Comparison of Antibody Responses to a Potential Combination of Specific Glycolipids and Proteins for Test Sensitivity Improvement in Tuberculosis Serodiagnosis

Esther Julián,1* Lurdes Matas,2 José Alcaide,3 and Marina Luquin1

Departament de Genètica i de Microbiologia, Facultat de Ciències, Universitat Autònoma de Barcelona, Bellaterra,1 Servei de Microbiologia, Hospital Universitari Germans Trias i Pujol, Badalona,2 and Programa de Control i Prevençió de la Tuberculosis, Departament de Salut i Seguretat Social, Barcelona,3 Spain

Received 1 August 2003/Returned for modification 25 September 2003/Accepted 21 October 2003

The humoral response to different proteinaceous antigens of Mycobacterium tuberculosis is heterogeneous among patients with active disease, and this has inspired the proposal to use a combination of several specific antigens to find an efficient serodiagnostic test for tuberculosis (TB). However, to date, comparisons of antibody responses to several antigens in the same population have been carried out without consideration of antigenic cell wall glycolipids. In the present study the presence of immunoglobulin G (IgG), IgM, and IgA antibodies to M. tuberculosis glycolipids (sulfolipid I, diacyltrehaloses, triacyltrehaloses, and cord factor) was compared with the response to four commercially available tests based on the 38-kDa protein mixed with the 16-kDa protein or lipoarabinomannan. Fifty-two serum samples from TB patients and 83 serum samples from control individuals (48 healthy individuals and 35 non-TB pneumonia patients) were studied. Three relevant results were obtained. (i) Smear-negative TB patients presented low humoral responses, but the sera which did react principally showed IgA antibodies to some glycolipidic antigens. (ii) TB patients exhibit heterogeneous humoral responses against glycolipidic antigens. (iii) Finally, test sensitivity is improved (from 23 to 62%) when IgG and IgA antibodies are detected together in tests based on different antigens (proteins and glycolipids). We conclude that it is possible to include glycolipidic antigens in a cocktail of specific antigens from M. tuberculosis to develop a serodiagnostic test.

Serodiagnosis has been considered the Holy Grail of tuberculosis (TB) diagnosis research. A serological test would be the ideal method for implementation worldwide due to its low cost and simplicity. To replace the “gold standard,” culture, a serological test should possess sensitivities of over 80% and test specificities of over 95%, according to the recommendations of the World Health Organization (30). To date no test has these features, even though researchers have been looking for one for more than 100 years.

The TB serodiagnosis history started in 1898, when Argoing agglutinated antibodies from TB patient sera (6). Since the introduction of the enzyme-linked immunosorbent assay (ELISA) in the 1970s, several groups of investigators have been trying to find an optimum antigen. Semipurified antigens such as purified protein derivative (PPD), A60, or Kp90 have been used. Purified antigens, that is, proteins, lipopolysaccharides, and glycolipids (e.g., antigen 5 [the 38-kDa protein], lipoarabinomannan [LAM], or diacyltrehaloses [DAT], respectively), have also been assayed. However, no test has shown sufficiently high sensitivity and specificity values for diagnostic purposes (2–5, 12, 13, 29). The third generation of assays originated with the introduction of recombinant proteins, but none of these new tests achieved good diagnostic characteristics either (1, 5, 21).

Nowadays, it is accepted that TB patients produce antibodies to more than one proteinaceous antigen (17, 18, 23, 24). A wide spectrum of humoral responses exist in TB patients, depending upon the disease stage, the patient’s immunological background, the antituberculous therapy, and/or the differential gene expression of different strains of Mycobacterium tuberculosis (17, 26). Thus, some investigators suggest the use of a combination of specific purified antigens for TB serodiagnosis (9). Combinations of antigens are evaluated by analyzing in parallel the presence of antibodies against them in sera from the same population (10, 11, 16, 18). However, previous studies analyzing the simultaneous response to several antigens did not consider the inclusion of antigenic cell wall glycolipids (10, 16–18, 23, 24).

