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Serodiagnosis of Tuberculosis: Comparison of Immunoglobulin A (IgA) Response to Sulfolipid I with IgG and IgM Responses to 2,3-Diacyltrehalose, 2,3,6-Triacyltrehalose, and Cord Factor Antigens

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Nonpeptidic antigens from the *Mycobacterium tuberculosis* cell wall are the focus of extensive studies to determine their potential role as protective antigens or serological markers of tuberculous disease. Regarding this latter role and using an enzyme-linked immunosorbent assay, we have made a comparative study of the immunoglobulin G (IgG), IgM, and IgA antibody responses to four trehalose-containing glycolipids purified from *M. tuberculosis*: diacyltrehaloses, triacyltrehaloses, cord factor, and sulfolipid I (SL-I). Sera from 92 tuberculosis patients (taken before starting antituberculosis treatment) and a wide group of control individuals (84 sera from healthy donors, including purified protein derivative-negative, -positive, healed, and vaccinated individuals, and 52 sera from nontuberculous pneumonia patients), all from Spain, were studied. The results indicated a significantly elevated IgG and IgA antibody response in tuberculosis patients, compared with controls, with all the antigens used. SL-I was the best antigen studied, showing test sensitivities and specificities for IgG of 81 and 77.6%, respectively, and of 66 and 87.5% for IgA. Using this antigen and combining IgA and IgG antibody detection, high test specificity was achieved (93.7%) with a sensitivity of 67.5%. Currently, it is widely accepted that it is not possible to achieve sensitivities above 80% in tuberculosis serodiagnosis when using one antigen alone. Thus, we conclude that SL-I, in combination with other antigenic molecules, could be a useful antigen for tuberculosis serodiagnosis.

To control tuberculosis (TB), it is still necessary to find a diagnostic method that is both more rapid to carry out and more sensitive than traditional methods (smear and culture) but which is simpler and less expensive than the new molecular diagnostic tests that are based on the amplification of nucleic acids. Serological methods seem to be the ideal choice and, thus, many mycobacterial antigens have been evaluated: cellular extracts, proteins, and glycolipidic molecules from the mycobacterial cell wall (31). In addition to the lipoarabinomannan antigen, the acylated trehalose family has been the most frequently investigated group of glycolipids. They are 2,3-diacyltrehalose (DAT), 2,3,6-triacyltrehalose (TAT), 2,3,6,6′-tetraacyltrehalose 2′-sulfate (sulfolipid I [SL-I]), and trehalose-6,6′-dimycolate (cord factor [CF]). Using the enzyme-linked immunosorbent assay (ELISA) technique, several studies were performed with these antigens, and an extensive variability in immunoglobulin G (IgG) or IgM titers (6, 7, 11, 18, 20, 24–26) was obtained, i.e., test sensitivities were as different as 11 to 88% for the DAT antigen (11, 25) and 51 to 93% for the TAT antigen (11, 26). These contradictory results are due to the ELISA protocol used (the different antigen concentrations or the use of detergent in washing buffers) (15, 30), or to the different sera groups analyzed (the number and type of patients and control subjects) (10). Thus, no definite conclusions as to their utility as serodiagnostic antigens have been reached.

In spite of the presence of high IgA antibody levels in sera from TB patients (9) and although different commercialized assays exist that detect IgA antibodies against cellular extracts and purified proteins (14, 16, 23), to date no analysis has been undertaken to determine the presence of specific IgA against the glycolipids mentioned above.

For the first time, the present study analyzed the IgA antibody response to mycobacterial glycolipids, compared with the IgG and IgM response, in a wide population of patients affected by TB and non-TB pulmonary infections, as well as in healthy people. Moreover, the study was carried out in parallel with four *M. tuberculosis* glycolipids, using an ELISA method specifically optimized for them (15).

MATERIALS AND METHODS

Study subjects. A total of 228 serum samples were studied. The demographic and clinical characteristics of the individuals involved are listed in Table 1.

