

Lipoarabinomannan-Reactive Human Secretory Immunoglobulin A Responses Induced by Mucosal Bacille Calmette-Guérin Vaccination

Robin M. Brown,¹ Orlando Cruz,¹ Michael Brennan,² Maria L. Gennaro,³ Larry Schlesinger,^{4,5,6,7,a} Yasir A. W. Skeiky,⁸ and Daniel F. Hoft¹

¹Saint Louis University Vaccine and Treatment Evaluation Unit, Division of Infectious Diseases and Immunology, Department of Internal Medicine, Saint Louis University, St. Louis, Missouri; ²Center for Biologics Evaluation and Research, US Food and Drug Administration, Bethesda, Maryland; ³Public Health Research Institute of New York, New York; ⁴Division of Infectious Diseases, Department of Medicine, ⁵Department of Microbiology, and ⁶Immunology Program, University of Iowa, and ⁷Veterans Affairs Medical Center, Iowa City; ⁸Corixa, Seattle, Washington

The ability of 17 recombinant mycobacterial proteins, native antigen 85 complex, lipoarabinomannan (LAM), and *Mycobacterium tuberculosis* lysate to detect antibody responses induced by bacille Calmette-Guérin (BCG) vaccination and active tuberculosis infection were studied in enzyme-linked immunosorbent assays. Only LAM-reactive serum immunoglobulin G responses were significantly increased in both BCG-vaccinated patients and patients with active tuberculosis ($P < .05$), and oral BCG vaccination also induced significant increases in LAM-reactive secretory immunoglobulin A ($P < .05$). LAM-reactive antibody assays can serve as markers of humoral and mucosal immunity in future trials of BCG and newer attenuated mycobacterial vaccines.

Intradermal vaccination with bacille Calmette-Guérin (BCG) is currently used in many countries to prevent severe disease associated with *Mycobacterium tuberculosis* infection. However,

widespread BCG use has not reduced the overall prevalence of *M. tuberculosis* infection worldwide [1], and more-effective tuberculosis (TB) vaccination strategies are needed. We have initiated a series of human trials designed to identify the partially protective immune responses induced by intradermal BCG vaccination and to investigate the possibility that changes in the method of BCG vaccination (e.g., route, dose, use of booster doses, and schedule of repeated vaccination) could result in enhanced efficacy. To optimize mucosal vaccine dosing, we need a relevant marker of vaccine-induced mucosal immunity that can be measured in human volunteers. Studies of secretory IgA are minimally invasive, and IgA responses generally are a good marker for induction of mucosal immunity, even if IgA may not be directly involved in protection. However, to our knowledge, there are no previous reports of human secretory IgA responses induced by BCG vaccination, and specific antigenic targets relevant for these measurements previously were unknown. We present comparisons of the ability of 20 different preparations of recombinant and native mycobacterial antigens to detect BCG-induced mucosal and systemic antibody responses.

Subjects and methods. Healthy individuals aged 15–50 years who had negative results of skin testing with purified protein derivative (PPD; 0 mm of induration) and of serological testing for human immunodeficiency virus (HIV) were enrolled in vaccine trials of intradermal and oral BCG vaccination [2]. Connaught strain BCG was administered intradermally ($\sim 3 \times 10^6$ cfu) or orally (2×10^{10} cfu ingested within 15 min of intake of 2 g of oral bicarbonate).

Serum was collected from coagulated blood. Feces (10 mg) were mixed with PBS (20 mL) overnight before collection of supernatants. Nasal washes were obtained by instilling 5 mL of PBS in each naris and collecting the exhaled fluid. Parotid secretions were collected with vacuum-cup devices [3]. Tears were induced with orange-peel mist and collected with disposable bulb syringes. Samples were clarified by centrifugation at 500 g and stored at -70°C before ELISA.

Serum samples also were collected from 10 patients who had recently begun therapy for active TB. Nine patients had sputum cultures that were positive for *M. tuberculosis*, and 1 patient had positive results of culture of a pleural biopsy specimen.

Recombinant MPT63, MPT64, KatG, MPT51, early secretory antigen 6 (ESAT-6), MTC28, 14-kDa protein, and 38-kDa protein were expressed in *Escherichia coli* and purified by fast protein liquid chromatography on a series of nickel, desalting, and anion-exchange columns at the Public Health Research Insti-

Received 9 August 2002; revised 16 October 2002; electronically published 24 January 2003.

