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Research paper

A novel application of affinity biosensor technology to detect antibodies to mycolic acid in tuberculosis patients

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Abstract

Tuberculosis has re-emerged as a global health problem due to co-infection with HIV and the emergence of drug-resistant strains of *Mycobacterium tuberculosis*. HIV co-infection introduced a 30% underestimation in TB diagnosis based on sputum analysis, calling for a reliable and fast serodiagnostic assay to assist in the management of TB in HIV-burdened populations. Serodiagnosis with mycobacterial lipid cell wall antigens gave promising results, in particular with LAM and cord factor. Free mycolic acids have also been considered because they are unique in structure to each species of *Mycobacterium* and can be economically extracted and purified. In a standard immunoassay such as ELISA, however, an unacceptable number of false positive and false negative test results were obtained. Here we report a much improved biosensor method to detect antibodies to mycolic acids in patient serum as surrogate markers of active tuberculosis. Mycolic acid (MA) liposomes were immobilized on a non-derivatized twin-celled biosensor cuvette and blocked with saponin. A high dilution of serum was used to calibrate the binding signal of the two cells, followed by contact with patient serum at a lesser dilution, but pre-incubated with either antigen-carrying, or empty liposomes. The serum, or the protein A purified IgG thereof, from sputum-positive tuberculosis patients could be inhibited from binding to the MA in the biosensor by prior incubation with MA-containing liposomes. The accuracy of the inhibition test was 84% if HIV-positive patients for whom a negative TB sputum analyses could not be relied upon to serve as a reference standard were excluded. If biosensor technology could be made suitable for high throughput screening, then it may provide the solution to the serodiagnosis of tuberculosis against a background of HIV.

Keywords: Antibodies; Mycolic acids; Biosensor; *Mycobacterium tuberculosis*; Serodiagnosis

1. Introduction

Tuberculosis (TB) is a chronic pulmonary disease caused by infection with *Mycobacterium tuberculosis*. It is a major scourge in developing countries as well as an increasing problem in many developed areas of the world, with about 8 million new cases and 3 million deaths each year (Hendrickson et al., 2000). Although tuberculosis is a curable disease that responds well to antibiotics, it has re-emerged as a growing, global health problem because of the development of drug-resistant strains. The resurgence of TB and the increased risk for TB in HIV-infected persons has magnified the need for rapid, inexpensive and accurate methods for the

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diagnosis of TB (Cheon et al., 2002). People with TB that are co-infected with HIV have a 5–15% risk yearly of developing active tuberculosis (Winkler et al., 2005). World Health Organization (WHO) guidelines suggest starting antiretroviral drugs within 2 months of tuberculosis treatment. Patients who start antiretroviral drugs too early in their TB treatment can be predisposed to immune reconstitution syndrome (IRS). Immune reconstitution syndrome has symptoms overlapping with worsening TB and can be life threatening to the patients (Lawn et al., 2005). A major challenge with immunological diagnosis of tuberculosis is to distinguish between mere physical exposure to TB, latent infection and chronic or severe active disease (Pai et al., 2006). Other factors that affect the performance of immune-based assays include previous BCG vaccination, exposure to non-tuberculous mycobacteria and co-infection with *M. tuberculosis* and HIV.

The tuberculin skin test, based on the cellular response to protein antigen, is currently the most generally used method of identifying TB infection. The specificity is low as purified protein derivative (PPD) contains many antigens widely shared among mycobacteria. Several studies have demonstrated that PPD cannot reliably distinguish between previous *Mycobacterium bovis* BCG vaccination, exposure to environmental mycobacteria, or infection with *M. tuberculosis* (Doherty et al., 2002; Chan et al., 2000). Sputum analysis for the presence of live *M. tuberculosis* is done in a variety of ways and is generally employed for the diagnosis of TB. Acid-fast microscopy is quick and easy, but needs confirmation by other tests, as some acid-fast bacilli are not *M. tuberculosis* (Hamasur et al., 2001). Culture of *M. tuberculosis* from sputum is the gold standard for the diagnosis of tuberculosis. The technique is very sensitive, such that even a few mycobacteria can be detected. However, primarily due to the slow growth of the bacteria, this method usually requires 4–8 weeks for completion (Samanich et al., 2000) and is subject to contamination. This often results in delayed diagnosis, adversely affecting patient care and TB control and allows for the spread of infection. In addition, HIV-infected patients often cannot produce sputum of the desired quality for TB detection (Frieden et al., 2003). PCR DNA detection kits are faster, more sensitive and accurate, but also require a sputum sample and a sophisticated laboratory with matching staff competency. Samples from other sources, in particular blood, showed much less sensitivity with PCR amplification tests (Saltini, 1998).

A simple diagnostic assay that does not require highly trained personnel or a complex technological infrastructure is essential for global control of TB (Foulds and O'Brien, 1998). A serologic test, such as ELISA, is a simple and inexpensive alternative to other TB diagnostic methods (Simonney et al., 1996; Moran et al., 2001). The disadvantage of ELISA is that it detects only the high affinity antibodies to the antigen, due to the need for washing steps after contact between the antigen and serum antibodies. Irrespective of the antigen(s) used, no single ELISA test has hitherto succeeded as a reliable test to confirm tuberculosis. Due to the limitations of conventional TB diagnostic tests, there is a widely felt need for a novel approach to develop a diagnostic test that is accurate and convenient to use, especially in HIV patients (Pavlou et al., 2004; Reid et al., 2006).