(i) TB patients. Sera were collected from adult patients who, according to clinical parameters, were suspected of having TB and who had been admitted to the "Germans Trias i Pujol" University Hospital (HUGTiP) in Badalona, Spain. However, the only serum samples included in this group were from patients in whom the disease had been confirmed by isolation of the tuberculous bacillus in cultures (Löwenstein-Jensen and the nonradiometric MB/Bact system [Organon

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TABLE 1. Demographic and clinical data for TB patients and controls

Source of serum samples	Patients (*n*)	Sex (men:women)	Age	
			Mean	Range
TB patients	92	62:30	35.7	1–87
Pulmonary adult TB				
HIV −	48	37:11	44.3	26–77
HIV +	10	5:5	33.8	26–48
Extrapulmonary adult TB				
HIV −	13	7:6	51.1	19–87
HIV +	3	2:1	31.3	27–35
Pulmonary child TB	18	11:7	9.1	0.8–17
Non-TB pneumonia patients	52	34:18	43.5	1–86
M. xenopi and *M. kansasii*	2	0:2	20	28–78
Chlamydia spp.	12	7:5	83	62–86
C. burnetii	6	5:1	40	33–45
L. pneumophila	11	7:4	54.3	26–74
M. pneumoniae				
Adults	9	4:5	23	20–28
Children	4	3:1	4.3	1–7
S. pneumoniae	8	8:0	63.5	33–75
Healthy subjects	84	52:32	20.5	1–80
PPD-negative adults	44	26:18	42.2	21–50
PPD-positive				
Adults	8	5:3	23	21–39
Children	14	12:2	11.1	3–16
Vaccinated	9	3:6	26.4	23–51
Healed				
Adults	3	1:2	52	40–80
Children	6	5:1	5.3	1–12
Total	228	148:80	33.5	1–87

Teknika, Durham, N.C.]). Fifty-eight of these adult patients were suffering from pulmonary TB (10 of them were coinfected with human immunodeficiency virus [HIV]), and 16 had extrapulmonary TB (three of them were HIV positive). The extrapulmonary localizations comprised disseminated TB (seven), pleural TB (two), lymphatic (two), puncture (two), pus abscess (one), bone (one), and cutaneous (one) cases. The patients had not yet started the antituberculosis treatment when the serum samples were taken. Eighteen serum samples from children with a clinical diagnosis of TB were also included in the study; for six children, the illness was subsequently confirmed by isolating *Mycobacterium tuberculosis* from gastric lavage or from respiratory specimens.

(ii) Control subjects. One hundred thirty-six HIV-seronegative serum samples were also included as negative controls (Table 1). Fifty of these were from non-TB pneumonia patients. These diseases were originated by *Chlamydia* spp. (12 serum samples), *Coxiella burnetii* (6 samples), *Legionella pneumophila* (11 samples), *Mycoplasma pneumoniae* (9 samples from adults and 4 from children), and *Streptococcus pneumoniae* (8 samples). They were all purified protein derivative (PPD) negative. Moreover, two serum samples included in this group were from patients with other pulmonary mycobacterial diseases: one produced by *Mycobacterium kansasii* and the other by *Mycobacterium xenopi*. The sera from these non-TB pneumonia patients were obtained from the Microbiology Service Seroteca at the HUGTiP.

Eighty-four sera were taken from healthy controls: 44 of these were PPD negative, 9 had been BCG vaccinated in the past, and 22 were PPD positive (8 adults and 14 children). Three serum samples were included from adults who had suffered from TB more than 5 years prior to sampling; they had received the standard treatment for TB and had completed it correctly. In addition, six sera were taken from children upon termination of standard treatment. These healthy control sera were obtained from employees of the HUGTiP or Ph.D. students, or they were collected from the Barcelona Tuberculosis Prevention and Control Programme. All sera were aliquoted and stored at −40°C until use.