Study protocols were approved by the Saint Louis University Institutional Review Board.

Financial support: National Institutes of Health (NO1-AI-45211 to D.F.H., Saint Louis University Vaccine and Treatment Evaluation Unit, and RO1-AI36989 to M.L.G.); Veterans Affairs Merit Review Award (to L.S.).

^a Present affiliation: Division of Infectious Diseases and Center for Microbial Interface Biology, Ohio State University, Columbus.

Reprints or correspondence: Dr. Daniel F. Hoft, Div. of Infectious Diseases and Immunology, Depts. of Internal Medicine and Molecular Microbiology, Saint Louis University Health Sciences Center, 3635 Vista Ave., FDT-8N, St. Louis, MO 63110 (hoftdf@slu.edu).

The Journal of Infectious Diseases 2003;187:513–7

© 2003 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2003/18703-0022\$15.00

tute of New York (New York). Recombinant CFP-10, TbDP (same as 45/47), TbH4, Ra3, and Ra11 (same as MPT64) were expressed in *E. coli* and purified by nickel-column chromatography at Corixa (Seattle). Recombinant heparin-binding hemagglutinin adhesin was expressed in *E. coli* and purified by nickel-column chromatography at the Center for Biologics Evaluation and Research (Bethesda, MD) [4]. Lipoarabinomannan (LAM) was purified by high-performance liquid chromatography from the H37Ra and H37Rv strains of *M. tuberculosis* at the University of Iowa (Iowa City), as described elsewhere [5]. The native antigen 85 (Ag85) complex was purified from log-phase culture filtrates of the *M. tuberculosis* Erdman strain at Colorado State University (Fort Collins; National Institute of Allergy and Infectious Diseases, National Institutes of Health, contract NO1 AI-75320). Whole lysates of *M. tuberculosis* Erdman strain were prepared from log-phase cultures grown in glycerol-alanine salts broth at Saint Louis University, as described elsewhere [6].

Immulon 2 plates (Dynatech Laboratories) were coated with recombinant proteins (1 $\mu\text{g}/\text{mL}$), Ag85 (5 $\mu\text{g}/\text{mL}$), or whole *M. tuberculosis* lysate (5 $\mu\text{g}/\text{mL}$) diluted in 0.1 M carbonate buffer (pH 9.0) and incubated overnight at 4°C. After blocking with 10% fetal calf serum, plates were washed, and samples (serum pools, individual serum samples, and mucosal specimens) were added to duplicate wells and incubated overnight at 4°C. Plates were washed and developed with goat anti-human IgG or goat anti-human IgA conjugated to horseradish peroxidase (Southern Biotechnology Associates) and then with ABTS substrate (Kirkegaard & Perry), and absorbance was read at 405 nm. Serum pools were added beginning at a dilution of 1:50 in PBS and further diluted 2-fold across plates. Individual serum and mucosal samples were used at dilutions of 1:50 and 1:100, respectively.

H37Ra LAM diluted in 100% ethanol (4 $\mu\text{g}/\text{mL}$) was added to Immulon 2 plates (100 $\mu\text{L}/\text{well}$). These plates were dried in a fume hood, washed with PBS and 0.05% Tween 20, blocked overnight at 4°C with 1% bovine serum albumin in PBS with 0.05% Tween 20, and developed as described above for protein ELISA.

Statistica (StatSoft) was used for data analyses. Pre- and post-vaccination responses were compared using Wilcoxon matched-pairs tests. Groups were compared using Mann-Whitney *U* tests. Proportional responses were compared using Fisher's exact 2-tailed tests. Repeated-measures analysis of variance was used to analyze LAM-reactive mucosal IgA after oral BCG vaccination.

Results. ELISAs with 20 different mycobacterial antigen preparations were completed using 3 serum pools prepared with individual serum samples from (1) 10 PPD-negative control subjects who were later vaccinated intradermally with BCG, (2) the same 10 individuals 2 months after vaccination with BCG, and (3) 10 patients with active TB disease (data not shown). ELISAs

were repeated at least 3 times and yielded similar results. The pooled serum from BCG-vaccinated subjects was found to have increased antibody reactivity only with H37Ra LAM. The pooled serum from patients with TB had increased antibody titers specific for recombinant 38-kDa protein, native Ag85 complex, and H37Ra LAM. Smaller increases were seen in the pooled serum from patients with TB of antibody specific for the 14-kDa, 45/47, ESAT-6, KatG, MPT64, and MPT70 recombinant proteins.