Mycolic acids are unique, 60–90 carbons long, branched α-alkyl, β-hydroxy fatty acids, which form an outer waxy lipid layer around the mycobacteria (Barry et al., 1998). Our previous studies (Schleicher et al., 2002) and those of others (e.g. Pan et al., 1999) have shown the prevalence of anti-mycolic acid antibody in TB patients with ELISA. Schleicher et al. (2002) investigated the diagnostic potential of an ELISA, based on the detection of antibodies to *M. tuberculosis* mycolic acids in the serum of HIV-seropositive and HIV-seronegative tuberculosis patients, in a population with a high prevalence of HIV. Although they observed a higher signal of antibody to mycolic acids in TB-positive patients than in TB-negative patients, they also found quite a number of false positive and false negative results. From their studies, they concluded that the ELISA has inadequate sensitivity and specificity to detect anti-mycolic acid antibody and is therefore not suitable as a reliable serodiagnostic assay for the diagnosis of pulmonary TB.

This study aimed to assess the potential of detecting anti-mycolic acid antibodies as a surrogate marker for tuberculosis with an IAsys affinity biosensor in the serum of patients with active pulmonary tuberculosis. The IAsys biosensor can monitor and quantify the binding of interacting analytes in real time, by detecting changes in refractive index in the vicinity of the immobilized ligand. The changes in refractive index values are proportional to the change in the accumulated mass (Cush et al., 1993; Buckle et al., 1993). The benefit of not having to label any of the reagents, combined with low sample consumption has made the optical biosensor a useful instrument in both research and commercial laboratories (Myszka, 1999). Although wave guide and SPR assays are currently still experimentally cumbersome, there is considerable technological development in this field such that one can realistically expect that the prevailing technical challenges can be overcome to

make such tests suitable for a routine diagnostic lab in the not too distant future.

Here, we describe how the technology could be applied in the detection of anti-mycolic acid antibodies as surrogate markers for active TB. To confirm that the active binding agents in the human sera were of antibody nature, the IgG antibodies were isolated from a healthy control and a TB-positive patient using protein A and tested in the biosensor assay. The advantages of protein A-based affinity chromatography (Verdoliva et al., 2002) were exploited to confirm that binding to immobilized mycolic acid on the IAsys cuvette surface was due to IgG from patient serum.

2. Materials and methods

2.1. Human sera

Serum samples collected for another study (Schleicher et al., 2002) were used; they were obtained from 61 patients (aged between 18 and 65 years), who were admitted to the general medical wards of the Helen Joseph Hospital, Johannesburg, South Africa, including a number with active pulmonary tuberculosis. The study population consisted of 32 tuberculosis-positive (TB^+) and 29 control tuberculosis-negative (TB^-) patients. The TB^+ group consisted of patients with newly diagnosed smear-positive pulmonary tuberculosis of which some were HIV-seropositive (23 patients). The TB^- patients that were used as controls had medical conditions other than TB and were recruited from the general medical wards. None of the TB^+ patients were on anti-TB chemotherapy at the time of serum collection.

For the purpose of IgG isolation and testing, a TB^+ patient serum was selected from a collection of sera supplied by the Medical Research Council (MRC) (Clinical and Biomedical Research Unit, King George V Hospital, Durban, 1994). Healthy volunteer sera were used as negative controls for IgG isolation.

2.2. ELISA of patient sera

Mycobacterial mycolic acids were isolated from a culture of *M. tuberculosis* H37Rv (American Type Culture Collection 27294) as described by Goodrum et al. (2001). Mycolic acids (250 μg) were dissolved in 4 ml of hot phosphate-buffered saline (PBS, pH 7.4) for 20 min at 85 °C and sonicated (Virsonic 600, United Scientific, USA) at 20% duty cycle and optimal output level for 1 min. The solution was kept at 85 °C during pipetting into ELISA plates (Sero-Well®, Bibby sterilin Ltd., UK), after which the plates were placed in plastic bags and incubated overnight at 4 °C. The final antigen load was approximately 3 μg/well. Control wells were coated with hot PBS only. After overnight incubation, the ELISA plates were flicked out and the wells blocked with 0.5% (m/v) carbohydrate-and fatty acid-free casein in PBS for 2 h at room temperature. The solution was flicked out, the wells were filled with 50 μl of serum, serum precipitate in triplicate and incubated for 1 h at room temperature, flicked out and washed three times with PBS/0.5% casein. The wells were aspirated to remove proteinaceous froth. The plates were incubated for 30 min at room temperature with peroxidase-conjugated goat anti-human IgG (whole molecule, Sigma) diluted 1/1000 in PBS/0.5% casein, flicked out, washed three times with PBS/0.5% casein and aspirated. The presence of antibody was revealed using 50 μl/well of hydrogen peroxide (40 mg) and *o*-phenylenediamine (50 mg) in 50 ml of 0.1 M citrate buffer (pH 4.5). Measurement of the yellow colour was done after 30 min at 450 nm using a Multiskan Ascent photometer (Thermo-Labsystems, Finland). To correct for background binding in the serum, the signal generated for the samples in PBS-coated wells was subtracted from that generated in mycolic acid-coated wells.

2.3. Preparation of liposomes

Stock solution of phosphatidylcholine (100 mg/ml) (Sigma, St Louis, MO) was prepared by dissolving the weighed amounts in chloroform. Mycolic acids-containing liposomes were prepared by adding 90 μl of phosphatidylcholine stock to 1 mg of dried mycolic acids. Empty liposomes, i.e. with no mycolic acids, were prepared by using 90 μl of phosphatidylcholine stock solution only. During pipetting, everything was kept on ice to avoid evaporation of chloroform. The liposome ingredients were dried with nitrogen gas in a heat block at 85 °C for about 10 min. Liposome formation was induced by addition of 2 ml of saline (0.9% NaCl) and placing in a heat block at 85 °C for 20 min, with vortexing every 5 min. The liposomes were then sonicated for 2 min at 30% duty cycle at an output of 3% with a Model B-30 Branson sonifier (Sonifer Power Company, USA). The sonicator tip was washed with chloroform and rinsed with distilled water before and after use. The liposomes (200 μl) were aliquoted into ten tubes and kept at −20 °C overnight before freeze-drying. After freeze-drying, 2 ml of phosphate-buffered saline (PBS) azide EDTA (Sigma, St Louis, MO) buffer (PBS/AE, pH 7.4) was added to each tube containing liposomes. The tubes were placed in a heat block for 20 min and sonicated as before.

