**Article III**

III.   **Julián, E.;** Luquin, M. (2001) "Serological diagnosis of tuberculosis using IgA detection against the mycobacterial Kp-90 antigen (letter)". *International Journal of Tuberculosis and Lung Diseases*. 5 (6): 585-586.

# Correspondence

## Serological diagnosis of tuberculosis using IgA detection against the mycobacterial kp-90 antigen

We read with interest the article by Julián et al. published in a recent issue of the International Journal concerning the evaluation of the Kreatech TB IgA enzyme-linked assay in Barcelona, Spain.[1] In their study, a sensitivity of 70.58% and a specificity of 68.68% were obtained. The authors attributed the overall low specificity to the inclusion of a high proportion of non-tuberculous pneumonia patients in the study, as this group gave a specificity of only 47.22%. Two published studies[2,3] that obtained high specificities of ≥90% included non-tuberculous chest diseases and healthy volunteers as controls. Amongst the former, there was about 10% of non-TB pneumonia patients only. In 1995, we evaluated 122 patients with active tuberculosis (smear-positive, culture-positive cases 90, histologically confirmed cases 18, clinical and radiographically compatible cases 14), 10 patients with inactive pulmonary TB, and 27 patients with non-tuberculous lung diseases (carcinoma of the lung, COPD, bronchiectasis) by a similar kit (unpublished data). The sensitivity and specificity of this method of serodiagnosis of TB were found to be respectively only 67.2% and 40.0%.

We used the cut-off points for positivity and negativity as recommended by the manufacturer at the time. Further evaluations were made by performing ROC analysis with a view to delineating a cut-off value with more optimal sensitivity and specificity. It was, however, found that the curve assumed a somewhat linear, rather than a parabolic configuration, thus precluding the possibility of obtaining more satisfactory sensitivity and specificity results. This is in contrast to the findings in one study,[2] but quite in keeping with that in another,[4] both of which used ROC analysis. In the latter, the optimal sensitivity and specificity obtained were also relatively low, i.e., 62.6% and 66.3%, respectively.

Thus, whilst serological tests can be a potentially attractive addition to the armamentarium in diagnosis of TB, perhaps especially in HIV-infected[5] and geriatric patients,[6] much more work has to be done to identify a test with replicative usefulness in clinical settings in different communities.[4,7]

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### References

1 Julián B, Matas L, Hernández A, Alcaide J, Luquin M. Evaluation of a new serodiagnostic tuberculosis test based on immunoglobulin A detection against Kp-90 antigen. Int J Tuberc Lung Dis 2000; 4: 1082–1085.
2 Alifano M, De Pascalis R, Sotia M, Faraone S, Del Pezzo M, Covelli I. Evaluation of IgA-mediated humoral immune response against the mycobacterial antigen P-90 in diagnosis of pulmonary tuberculosis. Chest 1997; 111: 601–605.
3 Arikan S, Tuncer S, Us D, Unal S, Ustaçelebi S. Anti-Kp-90 IgA antibodies in the diagnosis of active tuberculosis. Chest 1998; 114: 1253–1257.
4 Chiang I-H, Suo J, Bai K-J, et al. Serological diagnosis of tuberculosis. A study comparing three specific mycobacterial antigens. Am J Respir Crit Care Med 1997; 156: 906–911.
5 Chum H J, O'Brien R J, Chonde T M, Graf P, Rieder H. An epidemiological study of tuberculosis and HIV infection in Tanzania 1991–1993. AIDS 1996; 10: 299–309.
6 Davies P D O. Tuberculosis in the elderly. J Antimicrob Chemother 1994; 34 (Suppl A): 93–100.
7 Somi G R, O'Brien R J, Mfinanga G S, Ipuge Y A. Evaluation of the Myco-Dot™ test in patients with suspected tuberculosis in a field setting in Tanzania. Int J Tuberc Lung Dis 1999; 3: 231–238.

## In reply

We entirely agree with the comments from Yew et al. When the aim of the study is to evaluate a new commercial serological assay in order to diagnosis a disease (as with our cited paper[1]), inclusion of healthy donors only is not a realistic situation. The most problematic controls are people who are ill and who are susceptible to being confused with tuberculous patients. The sample studied has to be representative of the patients who are cared for in the hospitals of the given country or area. In addition to adult and child patients suffering from pulmonary and extrapulmonary disease, it would have been interesting to include patients with infectious diseases endemic to, or with a high prevalence in that particular area. The control population studied must be representative of the average type of person, healthy or otherwise, found in the environment.

In the case of commercial assays that are not subject to methodological differences, as in house tests, it is not possible to find results that are as divergent as those found in the literature. As we mention in our study,[1] bias in the control population has a consider-

able influence on test specificity, and that is extremely important, given that any new diagnostic test must reach specificities close to 100% in order to compete with existing standard diagnostic methods (microscopy and culture). There are only a few commercially available tests for the serodiagnosis of tuberculosis disease; nevertheless, none of them is yet in use as a standard diagnostic tool anywhere, as the results reported are discrepant with respect with their utility.[3] Currently, there is no commercial assay that provides a high sensitivity and specificity, but it is true that it is difficult to find work that strictly revises the characteristics of these assays with the optimal population.

On the other hand, serology is still one of the most promising current methods for detecting infected individuals, or even in providing trials for new candidate vaccines.[4] In these other approaches the group of people to be considered for checking these tests is very different from that in diagnostic tests. For all these reasons, as Small and Perkins remark,[5] in the evaluation of new serodiagnostic assays, it is indispensable to maintain a serious consensus with regard to the type and number of population studied, as a means of clarifying what occurs with each case.

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*References*

1 Julián E, Matas L, Hernández A, Alcaide J, Luquin M. Evaluation of a new serodiagnostic tuberculosis test based on immunoglobulin A detection against Kp-90 antigen. Int J Tuberc Lung Dis 2000; 4: 1082–1085.
2 Julián E, Cama M, Martinez P, Luquin M. An ELISA for five glycolipids from the cell wall of *Mycobacterium tuberculosis:* Tween 20 interference in the assay. J Immunol Methods 2001; 251: 21–30.
3 Potturnarthy S, Wells V C, Morris A J. A comparison of seven tests for serological diagnosis of tuberculosis. J Clin Microbiol 2000; 38: 2227–2231.
4 Gennaro M L. Immunologic diagnosis of tuberculosis. Clin Infect Dis 2000; 30 (Suppl 3): S243–246.
5 Small P M, Perkins M D. More rigour needed in trials of new diagnostic agents for tuberculosis. Lancet 2000; 356: 1048–1049.

## Evidence for the transmission of tuberculosis in a prison in Nakhon Ratchasima, Thailand

In industrialised countries strain characterisation has demonstrated tuberculosis transmission among prisoners.[1] However, such studies are relatively rare in developing countries.[2] This is the first report from Thailand providing evidence of tuberculous transmission in a prison.

The study was performed in a provincial prison in Nakhon Ratchasima, North-eastern Thailand, which had about 930 prisoners in four non-air-conditioned buildings. The prisoners shared nine sleeping units at night.

Every 3–5 months, from October 1996 to September 1997, all prisoners were requested to provide sputum specimens for microscopic examination and cultivation. *Mycobacterium tuberculosis* was isolated from 24 samples of the prisoners during this period. Nine were smear-positive, and all of the others were smear-negative. The mean age of patients was 39 years. Three patients were known to be HIV-positive. *M. tuberculosis* was identified by the niacin test as well as by analysis of the 16S–23S rDNA spacer.[3] Chromosomal DNA of 22 isolates was available for Southern hybridisation with IS6110-, DR- and PGRS-specific probes, which was performed as previously described.[4] Thirty-six *M. tuberculosis* isolates randomly selected among smear-positive patients with pulmonary tuberculosis from the general population in the province during the same period were also studied.

The distribution of 1S6110-hybridization patterns was similar to that previously described.[4] Respectively 11 and eight isolates belonged to the Beijing and Nonthaburi groups. Sixteen had a single copy of IS6110, while the others had heterogeneous hybridisation patterns.

Among 42 isolates with more than one copy of IS6110, two pairs of isolates with identical hybridisation patterns were found. Both isolates in the first pair contained two copies of IS6110 but had different DR- and PGRS-hybridisation patterns. One of the isolates was from a prisoner. These indicated that the isolates were not the same strain and confirmed the need to recharacterise isolates which contain few identical IS6110-hybridised bands by other methods before concluding the biological relationships between them.

Both isolates in the second pair had five hybridised bands and identical DR- and PGRS-hybridisation patterns. The first was isolated at the start of the study, while the second was isolated 4 months later from an HIV-infected patient who was imprisoned in the same unit as the first patient, indicating that transmission had occurred from the first patient to the second. HIV infection in the second patient would accelerate progression to the disease.[5] Measures to control tuberculosis transmission in prisons are clearly required.

Sixteen isolates had a single IS6110-hybridised band. On characterisation by DR- and PGRS-specific probes, most single-banded isolates had either unique DR- or PGRS-hybridised patterns, or both. However, four isolates shared identical DR- and PGRS-hybridisation patterns. One isolate in the group was from a prisoner, while the others were from the general population. There was no known epidemiological link between the four patients. This supports the contention that limited epidemiological inferences can be

**Article IV**

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# An ELISA for five glycolipids from the cell wall of *Mycobacterium tuberculosis*: Tween 20 interference in the assay

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## Abstract

*Mycobacterium tuberculosis* cell wall contains antigenic glycolipids: phenol-glycolipid (PGL), diacyltrehalose (DAT), triacyltrehalose (TAT), cord-factor (CF), and sulpholipid-I (SL-I). In the last decade, the usefulness of these antigens for the serodiagnosis of tuberculosis has been evaluated mainly using enzyme-linked immunosorbent assays (ELISA). Currently, there are no conclusive results about the utility of these glycolipidic antigens, because the results obtained by different groups are discrepant. In order to explain these discrepancies, we have investigated the methodological variations in the ELISAs used previously. Specifically, we have studied the following: the coating solvent, the optimum amount of glycolipid coated per well, the blocking agent, and the use of detergent (Tween 20) in the washing buffer. The most significant finding was that Tween 20 detaches PGL, DAT, TAT and SL-I from microtitre wells. However, Tween 20 does not remove CF from the wells. In addition, we have found that the best solvent for coating is *n*-hexane, that the optimum antigen coating concentration is 1000 ng/well, and that BSA and gelatin are equally effective blocking agents. We can therefore conclude that the use of Tween 20 as a detergent, and the lower antigen coating concentrations (100–200 ng/well), may well explain some of the discrepancies in previous studies. © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* ELISA; Glycolipid; Serology; Tuberculosis; Tween 20

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## 1. Introduction

Tuberculosis (TB) is still one of the greatest health care problems in the world. Prompt detection of the illness is essential if the fight against TB is to be successful. For this reason, one of the goals of the World Health Organization is the rapid detection of 70% of new infectious TB cases by 2010. In this way, one-quarter of TB deaths could be prevented in the next two decades (World Health Organization,

1998). Traditional diagnostic techniques based on the isolation of the tuberculous bacillus in culture media are time consuming, and it is necessary to wait several weeks to obtain a result. However, recent molecular biology techniques have made it possible to diagnose TB in a few hours. Another rapid diagnostic method would be the detection of specific antibodies in TB patients. One of the principal advantages of serological as opposed to molecular methods is the possibility of using blood, which is easier to obtain than respiratory samples or other organic fluids and tissues. However, at present there are no serological tests with sensitivities and specificities similar to those obtained using both molecular tests and traditional culture techniques.

Since the first serological trial in 1898 using an agglutination test, many assays to detect specific antibodies in TB patients have been tested (Daniel and Debanne, 1987). With the appearance of the enzyme-linked immunosorbent assay (ELISA) technique, many antigens have been evaluated in order to develop a rapid TB diagnostic test. First, there was the use of culture filtrates or sonicated extracts, either from *Mycobacterium tuberculosis* or from *Mycobacterium bovis*. Later, the purified protein derivative (PPD) from *M. tuberculosis* and other semipurified antigens such as A60 from *M. bovis* Bacille Calmette-Guérin were used. These complex antigens showed low specificities which were attributed to cross-reactions with similar antigens present in environmental mycobacteria or other micro-organisms (Wilkins, 1998). More purified proteins: Ag5, Ag6 or a variety of purified and recombinant proteins, showed better specificities but low sensitivities (reviewed by Daniel and Debanne, 1987; Bothamley, 1995; and Wilkins, 1998).

Reggiardo et al. (1980) described the first ELISA with purified glycolipids from the *M. bovis* cell wall, reporting sensitivities of up to 91.3%, and specificities of between 97.8 and 100%. Later, Papa et al. (1989) demonstrated the specificity of some of these glycolipids, because immune sera raised in rabbits reacted exclusively with the corresponding antigen and with crude extracts of *M. tuberculosis* complex, but not with crude extracts from 39 other mycobacterial species. These findings prompted the researchers to verify whether these molecules had a potential for TB serodiagnosis. Therefore, in the 90s

many studies using the ELISA technique were performed to test the utility of these glycolipids in TB diagnosis.

However, the results obtained showed a lack of uniformity. As an example of this, Savage et al. (1993) and Escamilla et al. (1996) using the diacyltrehalose (DAT) antigen both obtained a specificity of 98%, but sensitivities of 11.45% and 86.7%, respectively. We observed that these researchers used very different amounts of coated glycolipid per well (Table 1). Moreover, Torgal-García et al. (1988) obtained a sensitivity of 97.5% using phenol glycolipid (PGL) antigen, as against the 60% reported by Ridell et al. (1992). Their ELISA protocol only differs in the use of detergent in the washing buffer to avoid non-specific binding (Table 1). On the other hand, in almost all the previous assays, BSA was used in the saturating buffer (Table 1), but Simonney et al. (1995) questioned its use because high background levels were observed with the sera from TB patients.

We believe that the technical differences in the previous ELISAs may be responsible for some of the discrepancies between groups. As a result, in the present study we performed an ELISA under various conditions in order to clarify these issues: after selecting an antigen solvent, an optimal coating dose, and a blocking agent, we were able to investigate the effect of Tween 20, with five glycolipids from the *M. tuberculosis* cell wall.

## 2. Material and methods

### 2.1. Antigens

This study was performed using the following glycolipids: PGL antigen (phenol glycolipid, or triglycosyl phenolphthiocerol dimycocerosate) from the *M. tuberculosis* Canetti strain (CIPT 1400100-059); and, DAT (2,3-diacyltrehalose), TAT (2,3,6-triacyltrehalose), CF (cord factor, or trehalose-6,6′-dimycolate), and SL-I (2,3,6,6′-tetraacyl trehalose 2′-sulphate) antigens from *M. tuberculosis* H37Rv strain (ATCC 27294).

To isolate the glycolipids, *M. tuberculosis* was grown for 6 weeks at 37°C on Middlebrook 7H9 broth supplemented with ADC enrichment (Difco

Table 1

Survey of literature on ELISA procedures for measuring mycobacterial anti-glycolipid antibodies in sera, showing non-uniformity in the assay protocols

| Antigen | Reference | Solvent for adding to microtitre plate | Amount of glycolipid (per well) | Blocking agent | Detergent |
|---|---|---|---|---|---|
| DAT | Cruaud et al., 1990 | *n*-hexane | 100 ng | 5% BSA | None |
| | Saad et al., 1996 | *n*-hexane | 100 ng | 5% BSA | None |
| | Simonney et al., 1995 | *n*-hexane | 100 ng | 0.5% gelatin | None |
| | Escamilla et al., 1996* | *n*-hexane/ethanol (1/1) | 1000 ng | 3% BSA | None |
| | Ridell et al., 1992 | Ethanol/PBS | 500 ng | 5% BSA | 0.05% Tween 20 |
| | Sempere et al., 1995* | Methanol/carbonate buffer | 2000 ng | 0.2% BSA+0.05% Tween 20 | 0.05% Tween 20 |
| | Muñoz et al., 1997a* | *n*-hexane | 1000 ng | 1–2% BSA | None |
| TAT | Sempere et al., 1995* | Methanol/carbonate buffer | 2000 ng | 0.2% BSA+0.05% Tween 20 | 0.05% Tween 20 |
| | Escamilla et al., 1996* | *n*-hexane/ethanol (1/1) | 1000 ng | 3% BSA | None |
| | Muñoz et al., 1997a* | *n*-hexane | 1000 ng | 1–2% BSA | None |
| CF | Laszlo et al., 1992 | *n*-hexane | 100 ng | 5% BSA | None |
| | Maekura et al., 1993 | *n*-hexane | 2500 ng | 0.05% Tween 20 | 0.05% Tween 20 |
| | Chaicumpar et al., 1997 | *n*-hexane | 2500 ng | 0.05% Tween 20 | 0.05% Tween 20 |
| | Kawamura et al., 1997 | *n*-hexane | 2500 ng | 0.05% Tween 20 | 0.05% Tween 20 |
| SL-I | Cruaud et al., 1989 | *n*-hexane | 100 ng | 0.5% BSA | None |
| | Chaicumpar et al., 1997 | *n*-hexane | 2500 ng | 0.05% Tween 20 | 0.05% Tween 20 |
| | Pan et al., 1999 | *n*-hexane | 2500 ng | 0.05% Tween 20 | 0.05% Tween 20 |
| | Rojas-Espinosa et al., 1999 | ethanol | 2000 ng | 3% BSA | None |
| PGL | Torgal-García et al., 1988 | *n*-hexane | 250 ng | 5% BSA | None |
| | David et al., 1992 | *n*-hexane | 250 ng | 5% BSA | None |
| | Ridell et al., 1992 | *n*-hexane | 250 ng | 5% BSA | 0.05% Tween 20 |
| | Simonney et al., 1995 | *n*-hexane | 100 ng | 0.5% gelatin | None |
| | Chaicumpar et al., 1997** | *n*-hexane | 2500 ng | 0.05% Tween 20 | 0.05% Tween 20 |

*DAT or TAT from *M. tuberculosis* and/or *Mycobacterium fortuitum*.

**Investigators used PGLs with a similar structure to PGL-Tb 1.

Laboratories, Detroit, MI). The glycolipids were purified using column chromatography as previously described (Muñoz et al., 1997a, b). All the purified glycolipids were dissolved in chloroform, divided into 50 μg aliquots, and evaporated to dryness under a stream of nitrogen. All the aliquots were stored at 4°C and were checked before use.

### 2.2. Patients and control subjects

Sera from 10 patients with active pulmonary tuberculosis, who had been admitted to the Universitari Germans Trias i Pujol Hospital, Badalona (Spain), were obtained before anti-tuberculous chemotherapy was started. In all the TB cases, diagnosis was confirmed by isolation of tuberculous bacillus in cultures: Löwenstein-Jensen and the non-radiometric MB/Bact system (Organon Teknika, Durham, NC). Healthy control sera were obtained

from 10 PPD-negative healthy employees at the same hospital. These donors had no known history of mycobacterial disease. All sera were stored at −40°C before testing.

### 2.3. Solvent, coating doses and microtitre plate selection

The purified glycolipids used in this study are very easily re-dissolved with chloroform. However, as chloroform cannot be used with ELISA microtitre plates, we tested other candidate solvents for re-suspension of the glycolipids and for fixing them to microtitre plates. Dry glycolipids (10 μg) were re-suspended in *n*-hexane, ethanol, ethanol/*n*-hexane (1/1), and methanol, and the capacity of these solvents to recover glycolipids was compared with that of chloroform. Comparisons were made on TLC plates (G-60, 0.25 mm thick, Merck, Darmstadt,

Germany), developed with chloroform–methanol–water (30:8:1 vol/vol/vol), revealed by spraying the plates with 1% (wt/vol) anthrone in sulphuric acid, and heating at 120°C (Muñoz et al., 1997b).

Optimal coating doses for the ELISA protocol for each glycolipid were determined by coating microtitre wells with various amounts of each (from 0 ng/well to 3000 ng/well), and evaluated against the sera (from TB patients and controls). We chose the minimal concentration yielding absorbance values at the beginning of the stationary curve in our ELISA procedure on human TB sera as the optimal coating dose. For these assays different microtitre plates were studied (Immulon I M129B, from Dynatech Laboratories, USA; PolySorp from Nunc, Netherlands; Cliniplate from LabSystems, Finland; and Costar 3590 from Costar, USA).

### 2.4. Immunoblot analysis

In order to test cross-reactivity between serum samples and blocking agents we carried out an immunoblot analysis. Bovine serum albumin (8 μg; BSA, Sigma Chemical Co., St Louis, MO) and 16 μg gelatin (Sigma) were applied to a 7.5% SDS–PAGE gel (Bio-Rad laboratories, Richmond, CA, USA) and protein electro-transferred onto nitrocellulose membranes (Bio-Rad) using a Mini-PROTEAN II system (Bio-Rad), according to the manufacturer's protocol. Membranes were blocked in a solution of 3% (wt/vol) dry non-fat milk (Molico Sveltesse, Nestlé, Esplugues de Llobregat, Spain) in Tris buffer (0.02 M Tris–HCl, 0.15 M NaCl) overnight at 4°C.

Serum samples were used at a dilution of 1/200 and incubated for 2 h. After washing the sheets with Tris buffer, a 1/1500 dilution of goat anti-human IgG (H+L) alkaline phosphatase conjugate (SBA, Southern Biotechnology Associates, Inc., Birmingham, AL) was used. The antibody–antigen complex was detected using a solution of 1 ml of the nitro blue tetrazolium (NBT, 30 mg/ml in 70% dimethylformamide; Bio-Rad) and 1 ml of the 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 60 mg/ml in dimethylformamide; Bio-Rad) in 100 ml of the substrate buffer (0.1 M Tris, 0.5 mM MgCl$_2$, pH 9.5) until colour developed. The reaction was stopped by washing with water.

### 2.5. ELISA protocol

Purified 1000 ng of DAT, TAT, CF, SL-I, and PGL were each dissolved in 50 μl of *n*-hexane and the solutions were added to the wells of the plates (Immulon I, M129B), which were air dried and used the following day.

Before the blocking step, the microtitre plates were washed once with Tris-buffered saline (TBS, 0.02 M Tris–HCl, 0.5 M NaCl, pH 7.5). The plates were saturated for 1 h with 200 μl/well of blocking agent: 0.5% (wt/vol) gelatin or 1% (wt/vol) BSA solution in TBS. Then they were washed three times with TBS (three washes of 5 min each). Following this, 100 μl volumes of the serum samples, diluted 1/200 in each blocking buffer, were added in duplicate to the wells and incubated for 1 h. After another three washings with TBS, goat anti-human IgG alkaline phosphatase conjugate (SBA, Inc.) was added, at 1/3000 dilution in each blocking buffer, followed by a 1 h incubation period. The wells were then washed with TBS and developed with 200 μl of 1 mg/ml *p*-nitrophenyl phosphate substrate (pNPP, Sigma) in 10% diethanolamine buffer, pH 9.8, containing 0.5 mM MgCl$_2$. After 25 min the reaction was stopped with 50 μl of 3 M NaOH. Absorbance values were read at 405 nm with a microtitre reader (ELx 800, Automated Microplate Reader, Bio-Tech Instruments[®] Inc.).

All incubations were performed on a platform rocker (Titramax 100, Heidolph Elektro GmbH & Co. KG), and at room temperature (around 25°C). For each serum sample we used similarly treated wells, but without antigen, to check for non-specific absorption (zero point activity).

### 2.6. Washes with detergent

In order to minimize non-specific reactions and/or to improve the affinity of the antigen–antibody interaction, we carried out the ELISA previously described (2.5. ELISA protocol) using 0.5% gelatin in TBS as a blocking buffer, and TBS plus 0.5% polyoxyethylene sorbitan monolaureate, Tween 20, (wt/vol) (TBST) as a washing buffer.

Given that the result was entirely different with CF in contrast to the other glycolipids, the same

ELISA was performed using Tween 20 in the wash and antibody dilution buffers, with CF-coated plates.

### 2.7. Tween 20 influence

To investigate why the use of detergent modifies the ELISA results with these glycolipids, duplicate wells were coated with 1000 ng of each glycolipid diluted in *n*-hexane and evaporated at room temperature. After blocking with 0.5% gelatin in TBS for 1 h, the wells were incubated for 10, 20 or 60 min with 50 μl of TBS or TBST. Afterwards, the whole volume of each well was recovered and subjected to TLC on silica gel-coated plates. In order to recover the antigen remaining attached to the well, 50 μl of hexane were added to each well, and, following an interval of 20 min, the *n*-hexane was recovered and also monitored by TLC. The TLC plates were developed and revealed as described previously (see Section 2.3), in order to detect the presence of glycolipids.

