Antibody response to *Mycobacterium tuberculosis* 30 and 16kDa antigens in pulmonary tuberculosis with human immunodeficiency virus coinfection

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Abstract

There is urgent need for rapid diagnostic methods to identify tuberculosis among the HIV positive cases, since the mortality is high. We have isolated and evaluated the serodiagnostic potential of the 30kDa secreted antigen and 16kDa cytosolic antigen of *M. tuberculosis*, among the HIV-TB patients. Antibody response was studied using Enzyme linked immunosorbent assay. In the HIV-TB group, antibody was found to be 65%, 69% and 6% positive for IgG, A and M isotypes, respectively, against 30kDa. The sensitivity increased to 84%, upon combination of the results of 3 isotypes. Anti-16kDa was detected in 15% (G), 50% (A) and 3% (M) of cases. Combination of results improved the positivity to 57%. There was no difference in antibody response among the HIV-TB cases, related to CD4 counts. Thus the 30kDa antigen proved to be of diagnostic utility in HIV-TB. © 2003 Elsevier Inc. All rights reserved.

Keywords: Antibody-Isotypes; HIV-TB; ELISA; *M. tuberculosis*; 16kDa; 30kDa

1. Introduction

The increasing incidence of tuberculosis (TB) on account of its association with the Acquired immunodeficiency syndrome (AIDS) is undoubtedly a cause of global concern. TB is now the leading cause of death among persons with AIDS, killing 1 of every 3 people who die with AIDS (WHO, 1996). Delayed diagnosis of TB and initiation of appropriate treatment more than 3 weeks after presentation are associated with 45-85% of deaths in HIV infected patients (Barnes et al., 1991). Therefore accurate and early diagnosis of tuberculosis among the HIV-infected patients will aid in the better management and control of this disease and hence improve the survival rate of these patients.

The conventional acid fast staining method accounts for only 50% case detection among HIV-TB patients (Shafer & Edlin, 1996). Alternative methods for detection of *M. tuberculosis* based on serologic response to mycobacterial antigens are also reported. The major drawback in such assays is the extensive person-to-person variation to antibody response. In this scenario, the prospective approach would be to use more than a single antigen for the diagnosis of tuberculosis among the HIV-infected patients.

The 30kDa (Ag 85B) antigen is an early secreted, immunodominant antigen of *M. tuberculosis* and hence is a suitable candidate to be used in diagnosis. The 16kDa alpha crystallin protein is a cytosolic antigen, which is species-specific. The two antigens have been purified in our laboratory and evaluated in HIV seronegative pulmonary TB. Both the 30kDa antigen (Unpublished observation) and the 16kDa gave good results in HIV seronegative pulmonary TB (Raja et al., 2002).

The present study is focused on evaluating the diagnostic potential of the two antigen in HIV seropositive pulmonary TB, where diagnosis is difficult.

2. Materials and methods

2.1. Study subjects

2.1.1. HIV seropositive pulmonary TB patients (HIV-TB) (*N* = 68).

The HIV status was confirmed by 2 enzyme immuno assay (EIA) kits (Comb Aids-RS, Span Diagnostics, India...
and HIV TRI-DOT, J. Mitra & Co, India). When a serum was positive for both EIAs (Comb Aids and HIV TRI-DOT), it was considered HIV positive. If a serum was positive for only one EIA (which was rare), WB was done as confirmatory test.

Pulmonary tuberculosis was bacteriologically confirmed, by smear and culture positivity in 55 of the patients. Smear was found to be negative in the remaining 13 patients (but culture was positive).

In an additional set of patients, CD4 cells were enumerated. The patients’ characteristics were matched with those of unknown CD4 counts. Sera from 20 HIV-TB patients with CD4 counts <100cells/mm³ and 21 patients with CD4 counts between 500 and 1000cells/mm³ were selected and ELISA was repeated.

2.1.2. HIV seronegative TB patients (TB) (N = 175)

Sera from smear and culture positive patients were collected before treatment.

2.1.3. Normal healthy subjects (NHS) (N = 150)

They are laboratory and blood bank volunteers in whom HIV and TB infection were ruled out by 2 EIAs in serum and chest x-ray respectively.

2.2. Sera

Blood samples were collected and the serum was separated and stored sterile at -70°C until use.