2.4. Isolation of IgG using protein A Sepharose

A TB-positive patient serum was selected from a collection of sera supplied by the Medical Research Council (MRC) (Clinical and Biomedical Research Unit, King George V Hospital, Durban, 1994). The only criterion for selecting this serum for isolation was that it had previously given good responses to mycolic acid on ELISA (unpublished results). TB-negative samples, which consisted of pooled sera from healthy students working in the Biochemistry Department of the University of Pretoria, were used as negative controls. A modified method for IgG purification was used compared to that given in the literature (Seppala et al., 1990). Protein A, immobilized on Sepharose (3 ml; Fluka, BioChemika) was transferred to a 50-ml tube. The Sepharose was washed twice by centrifuging at 3000 rpm for 5 min, the supernatant removed by pipette and 10 mM Tris–HCl buffer (pH 8.08) added until the final volume in the tube was 20 ml. After the last washing step, the supernatant was removed, leaving the Sepharose just covered in buffer. Serum aliquots (500 µl each) were thawed at 37 °C for 30 min. Two aliquots (1 ml) were added to the Sepharose beads and the volume was adjusted to 20 ml with 10 mM Tris–HCl to effect a final serum dilution of 1/20. The serum was incubated with the Sepharose at room temperature with rotation for 2 h and then left to stand for the Sepharose to settle out. The supernatant was removed by pipette and transferred to a labelled 50-ml tube. This represented the IgG-depleted serum fraction.

The remaining bound IgG was removed from the protein A beads by washing with 100 mM Tris–HCl, keeping the final volume in each tube at 20 ml and then removing the supernatant after gentle mixing. This was repeated twice, after which the buffer was changed to 10 mM Tris–HCl for another three washes. The supernatant of the last washing step was measured at 280 nm against a suitable blank for the efficiency of washing. The Sepharose in the tube was finally washed once more with 10 mM Tris.

IgG was eluted with 0.1 M glycine (3 ml, pH 2.7) into tubes containing 1 M Tris–HCl (200 µl) to neutralize the acid. This process was repeated seven times using 0.1 M glycine (2 ml) and 2 min incubation before removing the next IgG supernatant. The efficiency of elution of protein from the beads was monitored at 280 nm against a suitable blank. The IgG fractions were pooled and dialyzed against 1.5 l of PBS with two exchanges after 12 h and 18 h. The IgG-depleted serum fraction isolated earlier was also dialyzed in the same manner. After dialysis, the solutions were transferred to 50 ml tubes, and 100 µl were removed for protein concentration determination. To the IgG (12 ml) and depleted serum fractions (12 ml), 99% glycerol (8 ml, 40% final concentration) was added and filtered through a 0.2-µm filter (Ministart Plus, CA-membrane and GF-prefilter) into sterile 50-ml tubes that were subsequently stored at 4 °C. This isolation process was repeated for the TB-negative serum sample. The protein A Sepharose beads were regenerated by washing twice with 0.1 M glycine (5 ml) and four times with 100 mM Tris–HCl (10 ml), until the pH was above 7. The Sepharose was stored in 100 mM Tris–HCl solution (10 ml, containing 1% benzyl alcohol).

2.5. Removal of glycerol for biosensor

To prepare the IgG and depleted serum fractions for IAsys biosensor analysis, the glycerol had to be removed. VetaSpin Micro columns (400 µl, Whatman) were pre-washed with PBS (400 µl) by centrifuging at $10\,000 \times g$ for 15 min. The glycerol-containing samples (400 µl) were then transferred to the spin columns and centrifuged at $10\,000 \times g$ for 40 min. The filtrate was removed and 200 µl of PBS added to the retentate and again centrifuged. This process was repeated another five times for each glycerol-containing sample. The final volume was reconstituted to the original sample volume (400 µl) with PBS. The concentration of IgG in the purified samples was determined by absorbance measurement at 280 nm using a Shimadzu spectrophotometer.

2.6. SDS-PAGE of isolated IgG and depleted serum

The protein concentration of the IgG fractions was determined using the absorbance at 280 nm and an extinction coefficient of 1.4, whereas the concentration of depleted serum and other protein fractions was determined using the Bradford assay. An amount corresponding to 10 µg per sample was aliquoted into Eppendorf tubes and four volumes of acetone added. The samples were then left to precipitate overnight at −70 °C. The next day the samples were centrifuged at $14\,000 \times g$ for 1 h at 4 °C. The supernatant was discarded and the samples dried under vacuum for 20 min. Deionized water (8 µl) was added to re-suspend the protein pellet and non-reducing sample buffer (8 µl) was added. The samples were boiled at 100 °C for 5 min. SDS-PAGE analysis of purified IgG fractions and depleted sera was performed according to Ey et al. (1978) in a MINIVE complete vertical electrophoresis system (Amersham Pharmacia Biotech AB, USA). Approximately 10 µg of protein was loaded per lane. The gel

consisted of a 10% separating gel and a 4% stacking gel. The low molecular weight markers contained 97, 66, 45, 30, 20.1 and 14.4 kDa standards. The loaded gel was first run at 60 V for 1 h and then at 120 V for 3 h until the indicator was 0.5 cm from the end of the gel.