## 3. Results

### 3.1. ELISA protocol optimization

We chose *n*-hexane as the antigen solvent because good (solid, uniform) glycolipid spots were observed on TLC plates for all the purified antigens when compared with antigen dissolved in chloroform. Some of these glycolipids were insoluble in the rest of the solvents: ethanol, ethanol/*n*-hexane or methanol; we observed that they were not recovered adequately, displaying either very diffuse spots on the TLC, or no spots at all. Moreover, optimal coating doses were established for all the glycolipids studied. A dose of 1000 ng of each antigen gave the greatest difference in absorbance values between the sera from TB patients and healthy controls. On the basis of these studies we performed the ELISA using 1000 ng of each antigen dissolved in 50 μl *n*-hexane per well.

We performed the assay with different plates specifically developed for these types of molecules. No differences were found for the various plates, although a more uniform and reproducible coating of the wells was obtained using Immulon I plates from Dynatech Laboratories.

### 3.2. Differences in blocking agents

In order to test for sero-reactive lineal epitopes in the blocking agents, which could react and produce a background signal when used in the saturating buffer, immunoblots were performed. The sera from the TB patients and healthy controls did not, apparently, react with the two blocking agents, gelatin and BSA (data not shown).

Subsequently, the ELISA procedure was carried out with the five glycolipid antigens using the two different blocking agents. The data obtained are shown in Table 2. We did not observe any non-specific reactions in the blank wells (without an-

Table 2

ELISA for five glycolipids from *M. tuberculosis*. Effect of two blocking agents on absorbance values[a]

| Antigen | Serum samples | Blocking agent | |
|---------|---------------|----------------|------|
| | | Gelatin | BSA |
| DAT | TB patients | 0.4451±0.1918 | 0.4887±0.0886 |
| | Control subjects | 0.1210±0.0415 | 0.1692±0.1237 |
| TAT | TB patients | 0.4588±0.1691 | 0.4724±0.2090 |
| | Control subjects | 0.0717±0.0768 | 0.0923±0.0533 |
| CF | TB patients | 1.0655±1.0771 | 1.1385±1.2568 |
| | Control subjects | 0.0705±0.0214 | 0.0123±0.0084 |
| SL-I | TB patients | 0.3213±0.1875 | 0.3022±0.1411 |
| | Control subjects | 0.0647±0.0186 | 0.0353±0.0552 |
| PGL | TB patients | 0.0681±0.0398 | 0.0601±0.0316 |
| | Control subjects | 0.0478±0.0196 | 0.0675±0.0420 |

[a] Values are expressed as mean absorbance values±S.Ds, obtained in the ELISA with sera from TB patients and controls. Results are shown as the average absorbance at 405 nm of samples from 10 TB patients and 10 healthy adults, assayed in duplicate.

tigen). The absorbance values obtained in all the assays were very similar for all the glycolipids when 1% BSA or 0.5% gelatin were used as blocking agents (see Table 2).

### 3.3. Diluting antibodies and washing buffer with detergent

The results obtained in the ELISA with or without detergent in the washing buffer are shown in Fig. 1. The absorbance values for patients who had tested positive with DAT, TAT, SL-I and PGL, became negative in the ELISA using TBST as a washing buffer. The sera that had previously given positive results, gave negative results when Tween 20 was used.

However, the absorbance values did not decrease in the sera which had reacted against the CF antigen (Fig. 1). Finally, to investigate whether the use of detergent had any effect at all on the CF antigen–antibody reaction, we performed the ELISA using TBST both as washing buffer and for antibody dilution. The results obtained were similar to those obtained using only TBS (data not shown), demonstrating that Tween 20 did not interfere in CF–antibody interactions.

### 3.4. Tween interference

The recovery of glycolipids as detected by TLC is illustrated in Fig. 2. When we incubated the wells with TBS (10, 20 or 60 min), there was no recovery of any glycolipid in the TLC (Fig. 2A1 and B1). This was confirmed when we subsequently added *n*-hexane to these wells, because we then recovered all of the antigens (Fig. 2A2 and B2).

When we incubated the wells with TBST, the results differed from antigen to antigen. The DAT and TAT were recovered from the plate after being in contact with TBST for only 10 min (Fig. 2B3). With SL-I and PGL, approximately 50% of the antigen was recovered after 10 min, and all the antigen was recovered after 10–20 min of incubation. However, the CF antigen remained attached to the microtitre plate even after being in contact with TBST for 1 h. This was confirmed by the recovery of all the antigen attached to the plate when we added *n*-hexane after washing the wells (Fig. 2A4).

## 4. Discussion

The selection of a particular ELISA procedure depends on the type of biomolecule being assayed. The development of immunoassays for mycobacterial glycolipids has been complicated by the characteristics of these molecules. In this study we attempted to establish some basic conditions suitable for the adequate development of a test using glycolipids, by analysing the antigen solvent, the concentration per well, and above all the blocking agent and the use of detergent in the washing buffer.

Blocking a surface to reduce non-specific binding is a compromise between low background and high sensitivity and specificity. The use of BSA, the most popular blocking agent (Table 1), is questionable because it gives a high background with sera from TB patients (Simonney et al., 1995). Moreover, Deshpande et al. (1994) have described a specific seroreactive protein (TB66) from the *M. tuberculosis* complex with an 85% homology with BSA in N-terminal sequence. In an earlier study Thorns and Morris (1985) also described common epitopes between mycobacterial antigens and BSA, based on the reaction with BSA of five monoclonal antibodies produced from a sonicated extract of *M. bovis*. These findings may have important implications for the serodiagnosis of mycobacterial infections. However, we found no evidence for cross-reactions with BSA using sera from TB patients in our experiments, neither by immunoblot nor ELISA. We obtained similar results (Table 2) using BSA or gelatin. However, gelatin is a cheaper choice (about 1000%) than BSA in ELISA and therefore we used gelatin in all the ELISA procedures.

The ability of Tween 20 to reduce non-specific binding has been amply established in many ELISA procedures when the nature of the antigen is proteinaceous. It does not interfere with the specific antigen–antibody interaction but minimises non-specific hydrophobic interactions between the added proteins and the microtitre plate (Carpenter, 1997). However, its use has been much discussed for assays in which the antigens were glycolipids or phospholipids (Cabral et al., 1994; Ravindranath et al., 1994; Black and Reynolds, 1998; Akimoto et al., 1999).

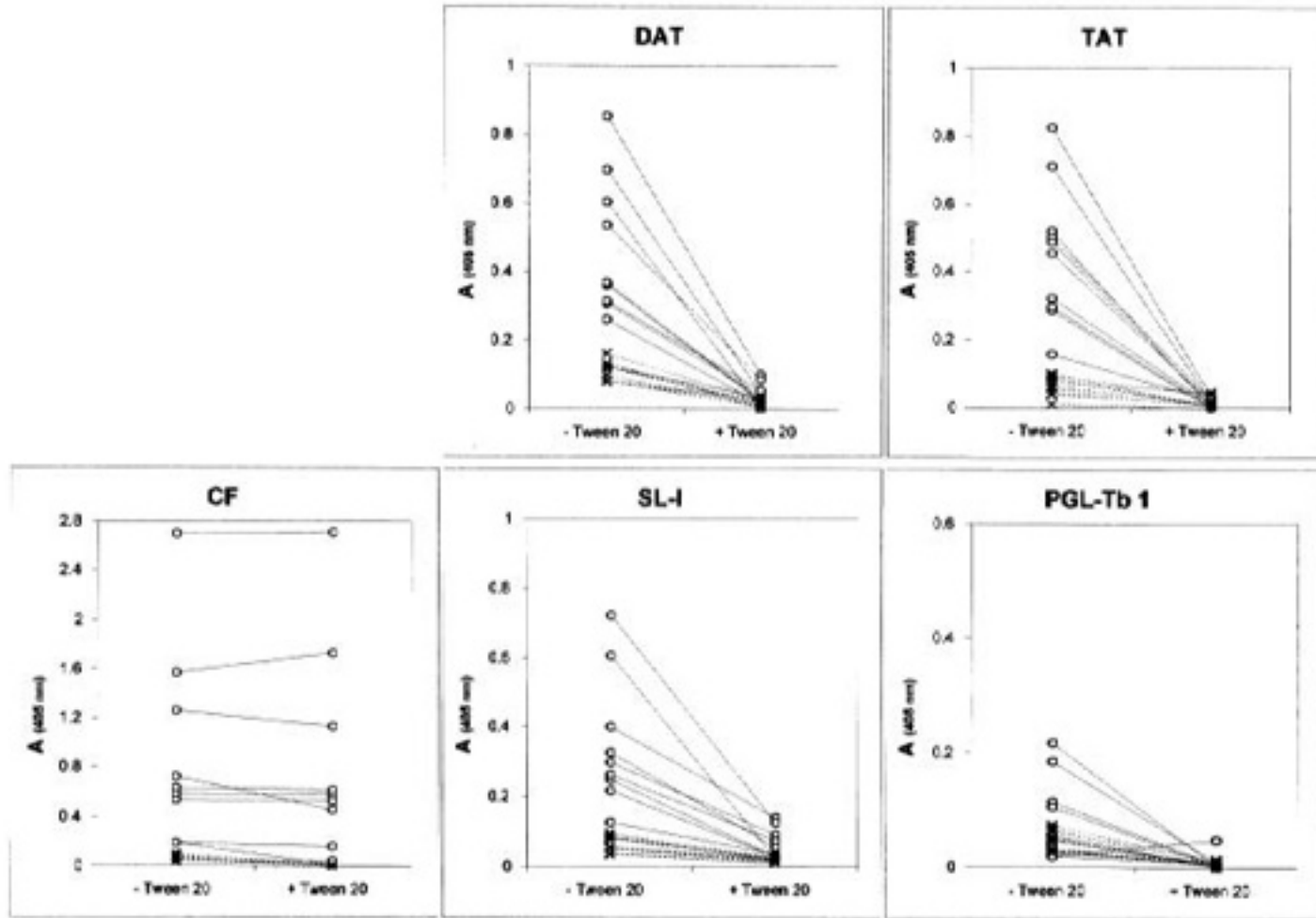In the case of the glycolipids from the myco-

Fig. 1. Effect of the presence (+Tween 20) and absence (− Tween 20) of detergent in the washing buffer used in the ELISAs for five glycolipids from *M. tuberculosis*. Reactivities of anti-glycolipid antibodies were tested with sera from 10 tuberculous patients (○), and sera from 10 healthy controls (×). Each plotted point represents the mean of two different measurements.
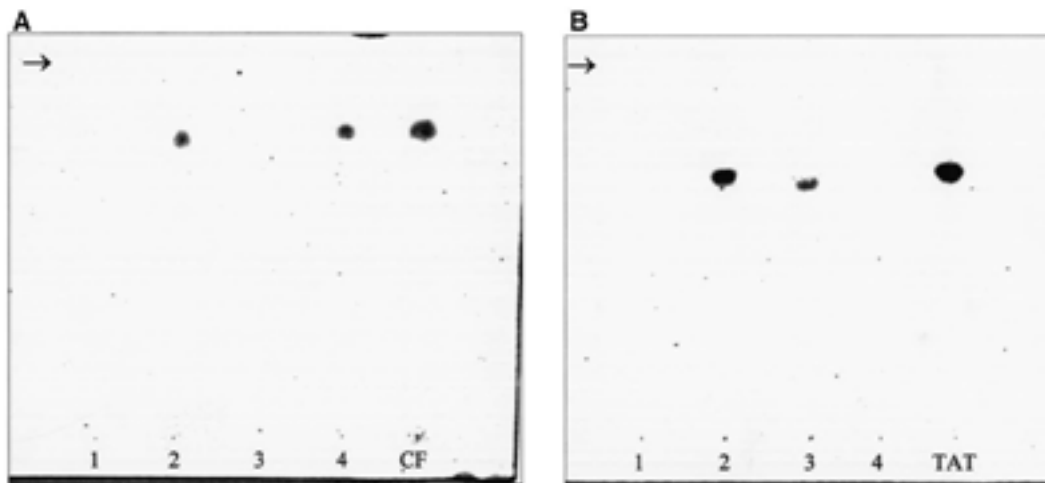
Fig. 2. Recovery of glycolipids, detected by TLC, with the following solutions: 1, TBS; 2, *n*-hexane after incubation with TBS; 3, TBST; 4, *n*-hexane after incubation with TBST. CF and TAT lanes show the glycolipidic patterns diluted with *n*-hexane. The TLC was developed with chloroform–methanol–water (30:8:1 vol/vol/vol). Arrows indicate the solvent front. (A) CF antigen; (B) TAT antigen.

bacterial cell wall, Cho et al. (1983) speculated that the PGL-I from *Mycobacterium leprae* was lost in the fluid phase when Tween was used. Rojas-Espinosa et al. (1999) also expressed doubts about the results obtained in assays with SL-I when using Tween 20. In our study we confirmed that detergent interference could occur in the assay (Fig. 1). The ELISA results obtained using Tween 20 could be due to the detergent interfering with the antigen–antibody reaction, because it disturbs the structure of the glycolipid molecule, or to the detergent detaching the antigen from the plate, thus impeding the subsequent reaction. In order to rule out one of the two possibilities we incubated the antigens attached to the plate with TBS or TBST for different periods of time, to determine whether they could be detached. After monitoring the results by TLC, we demonstrated that the fall in absorbance values was due to the ability of Tween 20 to detach the antigen bound to the plate (Fig. 2).

Our findings may explain the contradictory results obtained in previous studies. Using 2000 ng of TAT and DAT antigens and Tween in the assay, with sera from TB patients and healthy controls, Sempere et al. (1995) obtained specificities between 91 and 98.8%, and sensitivities between 9 and 51%. In contrast, in the absence of Tween 20, specificities of 96% and sensitivities between 80 and 93.3% have been re-

ported (Escamilla et al., 1996; Muñoz et al., 1997a). In another study Pan et al. (1999), with 2500 ng of SL-I antigen, but using detergent in the assay, hardly detected antibodies in TB patients.

In the case of CF, which is the only one of the glycolipids which does not become detached when washed with Tween 20 (Fig. 1), our data is in agreement with the reports of Maekura et al. (1993), Chaicumpar et al. (1997) and Pan et al. (1999), in which TBST was used in the wash and antibody dilution buffers (Table 1). Chaicumpar et al. (1997) followed the same ELISA protocol for CF, SL-I, and PGL, and found that all sera from TB patients reacted strongly with CF as compared with SL-I and PGL reactions.

The different behaviour of CF, compared with the rest of the glycolipids tested, is probably due to its structure. DAT and TAT are made up of a trehalose molecule esterified by two or three fatty acids, with lengths of up to 28 carbon atoms. SL-I is made up of four fatty acids with up to 33 carbon atoms (Goren et al., 1976). PGL is composed of two rhamnoses and one fucose, a phenol group, and two fatty acids of approximately 34 carbon atoms (Brennan and Nikaido, 1995). However the CF is a trehalose molecule esterified by two mycolic acids which are fatty acids with chains of 70–90 carbons atoms (Brennan and Nikaido, 1995), and hence far bigger

than the fatty acids in the rest of the glycolipids studied. This is probably what improves binding to the wells of microtitre plates and prevents displacement by TBST buffer.

Finally, we observed that the results obtained differed when using different coating doses of antigen. With the DAT antigen, researchers who used small amounts (100 ng) of antigen (Cruaud et al., 1989; 1990) obtained sensitivities of around 50–60%, whereas investigators using 1000 ng per well (Escamilla et al., 1996; Muñoz et al., 1997a) obtained sensitivities of around 80–90%. Laszlo et al. (1992) concluded that CF was not a good antigen since, using a coating dose of 200 ng, the difference in absorbance values between TB patients and control sera was only 0.201. However, using high concentrations (2000 ng) of the same antigen, Maekura et al. (1993) obtained differences of 1.13 in absorbance values. These results also highlight the importance of the choosing the right antigen concentration.

## 5. Conclusion

We can conclude that the discrepancies in antibody measurement that we observed in ELISAs using mycobacterial glycolipids were mainly due to the different amounts of glycolipid coating, and to the use of Tween 20 as a detergent. It is therefore very important to standardize ELISA conditions. Detergent should not be added to the washing buffer in assays with four of these antigens (DAT, TAT, SL-I, and PGL); only tests using CF as an antigen can be performed with Tween 20.

We recommend a coating concentration of 1000 ng using *n*-hexane as a solvent for these mycobacterial glycolipids. We also recommend the use of 0.5% gelatin as a blocking agent, and the avoidance of buffers containing Tween 20.

## Acknowledgements

## References

Akimoto, T., Akama, T., Kono, I., Yamane, K., Sumida, T., 1999. Detergent and antigen fragility affect the ELISA for measurement of anti-prothrombin autoantibodies. J. Rheumatol. 26, 580.

Black, C.L., Reynolds, E.C., 1998. An ELISA for a multiphosphorylated peptide, $\alpha_{s1}$-casein(59–79). J. Immunol. Methods 214, 63.

Bothamley, G.H., 1995. Serological diagnosis of tuberculosis. Eur. Respir. J. 8 (Suppl. 20), 676s.

Brennan, P.J., Nikaido, H., 1995. The envelope of mycobacteria. Annu. Rev. Biochem. 64, 29.

Cabral, A.R., Cabiedes, J., Alarcón-Segovia, D., 1994. Tween 20 detaches cardiolipin from ELISA plates and makes anticardiolopin antibodies undetectable regardless of he presence of b2-glycoprotein-I. J. Immunol. Methods 175, 107.

Carpenter, A.B., 1997. Enzyme-linked immunoassays. In: Rose, N.R., Conway de Macario, E., Fahey, J.L., Friedman, H., Penn, G.M. (Eds.), Manual of Clinical Laboratory Immunology, 6th Edition. American Society for Microbiology, Washington, DC, p. 20.

Chaicumpar, K., Fujiwara, N., Nishimura, O., Hotta, H., Pan, J.W., Takahashi, M., Abe, C., Yano, I., 1997. Studies of polymorphic DNA fingerprinting and lipid pattern of *Mycobacterium tuberculosis* patient isolates in Japan. Microbiol. Immunol. 41, 107.

Cho, S.-N., Yanagihara, D.L., Hunter, S.W., Gelber, R.H., Brennan, P.J., 1983. Serological specificity of phenolic glycolipid I from *Mycobacterium leprae* and use in serodiagnosis of leprosy. Infect. Immun. 41, 1077.

Cruaud, P.H., Berlie, C., Torgal-Garcia, J., Papa, F., David, H.L., 1989. Human IgG antibodies immunoreacting with specific sulfolipids from *Mycobacterium tuberculosis*. Zentraubl. Bakt. 271, 481.

Cruaud, P., Yamashita, J.T., Martín-Casabona, N., Papa, F., David, H.L., 1990. Evaluation of a novel 2,3-diacil-trehalose-2′-sulphate (SL-IV) antigen for case finding and diagnosis of leprosy and tuberculosis. Res. Microbiol. 141, 679.

Daniel, T.M., Debanne, S.M., 1987. The serodiagnosis of tuberculosis and other mycobacterial diseases by enzyme-linked immunosorbent assay. Am. Rev. Respir. Dis. 135, 1137.

David, H.L., Papa, F., Cruaud, P., Berlie, H.C., Maroja, M., Salem, J.I., Costa, M.F., 1992. Relationships between titres of antibodies immunoreacting against glycolipid antigens from *Mycobacterium leprae* and *M. tuberculosis*, the Mitsuda and Mantoux reactions, and bacteriological loads: implications in the pathogenesis, epidemiology and serodiagnosis of leprosy and tuberculosis. Int. J. Lepr. 60, 208.

Deshpande, R.G., Khan, M.B., Bhat, D.A., Navalkar, R.G., 1994. Purification and partial characterisation of a novel 66-kDa

seroreactive protein of Mycobacterium tuberculosis H$_{37}$Rv. J. Med. Microbiol. 41, 173.

Escamilla, L., Mancilla, R., Glender, W., López-Marín, L.-M., 1996. *Mycobacterium fortuitum* glycolipids for the serodiagnosis of pulmonary tuberculosis. Am. J. Respir. Crit. Care Med. 154, 1964.

Goren, M.B., Brokl, O., Roller, P., Fales, H.M., Das, B.C., 1976. Sulfatides of *Mycobacterium tuberculosis*: The structure of the principal sulfatide (SL-I). Biochemistry 15, 2728.

Kawamura, M., Sueshige, N., Imayoshi, K., Yano, I., Maekura, R., Kohno, H., 1997. Enzyme immunoassay to detect antituberculous glycolipid antigen (anti-TBGL antigen) antibodies in serum for diagnosis of tuberculosis. J. Clin. Lab. Anal. 11, 140.

Laszlo, A., Baer, H.H., Goren, M.B., Handzel, V., Barrera, L., de Kantor, I.N., 1992. Evaluation of synthetic pseudo cord-factor-like glycolipids for the serodiagnosis of tuberculosis. Res. Microbiol. 143, 217.

Maekura, R., Nakagawa, M., Hiraga, Y., Yamamura, Y., Ito, M., Ueda, E., Yano, S., He, H., Oka, S., Kashima, K., Yano, I., 1993. Clinical evaluation of rapid serodiagnosis of pulmonary tuberculosis by ELISA with Cord Factor (trehalose-6,6′-dimycolate) as antigen purified from *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. 148, 997.

Muñoz, M., Lanéelle, M.-A., Luquin, M., Torrelles, J., Julián, E., Ausina, V., Daffé, M., 1997a. Occurrence of an antigenic triacyl trehalose in clinical isolates and reference strains of *Mycobacterium tuberculosis*. FEMS Microbiol. Letters. 157, 251.

Muñoz, M., Luquin, M., García-Barceló, M., Julián, E., Ausina, V., Lanéelle, M.A., 1997b. Distribution of surface-exposed antigenic glycolipids in recent clinical isolates of *Mycobacterium tuberculosis*. Res. Microbiol. 148, 405.

Pan, J., Fujiwara, N., Oka, S., Maekura, R., Ogura, T., Yano, I., 1999. Anti-cord factor (trehalose 6,6′-dimycolate) IgG antibody in tuberculosis patients recognizes mycolic acid subclasses. Microbiol. Immunol. 43, 863.

Papa, F., Cruaud, P., David, H.L., 1989. Antigenicity and specificity of selected glycolipid fractions from *Mycobacterium tuberculosis*. Res. Microbiol. 140, 569.

Ravindranath, M.H., Ravindranath, R.M.H., Morton, D.L., Graves, M.C., 1994. Factors affecting the fine specificity and sensitivity of serum antiganglioside antibodies in ELISA. J. Immunol. Methods 169, 257.

Reggiardo, Z., Vazquez, E., Schnaper, L., 1980. ELISA test for antibodies against mycobacterial glycolipids. J. Immunol. Methods 34, 55.

Ridell, M., Wallerström, G., Minnikin, D.E., Bolton, R.C., Magnusson, M., 1992. A comparative serological study of antigenic glycolipids from *Mycobacterium tuberculosis*. Tuber. Lung Dis. 73, 101.

Rojas-Espinosa, O., Luna-Herrera, J., Arce-Paredes, P., 1999. Recognition of phenolic glycolipid-I (*Mycobacterium leprae*) and sulfolipid-I (*M. tuberculosis*) by serum from Mexican patients with leprosy or tuberculosis. Int. J. Tuberc. Lung Dis. 3, 1106.

Saad, M.H.F., Kritski, A.L., Werneck-Barroso, E., Cavalcante, S., Ferreira, M.A.S., Fonseca, L.d.S., 1996. Use of the 2,3-diacyl-trehalose and the purified protein derivative in the serodiagnosis of tuberculosis in AIDS. Mem. Inst. Oswaldo Cruz 91, 97.

Savage, C., Vincent, P., Leclerc, H., 1993. Serodiagnosis of tuberculosis. Evaluation of a sulpholipid antigen. Zentraubl. Bakt. 278, 49.

Sempere, M., Ariza, M., Sánchez-Montón, T., Rodriguez-González, T., Martín-Luengo, F., Valero-Guillén, P.L., 1995. Evaluación de aciltrehalosas de *Mycobacterium fortuitum* en el diagnóstico serológico de tuberculosis pulmonar. Enferm. Infecc. Microbiol. Clin. 13, 292.

Simonney, N., Molina, J.M., Molimard, M., Oksenhendler, E., Perronne, C., Lagrange, P.H., 1995. Analysis of the immunological humoral response to *Mycobacterium tuberculosis* glycolipid antigens (DAT, PGLTb1) for diagnosis in HIV-seropositive and -seronegative patients. Eur. J. Clin. Microbiol. Infect. Dis. 14, 883.

