

Research Article

Analysis of LAM and 38 kDa Antibody Levels for Diagnosis of TB in a Case-Control Study in West Africa

**Marie P. Gomez, Simon Donkor, Ifedayo M. Adetifa,
Martin O. C. Ota, and Jayne S. Sutherland**

Vaccinology Theme, Medical Research Council Unit, Atlantic Road, Fajara, P.O. Box 273, Banjul, Gambia

Correspondence should be addressed to Jayne S. Sutherland, jsutherland@mrc.gm

Received 10 October 2012; Accepted 28 October 2012

Academic Editors: C. M. Artlett, E. K. Kapsogeorgou, D. Lilic, and I. Mezzaroma

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CD4⁺ T cells are required for protection against tuberculosis (TB) disease progression, but interest in the role of antibodies in early protection, as biomarkers for disease status, and use in diagnostic tests has recently increased. In this study we analyzed plasma antibody levels in TB cases before and after treatment in both HIV-positive and -negative individuals and compared them with tuberculin skin test (TST+) (latently infected) household contacts (HHC). We also analyzed HHC that subsequently progressed to active disease within 2 years in order to see if antibodies play a role in protection against disease progression. We used a commercially available kit to 38 kDa antigen and lipoarabinomannan (LAM) and found that immunoglobulin (Ig) G levels were 4-fold higher in subjects with disease compared to latently infected controls ($P < 0.001$) and were 2-fold higher than pretreatment levels following successful TB treatment ($P < 0.001$ compared to both pretreated cases and latently infected controls). HIV infection resulted in low antibody levels regardless of disease status or treatment outcome. Furthermore, levels in disease progressors (incident cases) were similar to nonprogressors and were not elevated until just prior to disease progression confirming previous reports that IgG antibodies, at least in the periphery, do not confer protection against TB disease progression.

1. Introduction

Protective immunity to *Mycobacterium tuberculosis* (MTb) primarily requires CD4⁺ effector T cells as evidenced by a 6-fold increase in the likelihood of developing TB with decreasing CD4⁺ T cell counts in HIV-infected individuals [1]. However, there are limitations to the Th1/Th2 paradigm in TB [2] with recent work showing that antibodies may play a synergistic role in the protective immune response, particularly at lower pathogen burden [3], and should be explored in conjunction with T cell responses for future vaccine development. However, determining the precise role of antibodies in TB is problematic since TB antibody profiles are highly heterogeneous and influenced by multiple parameters such as bacillary burden [2], the stage of bacterial cycle [3], and HIV coinfection [4].

In this study, we used a commercially available kit to detect IgG levels to a recombinant 38 kDa protein and lipoarabinomannan (LAM), recently shown to distinguish

subjects with TB from other lung diseases in Tunisia [5]. 38 kDa antigen (also termed antigen 5 or 78, Rv0934) contains two MTb-specific B-cell epitopes and is highly specific for TB [6], whilst LAM is a glycolipid expressed on the bacterial cell wall that inhibits the bactericidal properties of macrophages [7]. Antibodies to LAM have been found in the urine of subjects with active TB disease, [8] and studies have shown an increase in antibody levels to different TB antigens following successful TB treatment [6, 9]. Interestingly, HIV infection does not appear to affect antibody levels to a number of different antigens [6, 8] with antibodies to LAM increasing in the urine of subjects with advanced immunosuppression [7] although a recent paper has shown differential antibody levels depending on HIV status [10]. However, there is a large range of sensitivity and specificity in studies assessing antibody levels, with heterogeneity of responses dependent on bacillary burden and geographical location. In this study we evaluated HIV-positive and HIV-negative TB cases before and after treatment and compared

TABLE 1: Subject information.