2.7. Detection of anti-mycolic acid antibody (IgG) with the IAsys affinity biosensor

The PBS/AE buffer (8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.05 g Na_2HPO_4 per litre, 1 mM EDTA and 0.025% (m/v) sodium azide) was prepared in double-distilled water and adjusted to pH 7.4. Cetyl-pyridinium chloride (CPC, 0.02 mg/ml) and saponin (1 mg/ml) were prepared in PBS/AE. The IAsys resonant mirror biosensor system and twin-cell non-derivatized cuvettes were from Affinity Sensors (Cambridge, UK). The sensor was set to a data-sampling interval of 0.4 s, a temperature of 25 °C and a stirring rate of 75% for all experiments. The cells were rinsed three times prior to use with 96% ethanol (Saarchem, SA), followed by extensive washing with PBS/AE. A 60-µl volume of PBS/AE was pipetted into each cell of the cuvette to obtain a stable baseline for 1 min. The PBS/AE was subsequently aspirated and the surface activated with 50 µl of CPC for 10 min. This was followed by washing five times with 60 µl of PBS/AE and then substituting with 25 µl of PBS/AE for a new baseline before immobilization of mycolic acids-containing liposomes to the surface for 20 min. The immobilized liposomes were then finally washed five times with 60 µl of PBS/AE, substituted with 50 µl of saponin and incubated for 10 min. This latter step was to avoid non-specific binding on the surface of the cuvette during the subsequent binding events. The cells were then washed five times with PBS/AE, the content of each cell substituted with 25 µl of PBS/AE and left for about 5–10 min to achieve a stable baseline. Inhibition studies were performed using patient serum that was first left at room temperature to thaw completely. After obtaining a stable baseline, a 1/1000 dilution of serum antibodies (10 µl) in PBS/AE was added to each cell, to compare the responses of the two cells over 10 min. A pre-incubation of 1/500 dilutions of serum with solutions of liposomes containing mycolic acids and empty liposomes (phosphatidylcholine alone) were allowed for 20 min. These were then added (10 µl) for binding inhibition studies in different cells, one with mycolic acid liposomes and the other with empty liposomes as a control, and allowed to bind for 10 min. Inhibition studies using IgG antibody were also performed, but differently. After obtaining a stable baseline, 10 µl of IgG solution (0.04 mg/ml) in PBS/AE were added to both cells and the response measured over 10 min as above. Subsequently, 10 µl of 0.08 mg/ml IgG diluted with either mycolic acids or phosphatidylcholine liposomes were added and the response measured for 10 min. Finally, dissociation of antibodies was effected by washing three times with PBS/AE and measurement of the response for 5 min.

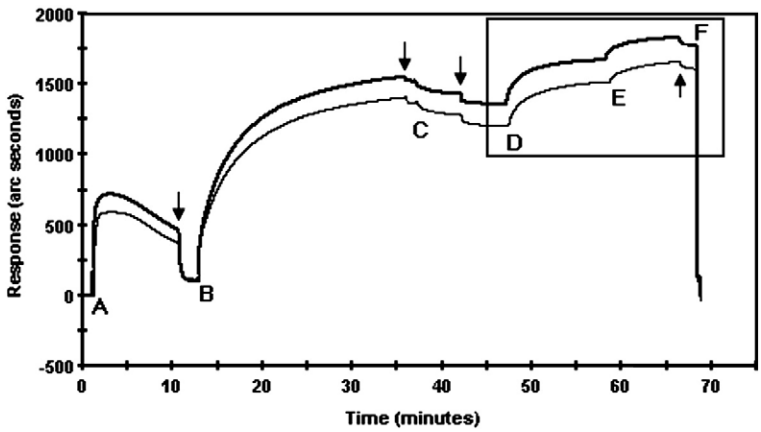

Fig. 1. A typical graph summarizing the process of measuring antibody binding or inhibition of binding by mycolic acid and empty (phosphatidylcholine only) liposomes, in the two cells of an IAsys biosensor cuvette surface coated with mycolic acid liposomes. The surface was activated with cetyl-pyridinium chloride (A), coated with mycolic acid liposomes (B), blocked with saponin (C), calibrated with a high dilution of serum (D), applied to measure the binding and dissociation of inhibited sera at lesser dilution (E), and regenerated with potassium hydroxide (12.5 M) and 96% ethanol (F). The arrows indicate washing with PBS/AE and the response from the two cells are differentiated by thick lines (channel 1, upper curve) and thin lines (channel 2, lower curve).

2.8. Regeneration of non-derivatized cuvettes

Regeneration was effected by three initial washes with 96% ethanol for 1 min, followed by washing seven times with 70 µl of PBS/AE for 1 min. The surface was then finally treated with 50 µl of potassium hydroxide (12.5 M) for 2 min followed by seven washes with 70 µl of PBS/AE for 1 min.

3. Results

The six main stages involved in measuring the binding of specific antibodies to lipid antigens in liposomes in real time on the biosensor are: (A) activation of the non-derivatized cuvette surface with CPC; (B) immobilization of the liposomes containing mycolic acids to the surface; (C) blocking with saponin to prevent non-specific protein binding; (D) binding (association) of antibodies from a high dilution of serum to calibrate the signal of the two cells of the cuvette; (E) binding and dissociation of inhibited patient serum at lesser dilution; and finally (F) surface regeneration (Fig. 1). The dilutions of serum used were estimated from a dilution range of one positive and one negative serum sample and are not necessarily optimal for all sera. The cuvette cell calibration curves for the high dilution serum in the two cells of one cuvette had to