Figure 1 presents ELISA data from assays measuring the reactivity of individual serum samples with antigens in which the largest differences among the 3 serum pools were detected. Serum samples diluted 1:50 from PPD-negative control subjects, BCG-vaccinated subjects, and patients with TB were incubated in ELISAs with H37Ra LAM, native Ag85 complex, 38-kDa recombinant protein, and whole *M. tuberculosis* lysate. The median values and ranges of individual responses to each of these antigens for all 3 groups of subjects are shown in figure 1.

LAM-reactive IgG responses were significantly increased among both BCG-vaccinated subjects ($P < .01$, by Wilcoxon matched-pairs test) and patients with TB ($P < .01$, by Mann-Whitney *U* test), compared with the PPD-negative control group. Ag85-specific IgG responses were significantly increased ($P < .05$, by Mann-Whitney *U* test) only among the patients with TB. High background reactivity to Ag85 was detected in some PPD-negative control subjects, including values as high as the highest responses in the group of patients with TB. Patients with TB had increased reactivity to the 38-kDa antigen, compared with the other 2 groups, although this increase did not achieve statistical significance. Median and maximum antibody reactions to *M. tuberculosis* whole lysate increased progressively from the PPD-negative control group to the BCG-vaccinated group to the TB group, but these differences were not statistically significant. Assays were repeated at least 3 times, with similar results. Further ELISAs were performed in which plates were coated with LAM purified from the *M. tuberculosis* H37Rv strain, which confirmed that BCG induced significantly increased levels of antibodies that were reactive with LAM expressed by a virulent *M. tuberculosis* strain (median OD, 0.68 for prevaccination serum samples and 1.09 for postvaccination samples; $P < .02$, by Wilcoxon matched-pairs test).

We analyzed the proportions of subjects with positive responses in each serum group for different ELISAs ("positive response" was defined as an OD ≥ 2 SD higher than the mean for PPD-negative control subjects). The proportion of subjects with positive responses for LAM-reactive IgG was significantly greater in the active TB group than in the PPD-negative control group (6 of 9 vs. 0 of 10 subjects, respectively; $P < .01$, by Fisher's exact 2-tailed test). In addition, the proportion of subjects with positive responses for LAM-reactive IgG was significantly increased after BCG vaccination (5 of 10 subjects before vaccination vs. 0 of 10 after vaccination; $P < .05$, by Fisher's

