TECHNICAL NOTE

Isolation and partial characterisation of the Triton X-100 solubilised protein antigen from Mycobacterium tuberculosis

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This report describes extraction of a new native antigen fraction from Mycobacterium tuberculosis without massive degradation of proteins by Triton X-100. The Triton X-100 solubilised protein (TSP) antigen showed a characteristic antigen profile and reproducible extraction pattern. To characterise the nature of their composition, the TSP antigen was fractionated by Triton X-114 phase partitioning. The TSP antigen contained a variety of lipids and glycoconjugates as well as diverse proteins. Most proteins were partitioned into the aqueous phase during phase fractionation, whereas non-protein molecules and lipoproteins were recovered in the detergent phase. The lymphoproliferative responses to the TSP aqueous fraction in healthy tuberculin reactors were significantly higher than those to the purified protein derivative (PPD) and unfractonated TSP. In contrast, the antibody responses to TSP aqueous fraction in tuberculosis patients showed weak reactivity. This study suggests that the TSP aqueous fraction can be used as a T-cell antigen associated with protective immunity against tuberculosis.

Introduction

Mycobacterial antigens recognised by T cells are believed to be important in inducing protective immune responses during infection. Although a significant number of studies have reported on cell-mediated immunity in human tuberculosis, no immunodominant protective antigens have been clearly identified [1]. Many studies have focused on secreted proteins [1–3] because live mycobacteria are more effective in inducing protective immunity than dead bacilli [3, 4]. The tuberculin purified protein derivative (PPD) has been used in most studies for immunological testing and in skin test reagent. However, PPD is a heat denatured mixture of proteins from Mycobacterium tuberculosis culture filtrates (CF), and its immunological activity may vary from lot to lot. Also, there is considerable variation in the antigenic content of the culture filtrates. Therefore, it is important to isolate a potential candidate antigen that can be substituted for PPD and to establish uniformity in its antigenic content.

A number of proteins are also found in association with the cell envelope of mycobacteria. Some of these are powerful immunogens [5–7] and may be involved in mycobacterial pathogenesis and immunity. Therefore the cell envelope-associated protein antigens were extracted from Mycobacterium tuberculosis with Triton X-100 (TX-100). Interestingly, the TX-100 solubilised protein (TSP) antigens seemed to comprise dominant T-cell antigen. However, mycobacteria express a variety of the hydrophobic glycoconjugates, such as oligosaccharides, glycolipids, lipo-arabinomannan (LAM), lipomannan (LM) and phosphatidylinositol mannosides (PIM) as well as proteins [8]. Thus more information about the component nature of the TSP antigen is required. This study used Triton X-114 (TX-114) phase partitioning, a procedure originally described by Bordier [9], to fractionate components of the TSP antigen according to their degree of hydrophobicity and to characterise the nature of their composition. Then the lymphoproliferative activities of the aqueous fraction from which the irrelevant non-protein molecules had been removed by TX-114 phase fractiona-
tion were examined and compared with those of PPD and the original TSP antigen.

Materials and methods

Preparation of culture filtrate and TSP antigen

*M. tuberculosis* H37Rv was grown for 6 weeks at 37°C as a surface pellicle on Sauton medium. The bacteria were harvested by centrifugation at 5000 g and washed twice with phosphate-buffered saline (PBS). The culture filtrates (CF) were sterile filtered and precipitated by ammonium sulphate 80%. The washed cells (100 g wet weight) were suspended in 200 ml of TX-100 1%/ImM mM phenylmethylsulphonyl fluoride (PMSF)/PBS and incubated with shaking at 200 rpm for 16 h at 37°C, and the precipitate was discarded by centrifugation. The TX-100 soluble extracts were sterile filtered, dialysed extensively against PBS, and then insoluble fractions were removed by centrifugation.

The concentrated extracts were precipitated with ammonium sulphate (10–90% saturation). The resulting precipitate was dialysed against PBS and used as the original TSP antigen. Protein concentrations were determined by a protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as the standard.

Phase partitioning of the TSP antigen with Triton X-114

Precondensation of TX-114 (Sigma) was performed by repeated dilution and phase separation as described by Bordier [9]. The detergent content in the stock of preconditioned TX-114 was 20% w/v.