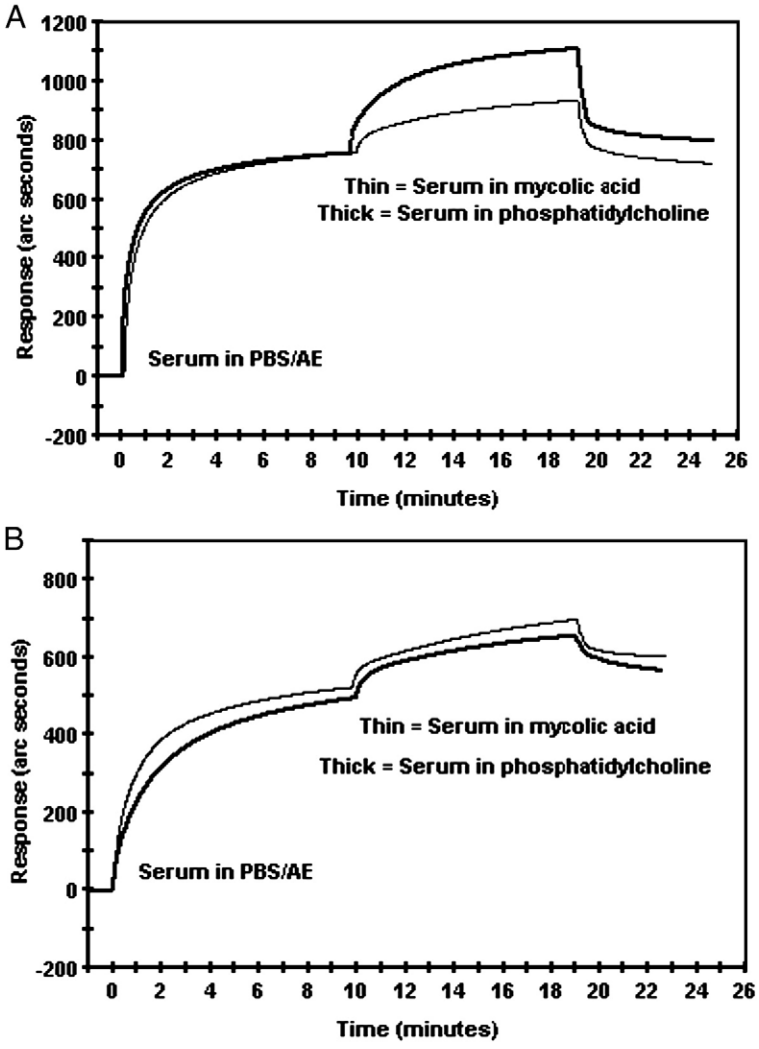

Fig. 2. Inhibition of human TB^+ (A) and TB^- (B) patient serum antibody binding with mycolic acid liposomes or empty liposomes on an IAsys cuvette surface coated with immobilized mycolic acid liposomes. For the first 10 min, a 1/1000 dilution of serum in PBS/AE was incubated in both cells. For inhibition studies, the pre-incubated serum in a dilution of 1/500 was then added with the thin line (A, lower curve; B, upper curve) representing the binding response of serum in mycolic acids and the thick line (A, upper curve; B, lower curve) representing that of serum in empty liposomes as control.

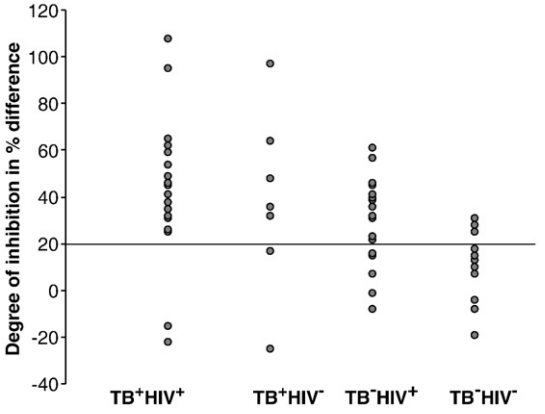

Fig. 3. The percentage of inhibition of binding of the biosensor signal for the 61 patient sera of TB^+ and TB^- controls after pre-incubation of sera with mycolic acid liposomes and empty liposomes before testing on mycolic acid-coated cuvettes.

fall within 90–100% identity in terms of the relative response amplitudes in order to be accepted. A limitation of the IAsys system was found to be the difference in quality from one cuvette to another when using liposomes as antigen coat. In rare cases, new cuvettes were not usable at all. Usually, new cuvettes were found to be reliable only after a succession of regenerations, while in other rare cases, new cuvettes could be reliably applied after a single regeneration cycle. The results (those in the rectangle, Fig. 1) were aligned using the Fastplot programme from IAsys.

3.1. Detection of anti-mycolic acid antibodies in human serum

Patient sera selected from the collection of Schleicher et al. (2002) were used to detect antibodies against mycolic acids on the optical IAsys biosensor. The ELISA experiments were performed as described in Schleicher et al. (2002). Of the 61 patient sera that were analyzed on the IAsys biosensor, 17 were re-analyzed on ELISA to confirm that the original antibody activity as reported by Schleicher et al. (2002) was still intact and to compare them with the results found on the IAsys biosensor during the same period of assessment. The inhibition studies on the IAsys were determined by pre-incubating test serum with mycolic acids-containing liposomes and applying these on biosensor cuvettes coated with mycolic acids. In the control experiments, sera were pre-incubated with empty liposomes. The pre-incubation of a sputum-positive TB patient serum with mycolic acid liposomes resulted in inhibition of antibody binding to mycolic acids when compared to the signal generated by the same serum pre-incubated with empty liposomes (Fig. 2A). This confirmed the specificity of binding of antibodies to mycolic acids in sputum-positive TB patient sera.