Torgal-García, J., David, H.L., Papa, F., 1988. Preliminary evaluation of a *Mycobacterium tuberculosis* phenolglycolipid antigen in the serologic diagnosis of tuberculosis. Ann. Inst. Pasteur/Microbiol. 139, 289.

Thorns, C.J., Morris, J.A., 1985. Common epitopes between mycobacterial and certain host tissue antigens. Clin. Exp. Immunol. 61, 323.

Wilkins, E.G.L., 1998. Antibody detection in tuberculosis. In: Davies, P.D.O. (Ed.), Clinical Tuberculosis. Chapman & Hall, London, p. 81.

World Health Organization, 1998. Tuberculosis is a global public health concern. In: Tuberculosis control and medical schools. Report of a WHO Workshop, Rome, Italy. 29–31 October 1997. World Health Organization, Geneva, Switzerland.

**Article V**

V.    **Julián, E.;**  Matas, L.; Pérez, A.; Alcaide, J.; Lanéelle, M.A.; Luquin, M. "Potential Role of IgA / SL-I Test for the Serodiagnosis of Tuberculosis. Comparison with IgG and IgM Responses and DAT, TAT and CF Antígens". [presentat al *Journal of Clinical Microbiology*].

# Potential Role of IgA / SL-I Test for the Serodiagnosis of Tuberculosis. Comparison with IgG and IgM Responses and DAT, TAT and CF Antigens.

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*Running title*: Humoral response to mycobacterial glycolipids

## ABSTRACT

Non-peptidic 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 comparatively studied the immunoglobulin G, M, and A antibody response to four trehalose-containing glycolipids purified from *M. tuberculosis*: diacyltrehaloses, triacyltrehaloses, cord factor, and sulpholipid-I (SL-I). Sera from 92 tuberculous patients (taken before starting antituberculous treatment) and a wide group of control individuals (84 sera from healthy donors including PPD-negatives, -positives, healed and vaccinated; and 52 from non-tuberculous pneumonia patients) from Spain, were studied. The results indicated a significantly elevated IgG and IgA antibody response in tuberculous patients compared with controls, with all the antigens studied. SL-I was the best antigen studied, showing test sensitivies and specificities for IgG of 81% and 77.6% respectively, and 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%. Nowadays it is widely accepted that it is not possible to achieve sensitivies above 80% in tuberculosis serodiagnostic using one antigen alone. Thus, we conclude that SL-I, in combination with other antigenic molecules, could be a useful antigen for tuberculosis serodiagnostic.

## INTRODUCTION

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, based on the amplification of nucleic acids. Serological methods seem to be the ideal choice, thus many mycobacterial antigens have been evaluated: cellular extracts, proteins, and glycolipidic molecules from the mycobacterial cell wall (30). In addition to the lipoarabinomannan (LAM) antigen, the family of the acylated trehaloses has been the most checked glycolipids. They are: the 2,3-diacyltrehalose (DAT), 2,3,6-triacyltrehalose (TAT), 2,3,6,6'-tetraacyltrehalose 2'-sulphate (sulpholipid 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 of immunoglobulin G or IgM titers (5, 6, 10, 17, 19, 23-25) was obtained, i.e. test sensitivity results as different as 11%-88% for the DAT antigen (24, 10) and 51-93% for the TAT antigen (25, 10). These contradictory results are due to the ELISA protocol used (the different antigen concentrations or the use of detergent in washing buffers) (14, 29), or to the different sera groups analyzed (quantity and type of patients and control subjects) (9). 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 (8) and, although different commercialized assays exist that detect IgA antibodies against cellular extracts and purified proteins (13, 15, 22), to date no analysis has been undertaken to determine the presence of specific IgA against the glycolipids mentioned above.

In order to evaluate the serological interest of DAT, TAT, SL-I and CF, the present work compares IgG, IgM and IgA antibody responses in a total of 228 sera, using the ELISA method recommended for these glycolipids (14).

## METHODS

### Study Subjects

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

*Tuberculous 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 non-radiometric MB/Bact system (Organon Teknika, Durham, NC). Fifty-eight of these adult patients were suffering from pulmonary TB (10 of them co-infected with the Human Immunodeficiency Virus, HIV), and 16 from extrapulmonary TB (3 of them HIV-positive). The extrapulmonary localizations comprised disseminated TB (seven), pleural TB (two), lymphatic (two), puncture (two), pus abscess (one), bone (one) and cutaneous (one). The patients had not yet started the antituberculous treatment when the serum samples were taken. Eighteen serum samples from children with a clinical diagnosis of tuberculosis were also included in the study; for six children, the illness was confirmed later

by isolating *M. tuberculosis* from gastric lavage or from respiratory specimens.

*Control Subjects.* One hundred and 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), *Legionella pneumophila* (11), *Mycoplasma pneumoniae* (9 from adults and 4 from children) and *Streptococcus pneumoniae* (8). They were all PPD-negative. Moreover, two sera samples were included in this group from patients with 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 five 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, Ph.D. students, or were collected from the Barcelona Tuberculosis Prevention and Control Programme. All sera were aliquoted and stored at -40ºC until use.

## ELISA glycolipids

For glycolipid isolation, a *M. tuberculosis* clinical isolated strain (21) was grown for 6 weeks at 37ºC on Middlebrook 7H10 supplemented with OADC enrichment (Difco Laboratories, Detroit, MI). DAT, TAT, SL-I and CF were purified using column chromatography as previously described by us (20, 21). ELISA was performed as we described (14). Briefly, purified DAT, TAT, CF or SL-I (1,000 ng each in 50 µl n-hexane/well) were coated to the plates (Immulon I from Dynatech Laboratories, USA). Sera were diluted in blocking buffer at 1/400, 1/200 and 1/100 for measuring IgG, IgM and IgA antibodies respectively. All points were duplicated. Goat anti-human IgG, IgM, and IgA alkaline phosphatase conjugates (Southern Biotechnology Associates, Inc., Birmingham, Ala) at 1/3000 dilution in blocking buffer were added. Absorbance was determined at 405 nm with a microtitre reader (ELx 800, Automated Microplate Reader, Bio-Tech Instruments , Inc.).

## Data analysis

Final calculations were performed as previously described (5) with slight modifications. To correlate the data for day-to-day variations, 3 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 non-specific 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 non-specific absorption (zero) was taken and the mean value was calculated. The normalized data were then calculated to establish the corrected 405 values by using the curve of standards.

## Statistical treatment of results

Arithmetic means and standard deviations (SD) from the mean were calculated for corrected optical density (OD) values. The cut-off points chosen were equal to the means of the OD corrected readings obtained with sera from all the healthy individuals plus 3 SD for each antigen and Ig. To adjust the cut-off value, we selected the mean plus 2 SD measured in the overall control population. The significance of the difference between means was calculated using the Mann-Whitney U. Comparison among several groups was made using the Kruskal-Wallis analysis (11). Sensitivity and specificity were calculated by standard methods. All statistical tests were conducted using SPSS for Windows (version 9.0, SPSS, Chicago) statistical software.

## RESULTS

The demographic and clinical data of the TB patients and controls studied are shown in Table 1. There was no statistically significant difference in the mean ± SD age of TB patients and control subjects (35.7 ± 22.1 *versus* 31.5 ± 23.5, 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), compared with those obtained in the overall control sera (healthy and non-TB pneumonia) (Figure 1). Differences in the IgM antibody levels were observed between TB patients and overall controls using DAT and TAT antigens, but not using 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 within each test (Figure 1).

In the child group, no significant differences were observed between the OD mean values from TB patients and overall controls for any antigen or antibody assayed (P>0.05). Although only four sera from children with non-TB pneumonia were studied, the results are higher than those from 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 with respect to smear negative, and in pulmonary TB with respect to extrapulmonary TB (Table 2). The highest test sensitivity was obtained when detecting IgG antibodies against the SL-I antigen (81%, with a specificity of 77.6%). Sensitivity values above 65% were obtained with TAT/IgA (74.3%), TAT/IgG (68.9%) and SL-I/IgA (66.2%) tests. The SL-I/IgG test was the most efficient for detecting smear negative TB cases. Among healthy adult subjects, only one or two 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 drop 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 was the most specific test (87.5%, with a sensitivity of 66.2%).

A test sensitivity of 58.1% with a specificity of 90.1% were obtained when detecting both IgG and IgA antibodies (i.e. the presence of both antibodies) against SL-I. One way to improve the results obtained consists of adjusting the cut-off. In our case, test sensitivity and specificity values of 67.56% and 93.75%, respectively, were achieved by adjusting it for 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% to 22.2%) (data not shown). Even using a different cut-off 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 both 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 with adult patients (3, 8, 26 and present results), and no differences between infected and ill children have been reported (26, 27, and current 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) (6, 19), and for purified proteinaceous antigens (28).

The best results were obtained when detecting IgG and IgA in adult TB patients. IgG was comparatively more reactive in giving the highest absorbance values and highest sensitivities (Figure 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 *M. tuberculosis* virulent strains (7), there are only two previous serological evaluations detecting IgG and IgM antibodies against SL-I (6, 23). Like us, Cruaud *et al*. (6) found 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 these 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 (14). The results obtained by Rojas-Espinosa *et al*. (23) are discrepant in the sense that they found a high reactivity for the IgM detection.

IgA was not tested either in these previous serological studies using SL-I or in the other studies using the other glycolipids. We show, for the first time, that adult TB patients have a specific IgA response against these glycolipids. In comparison with the 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 cut-off

value, we have achieved the best relation between sensitivity (67.5%) and specificity (93.7%).

With both Igs, elevated test specificity values were obtained in the healthy control group (between 96 and 100%); nevertheless it drops dramatically when sera from patients with non-TB pneumonia are considered. We have observed that this fact 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 (4, 15). The evaluation of new tests using mainly healthy subjects, and few (if any) patients infected by other agents is one of the common defects in TB serology studies (30). Unquestionably the inclusion of this population is crucial for clinical evaluation of new tests since they are susceptible of being confused with TB patients. For this reason we have included a large number (38.2% with respect to overall controls) of non-TB pneumonia patients in the current study.

We think that three strategies could be used to improve the specificity of TB serodiagnosis. First, by always using the specific antigens present only in *M. tuberculosis*; second, by avoing possible antigen contamination during the purification process and third, by preabsorbing the sera with mycobacteria other than *M. tuberculosis*, with other bacteria, or with molecules that share epitopes with the specific antigens. Very few studies (16) analyze the efficacy of absorbents in diminishing the cross-reactions and it would be very interesting to go into this by testing a wide range of absorbents.

Nowadays, it has been clearly accepted that TB patients produce antibodies against more than one antigen (18) and consequently a wide spectrum of humoral responses exists in these patients. Thus, some authors (12) 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 to be included in a TB serodiagnostic test.

## Acknowledgements

**REFERENCES**

1. **Alifano, M., R. De Pascalis, M. Sofia, S. Faraone, M. Del Pozzo, and I. Covelli.** 1997. Evaluation of IgA-mediated humoral immune response against the mycobacterial antigen P-90 in diagnosis of pulmonary tuberculosis. Chest. **111**:601-605.

2. **Arikan, S., S. Tuncer, D. Us, S. Ünal, and S. Ustaçelebi.** 1998. Anti-Kp 90 antibodies in the diagnosis of active tuberculosis. Chest. **114**:1253-1257.

3. **Bothamley, G., P. Udani, R. Rudd, F. Festestein, and J. Ivanyi.** 1998. Humoral response to defined epitopes of tubercle bacilli in adult pulmonary and child tuberculosis. Eur. J. Clin. Microbiol. Infect. Dis. **7**:639-645.

4. **Chiang, I. H., J. Suo, K. J. Bai, T. P. Lin, K. T. Luh, C. J. Yu, and P. C. Yang.** 1997. Serodiagnosis of tuberculosis. A study comparing three specific mycobacterial antigens. Am. J. Respir. Crit. Care Med. **156**:906-911.

5. **Cruaud, P., J. T. Yamashita, N. Martín-Casabona, F. Papa, and H. L. David.** 1990. Evaluation of a novel 2, 3-diacyl-trehalose-2'-sulphate (SL-IV) antigen for case finding and diagnosis of leprosy and tuberculosis. Res. Microbiol. **141**:679-694.

6. **Cruaud, P. H., C. Berlie, J. Torgal-Garcia, F. Papa, and H. L. David.** 1989. Human IgG antibodies immunoreacting with specific sulfolipids from *Mycobacterium tuberculosis*. Zbl. Bakt. **271**:481-485.

7. **Daffé, M., and P. Draper.** 1998. The envelope layers of mycobacteria with reference to their pathogenicity. Adv. Microbial Physiol. **39**:131-203.

8. **Daniel, T. M., and S. M. Debanne.** 1987. The serodiagnosis of tuberculosis and other mycobacterial diseases by enzyme-linked immunosorbent assay. Am. Rev. Respir. Dis. **135**:1137-1151.

9. **Daniel, T. M.** 1988. Antibody and antigen detection for the immunodiagnosis of tuberculosis: Why not? What more is needed? Where do we stand today? J. Infect. Dis. **158**:678-680.

10. **Escamilla, L., R. Mancilla, W. Glender, and L.-M. López-Marín.** 1996. *Mycobacterium fortuitum* glycolipids for the serodiagnosis of pulmonary tuberculosis. Am. J. Respir. Crit. Care Med. **154**:1964-1867.

11. **Fisher, L. D., and G. van Belle.** 1993. *In* J. Wiley & Sons, cop. (ed.). Biostatistics: a methodology for health sciences. New York.

12. **Gennaro, M. L.** 2000. Immunologic diagnosis of tuberculosis. Clin. Infect. Dis. **30**(Suppl 3):S243-246.

13. **Gupta, S., S. Kumari, J. N. Banwalikar, S. K. Gupta.** 1995. Diagnostic utility of the estimation of mycobacterial Antigen A60 specific immunoglobulins IgM, IgA and IgG in the sera of cases of adult human tuberculosis. Tuberc. Lung Dis. **76**:418-424.

14. **Julián, E., M. Cama, P. Martínez, and M. Luquin.** 2001. An ELISA for five glycolipids from the cell wall of *Mycobacterium tuberculosis*: Tween 20 interference in the assay. J. Immunol. Methods. **251**:21-30.

15. **Julián, E., L. Matas, A. Hernández, J. Alcaide, and M. Luquin.** 2000. Evaluation of a new serodiagnostic tuberculosis test based on the immunoglobulin A detection against Kp-90 antigen. Int. J. Tuberc. Lung Dis. **4**:1082-1085.

16. **Laal, S., K. M. Samanich, M. G. Sonnenberg, S. Zolla-Pazner, J. M. Phadtare, and J. T. Belisle.** 1997. Human humoral responses to antigens of *Mycobacterium tuberculosis*: immunodominance of high-molecular-mass antigens. Clin. Diag. Lab. Immunol. **4**:49-56.

17. **Laszlo, A., H. H. Baer, M. B. Goren, V. Handzel, L. Barrera, and I. N. de Kantor.** 1992. Evaluation of synthetic pseudo cord-factor-like glycolipids for the serodiagnosis of tuberculosis. Res. Microbiol. **143**:217-223.

18. **Lyashchenko, L., R. Colangeli, M. Houde, H. Al Jahdali, D. Menzies, and M. L. Gennaro.** 1998. Heterogeneous antibody responses in tuberculosis. Infect. Immun. **66**:3936-3940.

19. **Maekura, R., M. Nakagawa, Y. Hiraga, Y. Yamamura, M. Ito, E. Ueda, S. Yano, H. He, S. Oka, K. Kashima, and I. Yano.** 1993. Clinical evaluation of rapid serodiagnosis of pulmonary tuberculosis by ELISA with Cord Factor (trehalose-6,6'-dimycolate) as antigen purified from *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **148**:997-1001.

20. **Muñoz, M., M. A. Lanéelle, M. Luquin, E. Julián, V. Ausina, and M. Daffé.** 1997. Occurrence of an antigenic triacyl trehalose in clinical isolates and reference strains of *Mycobacterium tuberculosis*. FEMS Microbiol. Lett. **157**:251-259.

21. **Muñoz, M., M. Luquin, M. García-Barceló, E. Julián, V. Ausina, and M. A. Lanéelle.** 1997. Distribution of surface-exposed antigenic glycolipids in recent clinical isolates of *Mycobacterium tuberculosis*. Res. Microbiol. **148**:405-412.

22. **Pottumarthy, S., V. C. Wells, and A. J. Morris.** 2000. A comparison of seven tests for serological diagnosis of tuberculosis. J. Clin. Microbiol. **38**:2227-2231.

23. **Rojas-Espinosa, O., J. Luna-Herrera,**

and P. Arce-Paredes. 1999. Recognition of phenolic glycolipid-I (*Mycobacterium leprae*) and sulfolipid-I (*M. tuberculosis*) by serum from Mexican patients with leprosy or tuberculosis. Int. J. Tuberc. Lung Dis. **3**:1106-1112.

24. **Savage, C., P. Vincent, and H. Leclerc.** 1993. Serodiagnosis of tuberculosis. Evaluation of a sulpholipid antigen. Zbl. Bakt. **278**:49-57.

25. **Sempere, M., M. Ariza, T. Sánchez-Montón, T. Rodriguez-González, F. Martín-Luengo, and P. L. Valero-Guillén.** 1995. Evaluación de aciltrehalosas de *Mycobacterium fortuitum* en el diagnóstico serológico de tuberculosis pulmonar. Enferm. Infecc. Microbiol. Clin. **13**:292-296.

26. **Simonney, N., A. Bourrillon, and P. H. Lagrange.** 2000. Analysis of circulating immune complexes (CICs) in childhood tuberculosis: levels of specific antibodies to glycolipid antigens and relationship with serum antibodies. Int. J. Tuberc. Lung Dis. **4**:152-160.

27. **Tumeer, M., E. van Nerom, J. Nyabenda, A. Waelbroek, A. Duvivier, and M. Toppet.** 1994. Determination of

humoral immunoglobulins M and G directed against mycobacterial antigen 60 failed to diagnose primary tuberculosis and mycobacterial adenitis in children, Am. J. Respir. Crit. Care Med. **150**:1508-1512.

28. **Tumeer, M., J.-P. van Vooren, J. de Bruyn, E. Serruys, P. Dierckx, and J.-C. Yernault.** 1988. Humoral immune response in human tuberculosis: Immunoglobulins G, A, and M directed against the purified P32 protein antigen of *Mycobacterium bovis* Bacillus Calmette-Guérin. J. Clin. Microbiol. **26**:1714-1719.

29. **Vera-Cabrera, L., V. Handzel, and A. Laszlo.** 1994. Development of an enzyme-linked immunosorbent assay (ELISA) combined with a streptavidin-biotin and enzyme amplification method to detect anti-2, 3-di-o-acyltrehalose (DAT) antibodies in patients with tuberculosis. J. Immunol. Methods. **177**:69-77.

30. **Wilkins, E. G. L.** 1998. Antibody detection in tuberculosis, p.81-96, *In* P. D. O. Davies (ed.), Clinical tuberculosis. Chapman & Hall, London, UK

**FIGURE 1.** Results of the ELISA glycolipids in sera from adult subjects.

Individual absorbance values for each test. Each dot indicates an individual serum sample. The dotted line indicates cut-off values above which a test is positive: mean + 3SD 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 1. Demographic and clinical data of tuberculous patients and controls studied with the ELISA glycolipids.

| Source of serum samples | | Patients (n) | Sex (men : women) | Ages Mean | Ages Range |
|---|---|---|---|---|---|
| Tuberculous patients | | 92 | 62 : 30 | 35.7 | 1 - 87 |
| Pulmonary adult TB | HIV -- | 48 | 37 : 11 | 44.3 | 26 - 77 |
| | HIV + | 16 | 8 : 8 | 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 |
| *Mycobacterium xenopi* and *Mycobacterium kansasii* | | 2 | 0 : 2 | 29 | 28 - 78 |
| *Chlamydia pneumoniae* | | 12 | 7 : 5 | 83 | 62 - 86 |
| *Coxiella spp.* | | 6 | 5 : 1 | 40 | 33 - 45 |
| *Legionella pneumophila* | | 11 | 7 : 4 | 54.3 | 26 - 74 |
| *Mycoplasma pneumoniae* | Adults | 9 | 4 : 5 | 23 | 20 - 28 |
| | Children | 4 | 3 : 1 | 4.3 | 1 - 7 |
| *Streptococcus pneumoniae* | | 8 | 8 : 0 | 63.5 | 33 - 75 |
| Healthy subjects | | 84 | 52 : 32 | 29.5 | 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 |

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

| Test used | Source of serum samples (n=186) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pulmonary TB | | | Extrapulmonary TB | | | Non-TB pneumonia patients | Healthy subjects |
| | Total (n=58) | Smear positive (n=42) | Smear negative (n=16) | Total (n=16) | Smear positive (n=2) | Smear negative (n=14) | (n=48) | (n=64) |
| DAT antigen / IgG | 33 (56.89) | 25 (59.52) | 8 (50) | 7 (43.75) | 2 (100) | 5 (35.71) | 20 (41.66) | 0 (0) |
| TAT antigen / IgG | 41 (70.68) | 30 (71.42) | 11 (68.75) | 10 (62.5) | 2 (100) | 8 (57.14) | 28 (58.33) | 0 (0) |
| SE-I antigen / IgG | 48 (82.75) | 35 (83.33) | 13 (81.25) | 12 (75) | 2 (100) | 10 (71.42) | 24 (50) | 1 (1.56) |
| CF antigen / IgG | 35 (60.34) | 26 (61.90) | 9 (56.25) | 5 (31.25) | 1 (50) | 4 (28.57) | 12 (25) | 1 (1.56) |
| DAT antigen / IgA | 41 (70.68) | 33 (78.57) | 8 (50) | 8 (50) | 2 (100) | 6 (42.85) | 24 (50) | 2 (3.12) |
| TAT antigen / IgA | 45 (77.58) | 37 (88.09) | 8 (50) | 10 (62.5) | 2 (100) | 8 (57.14) | 25 (52.08) | 2 (3.12) |
| SE-I antigen / IgA | 40 (68.96) | 33 (78.57) | 7 (43.75) | 9 (56.25) | 2 (100) | 7 (50) | 14 (29.16) | 0 (0) |
| CF antigen / 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 antigen / IgM | 7 (12.06) | 4 (9.52) | 3 (18.75) | 1 (6.25) | 0 (0) | 1 (7.14) | 0 (0) | 1 (1.56) |
| TAT antigen / IgM | 16 (27.58) | 11 (26.19) | 5 (31.25) | 2 (12.5) | 0 (0) | 2 (14.28) | 4 (8.33) | 0 (0) |
| SE-I antigen / 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 antigen / IgM | 1 (1.72) | 1 (2.38) | 0 (0) | 1 (6.25) | 0 (0) | 1 (7.14) | 1 (2.08) | 1 (1.56) |