In the present work we have analyzed the patterns of antibody responses (immunoglobulin G [IgG], IgM, and IgA) of the same group of sera to glycolipids (DAT, triacyltrehaloses [TAT], sulfolipid I [SL-I], and cord factor [CF]) from M. tuberculosis using an in-house ELISA (14), in parallel with determination of the patterns of responses to proteinaceous and lipopolysaccharide antigens (the 38-kDa antigen combined with the 16-kDa antigen or LAM), using commercially available PATHOZYME kits.

MATERIALS AND METHODS

Patient sera. (i) Tuberculous patients. Fifty-two serum samples from human immunodeficiency virus-seronegative individuals (age range, 19 to 87 years) were studied. All these patients had been admitted to the Hospital Universitari Germans Trias i Pujol (HUTIP) in Badalona, Spain, and were clinically diagnosed with TB; this was subsequently confirmed bacteriologically by isolation of tuberculous bacilli in cultures of Löwenstein-Jensen medium and with the nonradioactive MB/Bact system (Organon Teknika, Durham, N.C.). Forty serum samples...
were from patients with pulmonary TB, and 12 serum samples were from patients with extrapulmonary TB. The extrapulmonary locations comprised disseminated locations (three patients), the lymphatic system (two patients), punctures (two patients), pleural fluid (two patients), a pus abscess (one patient), bone (one patient), and a cutaneous site (one patient). The patients had not yet started antituberculosis treatment when the serum samples were taken.

(ii) Control subjects. Eighty-three serum samples from human immunodeficiency virus-seronegative individuals (age range, 20 to 80 years) were included as negative controls. Thirty-five of these samples were from patients with non-TB pneumonia. These diseases were caused by Streptococcus pneumoniae (6 serum samples), Coxiella burnetii (6 serum samples), Chlamydia spp. (8 serum samples), Mycoplasma pneumoniae (8 serum samples), and Legionella pneumophila (7 serum samples). All of these patients were PPD negative. Sera from these patients were obtained from the Microbiology Service serum bank at HUGTiP. Forty-eight serum samples were from healthy adults. Eight of these had previously been vaccinated with Mycobacterium bovis BCG. All 8 were PPD positive, and 29 were PPD negative at the time that the serum samples were taken. Three samples were from individuals who had had TB more than 5 years earlier and who had received and completed the standard treatment for TB. Control sera were obtained from healthy employees of HUGTiP, Ph.D. students at the Universitat Autònoma de Barcelona, and the Barcelona Tuberculosis Prevention and Control Program. All serum samples were collected and stored at −40°C until use.

Serological tests. (i) ELISA with glycolipids. The glycolipidic antigens were purified from an M. tuberculosis clinical isolate as described previously (19, 20). The glycolipid fraction was extracted from M. tuberculosis cells by using a mixture of chloroform and methanol, and individual glycolipids were purified by silica-gel column chromatography. ELISA was performed as described previously (14). Briefly, microtiter plates (Immulon 1; Dynex Technologies, Inc., Chantilly, Va.) were coated with purified DAT, TAT, SL-I, or CF (1,000 ng each in 50 μl of n-hexane/well). Sera were diluted 1/400, 1/200, and 1/100 in blocking agent and added in duplicate to wells to measure the levels of IgG, IgM, and IgA antibodies, respectively. IgG-, IgM-, and IgA-specific conjugates labeled with alkaline phosphatase (Southern Biotechnology Associated, Inc., Birmingham, Ala.) were used at a 1/3,000 dilution in blocking agent. The absorbance at 405 nm was determined with a microtitre reader (EIAx800; Automated Microplate Reader; Bio-Tech Instruments, Inc.).

(ii) PATHOZYME kits. Four commercially available ELISAs were used: the PATHOZYME-TB complex kit (Omega Diagnostics, Alloa, Scotland) and the individual PATHOZYME-MYCO kits (Omega Diagnostics) for IgG, IgM, and IgA. All four of these tests basically use one compound, the 38-kDa antigen, which is obtained by recombinant technology. In the PATHOZYME-TB complex kit, the 38-kDa antigen is mixed with the 16-kDa recombinant protein, and in the PATHOZYME-MYCO tests it is mixed with the LAM antigen. The tests were performed according to the instructions of the manufacturer.