There was no inhibition of binding observed when a sputum-negative control serum (TB^-HIV^-) was pre-incubated with liposomes containing mycolic acids and tested on the biosensor to determine binding of antibodies to mycolic acids (Fig. 2B). This shows that specific anti-mycolic acid antibodies can be demonstrated in TB^+ patients, after pre-incubation of serum with mycolic acids. TB-negative sera from patients infected with HIV tested negative on the IAsys biosensor, with inhibition values of less than 20% (Fig. 3).

From 23 TB^+HIV^+ patient sera selected, two serum samples tested false negative on the biosensor (Fig. 3). Thirteen TB^-HIV^+ patient sera tested false positive, showing an inhibition of greater than 20% on the biosensor (Fig. 3). It is noteworthy that these patients were HIV-positive. Some patient sera that were false negative (e.g. Fig. 4A) and false positive (e.g. Fig. 4B) on the ELISA tested positive and negative respectively on the biosensor. The normalized signals on ELISA that were above two were regarded as positive and below two as

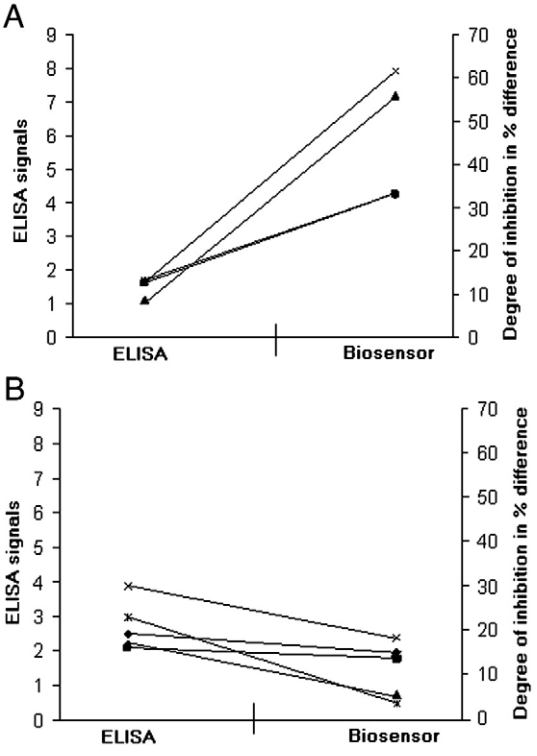

Fig. 4. Normalized ELISA signals and the percentage of inhibition of binding of the biosensor signal of false negative (A) and false positive (B) patients on ELISA who tested correctly on the biosensor.

negative. The TB^+ patients who showed truly positive responses of antibodies to mycolic acids on ELISA also tested truly positive on the biosensor. Our previous studies have also addressed the problems of detecting *M. tuberculosis*-specific antibodies to mycolic acid in TB patients co-infected with HIV on ELISA (Schleicher et al., 2002). Three of the patient sera tested from the 13 HIV^-TB^- tested false positive on the biosensor, and only two serum samples tested false negative in the TB^+HIV^- population (Table 1). An apparently lower specificity (27.8%) was observed in the TB^-HIV^+ subgroups. However, all these patients were hospitalized with diseases other than TB based on the prevailing diagnostic methods. The low specificity obtained amongst the HIV^+ population could reflect true positive results, since it is known that the sputum culture assay is not sensitive enough to detect TB in HIV-positive patients (Frieden et al., 2003). This may reflect the fact that the serum test is better able to detect TB in HIV^+ patients. The IAsys affinity biosensor was found to be more sensitive (91.3%) in detecting TB amongst the TB-positive patients co-infected with HIV. The overall specificity and sensitivity of the assay after analyzing 61 patient sera was 48.4% (15/31) and 86.7% (26/30), respectively.

It is known that the gold standard of sputum growth of mycobacteria does not measure accurately in the TB^-HIV^+ cohort (Table 1). As the serum collection was actually made for an earlier study, follow-up data were not available to determine the true TB status of the TB^-HIV^+ cohort tested here. When the 18 TB^-HIV^+ sera were omitted in the calculation of the performance parameters of the test based on the 61 data points, the accuracy of the assay was found to be 83.7% (36/43). The sensitivity (86.7%, 26/30) remained the same after exclusion of the TB^-HIV^+ population, and the specificity was 76.9% (10/13). The assay showed a high sensitivity (91.3%) in sera from patients who were TB-positive and co-infected with HIV. It is known that HIV-positive patients generally have lower levels of *M. tuberculosis*-specific antibodies to protein and certain lipid antigens than HIV-negative patients. This shows that the IAsys biosensor can detect anti-mycolic acid antibodies in an HIV endemic population.

Table 1
Specificity and sensitivity of the IAsys affinity biosensor assay for detecting anti-mycolic antibody in pulmonary TB and negative control patient sera

Patient group	No. of patients	False positive	False negative	Specificity (%)	Sensitivity
TB^+HIV^+	23	–	2	–	91.3 (21/23)
TB^+HIV^-	7	–	2	–	71.4 (5/7)
TB^-HIV^+	18	13	–	27.8 (5/18)	–
TB^-HIV^-	13	3	–	76.9 (10/13)	–
Total	61	16	4	48.4 (15/31)[a]	86.7 (26/30)[a]

[a] Accuracy = 81.8% (the data for the specificity of the TB^-HIV^+ group is omitted because of the known underestimation of TB positives by standard culture growth assays).

