

Evaluation of Lipoarabinomannan for the Serological Diagnosis of Tuberculosis

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The availability of highly purified lipoarabinomannan from *Mycobacterium tuberculosis* in its native acylated, highly antigenic state allowed its application to the serodiagnosis of tuberculosis in patients from the Republic of Mexico. Antilipoarabinomannan immunoglobulin G antibodies in sera from 66 patients with pulmonary, miliary, and pleural tuberculosis and tuberculosis lymphadenitis were measured by using the enzyme-linked immunosorbent assay against sera from a control population of healthy individuals, people with histoplasmosis, and people with lung diseases not caused by mycobacteria. The results pointed to an unexpectedly high degree of specificity of 91% and a sensitivity of 72%, comparable to figures from previous studies with other purified antigens; most of the false-positive results were for patients with histoplasmosis. Thus, lipoarabinomannan of *M. tuberculosis* is a potentially useful antigen for the serodiagnosis of tuberculosis.

Tuberculosis is still a problem in developing countries, where the prevalence can be extremely high (11); even developed countries show an increase in prevalence, as a consequence of the AIDS epidemic and other social factors (3). The diagnosis of tuberculosis relies on clinical and radiological findings which are not specific for the disease. In addition, it is necessary to follow up with the demonstration of acid-fast bacilli in smears followed by the culture of *Mycobacterium tuberculosis*. However, facilities for the latter are limited in most of the countries in which the disease is endemic. In some countries, tuberculin (purified protein derivative [PPD]) has some value for diagnosis; however, a high rate of false-positive results is seen in developing countries, and some patients with active tuberculosis have tested negative (4). For these reasons, serological tests for the diagnosis of tuberculosis have been widely explored. Recently, Daniel and Debanne reviewed the results of these studies by using the enzyme-linked immunosorbent assay (ELISA) (5). Several antigens (crude mycobacterial extracts, PPD, and some purified antigens such as antigen 5 and antigen 6) have been used. The sensitivity of different assays has been from 70 to 85%, with widely varying specificities; the greatest specificities have been obtained by using the purified antigens.

Recently, Hunter et al. purified lipoarabinomannan (LAM) from *M. tuberculosis* in its native acylated state (6) and tested it, obtaining promising results for efficacy in the serodiagnosis of leprosy (8). In the present study, we extended this approach to a population of patients with tuberculosis from the Republic of Mexico and demonstrated that most of these patients had antibodies against LAM, a finding which has diagnostic implications.

MATERIALS AND METHODS

Study groups. All of the patients under study were from the Instituto Nacional de Enfermedades Respiratorias, Mexico City, Mexico, and belonged to seven different groups.

Group I consisted of 31 patients with definitive clinical

symptoms of pulmonary tuberculosis. Only patients with pulmonary disease who showed sputum smears positive for acid-fast bacilli and from whom positive cultures for *M. tuberculosis* could be grown were included in this group.

Group II was composed of 17 patients with miliary tuberculosis. The diagnosis was based on evidence of a miliary pattern in chest X ray, the involvement of organs other than the lungs, a positive sputum smear, and a positive culture of *M. tuberculosis*. In some cases, the diagnosis was done by demonstration of granulomata by open lung biopsy and by a positive culture.

Group III was composed of 14 patients with confirmed pleural tuberculosis determined on the basis of the demonstration of granulomata in biopsy and of a positive culture for *M. tuberculosis*.

Group IV consisted of four patients with tuberculosis lymphadenitis determined on the basis of the demonstration of granulomata in biopsy and of a positive culture for *M. tuberculosis*.

Group V comprised a set of 75 patients with lung disease not due to tuberculosis. This group included patients with chronic bronchitis, pneumonia, lung cancer, pleural diseases, and other granulomatous diseases, such as allergic alveolitis. One patient with paracoccidioidomycosis was also included. In all of these patients, the presence of tuberculosis was excluded by demonstration of a negative sputum smear and negative culture for *M. tuberculosis*.

Group VI consisted of 10 patients with acute pulmonary histoplasmosis.