Data analysis. For the ELISA with glycolipids, the data analysis was performed as follows. To correlate the data for day-to-day variations, three titrated serum samples (standards) and a blank (blocking buffer alone) were included in each plate. A curve was drawn for each plate, and a comparison of their slopes was carried out. If these data were not satisfactory (slope less than 98%) the plate was rejected. The difference between the absorbance of the serum sample and nonspecific absorption (for wells treated with solvent alone, which were included for each serum sample tested) was noted, and the mean value was calculated. The normalized data were then calculated to establish the corrected change in the absorbance at 405 nm (ΔA405) by using the curve of the standards.

The cutoff was chosen as the mean plus 3 standard deviations (SDs) of the corrected optical density (OD) values obtained for the healthy population. A second cutoff (the mean plus 6 SDs, as shown in Table 1) was chosen for each test in order to distinguish the highest reactivities (8, 17).

The serum samples and the positive, negative, and cutoff controls included in the kits were tested in duplicate. For the IgG and IgA kits, all results are expressed as the number of serological units of specific IgG or IgA per milliliter and were read from a semilogarithmic reference curve, which was prepared by using the standard solutions included in the kit. For the IgM test, the low-positive control was used for the interpretation of the results. The calculation of cutoff values was carried out according to the recommendations of the manufacturer (Table 2).

RESULTS

The results for the antibody responses to different antigens were expressed as negative, low positive, and high positive, according to the different cutoff values described in Materials and Methods (Tables 1 and 2). The results obtained by each test are shown in Table 3.

Serum reactivities. To compare the results for TB patients, we chose the tests that provided high specificity values (greater than 90%) (Tables 3 and 4) after selection of the highest cutoff values (Tables 1 and 2). The tests eliminated were those with TAT (IgG), SL-I (IgG), DAT (IgA), and TAT (IgA). Finally, for comparative purposes, we excluded all tests based on the IgM response, since a high IgM antibody response was observed in very few TB patients (Table 3).

Antibody pattern. Using the tests selected, we analyzed the antibody response patterns, as shown in Tables 5 and 6 and as described below. In Table 5, a distinction is made between

| Table 1. Cutoff values chosen for the glycolipid tests |
|-----------------------------------------------|
| ELISA glycolipid | OD<sup>a</sup> |
|-------------------|-----------------|
| DAT (IgG) | <0.352 | 0.352–0.623 | >0.623 |
| TAT (IgG) | <0.141 | 0.141–0.243 | >0.243 |
| SL-I (IgG) | <0.111 | 0.111–0.189 | >0.189 |
| CF (IgG) | <0.130 | 0.130–0.214 | >0.214 |
| DAT (IgA) | <0.115 | 0.115–0.194 | >0.194 |
| TAT (IgA) | <0.063 | 0.063–0.113 | >0.113 |
| SL-I (IgA) | <0.099 | 0.099–0.171 | >0.171 |
| CF (IgA) | <0.103 | 0.103–0.179 | >0.179 |
| DAT (IgM) | <0.572 | 0.572–0.990 | >0.990 |
| TAT (IgM) | <0.149 | 0.149–0.256 | >0.256 |
| SL-I (IgM) | <0.168 | 0.168–0.292 | >0.292 |
| CF (IgM) | <0.331 | 0.331–0.561 | >0.561 |

<sup>a</sup> A sample is considered to have negative result when the corrected OD value is less than the mean plus 3 SDs for healthy donors by each test. A low-positive result is between the mean plus 3 SDs and the mean plus 6 SDs for healthy donors. A high-positive result is greater than the mean plus 6 SDs for healthy donors.