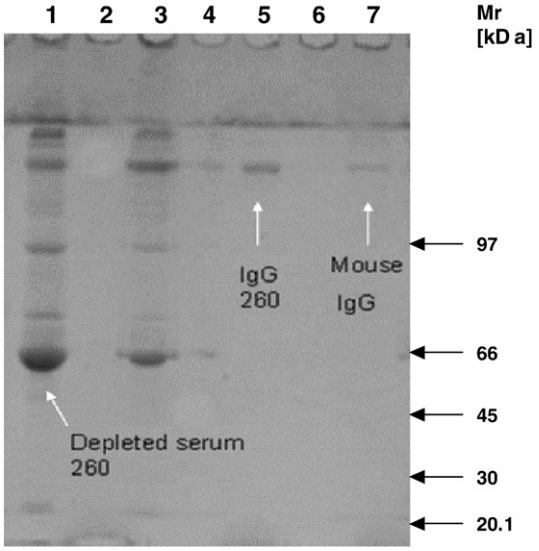

Fig. 5. SDS-PAGE gel indicating the purity of the IgG fraction in relation to a standard mouse IgG sample. The depleted serum fraction in lane 1 still contains IgG along with albumin at 66 kDa and other unidentified proteins. Samples were loaded in non-reducing buffer bovine serum albumin (BSA): (1) depleted serum no. 260, (2) blank, (3) whole serum no. 260, (4) blank, (5) IgG fraction from patient no. 260, (6) blank, (7) mouse IgG.

3.2. Isolation of IgG

To assess whether the inhibited signal on the IAsys affinity biosensor was due to the IgG in human sera, isolation of IgG was done using protein A Sepharose beads, which are specific for IgG but not for the other classes of immunoglobulin. The serum was not fully depleted of IgG as is evident from Fig. 5. The IgG fractions were expected to represent a repertoire of antigen binding specificities found in serum. The eluted IgG fractions were tested to bind mycolic acid on the IAsys biosensor.

3.3. Analysis of IgG on the IAsys biosensor

The isolated IgG from TB-positive serum no. 260 and TB-negative serum was analysed on the IAsys resonant mirror biosensor to confirm that the binding of

antibody to mycolic acid is due to the IgG. The liposomes containing mycolic acids were immobilized on non-derivatized cuvettes after activation with CPC as described previously. The response of IgG in PBS/AE to mycolic acid liposomes was measured in arc seconds at an IgG concentration of 0.04 mg/ml to detect high affinity binding. When comparing the binding of IgG from the TB-positive serum and IgG from negative serum to mycolic acid liposomes, there was a significant difference (paired Student's *t*-test with alpha = 0.05; Table 2).

Inhibition experiments are necessary in the biosensor analyses of sera, as no adequate difference in direct binding in PBS/AE was observed between TB-positive and TB-negative sera to provide improved diagnosis over that obtained by ELISA. This was thought to be due to serum components binding non-specifically to the liposomes. By incubating the serum in mycolic acid liposomes, the highly specific antibodies would be complexed and prevented from binding to the immobilized mycolic acid liposomes on the cuvette surface. We attempted to apply the same principle to the IgG samples. The purified IgG in PBS was mixed with either phosphatidylcholine or mycolic acid liposomes to afford a final concentration of 0.08 mg/ml of IgG in PBS/AE and the response was measured over 10 min as previously done for serum. The degree of binding of IgG to the immobilized mycolic acid liposomes after mycolic acid or phosphatidylcholine incubation was compared to determine whether the IgG could be inhibited.

The inhibition signals of IgG from patient 260 and the IgG negative control were measured on different channels of the IAsys biosensor. A significant difference in the ability of IgG 260 and IgG negative to be inhibited by mycolic acid liposomes was determined with Student's *t*-test using an alpha value of 0.05 (Table 2). These results correlate with previously obtained data using whole serum and confirm that it is the IgG fraction of serum that is inhibited from binding by preincubation with the mycolic acid antigen.

Table 2
Binding of IgG antibody purified from TB^+ and TB^- serum to mycolic acid liposomes

Biosensor signal	Patient (±SEM)	
	TB^+ (no. 260)	TB^- control
Serum binding (arc seconds)	462±35	248±24
IgG binding (arc seconds)	70±10	31±1
IgG binding inhibited with mycolic acid (%)	54±3.4	24±6

SEM, standard error of the mean; $n > 4$.

4. Discussion

In Africa, TB is often the first manifestation of HIV infection; it is the leading cause of death among HIV-infected patients. Corbett et al. (2006) stated that every opportunity should be taken to screen HIV-infected patients for active TB in order to prevent rapid death when both diseases manifest themselves in an individual, and to provide safe antiretroviral (ARV) treatment. The shorter the time from sampling to the diagnostic result, the more lives will be saved. Serodiagnosis with mycolic acids as antigen provides such an opportunity (Verschoor and Onyebujoh, 1999).

Pan et al. (1999) indicated that the anti-cord factor antibodies (IgG) in TB patients specifically recognized the mycolic acid structure, especially methoxy mycolic acid methyl esters. Mycolic acid is presented by antigen-presenting cells (APC) through a mechanism that does not involve major histocompatibility complex (MHC)-class I or MHC-class II molecules (Moody et al., 1999). The anti-mycolic acid immune response could therefore be independent of the participation of $CD8^+$ or $CD4^+$ T cells that respond to antigen presented on MHC I and MHC II surface proteins, respectively. Other than the MHC-presented protein antigens, mycolic acid is presented on CD1, with the ability to induce proliferation of T cell lines, with or without the CD4 or CD8 molecules (Beckman et al., 1994; Goodrum et al., 2001). The production of antibodies to protein antigens generally depends on the help of $CD4^+$ T cells. It is known that infection with HIV results in depletion of $CD4^+$ T cells and inhibition of the function of the remaining T cells (Price et al., 2001). Thus, Hendrickson et al. (2000) showed a decreased antibody specificity and sensitivity to a mycobacterial 30-kDa protein antigen with ELISA when screening patients in a population that had a high prevalence of HIV. Ratanasuwan et al. (1997) showed that when a lipoarabinomannan (LAM) was used in serological tests on HIV-negative and TB-positive patients, it showed sensitivities varying from 21% to 89%, but only 7% to 40% in HIV-positive patients. Antunes et al. (2002) described the MycoDot serological assay for tuberculosis, which is based on the detection of specific IgG antibodies against the LAM antigen, fixed onto a solid support consisting of a plastic comb designed to fit into the wells of a microtitre plate. The sensitivity values observed were definitely lower in cases of TB associated with HIV, which refuted the usefulness of the test in regions where HIV is highly endemic. They concluded that LAM as an antigen is only satisfactory in the serodiagnosis of TB as long as HIV is not highly prevalent in the population. Daniel