ELISA glycolipids. For glycolipid isolation, an *M. tuberculosis* clinically isolated strain (22) was grown for 6 weeks at 37°C on Middlebrook 7H10 supplemented with oleic acid-albumin-dextrose-catalase enrichment (Difco Laboratories, Detroit, Mich.). DAT, TAT, SL-I, and CF were purified using column chromatography as previously described (21, 22). ELISA was performed as described elsewhere (15). Briefly, plates (Immulon I; Dynatech Laboratories) were coated with purified DAT, TAT, CF, or SL-I (1,000 ng each in 50 μl of *n*-hexane/well). Sera were diluted in blocking buffer at predetermined optimal dilutions (1/400 for measuring IgG antibodies, 1/200 for IgM, and 1/100 for IgA). All points were duplicated. Goat anti-human IgG, IgM, and IgA alkaline phosphatase conjugates (Southern Biotechnology Associates, Inc., Birmingham, Ala.) at a 1/3,000 dilution in blocking buffer were added. Absorbance was determined at 405 nm with a microtiter reader (ELx 800 automated microplate reader; Bio-Tech Instruments, Inc.).

Data analysis. Final calculations were performed as previously described (6) with slight modifications. To correlate the data for day-to-day variations, three titrated sera having low, medium, and high levels (standards) and a blank (blocking buffer) were included in each plate. A curve was drawn for each plate, and the comparison of their slopes was carried out. If these data were not satisfactory (slope below 98%), the plate was rejected. Moreover, to detect nonspecific absorption, wells treated with solvent alone (i.e., without any antigen) were included for each serum tested and were used as a second negative control. The values of tested sera were corrected as follows: the difference between absorbance of serum and nonspecific absorption (zero) was taken and the mean value was calculated. The normalized data were then calculated to establish the corrected Δ405 values by using the curve of standards.

Arithmetic means and standard deviations (SD) from the mean were calculated for corrected optical density (OD) values. The cutoff points chosen were equal to the means of the OD corrected readings obtained with sera from all of the healthy individuals, plus 3 SD for each antigen-Ig combination. To adjust the cutoff value, we selected the mean plus 2 SD measured in the overall control population.

Statistical treatment of results. The significance of the difference between means was calculated using the Mann-Whitney U test. Comparison between several groups was made using the Kruskal-Wallis analysis (12). Sensitivity and specificity were calculated by standard methods. All statistical tests were conducted using SPSS for Windows statistical software (version 9.0; SPSS, Chicago, Ill.).

