower in AIDS patients than in patients without AIDS. Additional studies will be necessary to determine how useful this diagnostic test will be in this population. It is interesting that patients with AIDS have less antigenemia than patients without AIDS, since the load of mycobacteria is probably greater in AIDS patients. We believe that, because of their immunosuppression, patients with AIDS are not able to degrade mycobacteria and therefore do not release antigen in amounts necessary for detection by the test.

There are other interesting findings regarding the specificity of the test. The described test is not specific for *M. tuberculosis* infections. LAM is an antigen of all bacteria of the genus *Mycobacterium* (3), and arabinomannan is probably part of the walls of other fungi and major bacteria such as *Nocardia* spp. In this study, two serum samples from patients with paracoccidiodomycosis and nocardiosis yielded some degree of agglutination, which is probably related to the sharing of arabinomannan in their structure and some degree of cross-reaction with the antisera directed against LAM; however, the agglutination was present at a lower titer of sera (1:2) compared with that obtained by using sera from patients with tuberculosis (1:4).

In the present study we did not include patients with AIDS and other mycobacterial infections such as that due to *Mycobacterium avium* because in our population these infections are very unusual (26). We suspect that patients with AIDS and *M. avium* infection will give positive results identical to those obtained for patients with tuberculosis, and therefore the test could be simply a tool for detection of patients with any mycobacterial infection.

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