| Table 2. Cutoff values chosen for the PATHOZYME tests |
|------------------------------------------------------|
| Result | Serological unit or OD<sup>a</sup> |
|--------|---------------------------------|
| 38 kDa + 16 kDa (IgG) | 38 kDa + LAM (IgG) | 38 kDa + LAM (IgA) | 38 kDa + LAM (IgM) |
| Negative | <200 | <400 | <300 | OD less than that for low-positive control |
| Low positive | 200–450 | 400–900 | 300–600 | OD for low-positive control to OD for low-positive control × 1.5 |
| High positive | >450 | >900 | >600 | OD greater than that for low-positive control × 1.5 |

<sup>a</sup> Data are expressed in serological units according to the standard curve provided by the manufacturer for the tests for IgG and IgA. 38 kDa + 16 kDa, PATHOZYME-TB complex test; 38-kDa + LAM, PATHOZYME-MYCO test.
smear-positive and -negative pulmonary TB patients. The results showed that 24 serum samples from the 29 smear-positive pulmonary TB patients reacted to at least one of the glycolipidic antigens: 6 had only IgG antibodies to glycolipids, 9 had only IgA antibodies to glycolipids, and 9 had both IgG and IgA antibodies to glycolipids. However, only 16 serum samples from these patients reacted by commercially available tests: 10 serum samples had only IgG antibodies, one serum sample had IgA antibodies, and 5 serum samples had both IgG and IgA antibodies. In the case of 11 smear-negative pulmonary TB patients, the serum samples from only 4 patients were reactive in tests with glycolipidic antigens: all 4 reacted by the test with SL-I (IgA), 3 reacted by the test with CF (IgA), and 1 reacted by the test with CF (IgG). Only 3 serum samples from the 12 extrapulmonary TB patients showed antibodies by one test or another. In this group, only one serum sample belonging to a patient with disseminated TB showed antibodies by six different tests (Table 5).

With respect to the controls, no serum samples from healthy subjects reacted against glycolipidic antigens, although serum samples from three of them showed antibodies to proteins or the LAM antigen. The serum from one PPD-negative patient

### TABLE 3. Positive results obtained by all tests by use of two different cutoff values for each test

| Test | IgG | IgA | IgM |
|------|-----|-----|-----|
| 38 kDa + 16 kDa | &lt;100 | &lt;100 | &lt;100 |
| 38 kDa + LAM | &lt;100 | &lt;100 | &lt;100 |
| DAT | &lt;100 | &lt;100 | &lt;100 |
| SL-I | &lt;100 | &lt;100 | &lt;100 |
| CF | &lt;100 | &lt;100 | &lt;100 |

*Sensitivity and specificity of each test selected*
reacted with the 38-kDa antigen and the LAM antigen (IgG),
the serum from one PPD-positive patient reacted with the
38-kDa antigen and the LAM antigen (IgA), and the serum of
one vaccinated patient reacted with the 38-kDa antigen and
the 16-kDa protein (IgG). With reference to sera from patients
with non-TB pneumonia, the tests based on IgA antibody de-
tection were the most specific, and only two serum samples
were reactive. However, tests based on IgG antibody detection
were more nonspecific: 10 different serum samples reacted by
the four different tests with IgG. In this case no reactive serum
samples showed antibodies to more than one antigen (Table
6).

**Combinations of different tests.** The overall sensitivities
of each test were very low (Table 4). However, the combination of
antibody responses by different tests increased the test sensi-
tivity.

Sera from 26 of the 29 smear-positive pulmonary TB pa-
tients reacted by at least one of the seven different tests (Table
5): 10 serum samples showed reactivity only to glycolipids, 2
serum samples showed reactivity only by commercially avail-
able tests, and 14 serum samples showed reactivity by both
commercially available and in-house tests. Sera from 30 of the
40 pulmonary TB patients showed antibodies by at least one
test, with a sensitivity of 75%; however, the highest sensitivity
obtained by an individual test was 50% (SL-I [IgA]).