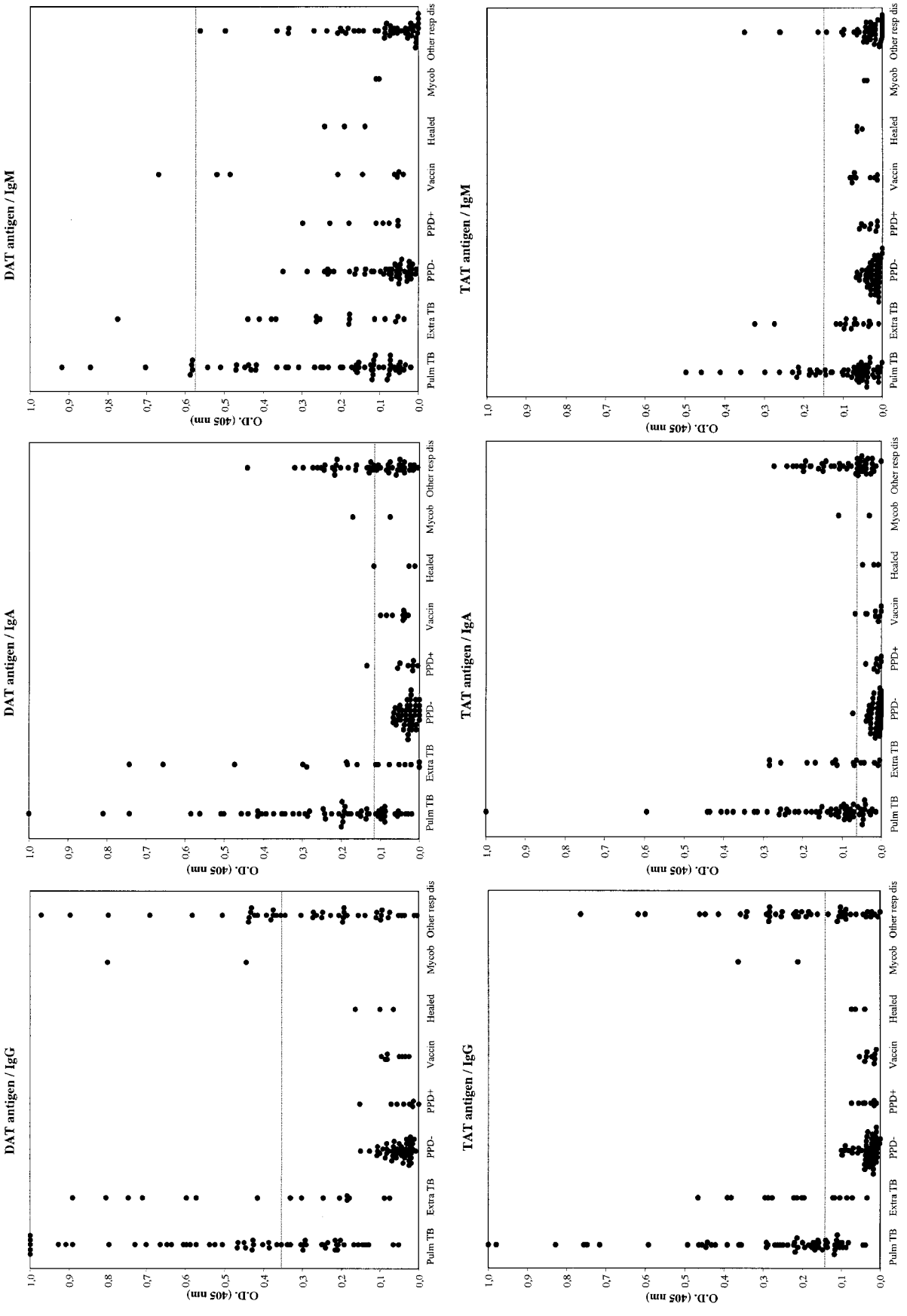
RESULTS

The demographic and clinical data for the TB patients and controls studied are shown in Table 1. There was no statistically significant difference in the mean age ± SD of TB patients and control subjects (35.7 ± 22.1 years versus 31.5 ± 23.5 years, respectively).

Mean and individual antibody levels. In TB adult patients, the mean values of IgG and IgA antibodies to each of the four glycolipid antigens tested were significantly elevated ($P < 0.001$) above those of the overall control sera (healthy and non-TB pneumonia) (Fig. 1). Differences in the IgM antibody levels were observed between the TB patients and the overall control groups for the DAT and TAT antigens, but not when using the SL-I and CF antigens. For all the antigens and Igs tested, no significant differences were observed between the mean values of the different groups of healthy controls within each test. However, the non-TB pneumonia patients did show higher levels ($P < 0.001$) than the rest of the controls in each test (Fig. 1).

In the child group, no significant differences were observed between the mean OD values of the TB patients and those of the overall control groups for any antigen or antibody assayed ($P > 0.05$). Although only four sera from children with non-TB pneumonia were studied, the results were higher than those of TB children in all the tests.

Sensitivity and specificity. In Table 2, the reactivity of pulmonary TB, extrapulmonary TB, smear-positive, and smear-negative samples are represented. This table shows that higher test sensitivities were obtained in smear-positive TB patients than in smear-negative patients, and in pulmonary TB with respect to extrapulmonary TB (Table 2). Considering all the adult populations in this study, the highest test sensitivity was