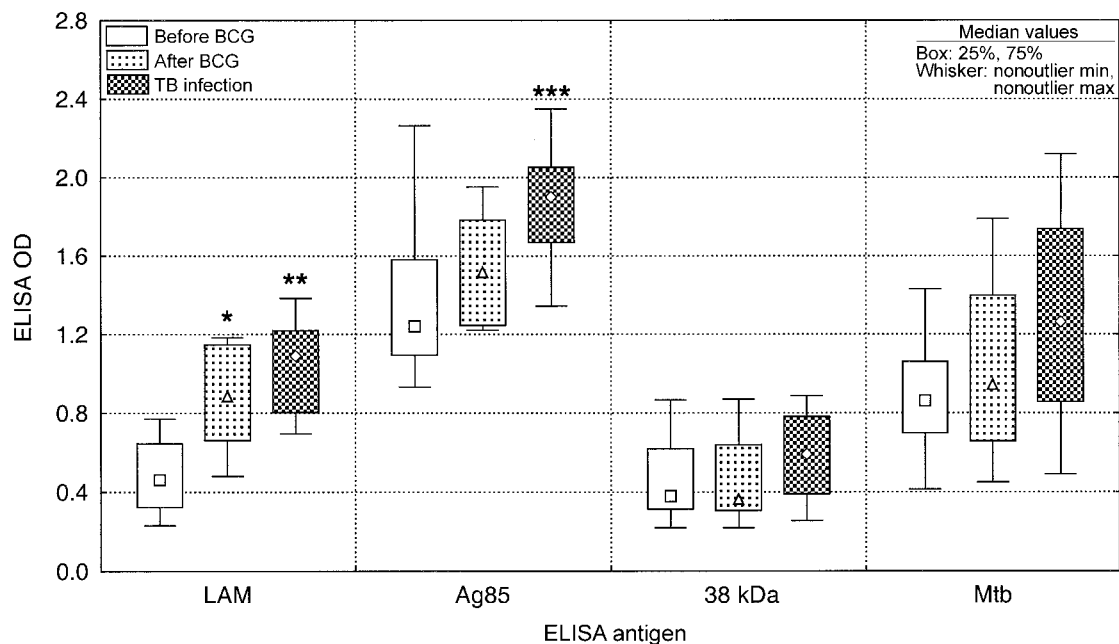


Figure 1. Individual serum IgG antibody responses. Comparison of lipoarabinomannan (LAM), antigen 85 (Ag85), 38-kDa protein, and whole *Mycobacterium tuberculosis* lysate (Mtb) ELISA reactivity in individual serum samples harvested from subjects before vaccination with bacille Calmette-Guérin (BCG; $n = 10$) and after BCG vaccination ($n = 10$) and from patients with tuberculosis (TB; $n = 9$). Median values (open symbols) with quartile ranges are shown for each group. * $P < .05$, postvaccination results vs. results for matching purified protein derivative (PPD)-negative control subjects (Wilcoxon matched-pairs test). ** $P < .05$, results from patients with TB vs. results from PPD-negative control subjects (Mann-Whitney U test). *** $P < .05$, results from patients with TB vs. both postvaccination results and results from PPD-negative control subjects (Mann-Whitney U test). Representative results from 5 similar experiments are shown. Max, maximum; min, minimum.

exact 2-tailed test). The proportion of subjects with positive LAM responses in the active TB group was not significantly different from that in the BCG-vaccination group (6 of 9 vs. 5 of 10, respectively; $P = .7$, by Fisher's exact 2-tailed test). Few samples were positive for antibodies reactive with Ag85, 38-kDa protein, or *M. tuberculosis* lysate, with no significant differences between groups.

Mucosal specimens (fecal extracts, nasal washes, parotid secretions, and tears) harvested from 8 volunteers before and after 2 oral vaccinations with 2×10^{10} cfu of BCG administered at an interval of 6 months were studied in H37Ra LAM, Ag85, and *M. tuberculosis* lysate ELISAs. All assays were repeated at least once, with similar results. Total IgA levels present in tears and parotid secretions were 100–1000-fold greater than total IgA levels detected in fecal extracts and nasal washes (data not shown). Figure 2 demonstrates that levels of LAM-reactive secretory IgA antibodies were significantly increased in tears harvested 6 months after primary oral BCG vaccination and on days 7 and 42 after the second oral BCG vaccination ($P < .05$, by Wilcoxon matched-pairs test). In addition, on a repeated-measures 1-way analysis of variance, LAM-reactive IgA responses in tears were found to be significantly increased, compared with prevaccination responses, on day 42 after the second vaccination ($P < .05$, by Tukey's honestly significant difference

post hoc comparison). Similarly, LAM-reactive secretory IgA responses were significantly increased ($P < .05$, by Wilcoxon matched-pairs tests) in parotid secretions after oral BCG vaccination (data not shown). Significant increases in LAM-reactive IgA were not detected in fecal extracts or nasal washes after oral BCG vaccination, and no significant increases in Ag85 or *M. tuberculosis* whole lysate-specific IgA responses were detected in any mucosal specimens after BCG vaccination (data not shown).

Discussion. In our studies, LAMs purified from *M. tuberculosis* H37Ra and H37Rv strains proved to be the most useful antigens for detection of BCG effects on mucosal and systemic B cell responses. LAM-reactive antibodies in serum were significantly increased after BCG vaccination when individual samples obtained before and after vaccination were compared (figure 1). Furthermore, LAM-specific secretory IgA responses were significantly increased after oral BCG vaccination (figure 2). These results demonstrate that measurements of LAM-reactive secretory IgA can be used to detect mycobacteria-specific mucosal immune responses in future vaccine trials. No other antigen tested in our experiments was able to detect mycobacteria-specific antibody responses after BCG vaccination.