Percentage of reacting sera is indicated in parentheses in each data column.
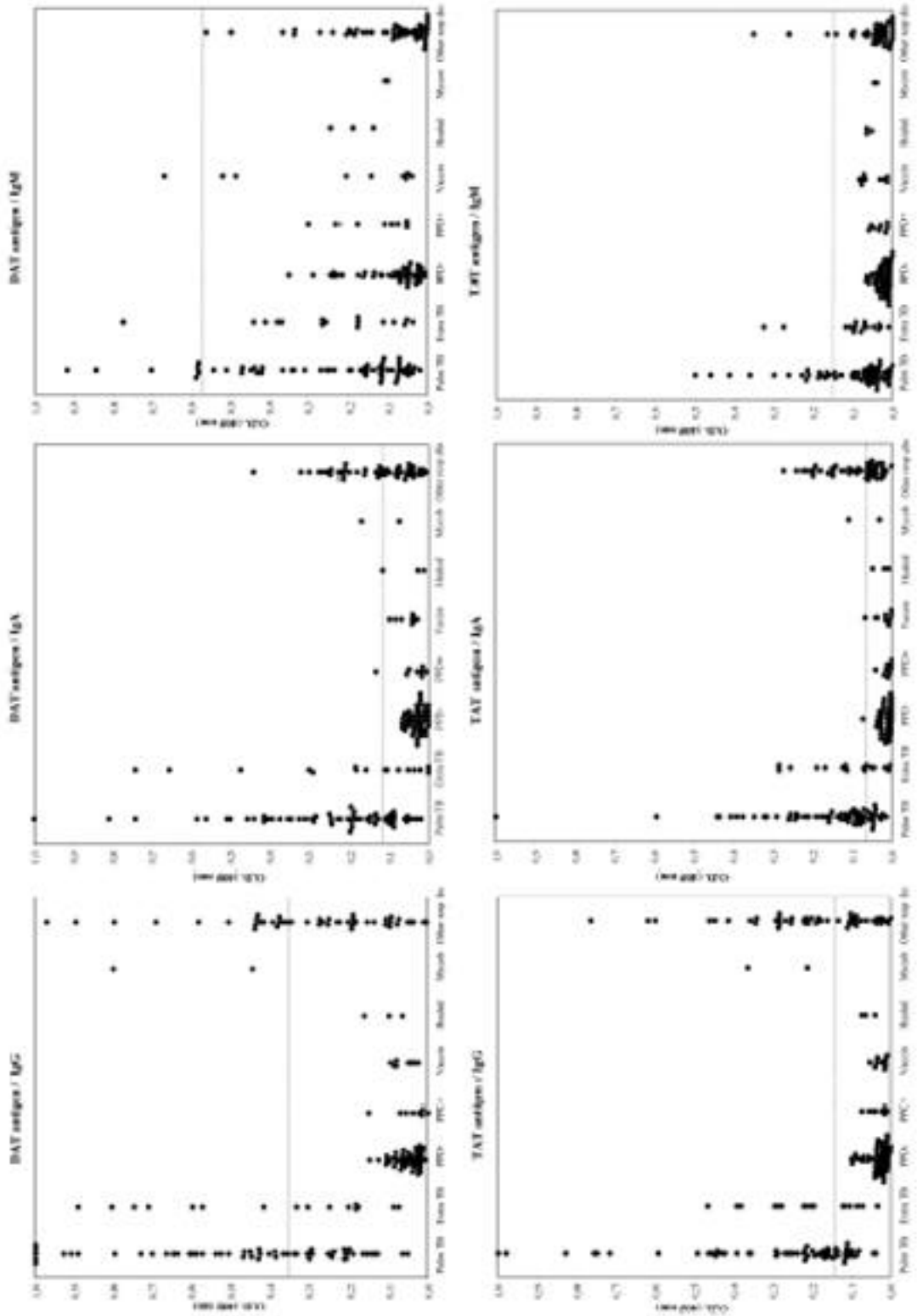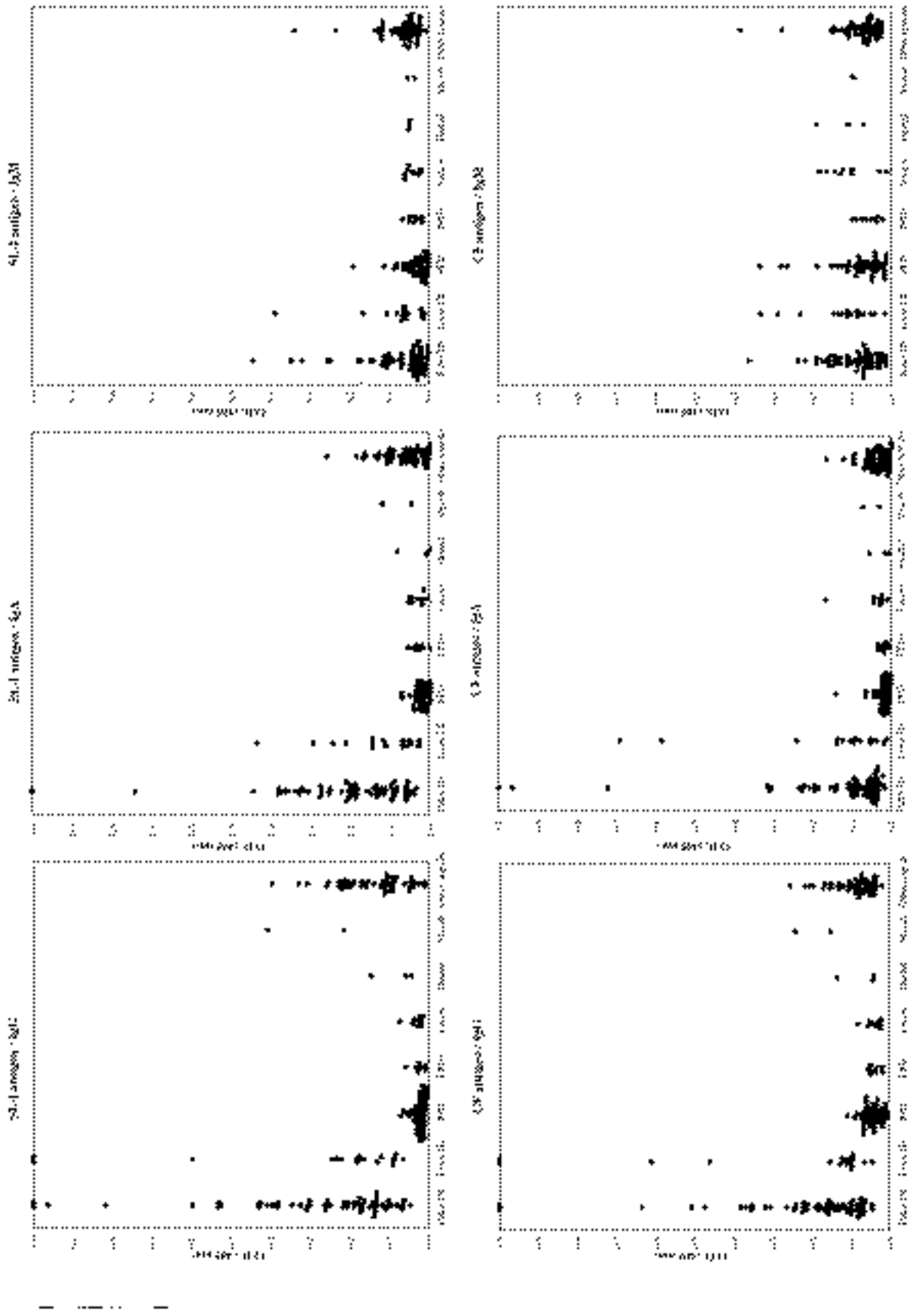
**Article VI**

**VI.**    **Julián, E.;**   Matas, L.; Alcaide, J.; Luquin, M. "Combination of purified glycolipids and proteins improve the serological diagnosis of tuberculosis". [presentat al *Journal of Infectious Diseases*].

# Combination of purified glycolipids and proteins improve the serological diagnostic of tuberculosis

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## ABSTRACT

The combination of several specific antigens has been proposed to improve the tuberculosis (TB) serodiagnosis tests. To date the comparison of antibody responses in the same population has been carried out using only proteinaceous antigens. In this study were compared the presence of Immunoglobulin G, IgM and IgA antibodies to *Mycobacterium tuberculosis* glycolipids (sulpholipid-I, diacylthrealoses, triacylthrealoses and cord-factor) with the response to the 38kDa antigen. Fifty-two sera from TB patients and 83 from control individuals (48 healthy and 35 non-TB pneumonia patients) were studied. The results showed three relevant facts. (1) More TB patients reacted to glycolipids than to protein-based tests. In 24 of the 29 smear-positive pulmonary TB patients, IgG and IgA antibodies were detected to one glycolipid at least; however, only 16 sera reacted to protein-based tests. (2) Glycolipid-based tests are more specific than proteins. Only one control subject showed IgAs to a glycolipid out of the 15 that reacted to some of the tests. (3) Finally, test sensitivity is improved (from 23% to 62%) when the glycolipids studied here are used together with specific proteins and IgG and IgA antibodies are detected.

## INTRODUCTION

Serodiagnosis has been considered the Holy Grail of tuberculosis (TB) diagnosis research. A serological test would be the ideal method for

worldwide implantation due to its low cost and simplicity. It should possess sensitivity values over 80% and test specificity over 95% to substitute gold standard culture, following the recommendations of the World Health Organization [1]. Nowadays there is no test with these features, despite the fact that researchers have been looking for one for more than 100 years.

The TB serodiagnosis history started in 1898 when Argoing and Courmant agglutinated antibodies from TB patient sera [2]. Since the introduction of the enzyme-linked immunosorbent assay (ELISA) in the 70s, several authors have been trying to find an optimum antigen. Semi-purified antigens such as purified protein derivative (PPD), A60 or Kp90 have been used. Purified antigens, both proteins and glycolipids (e.g. the antigen 5 (38kDa protein) or the lipoarabinomanan (LAM)), have also been assayed. However, no test showed sufficiently high sensitivity and specificity values for diagnosing purposes [3-8]. The third assay generation was originated with the introduction of recombinant proteins, but none of these new tests achieved good test diagnostic characteristics either [8-10].

Nowadays, it has been clearly accepted that a patient produces antibodies to more than one antigen [11-14]. A wide spectrum of humoral responses exists in TB patients, in terms of the disease stage, the immunological patient background, the antituberculous therapy, and/or the differential gene expression of different strains of *Mycobacterium tuberculosis* [11]. Thus, some authors suggest the combination of specific purified antigens for TB serodiagnosis [15]. It seems unquestionable that the only way to evaluate combinations of antigens is to analyze the presence of antibodies against them in parallel, using the same population [9, 12, 16, 17]. However, all previous studies simultaneously analyzing the response to several antigens, were made using purified proteins alone and detecting IgG antibodies [11-14, 16, 17]. To our knowledge, there is no research which comparatively studies the complete pattern of antibody responses against purified proteinaceous and glycolipidic antigens in the same population.

In the current work we have analyzed the IgG, IgM and IgA antibody response to proteinaceous antigens (38kDa and 16kDa), using commercially available PATHOZYME kits, in parallel to glycolipids (diacyltrehaloses (DAT), triacyltrehaloses (TAT), sulpholipid (SL-I) and cord factor (CF)), purified from *M. tuberculosis* by ourselves and using an in-house ELISA test [18].

MATERIAL AND METHODS

*SERA PATIENTS*

*Tuberculous patients*

Fifty-two serum samples taken from Human Immunodeficiency Virus (HIV)-negative persons, aged between 19 and 87, were studied. All these patients had been admitted to the Hospital Universitari Germans Trias i Pujol (HUGTiP) in Barcelona (Spain), and were clinically diagnosed as suffering from TB, which was subsequently confirmed bacteriologically by isolation of tuberculous bacillus in cultures: Löwenstein-Jensen and the nonradiometric MB/Bact system (Organon Teknika, Durham, NC). Forty serum samples were from patients suffering from pulmonary TB, and 12 from extrapulmonary TB. The extrapulmonary localizations comprised

disseminated TB (three), lymphatic (two), puncture (two), pleural (two), pus abscess (one), bone (one) and cutaneous (one). The patients had not yet started the antituberculous treatment when the serum samples were taken.

CONTROL SUBJECTS

Eighty-three HIV-seronegative serum samples (aged from 20 to 80) were included as negative controls. Thirty-five of these samples were from patients suffering from pneumonia other than TB. These diseases were originated by Streptococcus pneumoniae (6 serum samples), Coxiella burnetii (6), Chlamydia spp. (8), Mycoplasma pneumoniae (8) and Legionella pneumophila (7). All of them were PPD-negative. Sera from these patients were obtained from the Microbiology Service seroteca at the HUGTiP. Forty-eight sera were taken from healthy adults. Eight of these had been BCG-vaccinated in the past, eight were PPD-positive, and twenty-nine were PPD-negative at the time the serum samples were taken. Three samples were from persons who had suffered from TB more than five years previously, and who had received and completed the standard TB treatment. Healthy control sera were obtained from employees of the HUGTiP, Ph.D. students at the Universitat Autònoma de Barcelona and from the Barcelona Tuberculosis Prevention and Control Program. All sera were collected and stored at -40ºC until use.

SEROLOGICAL TESTS

ELISA with glycolipids

The glycolipidic antigens were purified from an M. tuberculosis clinical isolate as described previously [19, 20]. The glycolipidic fraction was extracted from M. tuberculosis cells using a mixture of chloroform and methanol, and individual glycolipids were purified using silica-gel column chromatography. ELISA was performed as described by us [18]. Briefly, microtiter plates (Immulon I from Dynex Technologies, Inc., Chantilly, VA) were coated with purified DAT, TAT, SL-I or CF (1,000 ng each in 50 µl n-hexane/well). Sera were diluted at 1/400, 1/200 and 1/100 in blocking agent and added in duplicate to wells, for measuring IgG, IgM and IgA antibodies, respectively. Specific IgG, IgM and IgA conjugates marked with alkaline phosphatase (Southern Biotechnology Associated, Inc., Birmingham, AL) at a 1/3000 dilution in blocking agent were used. Absorbance was determined at 405 nm with a microtiter reader (ELx 800, Automated Microplate Reader, Bio-Tech Instruments®, Inc.).

ELISA kits

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

Data analysis

For the ELISA using glycolipids, the data analysis was performed as follows. To correlate the data for day-to-day variations, three titrated sera (standards)

and a blank (blocking buffer alone) were included in each plate. A curve was drawn on each plate and the comparison of their slopes was carried out. If these data were not satisfactory (slope below 98%) the plate was rejected. The difference between absorbance of serum and non-specific absorption (wells treated with solvent alone included for each serum tested) was noted and the mean value was calculated. The normalized data were then calculated to establish the corrected 405 values by using the curve of standards.

The cut-off was chosen as the mean plus 3 standard deviation (SD) of the corrected optical density (OD) values obtained from the healthy population. For each test a second cut-off was chosen (mean plus 6 SD, as shown in Table 1) in order to distinguish the highest reactivities [11, 21].

The serum samples and the positive, negative, and cut-off controls included in the kits were tested in duplicate. For the IgG and IgA kits all results are expressed in serounits (U) of specific IgG or IgA/ml which are read from a semi-logarithmic reference curve, prepared with 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 cut-off values was carried out according to the recommendations of the manufacturer (Table 2).

RESULTS

Data from antibody responses to different antigens were expressed as negative, low and high positive results, according to the different cut-off values described in the Material and Methods Section (Table 1 and 2). All the results obtained in each test either with glycolipidic or proteinaceous antigens are shown in

Table 3.

Sera reactivity

To compare the results of TB patients, we chose the most specific antigens by selecting the highest cut-off values (Table 1 and 2). We discarded the tests that provided more than 6 control sera with high values (Table 3). The selected tests had a global specificity value of at least 92.7%. The eliminated tests were TAT/IgG, SL-I/IgG, DAT/IgA and TAT/IgA. Finally, for comparative purposes, we excluded all the tests based on IgM response since a high IgM antibody response was observed in very few TB patients (Table 3).

Antibody pattern

Using the selected tests we analyzed the antibody response pattern as shown in Tables 4, 5, and 6. In Table 4, a distinction is made between smear-positive and negative TB pulmonary patients. The results showed that 24 sera from the 29 smear-positive TB pulmonary patients reacted to at least one of the glycolipidic antigens: 6 had only IgG antibodies, 9 showed only IgA antibodies, and 9 showed both IgG and IgA antibodies to glycolipids. However, only 16 sera from these patients reacted to proteins: 10 sera presented only IgG antibodies, one serum IgAs, and 5 sera showed both IgG and IgA antibodies. In the case of 11 smear-negative TB pulmonary patients, only four reacted: all four to the SL-I/IgA test, three to the CF/IgA test and one to the CF/IgG.

Only three sera from the twelve extrapulmonary TB patients showed antibodies to proteins or glycolipids. In this group, only one serum belonging to a patient suffering from disseminated TB showed antibodies to six different tests (Table 4).

With respect to controls, no sera from

healthy subjects reacted against glycolipidic antigens, although three of them showed antibodies to proteins (Table 5). With reference to sera from patients affected with non-TB pneumonia, the IgA tests were the most specific, and only two sera reacted. However, tests based on IgG antibody detection were more unspecific: ten different sera reacted to the four different tests. In this case no reacting sera showed antibodies to more than one antigen (Table 6).

Combinations of different tests

Considering the seven different tests, 26 of the 29 smear-positive TB pulmonary patients reacted to at least one test (Table 4): 10 sera reacted only to glycolipids, two sera only to proteins, and 14 sera to both types of antigens. Thirty of the 40 pulmonary TB patients showed antibodies to at least one test, showing a sensitivity value of 75%, however, the highest sensitivity obtained with an individual test was 50% (SL-I/IgA).

Combining the results, we observed that 14 different control sera showed IgG or IgA antibodies to different antigens. Only one serum from a patient affected with pneumonia originated by *Streptococcus pneumoniae* showed IgG and IgA antibodies against two different antigens (Table 6). In general, 69 sera from the 83 controls did not show any response to mycobacterial antigens (a specificity value of 83.1%).

If the most unspecific test is not considered (38kDa + 16kDa/IgG), the overall test sensitivity is not lowered because the sera which reacted to 38kDa + 16kDa also reacted against the other tests. Thus, considering all the other tests together, the overall test specificity increased to 90.3%: 95.8% in healthy controls (Table 5), and 82.8% in non-TB pneumonia patients (Table 6).

DISCUSSION

We report here the antibody pattern obtained against four glycolipids from the *M. tuberculosis* cell wall and, simultaneously, against four commercially available tests based on the 38kDa antigen, using 135 serum specimens. Our results show a very heterogeneous antibody response in TB patients (Table 4). The majority of smear-positive pulmonary TB patients reacted to three or more different tests. Comparatively, the response was considerably reduced in smear-negative patients and extrapulmonary TB patients. In this latter group, one patient (number 83, Table 4) was the exception, reacting to 6 different tests. This patient had disseminated TB. Thus, in this study we found a clear relationship between the capacity to recognize a greater variety of antigens and the bacterial load in TB patients. Previous studies analyzing IgG antibodies to culture filtrate proteins showed that smear-positive pulmonary TB patients recognize more proteinaceous antigens than smear-negative TB patients [14, 22, 23]. Interestingly, we have found the same behavior in TB patients with respect to glycolipids.

One of the most attractive results of this comparative study has been that TB patients reacted more to glycolipids than to proteins (Table 4). Twenty-four of the 29 smear-positive pulmonary TB patients had IgG or IgA antibodies to at least one of the glycolipidic antigens. However only 16 of them reacted to proteins. In the most problematic cases to diagnose, smear-negative pulmonary TB patients (11 cases) and

extrapulmonary forms (12 cases), 7 patients reacted against glycolipids and only one to proteins. Regarding the controls, no sera from healthy subjects showed anti-glycolipid antibodies, but three showed antibodies to the IgG or IgA tests based on the 38kDa protein. Thus, the glycolipids studied here provide higher test sensitivity and specificity values than the test based on the 38kDa antigen.

For this comparative study we chose the 38kDa antigen (initially named antigen 5) because it is the most studied antigen for TB serodiagnosis, and it is easily available in commercial tests. The previous studies that used this antigen coincide with the high test specificity reported (between 88% and 100%). However, test sensitivity varies widely among the different reports: from 36 to 89% for smear-positive TB patients, from 16% to 54% for smear-negative, and between 12-56% for extrapulmonary TB patients [3, 4, 6, 11, 24]. Furthermore, in studies distinguishing between smear-positive and smear-negative pulmonary TB patients, antibodies to the 38kDa antigen were detected only from patients with sputum smear-positive for TB [14]. This observation is in agreement with our results since we did not find any smear-negative pulmonary TB patient who reacted to this antigen. Thus, it seems that antibodies to 38kDa are not produced in the early stages of TB disease and consequently that it is not a good antigen for detecting the most problematic smear-negative cases. In view of this fact, the 38kDa antigen has been combined with other proteins in order to increase its test sensitivity. Combining it with MTB48, a test sensitivity of 56.3% has been achieved (a gain of 12.9%) [16]; mixed with Mtb81, 68% of the TB patients analyzed reacted

[17]; or mixed with rCFP-10, antibody detection increased from 49% to 58% in smear-positive TB patients, and from 21% to 40% in smear-negative [25]. In this work we used two commercially available tests, the first combined the 38kDa antigen with the 16kDa antigen, and the second combined it with LAM, a lipopolysaccharide present in all mycobacteria, obtaining overall test sensitivities of 19% and 23%, respectively, when detecting IgG antibodies. None of these mixtures have yet attained enough sensitivity to diagnose TB.

Concerning glycolipids, we chose the most studied specific from *M. tuberculosis*. SL-I, DAT and TAT are antigenic glycolipids exposed on the surface of *M. tuberculosis* [26]. SL-I is exclusive to *M. tuberculosis*, and DAT and TAT are only present in *M. tuberculosis* and *M. fortuitum*. CF is located in dipper cell wall layers and is present in all mycobacteria, but it has a similar structure to the other three. Previous studies analyzing the serodiagnostic power of these antigens found significantly higher antibody titers in TB patients with respect to the control group, although the sensitivities and specificities reported varied widely among the different studies [18]. As we discussed above we found that more TB patients (both smear-positive and smear-negative) reacted to these glycolipids than to the 38kDa protein-based tests. Surprisingly, six of the seven sera from smear-negative patients that reacted only did so to glycolipids. The data indicate that IgA detection identifies more smear-negative pulmonary TB patients than does IgG detection (Table 4): among the four that reacted, IgA was detected in all of them, whereas IgG was detected in

only one patient. Furthermore IgA was more specific than IgG when glycolipids were detected. Out of the 15 control subjects that reacted to some tests, only one reacted to IgA detecting one glycolipid (SL-I/IgA).

The response to the most specific glycolipid could complement the antibody response to protein-based tests: combining SL-I/IgA and 38kDa+LAM/IgG tests, a test sensitivity of 62% in pulmonary TB patients has been achieved, with an overall test specificity of 96.3%. This supposes a gain of 12% and 32% respectively with respect to both tests.

Our data show that the glycolipidic antigens studied in this work induce a higher IgG and IgA humoral response than the 38kDa protein in TB patients who have not yet started the antituberculous treatment. However, neither the glycolipidic nor the proteinaceous antigens used in this study are useful for routine diagnostic application by themselves. The test sensitivity values improve using these glycolipids together with specific proteins and detecting IgG and IgA antibodies, but neither are good for diagnosing purposes.

We therefore conclude that it is necessary to find other specific antigens, or a combination of antigens, useful fundamentally for the diagnosis of smear-negative patients, since the majority of them did not react against the antigens studied here, and in general against the known antigens tested to date.

REFERENCES

1. Workshop Report. WHO tuberculosis diagnostics workshop: product development guidelines, Cleveland, Ohio. 1997:1-27. (www.who.int/tdr/publications)

2. Daniel TM, Debanne SM. The serodiagnosis of tuberculosis and other mycobacterial diseases by enzyme-linked immunosorbent assay. Am Rev Respir Dis 1987; 135:1137-51.

3. Bothamley GH. Serological diagnosis of tuberculosis. Eur Respir J 1995; 8(Suppl. 20):676s-88s.

4. Chan ED, Heifets L, Iseman MD. Immunologic diagnosis of tuberculosis: a review. Tuberc Lung Dis 2000; 80:131-40.

5. Chan ED, Reves R, Belisle JT, Brennan PJ, Hahn WE. Diagnosis of tuberculosis by a visually detectable immunoassay for lipoarabinomannan. Am J Respir Crit Care Med 2000; 161:1713-9.

6. Wilkins EGL. Antibody detection in tuberculosis. In: Davies PDO, ed. Clinical tuberculosis. London: Chapman & Hall, 1998:81-96.

7. Julián E, Matas L, Hernández A, Alcaide J, Luquin M. Evaluation of a new serodiagnostic tuberculosis test based on the immunoglobulin A detection against Kp-90 antigen. Int J Tuberc Lung Dis 2000; 4:1082-5.

8. Chiang I-H, Suo J, Bai K-J, *et al*. Serodiagnosis of tuberculosis. A study comparing three specific mycobacterial antigens. Am J Respir Crit Care Med 1997; 156:906-11.

9. Pottumarthy S, Wells VC, Morris AJ. A comparison of seven tests for serological diagnosis of tuberculosis. J Clin Microbiol 2000; 38:2227-31.

10. Al Zahrani K, Al Jahdali H, Poirier L, René P, Gennaro ML, Menzies D. Accuracy and utility of commercially available amplification and serologic tests for the diagnosis of minimal pulmonary tuberculosis. Am J Respir

Crit Care Med 2000; 162:1323-9.

11. Lyashchenko L, Colangeli R, Houde M, Al Jahdali H, Menzies D, Gennaro ML. Heterogeneous antibody responses in tuberculosis. Infect Immun 1998; 66:3936-40.

12. Moran AJ, Treit JD, Whitney JL, *et al*. Assessment of the serodiagnostic potential of nine novel proteins from *Mycobacterium tuberculosis*. FEMS Microbiol Lett 2001; 9875:1-6.

13. Samanich K, Belisle JT, Somnenberg MG, Keen MA, Zolla-Pazner S, Laal S. Delineation of human antibody responses to culture filtrate antigens of *Mycobacterium tuberculosis*. J Infect Dis 1998; 178:1534-8.

14. Samanich K, Belisle JT, Laal S. Homogeneity of antibody responses in tuberculous patients. Infect Immun 2001; 69:4600-9.