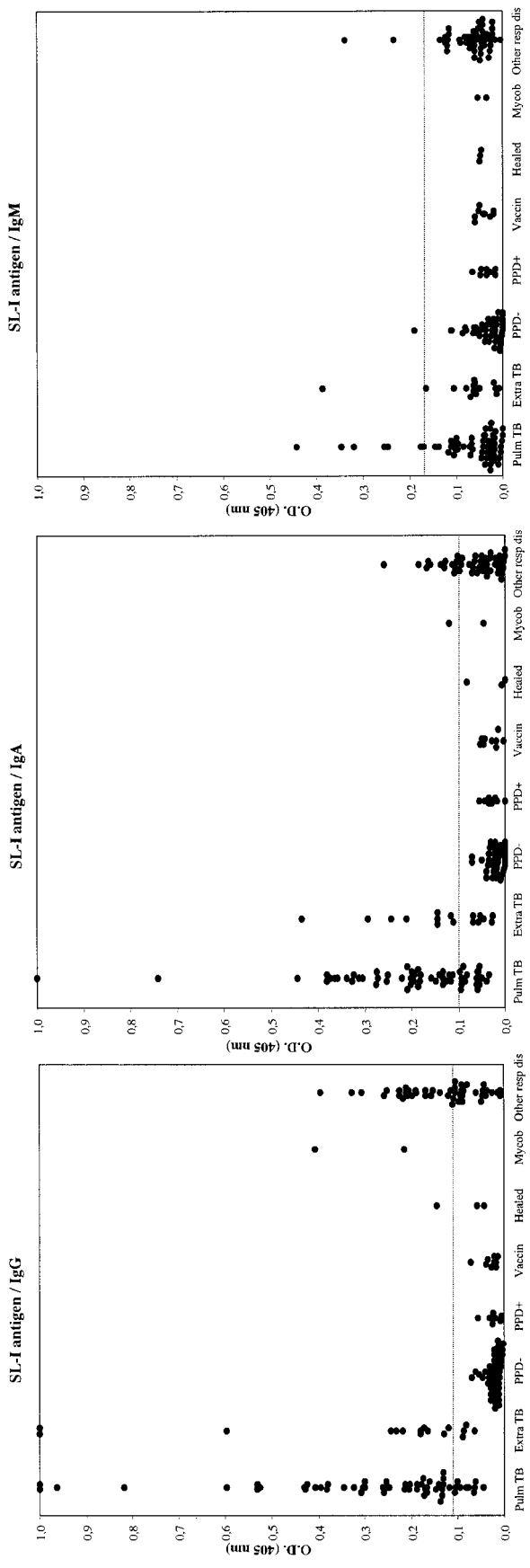


FIG. 1. Results of the ELISA for glycolipids in sera from adult subjects. Individual absorbance values for each test are shown. Each dot indicates an individual serum sample. The dotted line indicates cutoff values above which a test is positive (mean + 3 SD of the healthy population values). Pulm TB, pulmonary TB; Extra TB, extrapulmonary TB; PPD−, healthy PPD negative; PPD+, healthy PPD positive; Vaccin, healthy vaccinated; Healed, healthy healed; Mycob, other mycobacterial diseases; and Other resp dis, other respiratory diseases.