2.3. Antigens

2.3.1. Culture Filtrate Antigen (CFA) and 30kDa (Ag 85B) antigen

*M. tuberculosis* H37Rv was grown in Sauton’s liquid medium for 6 wks and the culture filtrate antigen (CFA) was prepared as described (Uma Devi et al., 2002). CFA was used as the starting material for the purification of 30kDa antigen.

Two-step chromatographic purification of the CFA involving anion exchange and hydrophobic interaction chromatography was carried out as described (Uma Devi et al., 2002).

2.3.2. Cytosol and 16kDa antigen

*M. tuberculosis* H37Rv sonicate and the cytosol were prepared as described (Uma Devi et al., 2002). Cytosol was used as the starting material for the purification of 16kDa antigen.

The 16kDa antigen was obtained from the cytosol fraction of *M. tuberculosis* H37Rv cell sonicate, using the gel filtration chromatography as described (Uma Devi et al., 2002).

The quantification of proteins was done with BCA protein assay kit (Pierce, Rockford, IL, USA).

2.4. Enzyme linked immunosorbet assay (ELISA)

ELISA was carried out to estimate the IgG, IgA and IgM antibody levels against the 30 and 16kDa antigens as described previously (Uma Devi et al., 2001). The plates (Nunc Maxisorp Certified, flat bottom) were coated with an optimal antigen concentration of 1μg/ml (30kDa) and 0.2μg/ml (16kDa). Sera were tested at 1:3200 dilution. Goat Anti-human IgG/IgA/IgM peroxidase conjugates (Jackson Laboratories, USA) were used at 1:1000 (G, M) and 1:500 (A) dilutions in Phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST) containing 1% BSA.

The samples to be assayed in a plate were randomly allocated to different wells within the plate and were also coded to conceal the identity of the specimens. Mean absorbance (O.D) of NHS + 2 SD was considered as the cut-off value. Any sample exhibiting absorbance above the cut-off value was classified as positive.

2.5. CD4 counts

CD4 T-lymphocyte enumeration in blood samples from HIV-TB patients was done by flow-cytometry (FACSort, Becton-Dickinson, San Jose, CA, USA). Briefly, CD3+/CD4+ cells from peripheral blood were labeled by the “Lysed whole blood” method, using the IMK-Lymphocyte kit (Becton-Dickinson, San Jose, CA, USA). Percentage of CD4 cells, among the total lymphocytes, was obtained from the FACSort. An automated hematology counter (ABX Micros, France) was used, to determine the total leukocyte counts (WBC) and lymphocyte percentages. The absolute CD4 counts were calculated as the product of the total WBC, percent lymphocytes and percent CD4 cells.

3. Results

3.1. Purification of 30 and 16kDa antigens

The 30 and 16kDa antigens were purified to homogeneity. The purified antigens were blotted with monoclonal antibodies (MAbs) from World Health Organization (WHO) bank, to establish the identity. Three MAbs, IT-27, IT-44 and IT-49 specifically reacting with 30/31kDa recognized the purified protein. Similarly, 16kDa antigen was recognized by IT-1 and IT-4, which are specific for the alpha crystallin protein (Uma Devi et al., 2002). The purity of the preparations (single molecular species) was confirmed by running on two dimensional (2-D) electrophoresis and reverse phase High-performance liquid chromatography (HPLC) (Uma Devi et al., 2002).
3.2. Antibody response to individual antigens in HIV-TB patients

3.2.1. 30kDa

Of the 68 cases of HIV-TB patients, 65% were positive for IgG, 69% for IgA and 6% for IgM antibodies (Table 1). The antigen detected more number of IgA positives (69%) in HIV-TB patients, when compared with HIV seronegative TB patients (15%). Among the HIV-TB cases, combination of IgG and IgA positivity increased the sensitivity to 84%. Addition of IgM results did not increase the sensitivity.

Since only 2 positives occurred in the NHS group, specificity also remained high (99%). By combination of IgG and IgA results, there were additional positives in the NHS group too, but the specificity was not compromised (97%).

The positive and negative predictive values (based on IgG + IgA positivity), were 0.92 and 0.93 respectively.