	HIV– TB cases	HIV+ TB cases	TST+ HHC	Incident cases
<i>n</i> =	76	77	135	35
Age	26 [21–37]	39 [32–47]	27 [20–34]	23 [20–32]
sex (M : F)	56 : 20	45 : 32	62 : 73	16 : 19
[Ab] Recruitment	13 [6–20]	5 [0.4–16]	3 [1–9]	7 [1–12]
[Ab] 6-months	25 [13–37]	6 [0.9–23]	n/a	7 [1–20]
[Ab] 18-months	n/a	n/a	n/a	16 [10–30]

M: male; F: female; [Ab]: Antibody concentration; TST+ HHC: tuberculin skin test positive household contact; incident case: HHC progressing between 3 months to 2 years following recruitment of a TB index case. Data expressed as median [interquartile range].

IgG antibody levels to subjects with latent TB infection. We also compared antibody responses in incident cases (household contacts (HHC) of index cases who progressed to active disease within 2 years) to determine the role of antibodies in protection from TB disease progression. Results are discussed in relation to our previous data on polyfunctional T cell responses (T cells producing more than 1 cytokine) and B cell levels before and after treatment of active TB cases compared to subjects with latent infection [11, 12].

2. Methods

2.1. Ethics Statement. This study was conducted according to the principles expressed in the Declaration of Helsinki. Ethical approval was obtained from the Gambia Government/Medical Research Council (MRC) Joint Ethics Committee. All patients provided written informed consent for the collection of samples and subsequent analysis.

2.2. Subjects. Archived plasma samples from 76-HIV negative and 77 HIV-positive TB cases together with 135 household contacts (nonprogressors) and 35 confirmed incident cases (progressors) were included in this study (Table 1). Incident cases were defined as progressing ≥ 3 months but ≤ 24 months following recruitment of the index case [13]. Only incident cases that were HIV-negative and >15 years were included in this study with 35 samples available at recruitment, 19 at 3 months, and 19 at 18 months. All household contacts were Tuberculin Skin Test positive (TST; 2 tuberculin unites [TU], PPD RT23, SSI, Denmark) with skin induration ≥ 10 mm. Plasma samples obtained from heparinised blood prior to TST were frozen at -70°C for subsequent evaluation of Mycobacterial antibodies by ELISA. All subjects were followed up at 6 and 18 months after-recruitment. Pleural fluid was obtained from subjects presenting with pleural effusion caused by TB ($n = 11$) or other respiratory disease ($n = 5$).

2.3. Pathozyme Myco IgG Kit for Detecting Antibodies to LAM and 38 kDa Antigen. Pathozyme Myco IgG kit (Omega Diagnostics, USA) components and test plasma were brought to room temperature. Plasma was diluted 1:5, 1:10, 1:100, 1:200, and 1:500 in specimen dilution buffer for initial titer experiments and then 1:10 only for all subsequent

experiments. 100 μL /well of samples and control serum (0, 2, 4, 8, and 16 IU/mL) were added into appropriate wells of precoated plate. Plates were incubated on moist absorbent paper at 37°C for 1 hour then washed 3 times with 1X wash buffer. 100 μL /well of anti-human horseradish peroxidase (HRP) conjugate was then added, plates incubated for 1 hour and washed. Substrate solution was then added at 100 μL /well and plates incubated at 37°C in the dark for 15 mins. The reaction was stopped by adding 100 μL /well of 0.2 M H_2SO_4 and optical density immediately measured at 450 nm using a spectramax-250 (Molecular Devices, USA).

2.4. Statistical Analysis. Data were analyzed using Mann-Whitney *U*-test or Kruskal-Wallis test followed by Dunn's post-test comparison. Receiver-Operator Curve analysis was performed to determine the Area under the Curve (AUC) and % sensitivity and % specificity for classification of TB disease and latent infection. *P* values <0.05 were considered significant.

3. Results

3.1. Preliminary Evaluation of Commercial Pathozyme Myco IgG Kit. Following titration of our plasma, we found that a dilution of 1:10 gave the most consistent results. The dilution of the plasma was 10-fold lower than indicated in the manufacturer's instructions but this is consistent with other immunological assays we have performed using West African subjects (unpublished data).