When combining the results, we observed that 14 different
control serum samples (3 from healthy people and 11 from
non-TB pneumonia patients) showed IgG or IgA antibodies to
some antigen. Only one serum sample from a patient with
pneumonia caused by *S. pneumoniae* showed IgG and IgA
antibodies simultaneously by two different tests (Table 6).
In general, 69 serum samples from the 83 controls did not show
any response to mycobacterial antigens (specificity, 83.1%).

If the results of the most nonspecific test (that with the
38-kDa antigen and 16-kDa protein [IgG]) is not considered,
the overall sensitivity remained about 75%, whereas the overall
test specificity increased to 90.3%; 95.8% for healthy controls
and 82.8% for non-TB pneumonia patients.

**DISCUSSION**

We report here on the patterns of antibody reactivities
against four glycolipids from the *M. tuberculosis* cell wall and,
simultaneously, the reactivities in four commercially available
tests based on the 38-kDa, 16-kDa, and LAM antigens ob-
tained for 135 serum specimens.

The first interesting result of this comparative study is that
the sera from TB patients reacted more strongly to glycolipids
than to the antigens in the commercially available tests. Twen-
ty-four of the 29 smear-positive pulmonary TB patients had
IgG or IgA antibodies to at least one of the glycolipidic anti-
gen. However, the sera from only 16 of them reacted to the
antigens in the commercially available tests (Table 5). Surpris-
ingly, among the most problematic cases in terms of diagnosis
(smear-negative pulmonary TB patients [11 patients] and pa-
tients with extrapulmonary forms of TB [12 patients]), the sera
from 7 patients reacted to the glycolipids and the serum from
only 1 patient showed reactivity by the commercially available
tests. The data also indicate that IgA detection identifies more
smear-negative pulmonary TB patients than does IgG detec-
tion (Tables 4 and 5): among the four serum samples that reacted, IgA was detected in all of them, whereas IgG was detected in only one.

None of the serum samples from healthy control subjects showed antiglycolipid antibodies, but three showed antibodies by the IgG or IgA test based on the 38-kDa protein. Furthermore, the IgA test was more specific than the IgG test when antibodies to glycolipids were detected. Of the 14 control subjects whose sera reacted by some tests, only the serum of 1 subject had IgA antibodies to glycolipidic antigens (by the SL-I [IgA] test).

In this work, we chose the glycolipids from *M. tuberculosis* that have been the most studied. SL-I, DAT, and TAT are antigenic glycolipids exposed on the surface of *M. tuberculosis* (28). SL-I is exclusive to *M. tuberculosis*, and DAT and TAT are present only in *M. tuberculosis* and *Mycobacterium fortuitum*. CF is located in the deeper cell wall layers and is present in the majority of mycobacteria. Previous studies analyzing the serodiagnostic powers of these antigens found significantly higher antibody titers in TB patients than in the control group. Although the reported sensitivities and specificities varied widely among the different studies (14), none of the tests provided enough sensitivity to be useful as a serodiagnostic tool by itself, as we have shown previously (13). However, the tests with glycolipids described here (SL-I and CF) provided higher sensitivities and specificities than the test based on the 38-kDa antigen (Table 4) with sera from the same population.

The humoral response to different antigens that we have observed by the commercially available test can only be inferred. We do not know the percentage of each antigen present in these kits. All of them are based on the 38-kDa antigen. This protein (which was initially named antigen 5) is the antigen that has been the most studied for the serodiagnosis of TB, and it is the basis of the majority of commercial tests for the serodiagnosis of TB (21). The specificities of the test reported previously coincide (from 88 to 100%). However, the sensitivities of the test reported previously vary widely: from 36 to 89% for smear-positive TB patients, 16 to 54% for smear-negative TB patients, and 12 to 56% for extrapulmonary TB patients (2, 3, 17, 27, 29). Samanich et al. (24) detected antibodies to the 38-kDa antigen only in sera from pulmonary TB patients with smear-positive sputum. This observation is in agreement with our results, since none of the sera from the smear-negative pulmonary TB patients in our study showed reactivity by the tests based on this antigen (Tables 4 and 5).