15. Gennaro ML. Immunologic diagnosis of tuberculosis. Clin Infec Dis 2000; 30(Suppl 3):S243-6.

16. Lodes MJ, Dillon DC, Mohamath R, *et al*. Serological expression cloning and immunological evaluation of MTB48, a novel *Mycobacterium tuberculosis* antigen. J Clin Microbiol 2001; 39:2485-93.

17. Hendrickson RC, Douglass JF, Reynolds LD, *et al*. Mass spectrometric identification of Mtb81, a novel serological marker for tuberculosis. J Clin Microbiol 2000; 38:2354-61.

18. Julián E, Cama M, Martínez P, Luquin M. An ELISA for five glycolipids from the cell wall of *Mycobacterium tuberculosis*: Tween 20 interference in the assay. J Immunol Methods 2001; 251:21-30.

19. Muñoz M, Luquin M, García-Barceló M, Julián E, Ausina V, Lanéelle MA. Distribution of surface-exposed antigenic glycolipids in recent clinical isolates of *Mycobacterium tuberculosis*. Res Microbiol 1997; 148:405-12.

20. Muñoz M, Lanéelle M-A, Luquin M, Torrelles J, Julián E, Ausina V, Daffé M. Ocurrence of an antigenic triacyl trehalose in clinical isolates and reference strains of *Mycobacterium tuberculosis*. FEMS Microbiol Letters 1997; 157:251-9.

21. Falla JC, Parra CA, Mendoza M, *et al*. Identification of B- and T-cell epitopes within the MTP40 protein of *Mycobacterium tuberculosis* and their correlation with the disease course. Infect Immun 1991; 59:2265-73.

22. Laal S, Samanich KM, Sonnenberg MG, Zolla-Pazner S, Phadtare JM, Belisle JT. Human humoral responses to antigens of *Mycobacterium tuberculosis*: immunodominance of high-molecular-mass antigens. Clin Diag Lab Immunol 1997; 4:49-56.

23. Samanich K, Keen MA, Vissa VD, *et al*. Serodiagnostic potential of culture filtrate antigens of *Mycobacterium tuberculosis*. Clin Diagn Lab Immunol 2000; 7:662-8.

24. Uma Devi KR, Ramalingam B, Brennan PJ, Narayanan PR, Raja A. 2001. Specific and early detection of IgG, IgM and IgA antibodies to *Mycobacterium tuberculosis* 38kDa antigen in pulmonary tuberculosis. Tuberculosis 2001; 81:249-53.

25. Dillon DC, Alderson MR, Day CH, *et al*. Molecular and immunological characterization of *Mycobacterium tuberculosis* CFP-10, an immunodiagnostic antigen missing in *Mycobacterium bovis* BCG. J Clin Microbiol 2000; 38:3285-90.

26. Vergné I, Daffé M. Interaction of mycobacterial glycolipids with host cells. Front. Bioscienc. 1998; 3:d865-76.

**Table 1.** Cut-off values chosen for the glycolipid tests.

| ELISA glycolipids | Result | | |
|---|---|---|---|
| | Negative* | Low** | High*** |
| 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 |

* A negative result is considered when a corrected OD value is lower than the mean plus 3 SD from healthy donors in each test

** a low positive is between the mean plus 3 SD and 6 SD from healthy donors

*** a high positive is greater than the mean plus 6 SD.

**Table 2.** Cut-off values chosen for the PATHOZYME tests.

| Result | PATHOZYME tests | | | |
|---|---|---|---|---|
| | 38kDa+16kDa IgG | 38kDa+LAM IgG | 38kDa+LAM IgA | 38kDa+LAM IgM |
| Negative | < 200 | < 400 | < 300 | < OD low positive control |
| Low positive | 200 to 450 | 400 to 900 | 300 to 600 | OD low positive control to OD low positive control x 1.5 |
| High Positive | > 450 | > 900 | > 600 | > OD low positive control x 1.5 |

Data were expressed in serounits according to the standard curve provided by the manufacturer, for IgG and IgA tests.

38kDa+16kDa PATHOZYME Complex *plus* test; 38kDa+LAM, PATHOZYME-MYCO test.

Table 3. Seropositive reactivities in all the tests using two different cut off values for each one.

| Tests | | Tuberculous patients (82) | | | | | | | | Cured subjects (83) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Pulmonary (40) | | | | Extrapulmonary (12) | | | | Healthy controls (48) | | Non-TB pneumonias (35) | |
| | | Smear + (29) | | Smear - (11) | | Smear + (1) | | Smear - (11) | | | | | |
| | | low | high | low | high | low | high | low | high | low | high | low | high |
| IgG | 38kDa+16kDa | 3 | 9 | .. | . | .. | . | .. | 1 | . | 3 | 6 | 5 |
| | 38kDa+LAM | 5 | 17 | 1 | . | . | . | 2 | . | 7 | 2 | 8 | 1 |
| | DAT | 6 | 9 | 5 | . | 1 | . | . | 2 | .. | . | 13 | 3 |
| | TAT | 7 | 13 | 5 | 3 | . | 5 | 3 | 2 | . | . | 6 | 15 |
| | SL-I | 8 | 24 | 4 | 6 | . | 5 | 6 | 1 | 1 | . | 6 | 13 |
| | CF | 0 | 11 | 5 | 7 | . | 5 | 1 | 1 | 1 | . | 6 | 1 |
| IgA | 38kDa+LAM | 0 | 6 | 1 | . | . | . | . | 1 | 2 | 3 | 0 | 1 |
| | DAT | 5 | 18 | 4 | 3 | 1 | . | 1 | 2 | 2 | - | 6 | 11 |
| | TAT | 5 | 17 | 1 | 5 | 1 | - | 2 | 3 | 1 | . | 5 | 13 |
| | SL-I | 7 | 16 | 3 | 4 | 1 | - | 2 | 1 | . | . | 16 | 1 |
| | CF | 4 | 8 | . | 2 | . | . | 3 | 1 | 2 | . | 1 | . |
| IgM | 38kDa+LAM | 1 | 3 | 2 | . | . | . | . | . | 3 | 2 | . | 2 |
| | DAT | 2 | . | 2 | . | .. | . | . | . | 3 | . | . | .. |
| | TAT | 4 | 1 | 1 | 1 | - | - | - | - | - | - | 1 | 3 |
| | SL-I | 1 | 3 | 1 | . | . | . | . | 1 | 1 | . | 1 | 1 |
| | CF | 1 | . | . | . | . | . | 1 | . | 1 | . | 1 | . |

Low and high results are calculated in terms of the values indicated in Tables 1 and 2.

The number of individuals belonging to each group is indicated in brackets.

38kDa+16kDa, PATHOZYME Complex plus test. 38kDa+LAM, PATHOZYME-MYCO test. -, no sera reacted in that test; smear, result of the Ziehl-Neelsen stain.

Table 4. Highest positive responses in sera from TB patients, using the most specific tests.

|  |  | Tuberculous patients |  |
|---|---|---|---|
|  |  | Pulmonary TB | Extrapulmonary TB |

Tests (rows):

- Smear
- IgG  38kDa+16kD
-      38kDa+LAM
-      DAT
-      CF
- IgA  38kDa+LAM
-      SL-I
-      CF

Pulmonary TB patient numbers: 3, 6, 7, 14, 15, 29, 31, 40, 43, 46, 97, 141, 142, 187, 188, 243, 245, 268, 269, 270, 271, 272, 273, 274, 366, 367, 368, 369, 370, 12, 34, 42, 66, 73, 79, 93, 137, 189, 189, 243

Extrapulmonary TB patient numbers: 44, 6, 28, 36, 60, 61, 83, 95, 96, 184, 246, 266

A dark square indicates a positive result, and a blank square indicates a negative result.

38kDa+16kDa, PATHOZYME Complex *plus* test; 38kDa+LAM, PATHOZYME-MYCO test; smear, result of the Ziehl-Neelsen stain.

153

**Table 5.** Highest positive responses in sera from healthy controls, using the most specific tests.

| Tests | Healthy controls — PPD negative | | | | | | | | | | | | | | | | | | | | | | | | | | | | Healthy controls — PPD positive | | | | | | | | Vaccinated | | | | | | | | Healed TB | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 185 | 192 | 199 | 200 | 201 | 202 | 203 | 204 | 205 | 206 | 208 | 209 | 211 | 212 | 213 | 214 | 216 | 217 | 219 | 220 | 221 | 223 | 224 | 226 | 227 | 228 | 229 | 230 | 169 | 180 | 218 | 222 | 225 | 249 | 254 | 262 | 207 | 210 | 252 | 253 | 255 | 257 | 258 | 264 | 181 | 197 | 248 |
| IgG 38kDa+16kD | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 38kDa+LAM | | | | | | | | | | | | | | | | | | | | | | | ■ | | | | | | | | | | | | | | | ■ | | | | | | | | | |
| DAT | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| CF | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| IgA 38kDa+LAM | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | ■ | | | | | | | | | | | | | | | |
| SL-I | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| CF | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

A dark square indicates a positive result, and a blank square indicates a negative result.

38kDa+16kDa, PATHOZYME Complex *plus* test; 38kDa+LAM, PATHOZYME-MYCO test.

Table 6. Highest positive responses in sera from non-TB pneumonia patients, using the most specific tests.

| Tests | Non-TB pneumonia patients | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 275 | 276 | 118 | 120 | 123 | 124 | 125 | 132 | 280 | 281 | 282 | 284 | 111 | 112 | 114 | 115 | 285 | 286 | 288 | 289 | 107 | 108 | 290 | 291 | 292 | 294 | 126 | 127 | 128 | 100 | 101 | 102 | 103 | 105 | 106 |
| IgG 38kDa+16kDa | | | | | | ■ | | | | | | | | | | | | | | | ■ | | | | | | | | | | ■ | | | ■ | ■ |
| 38kDa+LAM | | | | | | | | | | | | | | | ■ | | | | | | | | | | | | | | | | | | | | |
| DAT | | | | | ■ | | | | | | | | | | | | | | | | | | | | | | ■ | | | | | ■ | | | |
| CF | | | | ■ | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| IgA 38kDa+LAM | | | | | | | | | | | | | | | | ■ | | | | | | | | | | | | | | | | ■ | | | |
| SL-I | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| CF | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

A dark square indicates a positive result, and a blank square indicates a negative result.

38kDa+16kDa, PATHOZYME Complex *plus* test; 38kDa+LAM, PATHOZYME-MYCO test.

# CONCLUSIONS

# D. Conclusions

1. El protocol d'immunoassaig en suport de plàstic que hem trobat idoni per a l'ús de DAT, TAT, SL-I, CF i PGL de la paret de *M. tuberculosis* inclou una quantitat de 1.000 ng d'antigen dissolt en hexà i la no-utilització del detergent Tween 20.

2. La utilització del detergent Tween 20 en l'enzim immunoassaig amb antígens glicolipídics comporta una davallada de la sensibilitat en el cas de les DAT, TAT, SL-I i PGL, no així amb el CF. La incubació dels diferents reactius diluïts amb tampó més detergent, o la seva utilització en els rentats, determina que l'antigen es pugui desenganxar del suport de plàstic.

3. La inclusió d'una població de control formada per individus afectes d'altres infeccions respiratòries és indispensable per a l'avaluació dels nous tests serodiagnòstics per a la TB. La utilització únicament d'individus sans com a controls proporciona dades esbiaxades sobre la seva utilitat.

4. La resposta d'IgG enfront del LAM en individus amb una TB reincident és més elevada que la que s'obté en individus amb una nova TB.

5. Els malalts amb una TB reincident cal prendre'ls en consideració a l'hora d'avaluar un test de serodiagnòstic, a causa de la diferència de producció d'anticossos enfront dels casos de nova TB.

6. Cap dels nou tests comercialment disponibles avaluats (MycoDot™, Tuberculosis IgA test, ANDA-TB o Pathozyme) té una capacitat serodiagnòstica adient per a ser utilitzat en el nostre entorn. El més específic ha estat el test MycoDot™, amb un 100% d'especificitat però amb una sensibilitat del 18,5%.

7. Tot i que alguns dels dotze tests basats en antígens glicolipídics han presentat millors resultats d'especificitat que els tests comercialitzats

(Tuberculosis IgA test, ANDA-TB o Pathozyme), cap d'ells ha mostrat suficient criteri de discriminació entre la població de control i la tuberculosa com per a ser utilitzat per sí mateix com a eina diagnòstica.

8. La pre-absorció de les IgG dels sèrums prèviament a la detecció tant d'IgM com d'IgA no millora la sensibilitat dels tests basats en antígens glicolipídics.

9. Els resultats de sensibilitat obtinguts en el grup de nens amb TB són molt baixos (inferiors al 20%) per a qualsevol dels antígens i de les immunoglobulines assajats. Ni els antígens glicolipídics ni l'antigen Kp-90 son útils per al diagnòstic de la TB infantil.

10. La detecció d'anticossos IgM enfront dels antígens glicolípidics de la paret de *M. tuberculosis* no té utilitat serodiagnòstica.

11. La resposta IgA enfront dels glicolípids basats en una molècula de trealosa és més específica que per a la resta d'antígens avaluats (Kp-90, A60 i la proteïna 38 kDa) i les immunoglobulines assajades. Els tests MycoDot™ i SL-I/IgA han resultat ser els més específics dels vint-i-un tests avaluats.

12. La detecció d'anticossos enfront de diferents antígens de diferent naturalesa bioquímica millora la sensibilitat del test. La combinació del test SL-I/IgA amb tests basats en la proteïna 38 kDa proporciona un increment de la sensibilitat (del 62%) alhora que manté una especificitat elevada (del 96,3%).

# BIBLIOGRAFIA

# E. Bibliografia

Abe, C.; Hirano, K.; Tomiyama, T. (1999) "Simple and rapid identification of the *Mycobacterium tuberculosis* complex by immunochromatographic assay using anti-MPB64 monoclonal antibodies". *J. Clin. Microbiol.* 37: 3693-3697.

Al Zahrani, K.; Al Jahdali, H.; Poirier, L.; René, P.; Gennaro, M.L.; Menzies, D. (2000) "Accuracy and utility of commercially available amplification and serologic tests for the diagnosis of minimal pulmonary tuberculosis". Am. J. Respir. Crit. Care Med. 162: 1323-1329.

Albert, M.L.; Sauter, B.; Bhardwaj, N. (1998) "Dendritic cells acquire antigen from apoptotic cells and induce class I restricted CTLs". Nature. 392: 86-89.

Al-Hajjaj, M.S.; Gad-el-Rab, M.O.; Al-Orainey, I.O.; Al-Kassimi, F.A. (1999) "Improved sensitivity for detection of tuberculosis cases by a modified Anda-TB ELISA test". Tuber. Lung Dis. 79: 181-185.

Alifano, M.; De Pascalis, R.; Sofia, M.; Faraone, S.; Del Pezzo, M.; Covelli, I. (1998) "Detection of IgG and IgA against the mycobacterial antigen A60 in patients with extrapulmonary tuberculosis". *Thorax.* 53: 377-380.

Alifano, M.; De Pascalis, R.; Sofia, M.; Faraone, S.; Del Pezzo, M.; Covelli, I. (1997) "Evaluation of IgA-mediated humoral immune response against the mycobacterial antigen P-90 in diagnosis of tuberculosis". *Chest* 111: 601-605.

Altare, F.; Durandy, A.; Lammas, D.; Emile, J.F.; Lamhamedi, S.; Le Deist, F.; Drysdale, P.; Jouanguy, E.; Doffinger, R.; Bernaudin, F.; Jeppsson, O.; Gollob, J.A.; Meinl, E.; Segal, A.W.; Fischer, A.; Kumararatne, D.; Casanova, J.L. (1998) "Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency". *Science.* 280: 1432-1435.

Amicosante, M.; Houde, M.; Guaraldi, G.; Saltini, C. (1999) "Sensitivity and specificity of a multi-antigen ELISA test for the serological diagnosis of tuberculosis". *Int. J. Tuberc. Lung Dis* 3: 736-740.

Amicosante, M.; Richeldi, I.; Monno, L.; Cuboni, A.; Tartoni, P.L.; Angarano, G.; Orefici, G.; Saltini, C. (1997) "Serological markers predicting tuberculosis in human immunodeficiency virus-infected patients". *Int. J. Tuberc. Lung Dis* 1: 435-440.

Andersen, A.B.; Brennan, P.J. (1994) "Proteins and antigens of *Mycobacterium tuberculosis*". A: Tuberculosis: Pathogenesis, Protection, and Control. AMS Press. Bloom BR, ed. Washington, DC.

Andersen, P.; Munk, M.E.; Pollock, J.M.; Doherty, T.M. (2000) "Specific immune-based diagnosis of tuberculosis". *Lancet.* 356: 1099-1104.

Arend, S.M.; Andersen, P.; van Meijgaarden, K.E.; Skjøt, R.L.; Subronto, Y.W.; van Dissel, J.T.; Ottenhoff, T.H. (2000) "Detection of active tuberculosis infection by T cell responses to early-secreted antigennic target 6-kDa protein and culture filtrate protein 10". *J. Infec. Dis.* 181: 1850-1854.

Ärikan, S.; Tuncer, S.; Us, D.; Ünal, S.; Ustaçelebi, S. (1998) "Anti-Kp 90 antibodies in the diagnosis of active tuberculosis". *Chest* 114: 1253-1257.

Asselineau, J.; Lanéelle, G. (1998) "Mycobacterial lipids: a historical perspective". *Front. Biosci.* 3: e164-e174.

ATS. American Thoracic Society. (2000a) "Diagnostics standards and classification of tuberculosis in adults and children". *Am. J. Respir. Crit. Care Med* 161: 1376-1395.

ATS. American Thoracic Society. (2000b) "Targeted tuberculin testing and treatment of latent tuberculosis infection". *Am. J. Respir. Crit. Care Med* 161: S221-S247.

Bach, E.A.; Aguet, M.; Schreiber, R.D. (1997) "The IFN-gamma receptor: a paradigm for cytokine receptor signalling". *Annu. Rev. Immunol.* 15: 563-591.

Baer, H.H. (1993) "The structure of an antigenic glycolipid (SL-IV) from *Mycobacterium tuberculosis*". *Carbohydr. Res.* 240: 1-22.

Bardana, E.J.; McClatchy, J.K.; Farr, R.S.; Minden, P. (1973) "Universal occurrence of antibodies to tubercle bacilli in sera from nontuberculous and tuberculous individuals". *Clin. Exp. Immunol.* 13: 65-77.

Baulard, A.R.; Besra, G.S.; Brennan, P.J. (1999) "The cell-wall core of *Mycobacterium*: structure, biogenesis and genetics". Chapter 13. A: Mycobacteria. Molecular biology and virulence. Eds.: Ratledge C., i Dale J. Blackwell Science Ltd. UK. pp: 240-286.

Beckman, E.M.; Porcelli, S.A.; Morita, C.T.; Behar, S.M.; Furlong, S.T.; Brenner, M.B. (1994) "Recognition of a lipid antigen by CD1-restricted alpha beta+ T cells". *Nature*. 372: 691-694.

Behr, M.A.; Warren, S.A.; Salamon, H.; Hopewell, P.C.; Ponce de Leon, A.; Daley, C.L.; Small, P.M. (1999a) "Transmission of *Mycobacterium tuberculosis* from patients smear-negative for acid-fast bacilli". *Lancet*. 353: 444-449.

Behr, M.A.; Wilson, M.A.; Gill, W.P.; Salamon, H.; Schoolnik, G.K.; Rane, S.; Smal, P.M. (1999b) "Comparative genomics of BCG vaccines by whole-genome DNA microarray". *Science*. 284: 1520-1523.

Belisle, J.T.; Brennan, P.J. (1989) "Chemical basis of rough and smooth variation in mycobacteria". *J. Bacteriol.* 171: 3465-3470.

Besra, G.S.; Chatterjee, D. (1994) "Lipids and carbohydrates of *Mycobacterium tuberculosis*" (Chapter 20). A: Tuberculosis: Pathogenesis, Protection, and Control. AMS Press. Bloom BR, ed. Washington, DC.

Besra, G.S.; Khoo, K.H.; Belisle, J.T.; McNeil, M.R.; Morris, H.R.; Dell, A.; Brennan, P.J. (1994) "New pyruvylated, glycosilated acyltrehaloses from *Mycobacterium smegmatis* strains, and their implications for phage resistance in mycobacteria". *Carbohydr. Res.* 251: 99-114.

Besra, G.S.; Morehouse, C.B.; Rittner, C.M.; Waechter, C.J.; Brennan, P.J. (1997) "Biosynthesis of mycobacterial lipoarabinomannas". *J. Biol. Chem* 272: 18460-18466.

Bhattacharya, A.; Ranadive, S.N.; Kale, M.; Bhattacharya, S. (1986) "Antibody based enzyme-linked immunosorbent assay for determination of immune complexes in clinical tuberculosis". *Am. Rev. Respir. Dis* 134: 205-209.

Bloch, H. (1950) "Studies on the virulence of tubercle bacilli. Isolation and biological properties of constituent of virulent organisms". *J. Exp. Med* 91: 197-217.

Bloom, B.R.; Murray, C.J. (1992) "Tuberculosis: commentary on a reemergent killer". *Science*. 257: 1055-1064.

Boggian, K.; Fierz, W.; Vernazza, P.L.; The Swiss HIV Cohort Study. (1996) "Infrequent detection of lipoarabinomannan antibodies in Human Immunodeficiency Virus-associated mycobacterial disease". *J. Clin. Microbiol.* 34: 1854-1855.

Bosio, C.M.; Gardner, D.; Elkins, K.L. (2000) "Infection of B cell deficient mice with CDC1551, a clinical isolate of *Mycobacterium tuberculosis*: delay in dissemination and development of lung pathology". *J. Immunol.* 164: 6417-6425.

Bothamley, G.; Batra, H.; Ramesh, V.; Chandramui, A.; Ivanyi, J. (1992a) "Serodiagnostic value of the 19 Kilodalton antigen of *Mycobacterium tuberculosis* in indian patients". *Eur. J. Clin. Microbiol. Infect. Dis.* 11: 912-915.

Bothamley, G.H. (1995) "Serological diagnosis of tuberculosis". *Eur. Respir. J.* 8 (Suppl. 20): 676s-688s.

Bothamley, G.H.; Rudd, R.; Festenstein, F.; Ivanyi, J. (1992b) "Clinical value of the measurement of *Mycobacterium tuberculosis* specific antibody in pulmonary tuberculosis". *Thorax.* 47: 270-275.

Brennan, P.J. (1997) "Tuberculosis in the context of emerging and reemerging diseases". *FEMS Immunol. Med. Microbiol* 18: 263-269.

Brennan, P.J.; Nikaido, H. (1995) "The envelope of mycobacteria". *Annu. Rev. Biochem* 64:29-63.

Brozna, J.P.; Horan, M.; Rademacher, J.M.; Pabst, K.M.; Pabst, M.J. (1991) "Monocyte responses to sulphatide from *Mycobacterium tuberculosis*: inhibition of priming for enhance release of superoxide, associated with increased secretion of interleukin-1 and tumor necrosis factor alpha and altered protein phosphorilation". *Infect. Immun.* 59: 2542-2548.

Butler, W.R.; Guthertz, L.S. (2001) "Mycolic acid analysis by high-performance liquid chromatography for identification of *Mycobacterium* species". *Clin. Microbiol. Rev.* 14(4):704-726.

Camacho, L.R.; Constant, P.; Raynaud, C.; Lanéelle, M.A.; Triccas, J.A.; Gicquel, B.; Daffé, M.; Guilhot, C. (2001) "Analysis of the phthicerol dimycocerosate locus of *Mycobacterium tuberculosis*". *J. Biol. Chem* 276: 19845-19854.

Camacho, L.R.; Ensergueix, D.; Pérez, E.; Gicquel, B.; Guilhot, C. (1999) "Identification of a virulence gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis". *Mol. Microbiol* 34: 257-267.

Caminero, J.A. (1990a) "Diagnóstico serológico de la tuberculosis y otras micobacteriosis. I. Consideraciones generales". *Medic. Clin.* 94: 187-195.

Caminero, J.A. (1990b) "Diagnóstico serológico de la tuberculosis y otras micobacteriosis. II. Estado actual de los estudios serológicos de estas enfermedades". *Medic. Clin.* 94: 426-436.

Caminero, J.A.; Medina, M.V.; Rodríguez, F.; Cabrera, P. (1998) "Tuberculosis y otras micobacteriosis". A: Caminero JA, i Fernández L, eds. Manual de neumología y cirugía torácica. Madrid. Vol. 2: 1389-1419.