3.2. Anti-Myco IgG Levels before and after Treatment of Active TB Cases with or without HIV Coinfection. Analysis of plasma IgG antibody levels to LAM/38 kDa was performed in confirmed TB cases (HIV negative ($n = 76$) or HIV-positive ($n = 77$) before and after (6 months) of treatment and compared with TST+ (>10 mm induration) HIV-negative household contacts. We found absolute levels of IgG to be significantly higher in HIV-negative TB cases compared to TST+ HHC (Figure 1(a)) but not for HIV-positive subjects who had similar levels to the TST+ HHC (Figure 1(a)). After successful TB treatment, antibody levels in HIV-negative cases were significantly higher than pretreatment, HHC and HIV-negative cases (both before- and after-treatment) (Figure 1(a)); an average 2-fold increase was seen in each

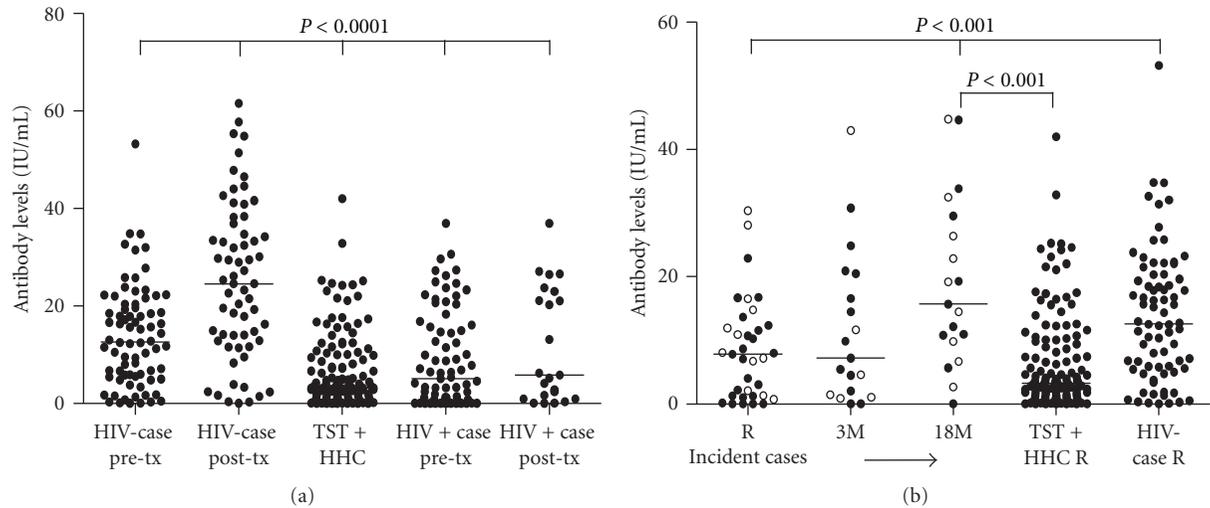


FIGURE 1: Analysis of plasma antibody levels in Tuberculosis (TB) infection and disease. (a) TB cases before and after treatment, with or without HIV coinfection were assessed for plasma antibody levels to Myco IgG and compared to Tuberculin skin test (TST)+ Household contacts (HHC). P values indicate significant differences between HIV-negative cases after treatment compared to all other groups and between HIV-negative cases before treatment compared to all other groups. (b) HHC were followed up for 2 years from recruitment and those that progressed to active disease (incident cases) were compared to those that did not progress (TST+ HHC) and active cases. R = recruitment; 3 M = 3 months after recruitment; 18 M = 18 months after recruitment.

HIV-negative subject ($P < 0.001$; Figure 1). Median [interquartile range (IQR)] antibody levels before treatment were 13 [6–20] IU/mL and 25 [13–37] after treatment compared to 3 [1–9] for TST+ HHC; 5 [0.4–16] for HIV-positive subjects before treatment, and 6 [0.9–23] for HIV-positive subjects after treatment. For the HIV-negative TB cases before treatment, there was a weak ($r^2 = 0.298$) but significant ($P = 0.007$) correlation with X-ray score but not with smear grade or culture time (data not shown). For the HIV positive subjects, those that successfully completed treatment ($n = 25$) had significantly higher antibody levels at recruitment compared to those that died during followup ($n = 6$; $P = 0.0043$; data not shown) indicating some degree of protection. However, these findings are inconclusive since there were a number of subjects where follow-up information was not available ($n = 33$) and no information on CD4 levels was available for any subject.