We initially thought that fecal samples would most likely contain detectable levels of mycobacteria-specific secretory IgA

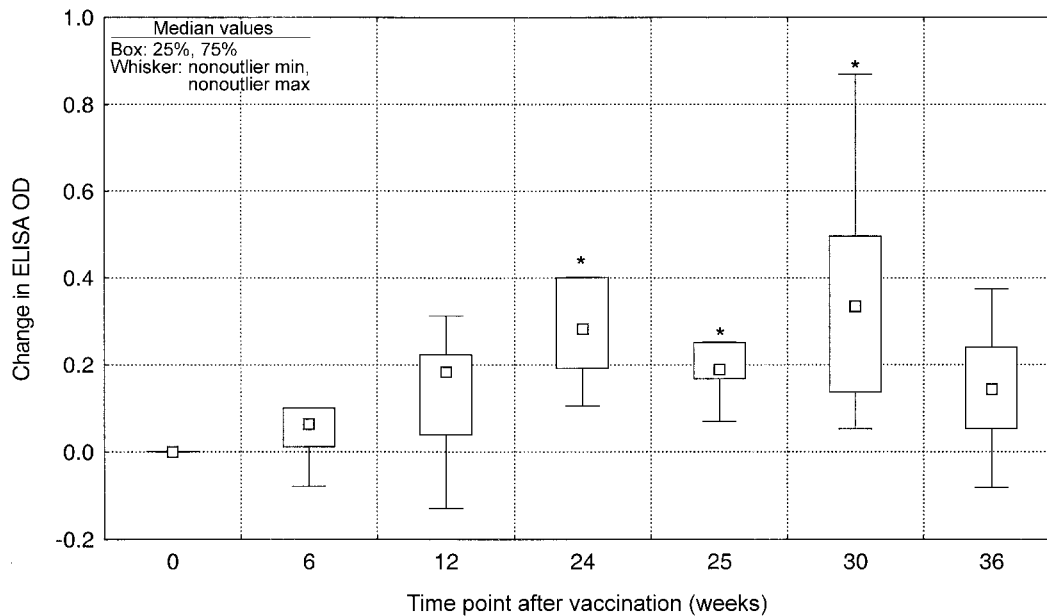


Figure 2. Lipoarabinomannan (LAM)-specific secretory IgA induced by bacille Calmette-Guérin (BCG) vaccination. Tears from volunteers who had received 2 oral vaccinations (2×10^{10} cfu of BCG) at an interval of 6 months were studied in LAM ELISAs. The optical density values detected in prevaccination samples from each volunteer were subtracted from postvaccination values to obtain the change in optical density results, which represents the increases in antibody reactivity induced by BCG vaccination. At week 24, a second oral BCG vaccination was administered to all of these volunteers. Median values (open symbols) with quartile ranges for the responses detected in all 8 individual volunteers are shown. * $P < .05$, postvaccination vs. prevaccination responses (Wilcoxon matched-pairs test). Representative results from 3 similar experiments are shown. Max, maximum; min, minimum.

after oral BCG vaccination and hoped to see mycobacteria-specific secretory IgA in nasal washes after vaccination because of the potential relevance for TB mucosal protection. However, fecal and nasal wash specimens contained 100–1000-fold less total IgA than did parotid samples and tears (data not shown). Therefore, the absence of detectable vaccine-induced IgA in fecal and nasal wash specimens was most likely due to dilutional effects, or perhaps to proteolytic degradation, rather than to a selective failure to develop mucosal immunity in gastrointestinal and nasopharyngeal tissues. The induction of gastrointestinal mucosal immunity was documented by the detection of BCG-induced IgA in parotid secretions. Furthermore, the presence of mycobacteria-specific IgA in tears indicated that oral BCG vaccination induced mucosal immune responses capable of circulating to other mucosal tissues via the common mucosal immune network.