Carpenter, A.B. (1997) "Enzyme-linked immunoassays". A: Manual of clinical laboratory immunology. Rose NR, Conway de Macario E, Folds JD, Lane HC, i Nakamura RM, eds. ASM Press. Washington, DC. pp. 20-29.

Carr, R.I.; Chakraborty, A.K.; Brunda, M.J.; Davidson, P.T.; Damle, P.B.; Hardtke, M.A.; Gilbride, K.J.; Minden, P. (1980) "Immune complexes and antibodies to BCG in sera from patients with mycobacterial infections". *Clin. Exp. Immunol.* 39: 562-569.

Catanzaro, A.; Davidson, B.L.; Fujiwara, P.I.; Goldberger, M.J.; Gordin, F.; Salfinger, M.; Sbabaro, J.; Schluger, N.W.; Sierro, M.F.; Woods, G.L. (1997) "Rapid diagnostic tests for tuberculosis. What is the appropriate use?". *Am. J. Respir. Crit. Care Med* 155: 1804-1814.

CDC. Centers for Disease Control and Prevention. (1999) "Tuberculosis elimination revisited: obstacles, opportunities, and a renewed commitment. Advisory Council for the Elimination of Tuberculosis (ACET)". MMWR *Morb. Mortal. Wkly. Rep* 48: 1-13.

Chadwick, M.V. (1981) "Tuberculosis- the killer disease". A: Mycobacteria. Ed. J. Wright & sons Ltd., Bristol, England.

Chaicumpar, K.; Fujiwara, N.; Nishimura, O.; Hotta, H.; Pan, J.W.; Takahashi, M.; Abe, C.; Yano, I. (1997) "Studies of polymorphic DNA fingerprinting and lipid pattern of *Mycobacterium tuberculosis* patient isolates in Japan". *Microbiol. Immunol.* 41: 107-119.

Chan, E.D.; Heifets, L.; Iseman, M.D. (2000a). "Immunologic diagnosis of tuberculosis: a review". *Tuberc. Lung Dis.* 80: 131-140.

Chan, E.D.; Reves, R.; Belisle, J.T.; Brennan, P.J.; Hahn, W.E. (2000) "Diagnosis of tuberculosis by a visually detectable immunoassay for lipoarabinomannan". *Am. J. Respir. Crit. Care Med* 161: 1713-1719.

Chan, J.; Fan, X.; Hunter, S.W.; Brennan, P.J.; Bloom, B.R. (1991) "Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages". *Infect. Immun.* 59: 1755-1761.

Chan, J.; Fujiwara, T.; Brennan, P.; McNeil, M.; Turco, S.J.; Sibille, J.C.; Snapper, M.; Aisen, P.; Bloom, B.R. (1989) "Microbial glycolipids: possible virulence factors that scavenge oxygen radicals". *Proc. Natl. Acad. Sci.* USA. 86: 2453-2457.

Chang, C.L.; Lee, E.Y.; Son, H.C.; Park, S.K. (2000) "Evaluating the usefulness of the ICT tuberculosis test kit for the diagnosis of tuberculosis". *J. Clin. Pathol.* 53: 715-717.

Chaparas, S.D.; Maloney, C.J.; Hedrick, S.R. (1970) "Specificity of tuberculins and antigens from various species of mycobacteria". *Am. Rev. Respir. Dis.*101: 74-83.

Charpin, D.; Herbault, H.; Gevaudan, M.J.; Saadjian, M.; de Micco, P.; Arnaud, A.; Vervloet, D.; Charpin, J. (1990) "Value of ELISA using A60 antigen in the diagnosis of active pulmonary tuberculosis". *Am. Rev. Respir. Dis* 142: 380-384.

Chiang, I.H.; Suo, J.; Bai, K.J.; Lin, T.P.; Luh, K.T.; Yu, C.J.; Yang, P.C. (1997) "Serodiagnosis of Tuberculosis. A study comparing three specific mycobacterial antigens". *Am. J. Respir. Crit. Care Med* 156: 906-911.

Chierakul, N.; Damrongchokpipat, P.; Chaiprasert, A.; Arjratanakul, W. (2001) "Antibody detection for the diagnosis of tuberculous pleuritis". *Int. J. Tuberc. Lung Dis* 5: 968-972.

Chin, D.P.; Osmond, D.; Page-Shafek, K.; Glassroth, J.; Rosen, M.J.; Reichman, L.B.; Kwale, P.A.; Wallace, J.M.; Poola, W.K.; Hopewell, P.C. (1996) "Reliability of anergy skin testing in persons with HIV infection". *Am. J. Respir. Crit. Care Med* 153: 1982-1984.

Cid, F.; Gorina, N.; Sánchez Aldeguer, J. (1993) "L'Hospital de l'Esperit Sant: un exemple de l'evolució assistencial a Catalunya". Ed. per Santa Coloma de Gramanet Fundació Hospital de l'Esperit Sant cop.

Cocito, C.G. (1991) "Properties of the mycobacterial antigen complex A60 and its applications to the diagnosis and prognosis of tuberculosis". *Chest* 100: 1687-93.

Cole, R.A.; Lu, H.M.; Shi, Y.Z.; Wang, J.; De-Hua, T.; Zhou, A.T. (1996) "Clinical evaluation of a rapid immunochromatographic assay based on the 38KDa antigen of *Mycobacterium tuberculosis* on patients with pulmonary tuberculosis in China". *Tuber. Lung Dis.*77: 363-368.

Cole, S.T.; Brosch, R.; Parkhill, J.; Garnier, T.; Churcher, C.; Harris, D.; Gordon, S.V.; Eiglmeier, K.; Gas, S.; Barry, C.E., 3rd.; Tekaia, F.; Badcock, K.; Basham, D.; Brown, D.; Chillingworth, T.; Connor, R.; Davies, R.; Devlin, K.; Feltwell, T.; Gentles, S.; Hamlin, N.; Holroyd, S.; Hornsby, T.; Jagels, K.; Krogh, A.; McLean, J.; Moule, S.; Murphy, L.; Oliver, K.; Osborne, J.; Quail, M.A.; Rajandream, M.A.; Rogers, J.; Rutter, S.; Seeger, K.; Skelton, J.; Squares, R.; Squares, S.; Sulston, J.E.; Taylor, K.; Whitehead, S.; Barrell, B.G. (1998) "Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence". *Nature.* 393: 537-544.

Collins, F.M. (1998) "Mycobacterial pathogenesis: a historical perspective". *Front. Biosci.* 3: e123-e132.

Collins, H.L.; Kaufmann, S.H.E. (2001) "The many faces of host responses to tuberculosis". *Immunology.* 103: 1-9.

Collins, H.L.; Kaufmann, S.H.E. (2002) "Acquired immunity against bacteria". A: Immunology of Infectious Diseases. Kaufmann, S.H.E; Sher, A., Ahmed, R. eds. ASM Press, Washington, DC. pp. 207-221.

Converse, P.J.; Jones, S.L.; Astemborski, J.; Vlahov, D.; Graham, N.M. (1997) "Comparison of a tuberculin interferon-gamma assay with the tuberculin skin test in high-risk adults: effect of human immunodeficiency virus infection". *J. Infect. Dis.* 176: 144-150.

Cox, J.S.; Chen, B.; McNeil, M.; Jacobs, W.R., Jr. (1999) "Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice". *Nature.* 402: 79-82.

Crick, D.C.; Mahapatra, S.; Brennan, P.J. (2001) "Biosynthesis of the arabinogalactan-peptidoglycan complex of *Mycobacterium tuberculosis*". *Glycobiology* 11: 107R-118R.

Cruaud, P.; Berlie, C.; Torgal García, J.; Papa, F.; David, H.L. (1989) "Human IgG antibodies immunoreacting with specific sulfolipids from *Mycobacterium tuberculosis*". *Zentralbl. Bakteriol.* 271: 481-485.

Cruaud, P.; Yamashita, J.T.; Casabona, N.M.; Papa, F.; David, H.L. (1990) "Evaluation of a novel 2,3-diacil-trehalose-2'-sulphate (SL-IV) antigen for case finding and diagnosis of leprosy and tuberculosis". *Res. Microbiol* 141: 679-694.

Da Costa, C.T.; Khanolkar-Young, S.; Elliott, A.M.; Wasunna, K.M.; McAdam, K.P. (1993) "Immunoglobulin G subclass reponses to mycobacterial lipoarabinomannan in HIV-infected and non-infected patients with tuberculosis". *Clin. Exp. Immunol.* 91: 25-29.

Daffé, M.; Cho, S.N.; Chatterjee, D.; Brennan, P.J. (1991) "Chemical synthesis and seroreactivity of a neoantigen containing the oligosaccharide hapten of the *Mycobacterium tuberculosis*-specific phenolic glycolipid". *J. Infect. Dis*. 163: 161-168.

Daffé, M.; Draper, P. (1998) "The envelope layers of mycobacteria with reference to their pathogenicity". *Adv. Microb. Physiol* 39: 131-203.

Daffé, M.; Lacave, C.; Lanéelle, M.A.; Gillois, M.; Lanéelle, G. (1988) "Polyphthienoyl trehalose, glycolipids specific for virulent strains of the tubercle bacillus". *Eur. J. Biochem.* 172(3):579-584.

Daleine, G.; Lagrange, P.H. (1995) "Preliminary evaluation of a *Mycobacterium tuberculosis* lipooligosaccharide (LOS) antigen in the serological diagnosis of tuberculosis in HIV seropositive and seronegative patients". *Tuber. Lung Dis.* 76: 234-239.

Daniel, T.M. (1980) "The immunology of tuberculosis". *Clin. Chest Med* 1: 189-201.

Daniel, T.M. (1988) "Antibody and antigen detection for the immunodiagnosis of tuberculosis: Why not? What more is needed? Where do we stand today?". *J. Infect. Dis.* 158: 678-680.

Daniel, T.M.; Bates, J.H.; Downes, K.A. (1994) "History of tuberculosis". A: Tuberculosis: Pathogenesis, Protection, and Control. Ed. Bloom, B.R. AMS Press. Washington, DC. pp. 13-24.

Daniel, T.M.; Debanne, S.M (1987) "The serodiagnosis of tuberculosis and other mycobacterial diseases by enzyme-linked immunosorbent assay". *Am. Rev. Respir. Dis* 135: 1137-1151.

Dannenberg, Jr., A.M. (1999) "Pathophysiology: basic aspects". Chapter 2. A: Tuberculosis and nontuberculous mycobacterial infections, 4th ed. Schlossberg D, ed. WB Saunders Company, Philadelphia, PA. pp. 17-47.

Del Prete, R.; Picca, V.; Mosca, A.; D'Alagni, M.; Miragliotta, G. (1998) "Detection of anti-lipoarabinomannan antibodies for the diagnosis of active tuberculosis". *Int. J. Tuberc. Lung Dis* 2: 160-163.

Deretic, V.; Fratti, R.A. (1999) "*Mycobacterium tuberculosis* phagosome". *Mol. Microbiol* 31: 1603-1609.

Devallois, A.; Goh, K.S.; Rastogi, N. (1997) "Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the hsp65 gene and proposition of an algorithm to differentiate 34 mycobacterial species". *J. Clin. Microbiol.* 35: 2969-2973.

Diagbouga, S.; Fumoux, F.; Zoubga, A.; Sanou, P.T.; Marchal, G. (1997) Immunoblot analysis for serodiagnosis of tuberculosis using a 45/47-Kilodalton antigen complex of *Mycobacterium tuberculosis*. *Clin. Diagn. Lab. Immunol* . 4: 334-338.

Dillon, D.C.; Alderson, M.R.; Day, C.H., Bement, T.; Campos-Neto, A.; Skeiky, J.A.; Vedvick, T.; Badaro, R.; Redd, S.G.; Houghton, R. (2000) "Molecular and immunological characterization of *Mycobacterium tuberculosis* CFP-10, an immunodiagnostic antigen missing in *Mycobacterium bovis* BCG". *J. Clin. Microbiol.* 38: 3285-3290.

Doetsch, R.N. (1978) "Benjamin Marten and his *new theory of consumption*". *Microbiol. Rev.* 42: 521-528.

Domínguez, J.; Galí, N.; Blanco, S.; Pedroso, P.; Prat, C.; Matas, L.; Ausina, V. (2001) "Detection of *Streptococcus pneumoniae* antigen by a rapid immunochromatographic assay in urine samples". *Chest* 119: 243-249.

Dubnau, E.; Chan, J.; Raynaud, C.; Mohan, V.P.; Lanéelle, M.A.; Yu, M.; Quémard, A.; Smith, I.; Daffé, M. (2000) "Oxygenated mycolic acids are necessary for virulence of *Mycobacterium tuberculosis* in mice". *Mol. Microbiol* 36: 630-637.

Dubocky, B.O.; White, F.C. (1969) "Further studies with the direct latex agglutination test in tuberculosis". *Am. Rev. Respir. Dis* 100: 364-371.

Dye, C.; Scheele, S.; Dolin, P.; Pathania, V.; Raviglione, M.C. (1999) "Consensus estatement. Global burden of tuberculosis. Estimated incidence, prevalence, and mortality by country. WHO global surveillance and monitoring project". *JAMA.* 282: 677-686.

Eisenach, K.D. (1999) "Molecular diagnostics" (Chapter 9). A: Mycobacteria. Molecular Biology and Virulence. Eds. Ratledge C.; Dale, J. Blackwell Science Ltd. UK. pp. 161-179.

Ellner, J.J. (1997) "Review: The immune response in human tuberculosis-implications for tuberculosis control". *J. Infect. Dis* 176: 1351-1359.

Enomoto, K.; Oka, S.; Fujiwara, N.; Okamoto, T.; Okuda, Y.; Maekura, R.; Kuroki, T.; Yano, I. (1998) "Rapid serodiagnosis of *Mycobacterium avium*-intracellulare complex infection by ELISA with cord factor (trehalose 6,6'-dimycolate), and serotyping using the glycopeptidolipid antigen". *Microbiol. Immunol.* 42: 689-696.

Ernst, J.D. (1998) "Macrophage receptors for *Mycobacterium tuberculosis*. Minireview". *Infect. Immun.* 66: 1277-1281.

Escamilla, L.; Mancilla, R.; Glender, W.; López-Marín, L-M. (1996) "*Mycobacterium fortuitum* glycolipids for the serodiagnosis of pulmonary tuberculosis". *Am. J. Respir. Crit. Care Med* 154: 1964-1867.

Espitia, C.; Cervera, I.; González, R.; Manzilla, R. (1989) "A 38-kD *Mycobacterium tuberculosis* antigen associated with infection. Its isolation and serologic evaluation". *Clin. Exp. Immunol.* 77: 373-377.

Fäldt, J.; Dahlgren, C.; Karlsson, A.; Ahmed, A.M.S.; Minnikin, D.E.; Ridell, M. (1999) "Activation of human neutrophils by mycobacterial phenolic glycolipids". *Clin. Exp. Immunol.* 118: 253-260.

Fauci, A. (1996) "Host factors and the pathogenesis of HIV-induced disease". *Nature.* 384: 529-534.

Ferrari, G.; Langen, H.; Naito, M.; Pieters, J. (1999) "A coat protein on phagosomes involved in the intracellular survival of mycobacteria". *Cell.* 97: 435-447.

Flynn, J.; Ernst, J.D. (2000) "Immune responses in tuberculosis". *Curr. Opin. Immunol.* 12: 432-436.

Flynn, J.L.; Chan, J. (2001) "Immunology of tuberculosis". *Annu. Rev. Immunol.* 19: 93-129.

Fournié, J.J.; Adams, E.; Mullins, R.J.; Basten, A. (1989) "Inhibition of human lymphoproliferative responses by mycobacterial phenolic glycolipids". *Infect. Immun.* 57: 3653-3659.

Freeman, R.; Magee, J.; Barratt, A.; Wheeler, J.; Steward, M.; Lee, M.; Piggott, N. (1999) "Rapid immunochromatographic assay for diagnosis of tuberculosis: antibodies detected may not be specific". *J. Clin. Microbiol.* 37: 2111-2112.

Froman, S.; Burge, R.; Gedebou, M.; Pickett, M.J. (1968) "Serologic testing for tuberculosis". *Am. Rev. Respir. Dis* 97: 201-205.

Gamboa, F.O. (1997) "Aplicabilidad de los métodos de amplificación genética en el diagnóstico de la tuberculosis". Tesi doctoral. Universitat Autònoma de Barcelona.

Gatfield J.; Pieters J. (2000) "Essential role for cholesterol in entry of mycobacteria into macrophages". *Science.* 288: 1647-1650.

Gennaro, M.L. (2000) "Immunologic diagnosis of tuberculosis". *Clin. Infect. Dis.* 30 (Suppl 3): S243-246.

Glatman-Freedman, A.; Casadevall, A. (1998) "Serum therapy for tuberculosis revisited: reappraisal of the role of antibody-mediated immunity against *Mycobacterium tuberculosis*". *Clin. Microbiol. Rev.* 11: 514-532.

Glickman, S.E.; Kilburn, J.O.; Butler, W.R.; Ramos, L.S. (1994) "Rapid identification of mycolic acid patterns of mycobacteria by high-performance liquid chromatography using pattern recognition software and a *Mycobacterium* library". *J. Clin. Microbiol.* 32: 740-745.

Goren, M.B.; Brokl, O.; Roller, P.; Fales, H.M.; Das, B.C. (1976) "Sulfatides of *Mycobacterium tuberculosis*: The structure of the principal sulfatide (SL-I)". *Biochemistry.* 15: 2728-2734

Grange, J.M.; Laszlo, A. (1990) "Serodiagnostic tests for tuberculosis: a need for assessment ot their operational predictive accuracy and acceptability". *Bull World Health Organ.* 68: 571-576.

Grobusch, M.P.; Schürmann, D.; Schwenke, S.; Teichmann, D.; Klein, E. (1998) "Rapid immunochromatographic assay for diagnosis of tuberculosis". *J. Clin. Microbiol.* 36: 3443.

Grubek-Jaworska, H.; Zwolska, Z.; Droszcz, P.; Rybus, L.; Dabrowski, A.; Droszcz, W. (1997) "Serum and bronchoalveolar IgG against A60 and 38kDa antigens in the diagnosis of tuberculosis". *Int. J. Tuberc. Lung Dis* 1: 556-562.

Guerrero, A.; Martín, N.; Moreno, S.; Nogales, M.C. (1999) "Diagnóstico microbiológico de las infecciones por micobacterias". A: Procedimientos en Microbiología Clínica. Recomendaciones de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Ed.: Picazo J.J.

Gupta, I.; Jain, A.; Singh, N.B.; Chaturvedi, V.; Agarwal, S.K. (1998) "Role of antigen specific

circulating immune complexes in diagnosis of tuberculosis". *Int. J. Tuberc. Lung Dis* 2: 456-461.

Gupta, S.; Bhatia, R.; Datta, K.K. (1997) "Serological diagnosis of childhood tuberculosis by estimation of mycobacterial antigen 60-specific immunoglobulins in the serum". *Tuber. Lung Dis.* 78: 21-27.

Gupta, S.; Kumari, S.; Banwalikar, J.N.; Gupta, S.K. (1995) "Diagnostic utility of the estimation of mycobacterial Antigen A60 specific immunoglobulins IgM, IgA and IgG in the sera of cases of adult human tuberculosis". *Tuber. Lung Dis.* 76: 418-424.

Gutiérrez, J.; Fernández, F.; Soto, M.J.; Maroto, M.C. (2001) "Control de calidad interno del inmunodiagnóstico microbiano para conseguir la calidad global". *Enferm. Infecc. Microbiol. Clin.* 19: 488-494.

Hale, Y.M.; Pfyffer, G.E.; Salfinger, M. (2001) "Laboratory diagnosis of mycobacterial infections: new tools and lessons learned". *Clin. Infect. Dis.* 33: 834-846.

Harrington, J.J., 3rd.; Ho, J.L.; Lapa e Silva, J.R.; Conde, M.B.; Kritski, A.L.; Fonseca, L.S.; Saad, M.H. (2000) "*Mycobacterium tuberculosis* lipid antigens: use of multi-antigen based enzyme immunoassay for free and complex dissociated antibodies". *Int. J. Tuberc. Lung Dis* 4: 161-167.

Hasegawa, N.; Miura, T.; Ishii, K.; Yamaguchi, K.; Lindner, T.H.; Merritt, S.; Matthews, J.D.; Siddiqi, S.H. (2002) New simple and rapid test for culture confirmation of *Mycobacterium tuberculosis* complex: a multicenter study. *J. Clin. Microbiol.* 40: 908-912.

Hassan, J.; Feighery, C.; Bresnihan, B.; Whelan, A. (1992) "Prevalence of anti-Fab antibodies in patients with autoimmune and infectious diseases". *Clin. Exp. Immunol.* 89: 423-426.

He, H.; Oka, S.; Han, Y.K.; Yamamura, Y.; Kusunose, E.; Kusunose, M.; Yano, I. (1991) "Rapid serodiagnosis of human mycobacteriosis by ELISA using cord factor (trehalose-6,6'-dimycolate) purified from *Mycobacterium tuberculosis* as antigen". *FEMS Microbiol. Immunol.* 76: 201-204.

Heifets, L.B.; Good, R.C. (1994) "Current laboratory methods for the diagnosis of tuberculosis" (Chapter 7). A: Tuberculosis: Pathogenesis, Protection, and Control. Ed. Bloom BR. AMS Press. Washington, DC. pp. 85-110.

Hendrickson, R.C.; Douglass, J.F.; Reynolds, L.D.; McNeill, P.D.; Carter, D.; Reed, S.G.; Houghton, R.L. (2000) "Mass spectrometric identification of Mtb81, a novel serological marker for tuberculosis". *J. Clin. Microbiol.* 38: 2354-2361.

Hinrikson, H.P.; Pfyffer, G.E. (1994) "Mycobacterial mycolic acids (mini-review)". Part I. *Med. Microbiol. Lett.* 3: 49-57.

Hoppe, H.; de Wed, B.J.; Cywes, C.; Daffé, M.; Ehlers, M.R. (1997) "Identification of phosphatidylinositol mannoside as a mycobacterial adhesin mediating both direct and opsonic binding to nonphagocytic mammalian cells". *Infect. Immun.* 65: 3896-3905.

Jackett, P.S.; Bothamley, G.H.; Batra, H.V.; Mistry, A.; Young, D.B.; Ivanyi, J. (1988) "Specificity of antibodies to immunodominant mycobacterial antigens in pulmonary tuberculosis". *J. Clin. Microbiol.* 26: 2313-2318.

Johnson, P.D.; Stuart, R.L.; Grayson, M.L.; Olden, D.; Clancy, A.; Ravn, P.; Andersen, P.; Britton, W.J.; Rothel, J.S. (1999) "Tuberculin-purified protein derivative-, MPT-64-, and ESAT-6-stimulated gamma interferon responses in medical students before and after *Mycobacterium bovis* BCG vaccination and in patients with tuberculosis". *Clin. Diagn. Lab. Immunol.* 6: 934-937.

Jouanguy, E.; Doffinger, D.; Dupuis, S.; Pallier, A.; Altare, F.; Casanova, J.L. (1999) "IL-12 and IFN-gamma in host defense against mycobacteria and Salmonella in mice and men". *Curr. Opin. Immunol.* 11: 346-351.

Kaplan, M.H.; Chase. M.W. (1980) "Antibodies to mycobacteria in human tuberculosis. I. Development of antibodies before and after antimicrobial therapy". *J. Infect. Dis.* 142: 825-834.

Kashima, K.; Oka, S.; Tabata, A.; Yasuda, K.; Kitano, A.; Kobayashi, K.; Yano, I. (1995) "Detection of anti-Cord factor antibodies in intestinal tuberculosis for its differential diagnosis from Crohn's disease and ulcerative colitis". *Digest. Dis. Sci.* 40: 2630-2634.

Katial, R.K.; Hershey, J.; Purohit-Seth, T.; Belisle, J.T.; Brennan, P.J.; Spencer, J.S.; Engler, E.J.M. (2001) "Cell-mediated immune response to tuberculosis antigens: comparison of skin testing and measurement of in vitro gamma interferon production in whole-blood culture". *Clin. Diagn. Lab. Immunol.* 8: 339-345.

Kaufmann, S.H. (2001a) "How can immunology contribute to the control of tuberculosis?" *Nat. Rev. Immunol.* 1: 20-30.

Kaufmann, S.H. (2001b) "Koch's dilemma revisited". *Scand. J. Infect. Dis.* 33: 5-8.

Kawamura, M.; Sueshige, N.; Imayoshi, K.; Yano, I.; Maekura, R.; Kohno, H. (1997) "Enzyme immunoassay to detect antituberculous glycolipid antigen (anti-TBGL antigen) antibodies in serum for diagnosis of tuberculosis". *J. Clin. Lab. Anal.* 11: 140-145.