Phase separation was performed as described by Hunter et al. [10] with a slight modification. Briefly, the TSP antigen was dialysed against 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl (TBS). TX-114 20% was added to the TSP antigen containing 10 mg of protein to give a final concentration of 2%, then mixed with TX-114 2%/TBS to produce a final volume of 10 ml. The mixed samples were chilled on ice, warmed to 37°C and then centrifuged at 7000 g for 10 min. The separated aqueous (Aq) and detergent (Dt) phases were then washed at least three times in the following manner. The Aq phase was cleansed by the repeated addition of TX-114 20% to a final concentration of 2% and then phase separated as described above. The Dt phase was diluted with nine parts TBS at 0°C, re-warmed, and centrifuged as described above. The resulting Aq phase was then dialysed against PBS and used as the TSP-Aq antigen.

Monoclonal antibodies (M Abs) and polyclonal antibodies

Specific M Abs for mycobacterial 17–19-kDa (HYT 6), 71-kDa (HAT1), 56-kDa (HBT3), 38-kDa (HBT12) and 65-kDa (CBA1) antigens were provided by the UNPD/World Bank/WHO Special Program for Research and Training in Tropical Diseases. Specific rabbit anti-30-kDa (antigen 85B or α-antigen) antisera have been described previously [11]. Polyclonal anti- *M. tuberculosis* (*M. tuberculosis*) antisera were produced by immunising two rabbits with ammonium sulphate 80% fraction of CF essentially as described previously [11].

SDS-PAGE and immunoblotting

The CF, TSP and phase-separated antigens were analysed by SDS-PAGE with polyacrylamide 12.5% resolving gel under reducing conditions [12]. The antigens separated in the gel were visualised by staining with Coomassie Blue or silver nitrate. For silver staining to enhance the appearance of LAM and PIM, a periodate oxidation step was added immediately following fixation [13]. To identify antibody reactivities, the separated proteins from SDS-polyacrylamide gels were transferred with 20 mM Tris-HCl buffer containing 192 mM glycine and methanol 20% to a nitrocellulose membrane (BioRad Laboratories, Richmond, CA, USA) with a semi-dry electroblotter [14]. The membranes were incubated with BSA 3%/PBS, and then with the M Abs or polyclonal antisera diluted in BSA 1%/PBS. Blots were also screened with pooled sera from six patients with bacteriologically diagnosed pulmonary tuberculosis. These patients were monitored in the Chungnam National University Hospital, Taejeon, Korea. After incubation with primary antibody, the blots were washed with PBS and 0.5 M NaCl/PBS/Tween 20 0.05%, and then incubated with appropriate peroxidase-conjugated secondary antibody (goat anti-human IgG, goat anti-rabbit IgG and goat anti-mouse Ig; Sigma). Colour development was accomplished by incubating washed blots in 4-chloro-1-naphthol (0.5 mg/ml) in 20 mM Tris-HCl buffer, pH 7.5, containing 500 mM NaCl and H2O2 0.06%.

Lymphocyte proliferation assays

Venous blood samples were collected from 15 PPD positive and 12 PPD negative healthy volunteers. Healthy individuals were students from the Chungnam National University, Taejeon, Korea. PPD positivity was established by skin induration after inoculation with 5 TU of PPD-RT23 (Statens Seruminstitut, Copenhagen, Denmark). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised blood by density sedimentation over Histopaque-1077 (Sigma). Cells from the interface were washed three times with RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) and resuspended at a concentration of 1 × 10⁶/ml in complete RPMI-1640 medium containing fetal bovine serum 10% (Gibco-BRL), penicillin G 100 U/ml, streptomycin 100 μg/ml and 5 × 10⁻⁵ 2-
mercaptoethanol. The cell suspensions were dispensed in 0.1-ml volumes in round-bottomed microtitration plate and cultured with 0.1 ml of antigens for 5 days at 37°C in air with CO₂ 5%. The TSP, PPD (Statens Seruminstitut) or TSP-Aq antigens were added at a concentration range of 0.1–10 μg/ml. All tests were performed in triplicate. At 18 h before harvest, 1 μCi of [³H]thymidine (Amersham, Buckinghamshire) was added to each well. The cells were harvested on to fibreglass paper and the incorporated radioactivity was measured in a liquid scintillation counter. The proliferative responses were expressed as stimulation index (defined as cpm in antigen-stimulated cultures/cpm in unstimulated cultures).