The overall results were split into those of smear positive and smear negative TB and analyzed. Of the 68 cases, 55 were presenting with smear positive tuberculosis. Thirty-seven of these were positive for anti-30kDa IgG and an additional 9 sera were positive for IgA, making the sensitivity in smear positive cases 83.6% (46/55). In 13 others with smear negative tuberculosis, eight were positive for anti-30kDa IgG and an additional 3 sera were positive for IgA, leading to the sensitivity of 84.6% (11/13) in smear negative cases (Table 2).

3.2.2. 16kDa

Among the HIV-TB patients, an extremely low positivity of 15% was observed for IgG (Table 3), 50% for IgA and 3% for IgM antibodies. An increased sensitivity of 57% was obtained, on combination of IgG+A+M results. The sensitivity however was lower, when compared with the HIV-seronegative TB patients.

The specificity of the assay was 100% when IgG isotype alone was considered, but dropped to 93% when IgG+IgA+IgM results were analyzed.

The positive and negative predictive values (based on IgG + IgA positivity) were 0.90 and 0.83 respectively.

Sensitivity in the smear positive cases for the 3 isotypes was 60% (33/55) and in smear negative cases, 46.1% (6/13) (Table 2).

3.2.3. Antibody response of HIV-TB based on CD4 counts

The ELISA, whose results are discussed above, was done using samples for which the CD4 status was not known. It was not possible to interpret the antibody response, based on the CD4 counts. Therefore, additional set of sera from 20 HIV-TB patients with CD4 counts <100 cells/mm³ and 21 HIV-TB patients with CD4 counts between 500 and 1000 cells/mm³ were selected and analyzed. There was no significant difference in the mean antibody levels between the 2 groups (Data not shown).

Table 1
Antibody positivity for 30kDa antigen

<table>
<thead>
<tr>
<th>ISOETYPE</th>
<th>TB (N = 175)</th>
<th>HIV-TB (N = 68)</th>
<th>NHS (N = 150)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No + ve</td>
<td>% SEN</td>
<td>No + ve</td>
</tr>
<tr>
<td>IgG</td>
<td>118</td>
<td>67</td>
<td>44</td>
</tr>
<tr>
<td>IgA</td>
<td>26</td>
<td>15</td>
<td>47</td>
</tr>
<tr>
<td>IgM</td>
<td>25</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>IgG + IgA</td>
<td>124</td>
<td>71</td>
<td>57</td>
</tr>
</tbody>
</table>

TB = Pulmonary tuberculosis
HIV-TB = HIV seropositive pulmonary tuberculosis
NHS = Normal Healthy subjects
No + ve = Number of positives
Sen = Sensitivity
Sp = Specificity
Positive Predictive value = 0.92
Negative Predictive value = 0.93

Table 2
ELISA positivity among HIV-TB based on smear status

<table>
<thead>
<tr>
<th>ISOETYPE</th>
<th>30kDa</th>
<th>16kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Smear + ve</td>
<td>% Sen.</td>
</tr>
<tr>
<td>IgG</td>
<td>37</td>
<td>67</td>
</tr>
<tr>
<td>IgA</td>
<td>37</td>
<td>67</td>
</tr>
<tr>
<td>IgM</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>IgG + A</td>
<td>46</td>
<td>84</td>
</tr>
<tr>
<td>IgG + A + M</td>
<td>46</td>
<td>84</td>
</tr>
</tbody>
</table>

Sen = Sensitivity
Table 3
Antibody positivity for 16kDa antigen

<table>
<thead>
<tr>
<th>ISOTYPE</th>
<th>TB (N = 175)</th>
<th>HIV-TB (N = 68)</th>
<th>NHS (N = 150)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No + ve</td>
<td>% SEN</td>
<td>No + ve</td>
</tr>
<tr>
<td>IgG</td>
<td>109</td>
<td>62</td>
<td>10</td>
</tr>
<tr>
<td>IgA</td>
<td>91</td>
<td>52</td>
<td>34</td>
</tr>
<tr>
<td>IgM</td>
<td>16</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>IgG + IgA</td>
<td>144</td>
<td>82</td>
<td>38</td>
</tr>
<tr>
<td>IgG + IgA + IgM</td>
<td>145</td>
<td>83</td>
<td>39</td>
</tr>
</tbody>
</table>

TB = Pulmonary tuberculosis
HIV-TB = HIV seropositive pulmonary tuberculosis
NHS = Normal Healthy subjects
No + ve = Number of positives
Sen = Sensitivity
Sp = Specificity
Positive Predictive value = 0.90
Negative Predictive value = 0.83

4. Discussion

The routine diagnostic methods are not of much use in HIV-TB, due to the asymptomatic picture it presents. As an alternative, serologic assays have been performed for anti-tuberculous antibody. Both crude and semi-purified antigens have been used.