Khan, E.A.; Starke, J.R. (1995) "Diagnosis of tuberculosis in children: increased need for better methods". *Emerging Infect. Dis.* 1: 115-123.

Kimura, M.; Converse, P.J.; Astemborski, J.; Rothel, J.S.; Vlahov, D.; Comstock, G.W.; Graham, N.M.; Chaisson, R.E.; Bishai, W.R. (1999) "Comparison between a whole blood Interferon-gamma release assay and tuberculin skin testing for the detection of tuberculosis infection among patients at risk for tuberculosis exposure". *J. Infect. Dis.* 179: 1297-1300.

Klausner, J.D.; Makonkawkeyoon, S.; Akarasewi, P.; Nakata, K.; Kasinrerk, W.; Corral, L.; Dewar, R.L.; Lane, H.C.; Freedman, V.H.; Kaplan, G. (1996) "The effect of thalidomide on the pathogenesis of human immunodeficiency virus type 1 and *M. tuberculosis* infection". *J. Acquir. Immune Defic. Syndr.* 11: 247-257.

Koch, R. (1882) "The aetiology of tuberculosis". *Berlin Klin. Wichenschr.* 19: 221-230. Traducció a l'anglès a *Am. Rev. Tuberc. Pulm. Dis* (1932) 25: 285.

Koneman, E.W.; Allen, S.D.; Janda, W.M.; Schreckenberger, P.C.; Winn, W.C., Jr. (1997) "Mycobacteria". (Chapter 17). A: Color atlas and textbook of diagnostic microbiology. (5th Edition). Eds.: Lippincott-Raven Publishers. Philadelphia, PA. pp.: 893-952.

Koyanagi, Y.; O'Brien, W.A.; Zhao, J.Q.; Golde, D.W.; Gasson, J.C.; Chen, I.S. (1988) "Cytokines alter production of HIV-1 from primary mononuclear phagocytes". *Science.* 241: 1673-1675.

Krambovitis, E. (1987) "Serodiagnosis of tuberculosis in perspective". *Serodiagnosis and Immunotherapy.* 1: 7-19.

Laal, S.; Samanich, K.M.; Sonnenberg, M.G.; Zolla-Pazner, S.; Phadtare, J.M.; Belisle, J.T (1997). "Human humoral responses to antigens of *Mycobacterium tuberculosis*: immunodominance of high-molecular-mass antigens". *Clin. Diagn. Lab. Immunol.* 4: 49-56.

Lalvani, A.; Pathan, A.A.; McShane, H.; Wilkinson, R.J.; Latif, M.; Conlon, C.P.; Pasvol, G.; Hill, A.V. (2001) "Rapid detection of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cells". *Am. J. Respir. Crit. Care Med* 163: 824-828.

Laszlo, A.; Baer, H.H.; Goren, M.B.; Handzel, V.; Barrera, L.; de Kantor, I.N. (1992) "Evaluation of synthetic pseudo cord-factor-like glycolipids for the serodiagnosis of tuberculosis". *Res. Microbiol* 143: 217-223.

Lawn, S.D.; Butera, S.T.; Folks, T.M. (2001) "Contribution of immune activation to the

pathogenesis and transmission of Human Immunodeficiency Virus Type 1 infection". *Clin. Microbiol. Rev.* 14:753-777.

Lawn, S.D.; Frimpong, E.H.; Nyarko, E. (1997) "Evaluation of a commercial immunodiagnostic kit incorporating lipoarabinomannan in the serodiagnosis of pulmonary tuberculosis in Ghana". *Trop. Med. Int. Health.* 2: 978-981.

Lim, J.H.; Park, J.K.; Jo, E.K.; Song, C.H.; Min, D.; Song, T.J.; Kim, H.J. (1999) "Purification and immunoreactivity of three components from the 30/32-kilodalton Antigen 85 complex in *Mycobacterium tuberculosis*". *Infect. Immun.* 67: 6187-6190.

Lima, V.M.F.; Bonato, V.L.D.; Lima, K.M.; Dos Santos, S.A.; Dos Santos, R.R.; Gonçalves, E.D.C.; Faccioli, L.H.; Brandao, I.T.; Rodrigues-Junior JM.; Silva CL. (2001) "Role of trehalose dimycolate in recruitment of cells and modulation of production of cytokines and NO in tuberculosis". *Infect. Immun.* 69: 5305-5312.

Liu, J.; Barry, C.E., 3rd.; Nikaido, H. (1999) "Cell wall: physical structure and permeability". (Chapter 12). A: Mycobacteria. Molecular Biology and Virulence. Eds.: Ratledge C, i Dale J. Blackwell Science Ltd. UK. pp:220-239.

Lodes, M.J.; Dillon, D.C.; Mohamath, R.; Day, C.H.; Benson, B.R.; Reynolds, L.D.; McNeill, P.; Sampaio, D.P.; Skeiky, Y.A.W.; Badaro, R.; Persing, D.H.; Reed, S.G.; Houghton, R.L. (2001) "Serological expression cloning and immunological evaluation of MTB48, a novel *Mycobacterium tuberculosis* antigen". *J. Clin. Microbiol.* 39: 2485-2493.

Lyashchenko, K.; Colangeli, R.; Houde, M.; Al Jahdali, H.; Menzies, D.; Gennaro, M.L. (1998) "Heterogeneous antibody responses in tuberculosis". *Infect. Immun.* 66: 3936-3940.

Maekura, R.; Nakagawa, M.; Nakamura, Y.; Hiraga, Y.; Yamamura, Y.; Ito, M.; Ueda, E.; Yano, S.; He, H.; Oka, S.; Kashima, K.; Yano, I. (1993) "Clinical evaluation of rapid serodiagnosis of pulmonary tuberculosis by ELISA with Cord Factor (trehalose-6,6'-dimycolate) as antigen purified from *Mycobacterium tuberculosis*". *Am. Rev. Respir. Dis.* 148: 997-1001.

Maekura, R.; Okuda, Y.; Nakagawa, M.; Hiraga, T.; Yokota, S.; Ito, M.; Yano, I.; Kohno, H.; Wada, M.; Abe, C.; Toyoda, T.; Kishimoto, T.; Ogura, T. (2001) "Clinical evaluation of anti-tuberculous glycolipid immunoglobulin G antibody assay for rapid serodiagnosis of pulmonary tuberculosis". *J. Clin. Microbiol.* 39: 3603-3608.

Maes, H.H.; Causse, J.E.; Maes, R.F. (1999) "Tuberculosis I: a conceptual frame for the immunopathology of the disease". *Med. Hypotheses* 52: 583-593.

Maes, R.; Homasson, J.-P.; Kubin, M.; Bayer, M. (1989) "Development of an enzyme immunoassay for the serodiagnostic of tuberculosis and mycobacterioses". *Med. Microbiol. Immunol.* 178: 323-335.

Mahairas, G.G.; Sabo, P.J.; Hickey, M.J.; Singh, D.C.; Stover, C.K. (1996) "Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*". *J. Bacteriol.* 178: 1274-1282.

Martín Casabona, N.; González Fuente, T.; Arcalis Arce, L.; Otal Entraigas, J.; Vidal Pla, R. (1989) "Evaluation of a phenolglycolipid antigen (PGL-Tb 1) from *M. tuberculosis* in the serodiagnosis of tuberculosis: comparison with PPD antigen". *Acta Leprol.* 7 (Suppl. 1): 89-93.

Martin Casabona, N.; González Fuente, T.; Papa, F.; Rosselló Urgell, J.; Vidal Pla, R.; Codina Grau, G.; Ruiz Camps, I. (1992) "Time course of anti-SL-IV immunoglobulin G antibodies in patients with tuberculosis and tuberculosis-associated AIDS". *J. Clin. Microbiol.* 30: 1089-1093.

Mathur, M.L.; LoBue, P.A.; Catanzaro, A. (1999) "Evaluation of a serologic test for the diagnosis of tuberculosis". *Int. J. Tuberc. Lung Dis.* 3: 732-735.

May, J.J.; Katilus, J.; Henson, P.M.; Dreisin, R.B. (1983) "The purification and identification of circulating immune complexes in tuberculosis". *Am. Rev. Respir. Dis* 128: 920-925.

Mazurek, G.H.; LoBue, P.A.; Daley, C.L.; Bernardo, J.; Lardizabal, A.A.; Bishai, W.R.; Iademarco, M.F.; Rothel, J.S. (2001) "Comparison of a whole-blood interferon gamma assay with tuberculin skin testing for detecting latent *Mycobacterium tuberculosis* infections". *JAMA* 286: 1740-1747.

McDonough, J.A.; Sada, E.; Sippola, A.A.; Ferguson, L.E.; Daniel, T.M. (1992) "Microplate and dot immunoassays for the serodiagnosis of tuberculosis". *J. Lab. Clin. Med* 120: 318-22.

McNeil, M.R.; Brennan, P.J. (1991) "Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis and drug resistance; some thoughts and possibilities arising from recent structural information". *Res. Microbiol* 142: 451-463.

Mehta, P.K.; Khuller, G.K. (1988) "Serodiagnostic potentialities of enzyme-linked immunosorbent assay (ELISA) using mannophosphoinositides of *Mycobacterium tuberculosis* H37Rv". *Med. Microbiol. Immunol.* 177: 285-292.

Mehta, P.K.; Khuller, G.K. (1989) "Comparative evaluation of the diagnostic significance of circulating immune complexes and antibodies to phosphatidylinositomannosides in pulmonary tuberculosis by enzyme-linked immunosorbent assay". *Med. Microbiol. Immunol* 178: 229-233.

Menzies, D. (1999) "Interpretation of repeated tuberculin test. Boosting, conversion, and reversion". *Am. J. Respir. Crit. Care Med* 159: 15-21.

Middlebrook, G.; Dubos, R.J. (1948) "Specific serum agglutination of erytrocytes sensitised with extracts of tubercle bacilli". *J. Exp. Med* 88: 521-528.

Minden, P.; McClartchy, J.K.; Cooper, R.; Bardana, E.J. Jr.; Farr, R.S. (1972) "Shared antigens between *Mycobacterium bovis* (BCG) and other bacterial species". *Science.* 176: 57-58.

Moran, A.J.; Treit, J.D.; Whitney, J.L.; Abomoelak, B.; Houghton, R.; Skeiky, Y.A.W.; Sampaio, D.P.; Badaró, R.; Nano, F.E. (2001) "Assessment of the serodiagnostic potential of nine novel proteins from *Mycobacterium tuberculosis*". FEMS Microbiol. Lett. 9875: 1-6.

Muñoz, M.; Lanéelle, M.A.; Luquin, M.; Torrelles, J.; Julián, E.; Ausina, V.; Daffé, M. (1997a) "Ocurrence of an antigenic triacyl trehalose in clinical isolates and reference strains of *Mycobacterium tuberculosis*". *FEMS Microbiol. Lett* 157: 251-259.

Muñoz, M.; Luquin, M.; García-Barceló, M.; Julián, E.; Ausina, V.; Lanéelle, M.A. (1997b) "Distribution of surface-exposed antigenic glycolipids in recent clinical isolates of *Mycobacterium tuberculosis*". *Res. Microbiol* 148: 405-412.

Muñoz-Elías, E.J.; McKinney, J.D. (2002) "Bacterial persistence: strategies for survival" (Chapter 23). A: Immunology of Infectious Diseases. Kaufmann, S.H.E.; Sher, A.; Ahmed, R.; eds. ASM Press, Washington, DC. pp 331-355.

Nassau, E.; Parsons, E.R.; Johnson, G.D. (1976) "The detection of antibodies to *Mycobacterium tuberculosis* by microplate enzyme-linked immunosorbent assay (ELISA)". *Tubercle.* 51: 67-70.

Ng, V.; Zanazzi, G.; Timpl, R.; Talts, J.; Salzer, J.L.; Brennan, P.J.; Rambukkana, A. (2000) "Role of the cell wall phenolic glycolipid-I in the peripheral nerve predilection of *Mycobacterium leprae*". *Cell* 103: 511-529.

Nigou, J.; Zelle-Rieser, C.; Guilleron, M.; Thurnher, M.; Puzo, G. (2001) "Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor". *J. Immunol.* 166: 7477-8485.

Noordhoek, G.T.; Kolk, A.H.; Bjune, G.; Catty, D.; Dale, J.W.; Fine, P.E.; Godfrey-Faussett, P.; Cho,

S.N.; Shinnick,T.; Svenson, S.B.; *et al.* (1996) "Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind comparison study among seven laboratories". *J. Clin. Microbiol.* 32: 277-284.

Nunn, P. (2001) "The global control of tuberculosis: what are the prospects?". *Scand. J. Infect. Dis.* 33: 329-332.

Orme, I.M.; Cooper, A.M. (1999) "Cytokine/chemokine cascades in immunity to tuberculosis". *Immunol. Today.* 20: 307-312.

Ortalo-Magné, A.; Dupont, M.A.; Lemassu, A.; Andersen, A.B.; Gounon, P.; Daffé, M. (1995) "Molecular composition of the outermost capsular material of the tubercle bacillus". *Microbiology.* 141: 1609-1620.

Ortalo-Magné, A.; Lemassu, A.; Lanéelle, M.A.; Bardou, F.; Silve, G.; Gounon, P.; Marchal G.; Daffé, M. (1996) "Identification of the surface-exposed lipids on the cell envelopes of *Mycobacterium tuberculosis* and other mycobacterial species". *J. Bacteriol.* 178: 456-461.

Ozeki, Y.; Kaneda, K.; Fujiwara, N.; Morimoto, M.; Oka, S.; Yano, I. (1997) "*In vivo* induction of apoptosis in the thymus by administration of mycobacterial cord factor (trehalose 6,6'-dimycolate)". *Infect. Immun.* 65: 1793-1799.

Pabst, M.J.; Gross, J.M.; Brozna, J.P.; Goren, M.B. (1988) "Inhibition of macrophage priming by sulfatide from *Mycobacterium tuberculosis*". *J. Immunol.* 140: 634-640.

Papa, F.; Cruaud, P.; Luquin, M.; Thorel, M.-F.; Goh, K.S.; David, H.L. (1993) "Isolation and characterization of serologically reactive lipooligosaccharides from *Mycobacterium tuberculosis*". *Res. Microbiol* 144: 91-99.

Papa, F.; Laszlo, A.; David, H.L. (1988) "Specificity of *Mycobacterium tuberculosis* phenolic glycolipid (PGL-tb 1) antiserum". *Ann. Inst. Pasteur Microbiol.* 139: 535-545.

Papa, F.; Laszlo, A.; David, H.L.; Daffé, M. (1989) "Serological specificity of *Mycobacterium tuberculosis* glycolipids". *Acta Leprol* 7 (Suppl. 1): 98- 101.

Papa, F.; Luquin, M.; David, H.L. (1992) "DOT- ELISA for detection of phenolic glycolipid PGL-Tb1 and diacyl- trehalose antigens of *Mycobacterium tuberculosis*". *Res. Microbiol* 143: 327-331.

Park, S.C.; Lee, B.I.; Cho, S.N.; Kim, W.J.; Lee, B.C.; Kim, S.M.; Kim, J.D. (1993) "Diagnosis of tuberculous meningitis by detection of immunoglobulin G antibodies to purified protein derivative and lipoarabinomannan antigen in cerebrospinal fluid". *Tuber. Lung Dis.* 74: 317-322.

Parlett, R.C.; Youmans, G.P. (1959) "An evaluation of the specificity and sensitivity of a gel double diffusion test for tuberculosis. A double-blind study". *Am. Rev. Respir. Dis* 80: 153-166.

Patil, S.A.; Gourie-Devi, M.; Anand, A.R.; Vijaya, A.N.; Pratima, N.; Neelam, K.; Chandramuki, A. (1996) "Significance of mycobacterial immune complexes (IgG) in the diagnosis of tuberculous meningitis". *Tuber. Lung Dis.* 77: 164-167.

Pereira, L.M.; Nguyen, L.N.; Ho, L.M.; Kuijer, S.; Jansen, H.M.; Kolk, A.H.J. (2000) "Development of antigen detection assay for diagnosis of tuberculosis using stupum samples". *J. Clin. Microbiol.* 38: 2278-2283.

Perkins, M.D. (2000) "New diagnostic tools for tuberculosis". *Int. J. Tuberc. Lung Dis* 4: S182-S188.

Pfaller, M.A. (1994) "Application of new technology to the detection, identification, and antimicrobial susceptibility testing of mycobacteria". *Am. J. Clin. Pathol.* 101: 329-337.

Porcelli, S.A.; Modlin, R.L. (1999) "The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids". *Annu. Rev. Immunol.* 17: 297-329.

Pottumarthy, S.; Morris, A.J.; Harrison, A.C.; Wells, V.C. (1999) "Evaluation of the tuberculin gamma interferon assay: potential to replace the Mantoux skin test". *J. Clin. Microbiol.* 37: 3229-3232.

Pottumarthy, S.; Wells, V.C.; Morris, A.J. (2000) "A comparison of seven tests for serological diagnosis of tuberculosis". *J. Clin. Microbiol.* 38: 2227-2231.

Qadri, S.M.; Smith, K.K. (1992) "Nonspecific of the Anda A60-tb ELISA test for serodiagnosis of mycobacterial disease". *Can. J. Microbiol.* 38: 804-806.

Raja, A.; Narayanan, P.R.; Mathew, R.; Prabhakar, R. (1995) "Characterization of mycobacterial antigens and antibodies in circulating inmune complexes from pulmonary tuberculosis". *J. Lab. Clin. Medic.* 125: 581-587.

Raja, A.; Uma Devi, K.R.; Ramalingam, B.; Brennan, P.J. (2002) "Immunoglobulin G, A, and M resposes in serum and circulating immuno complexes elicited by the 16-kilodalton antigen of *Mycobacterium tuberculosis*". *Clin. Diagn. Lab. Immunol*. 9: 308-312.

Rasolofo, V.; Rasolonavalona, T.; Ramarokoto, H.; Chanteau, S. (2000) "Predictive values of the ICT Tuberculosis test for the routine diagnosis of tuberculosis in Madagascar". *Int. J. Tuberc. Lung Dis.* 4:184-185.

Ratanasuwan, W.; Kreiss, J.K.; Nolan, C.M.; Schaeffler, B.A.; Suwanagool, S.; Tunsupasawasdikul, S.; Chuchottaworn, C.; Dejsomritrutai, W.; Foy, H.M. (1997) "Evaluation of the MycoDot™ test for the diagnosis of tuberculosis in HIV seropositive and seronegative patients". *Int. J. Tuberc. Lung Dis.* 1: 259-264.

Ravn, P.; Demissie, A.; Eguale, T.; Wondwosson, H.; Lein, D.; Amoudy, H.A.; Mustafa, A.S.; Jensen, A.K.; Holm, A.; Rosenkrands, I.; Oftung, F.; Olobo, J.; von Reyn, F.; Andersen, P. (1999) "Human T cell responses to the ESAT-6 antigen from *Mycobacterium tuberculosis*". *J. Infect. Dis.* 179: 637-645.

Reddy, J.R.; Kwang, J.; Lechtenberg, K.F.; Khan, N.C.; Prasad Reddy, B.; Chengappa, M.M. (2002) An immunochromatograhic serological assay for the diagnosis og *Mycobacterium tuberculosis. Comp. Immunol. Microbiol. Infect. Dis* 25: 21-27.

Reggiardo, Z.; Aber, V.R.; Mitchison, D.A.; Devi, S. (1981) "Hemagglutination tests for tuberculosis with mycobacterial glycolipid antigens. Results in patients with active pulmonary tuberculosis before and during chemotherapy and in healthy tuberculosis contacts". *Am. Rev. Respir. Dis* 124: 21-25.

Reggiardo, Z.; Vazquez, E. (1981) "Comparison of enzyme-linked immunosorbent assay and hemagglutination test using mycobacterial glycolipids". *J. Clin. Microbiol.* 13: 1007-1009.

Ridell, M.; Wallerström, G.; Minnikin, D.E.; Bolton, R.C.; Magnusson, M. (1992) "A comparative serological study of antigenic glycolipids from *Mycobacterium tuberculosis*". *Tuber. Lung Dis.* 73: 101-105.

Riedel, D.D.; Kaufmann, S.H. (1997) "Chemokine secretion by human polymorphonuclear granulocytes after stimulation with *Mycobacterium tuberculosis* and lipoarabinomannan". *Infect. Immun.* 65: 4620-4623.

Rius, C.; Caylà, J.A.; García de Ollala, P.; Vallès, X.; Jansà, J.M.; Tost, J. (2001) "La tuberculosi a Barcelona. Informe 2000". Programa de prevenció i control de la tuberculosi a Barcelona. Ed.: Institut Municipal de Salut Pública. Publicacions de l'Ajuntament de Barcelona.

Rojas-Espinosa, O.; Luna-Herrera, J.; Arce-Paredes, P. (1999) "Recognition of phenolic glycolipid-I (*Mycobacterium leprae*) and sulfolipid-I (*M. tuberculosis*) by serum from Mexican patients with leprosy or tuberculosis". *Int. J. Tuberc. Lung Dis* 3: 1106-1112.

Rook, G.A.; Seah, G.; Ustianowski, A. (2001) "*M. tuberculosis*: immunology and vaccination". *Eur. Respir. J* 17: 537-557.

Rufí, G.; García Rodríguez, J.A.; Solera, J.; Moreno, S. (2000) "Tuberculosis. A: Protocolos Clínicos SEIMC". Ed. Aguado JM, Almirante B, i Fortún J. (www.seimc.es)

Russell, D.G. (2001) "*Mycobacterium tuberculosis*: here today, and here tomorrow". *Nature Rev. Mol. Cell Biol* 2: 569-577.

Ryll, R.; Kumazawa, Y.; Yano, I. (2001) "Immunological properties of trehalose dimycolate (cord factor) and other mycolic acid-containing glycolipids. A review". *Microbiol. Immunol.* 45: 801-811.

Saad, M.H.; Kritski, A.L.; Werneck-Barroso, E.; Cavalcante, S.; Ferreira, M.A.S.; de Souza Fonseca, L. (1996) "Use of the 2,3-diacyl-trehalose and the purified protein derivative in the serodiagnosis of tuberculosis in AIDS". *Mem. Inst. Oswaldo Cruz* 91: 97-100.

Saavedra, R.; Segura, E.; Leyva, R.; Esparza, L.A.; López-Marín, L.M. (2001) "Mycobacterial di-O-acyl-trehalose inhibits mitogen- and antigen induced proliferation of murine T cells in vitro". *Clin. Diagn. Lab. Immunol*. 8: 1081-1088.

Sada, E.; Aguilar, D.; Torres, M.; Herrera, T. (1992) "Detection of lipoarabinomannan as a diagnostic test for tuberculosis". *J. Clin. Microbiol.* 30: 2415-2418.

Sada. E.; Brennan, P.J.; Herrera, T.; Torres, M. (1990) "Evaluation of lipoarabinomannan for the serological diagnosis of tuberculosis". *J. Clin. Microbiol.* 28: 2587-2590.

Salfinger, M.; Pfyffer, G.E. (1994) "The new diagnostic mycobacteriology laboratory". *Eur. J. Clin. Microbiol. Infect. Dis.* 13: 961-979.

Samanich, K.; Belisle, J.T.; Laal, S. (2001) "Homogeneity of antibody responses in tuberculous patients". *Infect. Immun.* 69: 4600-4609.

Samanich, K.M.; Belisle, J.T.; Sonnenberg, M.G.; Keen, M.A.; Zolla-Pazner, S.; Laal, S. (1998) "Delineation of human antibody responses to culture filtrate antigens of *Mycobacterium tuberculosis*". *J. Infect. Dis.* 178: 1534-1538.

Samanich, K.M.; Keen, M.A.; Vissa, V.D.; Harder, J.D.; Spencer, J.S.; Belisle, J.T.; Zolla-Pazner, S.; Laal, S. (2000) "Serodiagnostic potential of culture filtrate antigen of *Mycobacterium tuberculosis*". Clin. Diag. Lab. Immunol. 7: 662-668.

Samuel, A.M.; Ashtekar, M.D.; Ganatra, R.D. (1984) "Significance of circulating immune complexes in pulmonary tuberculosis". *Clin. Exp. Immunol.* 58: 317-324.

Savage, C.; Vincent, P.; Leclerc, H. (1993) "Serodiagnosis of Tuberculosis. Evaluation of a sulpholipid antigen". *Zentralbl. Bakteriol.* 278: 49-57.

Schlesinger, L.S.; Horwitz, M.A. (1991) "Phenolic glycolipid-1 of *Mycobacterium leprae* binds complement component C3 in serum and mediates phagocitosis by human monocytes". *J. Exp. Med.* 174: 1031-1038.