3.3. Anti-Myco IgG Antibodies Do Not Protect against Disease Progression. In order to assess the role of TB antibodies in protection against development of active disease, we analyzed changes in antibody levels of subjects who progressed to active disease between 3 and 24 months after recruitment of the household index case and compared to those who did not progress (TST+ HHC controls; Figure 1(b)). Median [IQR] plasma antibody levels to Myco IgG at recruitment, 3 months, and 18 months were 7 [1–12], 7 [1–20], and 16 [10–30] IU, respectively, for incident cases. Recruitment levels were comparable to the TST+ HHC group (nonprogressors) but significantly lower than the HIV-negative TB cases at recruitment. No change was seen at 3 months after recruitment but levels were significantly higher at 18 months after recruitment compared to R and 3 M time-points and

the HHC controls ($P < 0.001$) but were similar to subjects with active TB disease at R.

3.4. Antibody Levels Do Not Allow Accurate Discrimination between TB Infection and Disease. Receiver operated curve analysis was performed to determine the ability of antibody levels to discriminate between active TB disease and latent infection (Figure 2). Analysis of HIV-negative TB cases showed a sensitivity and specificity of 70% with an AUC of 0.74 (Figure 2(a)). For HIV-positive subjects, sensitivity was only 49% with specificity of 65% (AUC = 0.53; Figure 2(b)).

3.5. Analysis of IgG Antibodies in Lung and Urine Samples. We analyzed 30 urine samples from active TB cases and found no detectable antibodies in any sample (data not shown). We also analyzed pleural effusions from 11 confirmed TB cases and 5 subjects with other causes [13] and found a higher level of antibodies in the TB patients compared (median [IQR] = 5.1 [0.4–12] and 1.2 [0.7–16], resp., Figure 3), but this was not significant.

4. Discussion

Our findings show significantly higher levels of IgG antibodies to LAM/38 kDa antigen in subjects with active TB disease compared to TST+ (latently infected) household contacts, and these were increased even further following successful TB treatment. Antibody levels in urine were undetectable and, despite sequestration of other cells to the lung [14], no discernible increase in antibody levels was seen in pleural effusions. Furthermore, subjects with HIV coinfection showed low antibody levels before and after treatment (comparable to latent infection). Our findings