TABLE 2. Seropositive adult subjects with each of the 12 serological tests

Test used (antigen/Ig)	No. (%) seropositive (n = 186 serum samples)							
	Pulmonary TB patients			Extrapulmonary TB patients			Non-TB pneumonia patients (n = 48)	Healthy subjects (n = 64)
	Total (n = 58)	Smear positive (n = 42)	Smear negative (n = 16)	Total (n = 16)	Smear positive (n = 2)	Smear negative (n = 14)		
DAT/IgG	33 (56.89)	25 (59.52)	8 (50)	7 (43.75)	2 (100)	5 (35.71)	20 (41.66)	0 (0)
TAT/IgG	41 (70.68)	30 (71.42)	11 (68.75)	10 (62.5)	2 (100)	8 (57.14)	28 (58.33)	0 (0)
SL-I/IgG	48 (82.75)	35 (83.33)	13 (81.25)	12 (75)	2 (100)	10 (71.42)	24 (50)	1 (1.56)
CF/IgG	35 (60.34)	26 (61.90)	9 (56.25)	5 (31.25)	1 (50)	4 (28.57)	12 (25)	1 (1.56)
DAT/IgA	41 (70.68)	33 (78.57)	8 (50)	8 (50)	2 (100)	6 (42.85)	24 (50)	2 (3.12)
TAT/IgA	45 (77.58)	37 (88.09)	8 (50)	10 (62.5)	2 (100)	8 (57.14)	25 (52.08)	2 (3.12)
SL-I/IgA	40 (68.96)	33 (78.57)	7 (43.75)	9 (56.25)	2 (100)	7 (50)	14 (29.16)	0 (0)
CF/IgA	19 (32.75)	16 (38.09)	3 (18.75)	6 (37.5)	0 (0)	6 (42.85)	2 (4.16)	2 (3.12)
DAT/IgM	7 (12.06)	4 (9.52)	3 (18.75)	1 (6.25)	0 (0)	1 (7.14)	0 (0)	1 (1.56)
TAT/IgM	16 (27.58)	11 (26.19)	5 (31.25)	2 (12.5)	0 (0)	2 (14.28)	4 (8.33)	0 (0)
SL-I/IgM	7 (12.06)	6 (14.28)	1 (6.25)	1 (6.25)	0 (0)	1 (7.14)	2 (4.16)	1 (1.56)
CF/IgM	1 (1.72)	1 (2.38)	0 (0)	1 (6.25)	0 (0)	1 (7.14)	1 (2.08)	1 (1.56)

obtained when detecting IgG antibodies against the SL-I antigen (81%, with a specificity of 77.6%). Sensitivity values above 65% were obtained for the following test combinations: TAT IgA (74.3%), TAT IgG (68.9%), and SL-I IgA (66.2%). The SL-I IgG test was the most efficient for detecting smear-negative TB cases. Among healthy adult subjects, only 1 or 2 serum samples out of 64 were positive for some of the tests, so test specificities between 96.8 and 100% were obtained. However, these percentages dropped in non-TB pneumonia patients. Around 50% of non-TB pneumonia patients reacted to the SL-I IgG, TAT IgG, and TAT IgA tests (Table 2). On the other hand, only 29% of these patients reacted to SL-I IgA. Taking this control population (non-TB pneumonia patients) into account, the SL-I IgA test was the most specific (87.5%, with a sensitivity of 66.2%).

Considering all the adult populations, including the non-TB pneumonia patients, a test sensitivity of 58.1% with a specificity of 90.1% was obtained for detecting both IgG and IgA antibodies against SL-I. One way of improving these results is to adjust the cutoff. In our case, test sensitivity and specificity values of 67.56 and 93.75%, respectively, were achieved by adjusting for the SL-I IgA and IgG tests and combining them.

Test sensitivities lower than 25% were obtained in all the tests when detecting IgM antibodies in adult patients. With respect to child TB, very low sensitivity values were obtained in all tests (between 0 and 22.2%) (data not shown). Even if using a different cutoff from that selected, it is not possible to discriminate between true and false positives in any test.

DISCUSSION

To determine the best test for potential use in TB serodiagnosis, we have accurately analyzed the complete antibody response to each one of these antigenic molecules: DAT, TAT, SL-I, and CF, using a wide population.

The results obtained throughout in the child group and when detecting IgM antibodies in adult sera are particularly bleak. In child populations few studies have been carried out throughout the history of TB serodiagnosis. In general, the observed humoral response is lower than that with adult patients (references 3, 9, and 27 and present results), and no differences between infected and sick children have been reported (references 27 and 28 and the present work). Thus, no antigen tested would seem to be useful as a diagnostic tool in child TB patients. In the case of IgM detection in adults, these discouraging results (Table 2) have already been described for three of the four glycolipids (DAT, SL-I, and CF) (7, 20) and for purified proteinaceous antigens (29).