It has been demonstrated elsewhere [7] that increased LAM-reactive antibody responses are present during active TB and lepromatous leprosy disease. Sada et al. [8] found that detection of LAM-reactive serum IgG by ELISA had a specificity of 91% and sensitivity of 72% for the diagnosis of active TB among Mexicans in a group with a high TB prevalence. In another study, the presence of LAM-reactive IgG in cerebrospinal fluid was found to have a sensitivity of 85% and a specificity of 96%

for the diagnosis of TB meningitis [9]. Commercially available serological assays for LAM reactivity have been developed that discriminate between healthy PPD-negative individuals and patients with TB who are not coinfecting with HIV [10, 11]. All of these reports indicate that serologic tests for LAM reactivity can be useful for the diagnosis of active TB. However, there are special circumstances in which diagnostic tests for LAM reactivity need to be interpreted with caution. Coinfection with HIV can lower the sensitivity of LAM-specific serological tests [12, 13]. In addition, serological testing for LAM reactivity was less sensitive for the detection of newly acquired TB than for relapse of TB disease [14]. Our results indicate that BCG vaccination also can induce production of LAM-reactive antibodies, which could be interpreted erroneously as evidence for ongoing active TB. False-positive LAM-reactive antibody responses induced by BCG vaccination are most likely to occur in persons who have recently been vaccinated with BCG and perhaps also in persons who have been vaccinated multiple times with BCG.

In summary, we have studied a panel of mycobacterial antigens for the ability to detect antibody responses induced by BCG vaccination and/or active TB. Only the LAM ELISA could detect significant increases in serum IgG induced by either BCG vaccination or active TB, as well as increases in secretory IgA induced

by mucosal BCG vaccination. Measurements of LAM-specific antibody responses will be useful in future vaccine trials for detection of vaccine-induced humoral and mucosal immunity.

Acknowledgment

We acknowledge Davin C. Dillon (Corixa, Seattle) for his help in providing recombinant antigens.

References

1. Scheele DC, Dolin P, Pathania V, Raviglione MC. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA* **1999**; 282:677–86.
2. Hoft DF, Brown R, Belshe RB. Mucosal BCG vaccination in humans inhibits delayed-type hypersensitivity to PPD, but induces mycobacterial specific IFN- γ responses. *Clin Infect Dis* **2000**; 30:S217–22.
3. Schaefer ME, Rhodes M, Prince S, Michalek SM, McGhee JR. A plastic intraoral device for the collection of human parotid saliva. *J Dent Res* **1977**; 56:728–33.
4. Delogu G, Brennan MJ. Functional domains present in the mycobacterial hemagglutinin, HBHA. *J Bacteriol* **1999**; 181:7464–9.
5. Schlesinger LS, Kaufman TM, Iyer S, Hull SR, Marchiando LK. Differences in mannose receptor-mediated uptake of lipoarabinomannan from virulent and attenuated strains of *Mycobacterium tuberculosis* by human macrophages. *J Immunol* **1996**; 157:4568–75.
6. Hoft DF, Brown R, Roodman S. Bacille Calmette-Guérin vaccination enhances human $\gamma\delta$ T cell responsiveness to mycobacteria suggestive of a memory-like phenotype. *J Immunol* **1998**; 161:1045–54.
7. Hunter SW, Gaylord H, Brennan PJ. Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. *J Biol Chem* **1986**; 261:12345–51.
8. Sada E, Brennan PJ, Herrera T, Torres M. Evaluation of lipoarabinomannan for the serological diagnosis of tuberculosis. *J Clin Microbiol* **1990**; 28:2587–90.
9. Park SC, Lee BI, Cho SN, et al. Diagnosis of tuberculous meningitis by detection of immunoglobulin G antibodies to purified protein derivative and lipoarabinomannan antigen in cerebrospinal fluid. *Tuber Lung Dis* **1993**; 74:317–22.
10. Del Prete R, Picca V, Mosca A, D'Alagni M, Miragliotta G. Detection of anti-lipoarabinomannan antibodies for the diagnosis of active tuberculosis. *Int J Tuberc Lung Dis* **1998**; 2:160–3.
11. 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.
12. Da Costa CT, Khanolkar-Young S, Elliott AM, Wasunna KM, McAdam KP. Immunoglobulin G subclass responses to mycobacterial lipoarabinomannan in HIV-infected and non-infected patients with tuberculosis. *Clin Exp Immunol* **1993**; 91:25–9.
13. Lawn SD, Frimpong EH, Nyarko E. Evaluation of a commercial immunodiagnostic kit incorporating lipoarabinomannan in the serodiagnosis of pulmonary tuberculosis in Ghana. *Trop Med Int Health* **1997**; 2:978–81.
14. Julian E, Matas L, Ausina V, Luquin M. Detection of lipoarabinomannan antibodies in patients with newly acquired tuberculosis and patients with relapse tuberculosis. *J Clin Microbiol* **1997**; 35:2663–4.