Among the available purified protein antigens, 38kDa and its epitopes have been well studied for the diagnostic utility in HIV-TB (Verbon et al., 1993; Wilkinson et al., 1997; Hendrickson et al., 2000). The results obtained with different studies have been variable and this antigen again has not proved to be useful uniformly.

The 30kDa (Ag 85B) antigen being an early secreted antigen, and found in the culture as early as Day 3, is a suitable candidate to be used in diagnosis. The serodiagnostic potential of this antigen has been extensively studied in adult TB cases by various investigators with the sensitivity ranging from 41-94% and specificity of 75-100% (Raja et al., 1994; Lim et al., 1999). This antigen, along with the species-specific alpha crystallin 16kDa have been purified in our laboratory and found to be useful in HIV seronegative pulmonary TB (Unpublished observation; Raja et al., 2002).

The 30kDa (Ag 85B) has been tried in different populations. McDonough et al. (1992), failed to detect antibodies to antigen 85B in patients with HIV-TB. Daniel et al. (1994) evaluated the diagnostic potential of this antigen among the HIV-TB patients and non-HIV infected TB patients in Uganda and reported 28% and 62% sensitivity respectively.

However, in our population, using 30kDa antigen alone, it was possible to obtain a reasonably good sensitivity of 84% with a specificity of 96.73% by combination of all three isotypes. The reasons may be the geographical variations in study population, as well as the fact that in our study isotypes other than IgG also was measured. In fact we found IgA positivity in several sera, which were IgG negative. Moreover, a high positivity 84.6% was obtainable in sputum smear negative HIV-TB group also, which is a diagnostic challenge.

The only other antigen reported in literature to have > 80% sensitivity for HIV-TB is the MTB81, malate synthase recombinant antigen (Hendrickson et al., 1993). The study reports 92% sensitivity in a cohort of a rather small number of 27 patients, majority of them from Uganda. The same paper also reports that in another cohort of 37 patients from South Africa, the same antigen offered only 70% sensitivity. Moreover the study group included mainly smear positive cases, except for 2 smear negative HIV-TB cases.

The 16kDa antigen had a much lesser sensitivity of 57%. Moreover, the 16kDa did not react with any of the sera, which were negative for anti-30kDa. Therefore, testing these 2 antigens together will not improve the test outcome. Verbon et al (1993) also reported a very low sensitivity of 7% and specificity of 97% for HIV-TB patients. Other than this no other studies have been carried out to evaluate the 16kDa antigen among HIV-TB cases.

Difference in antibody response based on the CD4 count has been observed for other antigens, for (e.g.), Lipoarabinomannan (LAM). Ratansuwan et al. (1997) used a MycoDot test for the diagnosis of tuberculosis in the HIV seropositive and negative patients. The sensitivity of the test in patients with CD4 counts > or = 200 cells/mm³ was significantly higher than in those with CD4 counts < 200 cells/mm³, Some other immune parameters like delayed hypersensitivity, were found not to be dependent on CD4 numbers. Diagbouga et al. (1998) have carried out studies to understand the correlation between the CD4 T-lymphocyte counts and induration sizes of the tuberculin skin test in HIV patients and could not find any difference.

In the present study, there was no significant difference in the mean antibody levels between the two groups of HIV-TB based on CD4 numbers. Thus, the antibody response did not seem to correlate with the T-cell help. This may also be due to the persistence of the earlier formed antibodies (before the CD4 counts dropped) in circulation.

Thus the 30kDa antigen alone was found to have a good sensitivity for diagnosis of HIV-TB, both smear negative and positive. The sensitivity was high irrespective of the
immunosuppression evidenced by CD4 counts. Also, combination of isotype response could be a promising approach.

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References