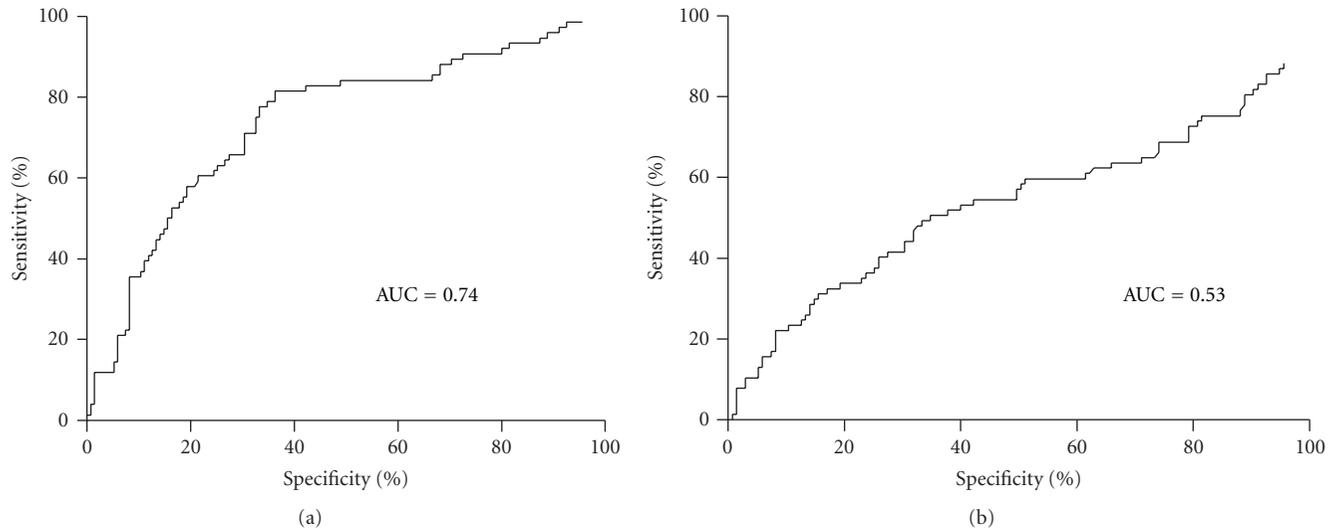


FIGURE 2: Classification of TB cases and latently infected contacts. (a) HIV-negative cases compared to TST+ HHC and (b) HIV-positive cases compared to TST+ HHC. Sensitivity and specificity of Anti-Myco IgG antibodies for classification of active disease were assessed in HIV-positive and HIV-negative TB cases.

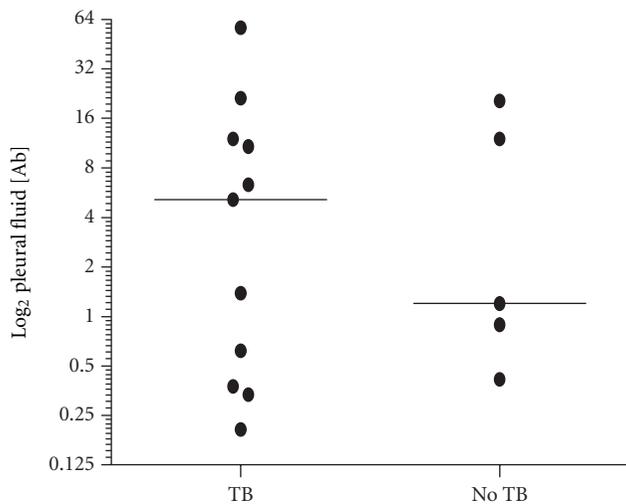


FIGURE 3: Analysis of Myco IgG antibody levels in pleural fluid from TB and non-TB patients. Antibody levels in pleural effusions were measured and compared between subjects with TB and those with pleural effusion caused by another illness. While subjects with TB had a broad range of values, there was no significant difference between the groups.

support a recent meta-analysis [8] showing that whilst TB antibodies may be used as part of a clinical algorithm, they cannot be used on their own for diagnosis of active TB thereby limiting their merit as a rapid diagnostic test.

It is well known that antibody responses in TB are highly heterogeneous [3]; thus it is not surprising that we saw differential responses in this study, particularly for HIV coinfection. Furthermore, previous studies have suggested limited sensitivity with the commercial kit we used in this

study due to the fact it is based on antigens that predominantly elicit antibody responses in HIV uninfected patients with advanced TB, such as the 38 kDa protein (reviewed in [15]). This suggests that latently infected individuals including those who later progress to disease (i.e., both protected and unprotected cohorts) respond to different antigens at the different stages of disease further restricting the use of antibodies for diagnostic purposes. Another study suggested that the early stages of infection are likely associated with low bacterial burden resulting in low antibody titers to most antigens [4, 16, 17]. However, bacterial load should also be low following successful TB treatment, yet we saw a 2-fold increase in antibody levels in the same subjects after treatment compared to before treatment, suggesting that it is not the quantity of bacteria but treatment-associated dissemination that elicits antibody responses as the bacteria will be accessible. It is also possible that the extremely low levels of IgG in latent infection reflect epitope masking (i.e., a survival mechanism of the bacteria) or simply that other antigens are being recognised instead. It has also been shown that HIV-infected TB patients develop antibody responses to a smaller repertoire of antigens compared to HIV-uninfected subjects [18, 19], which may explain why we saw a significantly lower response in HIV-positive compared to HIV-negative subjects, although immunosuppression is clearly a contributing factor. Unfortunately the immunocompetence of subjects in this study was not known and there was a lack of follow-up data making it difficult to draw any conclusions; this should be addressed in future studies.