Surprisingly, the test which included the 16-kDa antigen turned out to be the most nonspecific for the control non-TB pneumonia population (Table 4), even though this antigen is exclusive to *M. tuberculosis* complex species. Our results coincide with those recently obtained by Raja et al. (22). Those investigators found that 31% of the individuals with non-TB lung diseases has antibodies to the 16-kDa antigen.

The other attractive result obtained in this study was the heterogeneous antibody response in smear-positive pulmonary TB patients. The majority of the serum samples from these patients showed reactivity by three or more different tests (Table 5). The antibody response was considerably reduced for smear-negative patients compared with that for smear-positive patients, as described above. One patient (Table 5, patient 83) in the extrapulmonary TB group was the exception, with the
serum from this patient being reactive by six different tests. This patient had disseminated TB. Thus, the results may show a relationship between the capacity to recognize a greater variety of antigens and the bacterial load in TB patients. Previous studies analyzing IgG antibodies showed that smear-positive pulmonary TB patients recognize more proteinaceous antigens than smear-negative TB patients (15, 18, 25). We obtained the same result using the commercially available tests, and interestingly, the same behavior with respect to glycolipids has been found in TB patients.

The heterogeneous humoral responses found by different investigators using proteinaceous antigens provide the rationale for the development of a multiantigen test. In this way, the 38-kDa antigen has been combined with other proteins to increase the sensitivity of the test. A test sensitivity of 56.3% has been achieved when the 38-kDa antigen is combined with MTB48 (a gain of 12.9%) (16); when the 38-kDa antigen is mixed with Mtb81, the sera from 68% of the TB patients analyzed reacted (10); and when the 38-kDa antigen is mixed with recombinant CFP-10, the rates of antibody detection increased from 49 to 58% for smear-positive TB patients and 21 to 40% for smear-negative patients (7). In the kits used here, the 38-kDa antigen was mixed with the 16-kDa and LAM antigens, and overall sensitivities for IgG antibody detection of 19 and 23%, respectively, were obtained (Table 4). None of these mixtures has yet attained enough sensitivity for use for the diagnosis of TB.

The heterogeneous antibody response to glycolipids that we observed led us to test the possibility of combining the results of the tests with glycolipids with those obtained with the other antigens. The response to the most specific glycolipid could complement the antibody response to protein-based tests: by use of a combination of the SL-1 (IgA) test and the test with the 38-kDa antigen and LAM (IgG), a test sensitivity of 62% for pulmonary TB patients was achieved, with an overall test specificity of 96.3%. This results in gains in sensitivity of 12 and 32%, respectively.

Thus, our data show that the glycolipidic antigens studied in this work induce higher IgG and IgA humoral responses than the tests based on the 38-kDa protein for TB patients who have not yet started antituberculous treatment. However, neither the glycolipid-based tests nor the commercially available tests used in this study are useful for routine diagnostic application by themselves. The sensitivities of the tests improve when the glycolipids are used together with the 38-kDa and LAM antigens and when IgG and IgA antibodies are detected, but neither type of test is good for diagnostic purposes. However, the results presented here support the continued evaluation of glycolipidic antigens in combination with other antigens for the diagnosis of TB.

In view of the results obtained in the present study, we conclude that subsequent studies on the serological diagnosis of TB must take into account three recommendations. First, it will be necessary to test a large number of new potential antigens in parallel to evaluate their real contributions to the serodiagnosis of TB. Second, the glycolipidic antigens must not be excluded in such studies. Finally, in subsequent studies it will be essential to focus on finding combinations of antigens fundamentally useful for the diagnosis of TB in smear-negative patients, since the sera from these subjects did not react against the antigens studied here, nor, in general, did they react against the known antigens tested to date.

ACKNOWLEDGMENTS

This work was supported by grants from the Fondo de Investigaciones Sanitarias de la Seguridad Social (grant 96/1422) and from the Generalitat de Catalunya (grant 2002SGR-00099). ELISA kits were kindly provided by Omega Diagnostics.

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