In the present study, the best results were obtained when detecting IgG and IgA in adult TB patients. IgG was comparatively more reactive, giving the highest absorbance values and highest sensitivities (Fig. 1 and Table 2), whereas IgA was found to be more specific. Among the antigens, SL-I showed the best relation between test sensitivity and specificity: 81 and 77.6%, respectively, for IgG, and 66.2 and 87.5% for IgA. Despite it being an exclusive antigen to the virulent *M. tuberculosis* strains (8), there are only two previous serological evaluations of IgG and IgM antibodies against SL-I (7, 24). Cruaud et al. (7) found, as we did, that the reaction of sera from TB patients was significantly higher than that from healthy controls when detecting IgG antibodies to SL-I, and that IgM reactivity was negligible. However, we achieved a test sensitivity of 81%, in contrast to only 33% reported by those authors. We think this is due to differences in the ELISA procedure, specifically, the low quantity of antigen used by them (only 100 ng per well) while we found 1,000 ng per well for SL-I to be an optimum coating (15). The results obtained by Rojas-Espinosa et al. (24) are discrepant in the sense that they found a high reactivity for the IgM detection. IgA was not tested in either of these previous serological studies using SL-I or in the other studies using the other glycolipids. We have shown, for the first time, that adult TB patients have a specific IgA response against these glycolipids. In comparison with IgG, IgA is less sensitive, but its attractiveness lies in that it is more specific. Thus, by combining the detection of IgA and IgG antibodies against SL-I and adjusting the cutoff value, we have achieved the best relation between sensitivity (67.5%) and specificity (93.7%), taking all the adult populations of this study into account, including the non-TB pneumonia patients.

With both Ig's, elevated test specificity values were obtained in the healthy control group (between 96 and 100%); never-

theless, the specificity drops dramatically when sera from patients with non-TB pneumonia are considered. We have observed that this is a constant in TB serological studies. Reports showing high test specificities (90 and 95%) studied few samples belonging to non-TB pneumonia patients (1, 2). When the same test was carried out with a larger number of non-TB pneumonia patients, the specificity went down markedly (5, 16). The evaluation of new tests using mainly healthy subjects, and with few (if any) patients infected by other agents, is one of the common defects in TB serology studies (31). Unquestionably, the inclusion of this population is crucial for clinical evaluation of new tests, since they are susceptible to being confused with TB patients. For this reason, we have included a large number of non-TB pneumonia patients (38.2% with respect to overall controls) in the present study.

The unspecific hyperglobulinemia associated with some non-TB respiratory diseases (4) and the fact that the majority of adults have been in contact with environmental mycobacteria all their lives could be reasons for the false positive results in some non-TB pneumonia patients. As mentioned, these disappointing results have been borne out by other authors using other antigens. By including such a high percentage of non-TB pneumonia patients in the control population, we were able to choose the most specific antigen-antibody combination. However, in the daily routine of a microbiology laboratory a high percentage of these patients will be correctly diagnosed using specific microbiological tests for each of the remaining infectious agents. Thus, in a clinical setting, the usefulness of the SL-I IgA test will be greater than is shown by the overall results obtained in this study. Nevertheless, a positive result using the SL-I IgA test would not indicate starting an antituberculosis treatment, since it would be necessary to have other clinical or microbiological suggestions of TB.

We think that three strategies could be used to improve the specificity of TB serodiagnosis: first, always using the specific antigens present only in *M. tuberculosis*; second, avoiding possible antigen contamination during the purification process; and third, preabsorbing the sera either with mycobacteria other than *M. tuberculosis*, with other bacteria, or with molecules that share epitopes with the specific antigens. Very few studies (17) have analyzed the efficacy of absorbents in diminishing the cross-reactions, and it would be very interesting to investigate this by testing a wide range of them.

It has been clearly accepted that TB patients produce antibodies against more than one antigen (19) and, consequently, a wide spectrum of humoral responses exists in these patients. Thus, some authors (13) and ourselves propound the combination of specific purified antigens to increase the sensitivity of serodiagnosis. In this way SL-I, combined with other specific antigens, could be a useful antigen for inclusion in a TB serodiagnostic test.

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