In regard to development of an optimal vaccine, it is important to assess both cellular and humoral immune responses to TB antigens. We have previously published data on a subgroup of these subjects in regard to immune cell phenotype and function before and after TB treatment

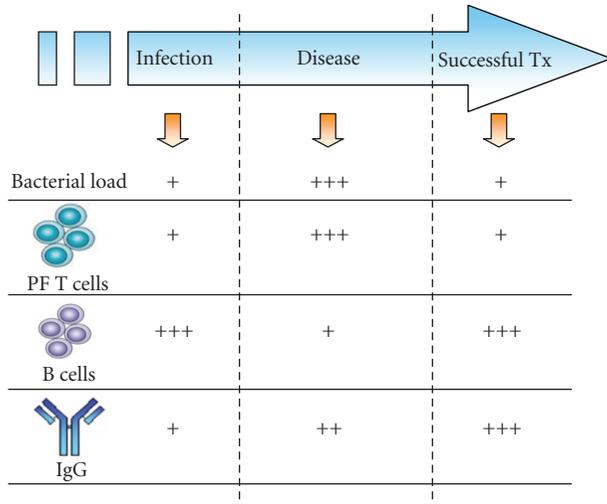


FIGURE 4: Diagram of antibody changes compared with T and B cell changes in TB infection and disease. Data for antibody levels was obtained from this study and compared with our previous studies assessing B cell and polyfunctional (PF) T cell responses in TST+HHC and TB cases before and after treatment. Antibody levels show distinct kinetics. Tx = treatment.

compared to latently infected subjects [10, 11]. We found that B cell levels were significantly reduced in TB cases compared to TST+ HHC and restored after treatment [12]. We assumed this was due to sequestration of the cells to the lungs but found no increase when blood and lung were compared at an acute stage of disease in pleural TB [14]. In contrast, polyfunctional T cell responses (cells producing more than one cytokine simultaneously) were increased prior to treatment and were reduced after treatment [11]. Putting these data together (Figure 4), IgG antibody levels to LAM and 38 kDa antigen are low prior to disease and correlate with a low bacterial load. However, whilst they increase 4-fold with onset of active disease (correlating with an increase in bacterial load/virulence), they are highest following successful TB treatment when bacterial load is low, likely due to accessibility induced by drug treatment. Interestingly, whilst circulating antibody levels do not correlate with circulating B cell levels at disease onset, both B cells and IgG antibodies in the peripheral blood are significantly increased following successful treatment, although B cells remain at similar levels to their latently infected counterparts whereas IgG levels are increased 10-fold compared to latent infection.

Although antibodies do provide a level of protection in some mouse models [20], in humans (at least in our setting) antibodies to two highly immunogenic antigens do not appear to be protective. Analysis of subjects in close contact with TB index cases provides a unique platform for studying requirements for protection from progressing to active disease. Surprisingly, we found no difference in antibody levels between incident cases and controls until just prior to disease progression where levels in incident (i.e., nonprotected group) increased significantly. This is

likely due to two main factors. Firstly, antigenic variation is seen between latent and reactivation states of the bacteria; therefore, different antibodies will be required for different stages of infection and disease. Secondly, B cells require extracellular bacilli for optimal activation, which only occurs when the bacterial load is high enough, just prior to disease progression. Following successful drug treatment, antigenic fragments will increase further and is reflected by the 10-fold increase in antibody levels we saw following treatment. Whether an increased antibody response following treatment may help in defense mechanisms upon reinfection remains to be seen but mouse models of passive immunity suggest that this will be beneficial [20]. The major limitation of this study was analysis of only two antigenic targets for antibody responses. The diversity of bacterial antigens associated with distinct disease stages requires analysis using proteome arrays of multiple targets to properly evaluate the role of antibodies in tuberculosis immunity.

Acknowledgments

This research was conducted through funding by the MRC Unit, Gambia. The authors thank all the staff at the TB clinic, outpatient's clinic, and MRC ward for subject enrolment and consent. They also thank Professor Gerhard Walzl for critical review of the paper. Finally, they thank all study subjects and their families.

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