**Statistical methods**

Duncan's multiple range test was used to compare three samples; p < 0.05 was considered as significant. Student's t test was used to compare the two groups.

**Results**

**Isolation and TX-114 phase separation of the TSP antigen**

Primary studies showed that the TSP antigen extracted from *M. tuberculosis* H37Rv with TX-100 was a strong inducer of the T-cell response in healthy tuberculin reactors. To further characterise the composition of the TSP antigen, it was subjected to phase partitioning with TX-114. Fig. 1 shows the staining patterns of the Aq and Dt phases of the TSP antigens before and after TX-114 phase separation on SDS-polyacrylamide gels. While Coomassie Blue staining was specific for proteins, silver staining revealed the presence of both polysaccharides and proteins. The TSP antigen contained various components such as proteins, lipids, carbohydrates, LAM and LM.

Immunoblot analyses were performed to examine the distribution of antigenic determinants recognised by

![Fig. 1. SDS-PAGE analysis of the TSP antigen extracted from M. tuberculosis H37Rv.](image-url)

The TSP antigen was subjected to phase partitioning with Triton X-114 and analysed by SDS-PAGE. The gels were stained with (A) silver only, (B) silver stain containing periodic acid (periodate silver stain), and (C) Coomassie Brilliant Blue (CB stain). Lanes: 1, mol. wt standard; 2, original TSP antigen; 3, Aq phase of the TSP antigen (TSP-Aq); 4, Dt phase of TSP antigen (TSP-Dt); LAM, lipo-arabinomannan; LM, lipomannan. D, comparison of the TSP antigen and 6-week-old culture filtrate (CF).
different MAbs and polyclonal antibodies in 6-week-old CF and TSP antigen (Fig. 2). All screened antigens were detected in the CF and original TSP (Fig. 2IIIA and IIIB). However, the 65-kDa antigen (which is known to be easily degraded, producing multiple bands immunoblotting, as an indicator of the extent of lysis) appeared with more multiple bands in the CF than the original TSP.

More than 95% of the protein present in the TSP antigen was recovered in the Aq phase during TX-114 phase separation. The numbers in Fig. 1A indicate major distinct bands of the TSP antigen. Some antigen bands (nos. 15, 16, 17 and 18) were more distinct in the TSP-Aq fraction than the original TSP antigen. Antigen band 4 was present in both phases. The 30-kDa antigen (band 18) was detected in the Aq phase (Fig. 2IIIC, lane 5). The antigen band around 23-kDa (band 12), which was not stained clearly with Coomassie Blue, was most strongly stained with silver and was also present in large quantities in the TSP antigen (Fig. 1A).

**Fig. 2.** Immunoblotting analysis of the TSP antigen and CF of *M. tuberculosis*. The CF (lane a and panel A), original TSP antigen (lane b and panel B), TSP-Aq fraction (lane c and panel C) and TSP-Dt fraction (lane d and panel D) were subjected to SDS-PAGE and analysed by immunoblotting for the presence of various antigens. Nitrocellulose membranes were incubated with polyclonal anti-M.tbc antisera (I), the pooled sera from six patients with bacteriologically diagnosed pulmonary tuberculosis (II), or the following MAbs and polyclonal antibodies (III) (designations of the antigen recognised by MAbs are given in parentheses): lane 1, HYT6 (17–19 kDa); 2, HAT1 (71 kDa); 3, HBT3 (56 kDa); 4, HBT12 (38 kDa); 5, specific rabbit anti-30 kDa antisera; 6, CBA1 (65 kDa); MW, mol. wt standard.
Large amounts of LAM and LM were observed as diffuse bands in the 33–40-kDa region and the 23-kDa region, respectively, on treating gels with periodic acid during silver staining and were recovered exclusively in the Dt phase (Fig. 1B). In addition, prominent yellow-stained bands such as nos. 9, 11 and 13 on silver staining were also recovered in the Dt phase fraction (Fig. 1A, lane 4) and were not stained with Coomassie Blue (Fig. 1C). Immunoblot analyses showed that bands 9 and 13 were recognised by MAb HBT12, which recognises the well-defined 38-kDa lipoprotein and MAb HYT6 recognising the 19-kDa lipoprotein (Fig. 2IID, lanes 1 and 4), respectively. Thus, the application of extensive TX-114 phase separation to the TSP antigen allowed removal of lipids, carbohydrates, LAM and LM.