et al. (1994) performed a field test in Mexico, and showed that an ELISA based on the mycobacterial 30-kDa protein antigen had a sensitivity of 70% in patients with culture-positive or smear-positive pulmonary TB and a specificity of 100% in 125 control donors. The same test was evaluated with HIV-positive and -negative patients in Uganda. Although the sensitivity and specificity in HIV-negative donors were similar to the results of the Mexico test, the ELISA gave a sensitivity of 28% in 128 sera from HIV-positive donors. However, the immune response to mycolic acid could in principle proceed independently of the $CD4^+$/$CD8^+$ T cells. The human CD1 protein is known to mediate T cell responses by presenting at least three classes of mycobacterial lipids, i.e. free mycolates, glycosylated mycolates and diacylglycerol-based glyco-phospholipids. The alkyl chains of the mycolic acid antigen have been proposed to bind directly within the hydrophobic groove of CD1 resulting in presentation of the hydrophilic caps to the T cell's antigen receptor (Porcelli et al., 1996; Moody et al., 1999). The CD1-restricted lipid antigen presentation pathway could provide a possible explanation why the antibody response to mycolic acids is maintained in HIV-seropositive patients, despite a declining CD4 T lymphocyte count (Schleicher et al., 2002). Simonney et al. (2007) also suggested that the CD1-restricted lipid antigen presentation pathway is the likely mechanism accounting for the perseverance of high circulating antibody responses to PGL-Tb1 antigen in HIV-infected patients with TB. Simonney et al. (2007) showed that about half of HIV-positive individuals produce specific anti-glycolipid antibody several months before a diagnosis of TB disease can be made.

Here, a significant increase in sensitivity and specificity was shown for anti-mycolic acid antibody detection in patient serum with the inhibition assay on the biosensor, compared to that reported in our previous study using an ELISA assay (Schleicher et al., 2002). The false positive results observed amongst the TB^-HIV^+ population could show that the patients were true positive on the IAsys biosensor, since a sputum culture was used as the gold standard method for confirming their TB status. However, it is known that sputum culture of HIV-infected patients needs more incubation time than that of patients without HIV infection, which is consistent with the lower bacillary load seen in the sputum of HIV-infected patients (Brindle et al., 1993). The culture requires 10–100 viable *M. tuberculosis* per millilitre of sputum to give positive results (Colebunders and Bastian, 2000). It has also been shown that 15–20% of adults with pulmonary TB whose diagnosis was based on clinical, radiographic, and histopathological findings and response to anti-TB treatment have negative sputum cultures (Frieden et al., 2003).

The IAsys affinity biosensor was able to detect low affinity antibody binding to mycolic acids, in addition to high affinity antibody, which the conventional methods cannot generally achieve. In an ELISA, these antibodies would have been washed away before the final step and the patient would have tested false negative. The advantage of the biosensor lies in its real-time detection of antibody binding, without the need for prior washing away of the unbound antibody excess. In addition, the inhibition of binding as an endpoint eliminates much non-specific binding interference, which adds to the increased specificity of the biosensor assay. A disadvantage of the biosensor is that it is blind to the identity of the binding ligand from the serum sample. Here, the binding of IgG to mycolic acids was confirmed by showing that its binding inhibition could be reproduced with purified IgG from the same serum sample.

The few false negative results that still remain with the biosensor analysis are probably due to the inhibition of antibody activity by circulating mycolic acid antigen. Should this be the case, one can envisage that a duplicate test be run that is spiked with a stable source of anti-mycolic acid antibodies, such as monoclonal antibodies. A true negative will then return the spike signal, whereas a false negative will consume the signal. False positive results pose a more daunting technical challenge, but may be due to the cross-reactivity of antibodies to mycolic acids of non-tuberculous pathogenic mycobacteria, e.g. *M. avium*, which do occur at low frequency, especially in HIV-positive patients. More work is required to manage the specificity of the assay by, for instance, screening sera from patients that are TB negative, but test positive for *M. avium* disease. This work is currently underway.

Many serological assays have been developed for detection of specific antibody to lipid cell wall antigens in tuberculosis patients (Lyashchenko et al., 1998; Pan et al., 1999; Pottunarthy et al., 2000; Julian et al., 2002; Schleicher et al., 2002; Lopez-Marin et al., 2003; Fujita et al., 2005), but generally they do not meet the requirements on specificity and sensitivity (Attalah et al., 2005). The biosensor approach may improve that by means of its unique benefits reported here. However, the technique is technically quite difficult to perform in the laboratory and the technology is not yet suitable for large scale screening of patients.

Since only 61 patients were analyzed with the biosensor in this study, more patient and control sera will have to be analyzed to properly validate it as a reliable technique to determine anti-mycolic acid antibodies as surrogate markers for active tuberculosis. However, the

detection of anti-mycolic acid antibodies with the IAsys affinity biosensor appears to be technically feasible, quick and may also be made affordable by further optimization and innovation of the biosensor hardware. Moreover, the biosensor assay may even prove to be more sensitive than the microbiological sputum growth assay, as suggested here with the serum samples from HIV^+ patients that tested positive with the biosensor, but negative with the sputum assay.

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