Group VII consisted of 32 healthy subjects, 10 of whom were PPD positive and 10 of whom were PPD negative; the PPD status of the remaining 12 was not known. The first 20 of these subjects had attended the hospital for evaluation since they had been in contact with tuberculosis patients; however, all had normal chest X rays. The remaining 12 serum samples were obtained from a blood bank.

In all of the patients with tuberculosis, sera were obtained prior to the onset of any treatment. All sera were stored at -20°C until use.

Antigen. LAM was purified from *M. tuberculosis* by anion-exchange and gel filtration chromatography in deter-

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gent as described by Hunter et al. (6). The LAM purification was done in the Department of Microbiology, Colorado State University, by P.J.B.

ELISA. LAM was used as the solid-phase antigen at a concentration of 10 µg/ml in carbonate bicarbonate buffer. The coating amount was 100 µl (1 µg) placed onto U-bottom polystyrene microtiter plates (Dynatech, Alexandria, Va.) which were kept overnight at 40°C. After three washes with phosphate-buffered saline containing 2% Tween-80 (PBS-Tween), the plates were blocked for 2 h at 37°C with 200 µl of 1% albumin (Sigma, St. Louis, Mo.) in PBS. The 100 µl of the test sera diluted 1:500 in PBS-Tween was incubated for 1 h at 37°C, washed three times with PBS-Tween, and incubated with biotinylated anti-human immunoglobulin G (1:500 dilution in PBS-Tween) (Dynatech) for 1 h at 37°C. The plates were washed, 100 µl of streptavidin peroxidase was added, and the plates were incubated for 1 h at 37°C. Finally, the plates were washed, 100 µl of substrate (*ortho*-phenyldiamine [Sigma]; 2 mg in 10 ml of citrate buffer containing 0.1 mM H₂O) was added per well, and after 20 min at room temperature the reaction was stopped by adding 50 µl of 5 N H₂SO₄. The A₄₉₀ was read in an ELISA reader (Minireader; Dynatech). The selection of 100 µg of antigen per ml and of a serum dilution of 1:500 was arrived at from preliminary experiments. This concentration of antigen and dilution of sera were considered to be optimal for the assay. The use of just one serum dilution was chosen because of a good correlation between the titers of sera and the A₄₉₀ value of one dilution of the same sera ($r = 0.90$).

Expression of results and statistical methods. Because the antibody levels of a single specimen, as reflected by the A₄₉₀, varied from day to day, the assay was standardized by comparing the A₄₉₀ of the test serum to that of one positive control serum. The A₄₉₀ of each serum divided by the A₄₉₀ of the positive control for that day determined an absorption index.

An ELISA was considered positive if the results showed an absorption index value higher than 0.200. This value was selected by using the 95% confidence interval of group VI (control group with lung diseases other than tuberculosis); if the mean plus 2 standard deviations of the control group were chosen, a great number of sera from patients with tuberculosis would be lost; therefore, the sensitivity of the assay was lowered, but the specificity did not improve.

We calculated the sensitivity, specificity, and positive and negative predictive values for ELISA results (10) for each group as well as for the two critical groups (patients with tuberculosis and controls). For the calculation of positive and predictive values, we used different prevalences: one at 20% and another at 50%. The reason we selected 50% is because in our hospital, approximately 50% of the sera that are sent for serological diagnosis are from patients with the disease.

Differences in mean antibody concentrations between the groups of sera were determined by a variance analysis, using the Tukey test for analysis post hoc, at a significance of 0.05. These analyses were done by using a statistics packet for microcomputers (Systat).