Schluger, N.W. (2001a) "Changing approaches to the diagnosis of tuberculosis". *Am. J. Respir. Crit. Care Med.* 164: 2020-2024.

Schluger, N.W. (2001b) "Recent advances in our understanding of human host responses to tuberculosis". *Respir. Res* 2:157-163.

Schluger, N.W.; Rom, W.N. (1998) "The host immune response to tuberculosis". *Am. J. Respir. Crit. Care Med.* 157: 679-691.

Seah, G.T.; Scott, G.M.; Rook, G.A. (2000) "Type 2 cytokine gene activation and its relationship to

extent of disease in patients with tuberculosis". *J. Infect. Dis.* 181: 385-389.

Sempere, M.; Ariza, M.; Sánchez-Montón, T.; Rodríguez-González, T.; Martín-Luengo, F.; Valero-Guillén, P.L. (1995) "Evaluación de aciltrehalosas de *Mycobacterium fortuitum* en el diagnóstico serológico de tuberculosis pulmonar". *Enferm. Infecc. Microbiol. Clin.* 13: 292-296.

Sepkowitz, K.A.; Raffalli, J.; Riley, L.; Kiehn, T.E.; Armstrong, D. (1995) "Tuberculosis in the AIDS era". *Clin. Microbiol. Rev.* 8: 180-199.

Shinnick, T.M. (2000) "Diagnostic test needs for evaluating antituberculosis vaccines". *Clin. Infect. Dis.* 30 (Suppl 3): S276-278.

Simonney, N.; Bourrillon, A.; Lagrange, P.H. (2000) "Analysis of circulating immune complexes (CICs) in childhood tuberculosis: levels of specific antibodies to glycolipid antigens and relationship with serum antibodies". *Int. J. Tuberc. Lung Dis.* 4: 152-160.

Simonney, N.; Molina, J.M.; Molimard, M.; Oksenhendler, E.; Lagrage, P.H. (1996). "Comparison of A60 and three glycolipid antigens in an ELISA test for tuberculosis". *Clin. Microbiol. Infect.* 2: 214-222.

Simonney, N.; Molina, J.M.; Molimard, M.; Oksenhendler, E.; Lagrange, P.H. (1997) "Circulating immune complexes in human tuberculosis sera: demonstration of specific antibodies against *Mycobacterium tuberculosis* glycolipid (DAT, PGL-tb 1, LOS) antigens in isolated circulating immune complexes". *Eur. J. Clin. Invest.* 27: 128-134.

Simonney, N.; Molina, J.M.; Molimard, M.; Oksenhendler, E.; Perronne, C.; Lagrange, P.H. (1995) "Analysis of the immunological humoral response to *Mycobacterium tuberculosis* glycolipid antigens (DAT, PGLTb1) for diagnosis in HIV-seropositive and -seronegative patients". *Eur. J. Clin. Microbiol. Infect. Dis.* 14: 883-891.

Sirakova, T.D.; Thirumala, A.K.; Dubey, V.S.; Sprecher, H.; Kolattukudy, P.E. (2001) "The *Mycobacterium tuberculosis pks2* gene encodes the synthase for the hepta- and octamethyl-branched fatty acids required for sulfolipid synthesis". *J. Biol. Chem.* 276: 16833-16839.

Small. P.M.; Perkins, M.D. (2000) "More rigour needed in trials of new diagnostic agents for tuberculosis". *Lancet.* 356: 1048-1049.

Soini, H.; Musser, J.M. (2001) "Molecular diagnosis of mycobacteria". *Clin. Chem.* 47: 809-814.

Somi, G.R.; O'Brien, R.J.; Mfinanga, G.S.; Ipuge, Y.A. (1999) "Evaluation of the MycoDot[TM] test in paients with suspected tuberculosis in a field setting in Tanzania". *Int. J. Tuberc. Lung Dis.* 3: 231-238.

Sorensen, A.L.; Nagai, S.; Houen, G.; Andersen, P.; Andersen, A.B. (1995) "Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*". *Infect. Immun.* 63: 1710-1717.

Sousa, A.O.; Henry, S.; Marója, F.M.; Lee, F.K.; Brum, L.; Singh, M.; Lagrange, P.H.; Aucouturier, P. (1998) "IgG subclass distribution of antibody responses to protein and polysaccharide mycobacterial antigens in leprosy and tuberculosis patients". *Clin. Exp. Immunol.* 11: 48-55.

Sousa, A.O.; Wargnier, A.; Poinsignon, Y.; Simonney, N.; Gerber, F.; Lavergne, F.; Herrmann, J.L.; Lagrange, P.H. (2000) "Kinetics of circulating antibodies, immune complex and specific antibody-secreting cells in tuberculosis patients during 6 months of antimicrobial therapy". *Tuber. Lung Dis.* 80: 27-33.

Stender, H.; Lund, K.; Petersen, K.H.; Rasmussen, O.F.; Hongmanee, P.; Miorner, H.; Godtfredsen, S.E. (1999) "Fluorescence in situ hibridisation assay using peptide nucleic acid probes for differentiation between tuberculous and nontuberculous *Mycobacterium* species in smears of

*Mycobacterium* cultures". *J. Clin. Microbiol.* 37: 2760-2765.

Stenger, S.; Hanson, D.A.; Teitelbaum, R.; Dewan, P.; Niazi, K.R.; Froelich, C.J.; Ganz, T.; Thoma-Uszynski, S.; Melian, A.; Bogdan, C.; Porcelli, S.A.; Bloom, B.R.; Krensky, A.M.; Modlin, R.L. (1998) "An antimicrobial activity of cytolytic T cells mediated by granulysin". *Science.* 282: 121-125.

Streeton, J.A.; Desem, N.; Jones, S.L. (1998) "Sensitivity and specificity of gamma interferon blood test for tuberculosis infection". *Int. J. Tuberc. Lung Dis* 2: 443-450.

Struyf, F.; Lemmens, A.; Valadas, E.; Verhaegen, J.; Van Ranst, M. (1999) "Usefulness of immunochromatographic detection of antibodies to *Mycobacterium tuberculosis* as an adjunct to auramine staining for rapid diagnosis of tuberculosis in a low-prevalence setting". *Eur. J. Clin. Microbiol. Infect. Dis* 18: 740-742.

Sturgill-Koszycki, S.; Schlesinger, P.H.; Chakraborty, P.; Haddix, P.L.; Collins, H.L.; Fok, A.K.; Allen, R.D.; Gluck, S.L.; Heuser, J.; Russell, D.G. (1994) "Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase". *Science.* 263: 678-681.

Teitelbaum, R.; Glatman-Freedman, B.; Chen, B.; Robbins, J.B.; Unanue, E.; Casadevall, A.; Bloom, B.R. (1998) "A mAb recognising a surface antigen of *Mycobacterium tuberculosis* enhances host survival". *Proc. Natl. Acad. Sci. USA.* 95: 15688-15693.

Tenu, J-P.; Sekkaï, D.; Yapo, A.; Petit, J-F.; Lemaire, G. (1995) "Phosphatidylinositolmannoside-based liposomes induce no synthase in primed mouse peritoneal macrophages". *Biochem. Biophys. Res. Commun* 208: 295-301.

Tessema, T.A.; Hamasur, B.; Bjun, G.; Svenson, S.; Bjorvatn, B. (2001) "Diagnostic evaluation of urinary lipoarabinomannan at an Ethiopian tuberculosis centre". *Scand. J. Infect. Dis.* 33: 279-284.

Thole, J.; Janssen, R.; Young, D. "Mycobacterial antigens". (Chapter 18). A: Mycobacteria. Molecular Biology and Virulence. Eds.: Ratledge C, i Dale J. Blackwell Science Ltd. UK. pp:220-239.

Thorns, C.J.; Morris, J.A. (1985) "Common epitopes between mycobacterial and certain host tissue antigens". *Clin. Exp. Immunol.* 61: 323-328.

Tisdall, P.A.; Roberts, GD.; Anhalt, JP. (1979) "Identification of clinical isolates of mycobacteria with gas-liquid chromatography alone". *J. Clin. Microbiol.* 10: 506-514.

Toossi, Z.; Xia, L.; Wu, M.; Salvekar, A. (1999) "Transcriptional activation of HIV by *Mycobacterium tuberculosis* in human monocytes". *Clin. Exp. Immunol.* 117: 324-330.

Torgal-García, J.; David, H.L.; Papa, F. (1988) "Preliminary evaluation of a *Mycobacterium tuberculosis* phenolglycolipid antigen in the serologic diagnosis of tuberculosis". *Ann. Inst. Pasteur Microbiol.* 139: 289-294.

Torgal-García, J.; Papa, F.; David, H.L. (1989a) "Immunological response to homologous and heterologous phenolic glycolipid antigens in tuberculosis and leprosy". *Acta Leprol* 7: 102-106.

Torgal-García, J.; Papa, F.; David, H.L. (1989b) "The use of PGL-Tb 1 from *M. tuberculosis* in the serological diagnosis of tuberculous meningitis: a preliminary report". *Acta Leprol* 7(Suppl. 1): 136-137.

Tórtola, M.T.; Lanéelle, M.A.; Martín-Casabona, N. (1996) "Comparison of two 2,3-diacyl trehalose antigens from *Mycobacterium tuberculosis* and *Mycobacterium fortuitum* for serology in tuberculosis patients". *Clin. Diagn. Lab. Immunol.* 3: 563-566.

Tortoli, E.; Nanetti, A.; Piersimoni, C.; Cichero, P., Farina, C.; Mucignat, G.; Scarparo, C.; Bartolini, L.; Valentini, R.; Nista, D.; Gesu, G.; Tosi, C.P.; Crovatto, M.; Brusarosco, G. (2001) "Performance assesment of new multiplex probe assay for identification of mycobateria". *J. Clin. Microbiol.* 39:

1079-1084.

Turneer, M.; Van Nerom, E.; Nyabenda, J.; Waelbroek, A.; Duvivier, A.; Toppet, M. (1994) "Determination of humoral immunoglobulins M and G directed against mycobacterial antigen 60 failed to diagnose primary tuberculosis and mycobacterial adenitis in children". *Am. J. Respir. Crit. Care Med.* 150: 1508-1512.

Turneer, M.; Van Vooren, J-P.; De Bruyn, J.; Serruys, E.; Dierckx, P.; Yernault, J.C. (1988) "Humoral immune response in human tuberculosis: Immunoglobulins G, A, and M directed against the purified P32 protein antigen of *Mycobacterium bovis* Bacillus Calmette-Guérin". *J. Clin. Microbiol.* 26: 1714-1719.

Ulrichs, T.; Kaufmann, S.H.E. (2002) "Mycobacterial persistence and immunity". *Front. Biosc.* 7: 458-469.

Uma Devi, K.R.; Ramalingam, B.; Brennan, P.J.; Narayanan, P.R.; Raja, A. (2001). "Specific and early detection of IgG, IgM and IgA antibodies to *Mycobacterium tuberculosis* 38kDa antigen in pulmonary tuberculosis". *Tuberculosis.* 81: 249-253.

van Soolingen, D.; Hoogenboezem, T.; de Haas, P.E.; Hermans, P.W.; Koedam, M.A.; Teppema, K.S.; Brennan, P.J.; Besra, G.S.; Portaels, F.; Top, J.; Schouls, L.M.; van Embden, J.D. (1997) "A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex, Canetti: characterization of an excepcional isolate from Africa". *Int. J. Syst. Bacteriol* 47: 1236-1245.

Vera-Cabrera, L.; Handzel, V.; Laszlo, A. (1994) "Development of an enzyme-linked immunosorbent assay (ELISA) combined with a streptavidin-biotin and enzyme amplification method to detect anti-2, 3-di-o-acyltrehalose (DAT) antibodies in patients with tuberculosis". *J. Immunol. Methods* 177: 69-77.

Vera-Cabrera, L.; Rendon, A.; Díaz-Rodríguez, M.; Handzel, V.; Laszlo, A. (1999) "Dot blot assay for detection of antidiacyltrehalose antibodies in tuberculous patients". *Clin. Diagn. Lab. Immunol.* 6: 686-689.

Verbon, A.; Weverling, G.J.; Kuijper, S.; Speelman, P.; Jansen, H.M.; Kolk, A.H.J. (1993) "Evaluation of different test for the serodiagnosis of tuberculosis and the use of likelihood ratios in serology". *Am. Rev. Respir. Dis* 148: 378-384.

Vercellone, A.; Nigou, J.; Puzo, G. (1998) "Relationships between the structure and the roles of lipoarabinomannans and related glycoconjugates in tuberculosis pathogenesis". *Front. Biosci* 3: e149-e163.

Vercellone, A.; Riviere, M.; Fournié, J.J.; Puzo, G. (1992) "Specific binding of phenolic antigens from *Mycobacterium bovis* BCG with antibodies". *FEBS Lett* 303: 22-26.

Vergne, I.; Daffé, M. (1998) "Interaction of mycobacterial glycolipids with host cells". *Front. Biosci* 3: d865-876.

Via, L.E.; Deretic, D.; Ulmer, R.J.; Hibler, N.S.; Huber, L.A.; Deretic, V. (1997) "Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7". *J. Biol. Chem* 272: 13326-13331.

Wadsworth, A.B.; Maltaner, E.J.; Stevens, B.S. (1930) "The complement fixation test in tuberculosis: a report of the results obtained in a series of 1,002 cases". *Am. Rev. Tuberc* 22: 539-548.

Watanabe, M.; Honda, I.; Kawajiri, K.; Niinuma, S.; Kudoh, S.; Minnikin, D.E. (1995) "Distribution of antibody titres against phenolic glycolipids from *Mycobacterium tuberculosis* in the sera from tuberculosis patients and healthy controls". *Res. Microbiol* 146: 791-797.

Watterson, S.A.; Drobniewski, F.A. (2000) "Modern laboratory diagnosis of mycobacterial infections". *J. Clin. Pathol.* 53:727-732.

WHO. Tuberculosis Diagnosis Workshop. (1997) Product development guidelines. Workshop report. WHO global tuberculosis programme. p: 1-27. (www.who.int)

WHO. World Heath Organization. Global Tuberculosis Control. WHO Report 2002. WHO/CDS/TB/2002. 275. Geneva: WHO, 2002. (www.who.int)

WHO. World Heath Organization. Global Tuberculosis Control. WHO Report 2000. WHO/CDS/TB/2000. 275. Geneva: WHO, 2000. (www.who.int)

WHO. World Heath Organization. Tuberculosis. WHO Press Releases. Fact Sheet nº 104. Geneva: WHO, 2000. (www.who.int.inf-fs/en/fact104.html)

Wilkins, E.G.L. (1998) "Antibody detection in tuberculosis". Chapter 6. In Clinical tuberculosis. Ed. PDO Davies. Published by Chapman & Hall, London. pp. 81-96.

Wilkinson, R.J.; Haslov, K.; Rappuoli, R.; Giovannoni, F.; Narayanan, P.R.; Desai, C.R.; Vordermeier, H.M.; Paulsen, J.; Pasvol, G.; Ivanyi, J.; Singh, M. (1997) "Evaluation of the recombinant 38-Kilodalton antigen of *Mycobacterium tuberculosis* as a potencial immunodiagnostic reagent". *J. Clin. Microbiol.* 35: 553-557.

Winters, W.D.; Cox, R.A. (1981) "Serodiagnosis of tuberculosis by radioimmunoassay". *Am. Rev. Respir. Dis* 124: 582-585.

Zhang, L.; Goren, M.B.; Holzer, T.J.; Andersen, B.R. (1988) "Effect of *Mycobacterium tuberculosis*-derived sulfolipid I on human phagocytic cells". *Infect. Immun.* 56: 2876-2883.

Zhou, A.T.; Ma, W.L.; Zhang, P.Y.; Cole, R.A. (1996) "Detection of pulmonary and extrapulmonary tuberculosis patients with the 38-kilodalton antigen from *Mycobacterium tuberculosis* in a rapid membrane-based assay". *Clin. Diagn. Lab. Immunol.* 3: 337-341.

Zink, A.; Haas, C.J.; Reischl, U.; Szermies, U.; Nerlich, A.G. (2001) "Molecular analysis of skeletal tuberculosis in an ancient Egyptian population". *J. Med. Microbiol* 50: 355-366.

Zou, Y.L.; Zhang, J.D.; Chen, M.H.; Shi, G.Q.; Prignot, J.; Cocito, C. (1994) "Serological analysis of pulmonary and extrapulmonary tuberculosis with enzyme-linked immunosorbent assays for anti-A60 immunoglobulins". *Clin. Infect. Dis.* 19: 1084-1091.

# ANNEX

# F. Annex

**VII.** Muñoz, M.; Luquin, M.; García-Barceló, M.; **Julián, E.;** Ausina, V.; Lanéelle, M.A. (1997) "Distribution of surface-exposed antigenic glycolipids in recent clinical isolates of *Mycobacterium tuberculosis*". *Research in Microbiology.* 148: 405-412.

**VIII.** Muñoz, M.; Lanéelle, M.A.; Luquin, M.; Torrelles, J.; **Julián, E.;** Ausina, V.; Daffé, M. (1997) "Occurrence of an antigenic triacyl trehalose in clinical isolates and reference strains of *Mycobacterium tuberculosis*". *FEMS Microbiology Letters* 157: 251-259.

**Article VII**

**VII.** Muñoz, M.; Luquin, M.; García-Barceló, M.; **Julián, E.;** Ausina, V.; Lanéelle, M.A. (1997) "Distribution of surface-exposed antigenic glycolipids in recent clinical isolates of *Mycobacterium tuberculosis*". *Research in Microbiology.* 148: 405-412.

**Aricle VIII**

**VIII.**   Muñoz, M.; Lanéelle, M.A.; Luquin, M.; Torrelles, J.; **Julián, E.;**   Ausina, V.; Daffé, M. (1997) "Occurrence of an antigenic triacyl trehalose in clinical isolates and reference strains of *Mycobacterium tuberculosis*". *FEMS Microbiology Letters* 157: 251-259.

# G. Agraïments

Bé, arribats a aquest punt.... per fi!, em resta un dels apartats més importants de la feina: agrair als qui directa o indirectament han contribuït a que la pugués dur a terme.

L'haver treballat en el món de la tuberculosi pot ser una de les coses més interessants que poden passar-li a qualsevol principiant de científic. Et penses que malgrat aquesta malaltia porti fins 4.000 anys amb nosaltres, i més de 100 anys els científics tractant de tu a tu al bacil, ... arribaràs tu i ho solucionaràs tot. Però només cal trobar-se a una jefa com la Marina per a adonar-te de que això no és el que et penses. Primer m'inculcà el "...*no t'has de creure res del que llegeixes, res del que et diuen i només una petita part del que veus*" us enrecordeu a la tesina?, doncs després de conviure dia a dia més de set anys amb ella (!!!), encara he aprés més coses ... Tot i el seu escepticisme i fer-te creure fins el final el que fas per a demostrar-li a ella, és de les poques persones que he vist emocionar-se i mostrar humanitat en aquest món de la recerca, malgrat que "*estem aquí només per a divertir-nos*". Així, hi ha molts moments que no podré oblidar mai, com veure-la totalment cagada (gairebé diria que més que jo) abans de sortir a xerrar a Berlin, la cara girada quan li han tombat un projecte o un article, la celebració absolutament sempre dels petits "èxits" amb cava, ... diria que m'ha semblat més un company de laboratori que un "jefa". Gràcies per tot. Em penso que ets de les poques persones que ha patit i sap valorar el que ha costat aquesta tesi.

Després de la Marina, el Manolo és amb qui més he conviscut al lab, els primers 4 anys i, fins i tot, una reentré que va fer d'un any amb la que va ser capaç de regirar de nou el ritme del laboratori. Ell em va ensenyar a tenir paciència amb els micobacteris, a fer columnes, a fer plaques de cromatografia de tota mena, ... a com tractar a les cambreres de Can Ruti, a les del Quiñonero, quin és el millor lloc del congelador per a guardar els gelats, com pot arribar a estar de bó un pastís de xocolata, etc, etc, etc. Gracias por todo Manolo!

Els que estan heretant tot això ara són el Pep i la Mercè. Quan treballes amb el Pep t'encomanes d'un esperit científic perfeccionista horrorós però amb el que aprens un munt. Malgrat potser sí és una mica-massa pessimista, és un plaer poder comptar amb ell al lab, només cal demanar-li ajut i sempre està disposat. La Mercè: Hola què taaal!!!! És més distesa.. però tot i que sembla que passa de tot, no és així... ja us ho dic jo!: ho viu!; encara recordo el dia que ens vam posar a saltar al lab perquè veiem el CF a la CCF, després del rentat amb Tween. A tots dos us vull donar gràcies primer per haver estat capaços de llegir-vos i corregir-me tota la tesi!, i a més dornar-vos les gràcies pel que hem compartit, per la complicitat, i recomanar-vos molta molta paciència i empenta en les vostres respectives tesis. Ja

sabeu que si hem passeu un manuscrit jo us el corregeixo, eh?. Els darrers anys també hem conviscut al laboratori amb el Carlos Yesid al qui dessitjo molta sort en la seva tesi, i darrerament amb la Núria que tot just comença ara.

Quan vaig arribar a la UAB em vaig trobar tot un grapat de tesinandos amb els que vaig compartir moltes hores de menjador i una bona convivència. Amb ells em vaig introduir en el món submarí, en les classes de salsa, en les festes mexicanes, ... A tots ells: l'Esther, l'Albert, el Chechi, l'Anna, el Chapi, el Calin, el David, la Nathalie, el Sebi, la Laura, el Rodri, l'Emilio, la Teresa, l'Olga, i el Francesc, que han anat seguint diferents camins, els agraeixo tots els moments viscuts, l'ajut quan ha calgut i la seva amistat que espero que es mantingui molts anys. Faig extensiu aquest agraïment a les noves generacions (potser ja no tan noves...), els "nens" de l'Isidre: la Irma i l'Ignasi per ajudar-me sempre que ho he necessitat, per les correccions d'anglès, per avisar-me sempre que venien els bascos de Burdinola, en fi: per tractar-me tant bé. Igual que a la Mercé i al Pep, us dessitjo sort en les vostres tesis, que ja veureu com tard o d'hora arribaran; a més, tinc entés que això de les RNR és molt interessant i hi ha molta feina per fer.

Per una altra banda, he de donar les gràcies a tota la gent amb la que durant els darrers quatre anys he compartit la dificil feina de sobreviure donant pràctiques. Agrair la feina de la senyora Virginia, a més de tots els consells rebuts i xerrades compartides, i de la Lidia que, a més, ha estat capaç de soportar la correcció de la mega-bibliografia d'aquesta tesi. A les secres, la Conchi i la Júlia, per estar disposades sempre a ajudar-me encara que estiguin fins al sostre de feina, i a la Elisa per la seva complicitat. I agrair a la Silvia i la Mª José de Genètica per la paciència quan les he atabalat a preguntes de la tesi.

Tampoc hagués pogut realitzar la feina tots aquests anys sense l'ajut d'un munt de persones que desinteressadament s'han preocupat i col·laborat en els diferents petits projectes. Primer de tot li he d'agrair a la gent del Servei de Microbiologia de Can Ruti el seu ajut. Amb ells vaig conviure els primers mesos abans de treslladar-nos a la UAB, i posteriorment han estat continues les meves trucades demanant coses.... He d'agrair la seva disponibilitat al Jose, el Joan Lonca, l'Eduardo, o el PereJoan que sempre responen amablement a les meves comandes!!. Al Manterola que em va ajudar molt al congrés de la SEIMC a Barcelona. Però sobretot he d'agrair el seu ajut al Vicenç i a la Lurdes, al començament en la recollida de tots els sèrums i posteriorment en l'assessorament al llarg d'aquests darrers anys.

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en el món dels ELISAs i col·laborar en la posada en marxar d'aquesta tesi. Igualment, a l'inici d'aquest projecte vam rebre el suport econòmic i l'assessorament tècnic de l'empresa Biokit, S.A.; he d'agrair en especial la col·laboració i l'interés de l'Angels Díaz, l'Angels Mor, i el Joaquin Ortiz. Al Xavi Carbo del laboratori de Micro de l'IBB per intentar fer-me entendre els problemes informàtics i resoldre-me'ls sempre!. I a tota la gent del laboratori de micro, de biologia molecular i d'immunologia de l'IBB pel suministrament de tot el què he necessitat urgentment i no arribava mai: BSA, substrate per la fosfatasa, plaques d'ELISA, ... fins i tot es van prestar a donar-me la seva pròpia sang! Gràcies a tots.

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Qui ho anava a dir, eh?