RESULTS

The mean absorption index and the standard deviation for each group of patients are given in Table 1. When the results were subjected to analysis of variance, we showed a statistically significant difference between the groups of patients with tuberculosis (groups I through IV) and the control

TABLE 1. Estimation of the degree of sensitivity or specificity of the various population groups in the anti-LAM ELISA

Study group	No. of sera tested	No. positive	Sensitivity/specificity ^a (%)	Absorption index (mean ± SD)
I (pulmonary tuberculosis)	31	25	80.6	0.739 ± 0.529
II (miliary tuberculosis)	17	14	82.3	0.854 ± 0.562
III (pleural tuberculosis)	14	6	42.8	0.649 ± 0.842
IV (tuberculous lymphadenitis)	4	3	75	0.416 ± 0.461
Avg			72.7	
V (lung diseases other than tuberculosis)	75	5	93.4	0.104 ± 0.222
VI (acute pulmonary histoplasmosis)	10	4	60	0.431 ± 0.527
VII (healthy controls)	32	1	96.9	0.555 ± 0.193
Avg			91.5	

^a Sensitivity value applies to groups I through IV, i.e., persons with tuberculosis. Specificity applies to the control groups, V through VII.

groups (groups V and VII); the patients with histoplasmosis (group VI), however, did not display any significant difference from the groups of patients with pulmonary tuberculosis (group I), pleural tuberculosis (group IV), and lymphadenitis (group III) but did display a significant difference from patients with miliary tuberculosis (group II). When we analyzed the differences between the various groups of patients with tuberculosis, we did not observe differences between the patients with pulmonary, miliary, and pleural tuberculosis. The patients with tuberculous lymphadenitis had an absorption index significantly lower than those of patients with pulmonary and miliary tuberculosis.

The absorption index for each serum is shown in Fig. 1. With a cutoff value at 0.200, we calculated the sensitivity and specificity of the assay. The results are shown in Table 1. The greatest sensitivity was shown for patients with miliary tuberculosis, and the lowest was shown for patients with pleural tuberculosis. Regarding the specificity of the assay, the highest was observed for healthy controls, and the lowest was observed for patients with histoplasmosis. For the patients with lung diseases not caused by mycobacteria, the false-positive results were for one patient with paracoccidioidomycosis, two with adenocarcinoma of the lung, and two with bronchiectasia of unknown origin. When we joined the results obtained from patients with histoplasmosis and lung diseases other than tuberculosis (groups V and VI), the specificity was 88%.

The positive predictive values were 66% at 20% of prevalence and 88% at 50% of prevalence. The negative predictive values were 92% at 20% of prevalence and 76% at 50% of prevalence.

DISCUSSION

This is the first study in which LAM from *M. tuberculosis* in its acylated, highly antigenic state was applied to the serodiagnosis of tuberculosis. The results are most promising in that the outcome is comparable to or better than the results from other studies using different mycobacterial antigens (5). In addition, the present study has some other characteristic features. It was done in a country in which tuberculosis is endemic. The hospital of the Instituto Nacio-

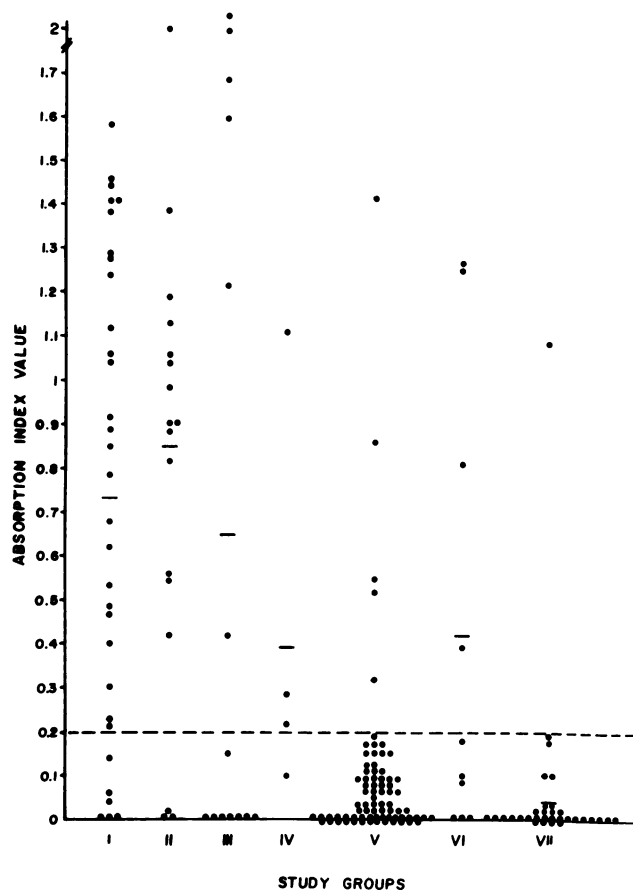


FIG. 1. Compilation of the results from application of LAM-based ELISA to sera from groups of patients with tuberculosis and from control groups. The results are expressed as the absorption index for each serum sample. The broken line represents the cutoff value. Horizontal bars represent means of the absorption index.

nal de Enfermedades Respiratorias corresponds to a reference hospital for lung diseases in Mexico City, and the patients are referred here only when there are complications in the diagnosis of tuberculosis. The selection of patients in each group was strict, and for most of them, the diagnosis was confirmed by culture of mycobacteria or by histopathologic study. For the population that we studied, we can appropriately divide the groups of patients with tuberculosis and evaluate the results of the assay for each group (pulmonary, miliary, and pleural tuberculosis and tuberculosis lymphadenitis). Finally, the control group was excellent since it included both a number of patients with lung diseases in which the diagnosis of tuberculosis was suspected clinically and a second group of healthy subjects who had a similar epidemiological background to the group of patients with tuberculosis.

It is well known that the results of any serological study in infectious diseases depend on the quality of the antigen used. Tuberculosis is not an exception in this sense, and the greatest sensitivity and specificity have been obtained by using purified antigens, such as antigen 5 (9). In the present study, the sensitivity was superior to those obtained in some studies using antigen 5 (1) and similar to those obtained in studies in which an undifferentiated group of tuberculosis patients was examined with PPD (13); the specificity was intermediate between the 100% reported for antigen 5 and the 84% reported for PPD (1, 13).

It is striking that no significant difference was observed in the levels of antibodies in individuals with the two polar forms of tuberculosis, namely, pulmonary and miliary tuberculosis. Yet, in the case of leprosy, there are marked differences in the antibody load in tuberculoid versus lepromatous disease, reflecting the different bacterial loads (8). Miliary tuberculosis is a disease in which the diagnosis is difficult, since a negative smear is frequently encountered and since differential diagnosis raises possibilities other than tuberculosis. Indeed, in some cases, the diagnosis has to be done by invasive methods and sometimes even by open lung biopsy. Accordingly, for this group of patients, the surprising sensitivity of the assay based on LAM is a decided boon. Another interesting group are the patients with pleural tuberculosis. As in miliary tuberculosis, the diagnosis of pleural tuberculosis is not easy because the sensitivity of positive smears in pleural fluid is lower than 10% (2) and because the diagnosis has to be done by invasive procedures such as pleural biopsy, in which evidence of granulomas is sought. Although the sensitivity of the LAM-based ELISA for this group is lower than for the pulmonary or miliary tuberculosis group, a positive assay would obviate invasive procedures. It is also significant that healthy contacts of patients with tuberculosis, even if they have a positive PPD, have a negative test for LAM, suggesting that this assay could be useful for the study of contacts with tuberculosis. Of course, it will be necessary to initiate a separate study to determine whether contacts with tuberculosis who develop a clinical disease have antibodies against LAM.

Regarding the issue of specificity, it is important to make a few points. LAM is not an *M. tuberculosis*-specific antigen, being present in all members of the genus *Mycobacterium* (7). The high anti-LAM reactivity in sera from patients with histoplasmosis and paracoccidioidomycosis also suggests cross-reaction with eucaryotic microorganisms, at least with these two fungi. Wheat et al. have reported previously that some sera from patients with tuberculosis reacted falsely with *Histoplasma capsulatum* antigens (12); it is feasible that the responsible fungal antigen shares determinants with LAM. Clearly, the problem of false-positives due to histoplasmosis or paracoccidioidomycosis or both is a matter of some concern in tropical regions. We did not include in our study patients with other mycoses such as coccidioidomycosis and blastomycosis, and we do not know if these patients give a false-positive result with LAM. Studies in areas in which these mycoses are endemic have to be done in order to answer this question.

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