Comparison of the TSP and culture filtrate

The antigens present in the TSP antigen were compared with the antigens present in the 6-week-old CF of M. tuberculosis (Fig. 1D). Although the antigens >50 kDa showed similar patterns in both preparations, their antigen bands <43 kDa differed markedly.

Antibody reactivities to both antigen preparations in tuberculosis patients were also compared by immunoblotting (Fig. 2II). The pooled sera from six patients were used to exclude heterogeneity among donors in antibody responses to mycobacterial antigens. The antigen of 23 kDa (band 12 in Fig. 1A) was also detected in the CF on silver-stained gel (Fig. 1D, lane 2) and antibody reactivity to the 23-kDa antigen in tuberculosis patients was observed (Fig. 2II). The multiple reactive bands in the 25–80-kDa range were found in the TSP antigen and CF, but their relative patterns differed markedly (Fig. 2II, lanes a and b). Interestingly, no strong reactive bands were observed in the TSP-Aq fraction, except weak reactivity against the 23-kDa molecules and >50-kDa region (Fig. 2II, lane c). The multiple condensed bands in the 35–40-kDa region (probably corresponding to LAM) were found in the TSP antigen and a similar pattern of reactivity was also observed in immunoblotting with polyclonal anti-M. tuberculosis sera. It has been shown previously that the 30-kDa and 38-kDa antigens are major targets in the antibody response in tuberculosis [7, 11, 15]. These results suggest that the antigens present in the TSP antigen are considerably different from the antigen present in the CF.

Proliferative response to the TSP antigen

The biological activity of TSP-Aq antigen which removed the irrelevant non-protein molecule was monitored by T-cell proliferation and compared with those of PPD and the original TSP. Although considerable variability among donors was observed, the individual response to each antigen showed similar patterns (Table 1). The proliferative responses to all antigens were significantly greater in tuberculin-positive subjects than in tuberculin-negative subjects. The tuberculin-negative subjects produced either no stimulation or marginal increase in the lymphocyte response. Comparing the proliferative response with the three antigens at a concentration of 1.0 μg/ml, proliferative responses to the TSP-Aq were significantly greater than those to the PPD and the original TSP. Furthermore, at a concentration of 0.1 μg/ml, the TSP-Aq antigen stimulated T-cell proliferation, whereas the PPD failed to do so. While the original TSP at a concentration of 10 μg/ml failed to stimulate T lymphocytes, TSP-Aq induced significant proliferation (data not shown).

Discussion

In this work, a cell envelope-associated protein designated TSP antigen from M. tuberculosis H37Rv was isolated and partially characterised. It contains major antigens recognised by human T cells. The TSP-Aq antigen is a promising new antigen preparation which may be of value in studies for immunological testing. The mycobacterial cell extracts perhaps vary to a lesser extent in antigenic content than do CF. However, little is known of the composition, function and immunoreactivity of the protein antigens associated with the cell envelope of mycobacteria [6, 10], mostly because of the difficulty of resolution of proteins from a highly complex cell wall with an abundance of lipids and

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<tr>
<th>Subjects</th>
<th>Number tested</th>
<th>Mean (SD) stimulation index</th>
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<tr>
<td></td>
<td></td>
<td>PPD</td>
</tr>
<tr>
<td>Tuberculin-positive</td>
<td>15</td>
<td>6.87 (7.59)</td>
</tr>
<tr>
<td>Tuberculin-negative</td>
<td>12</td>
<td>2.20 (1.08)</td>
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<tr>
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*aPeripheral blood mononuclear cells were stimulated for 5 days with each antigen at a concentration of 1 μg/ml.

1 Significant difference (p < 0.05) among the antigens within tuberculin-positive subjects as determined by Duncan's test for multiple comparison of means.

2 Calculated by Student's t test.
lipo polysaccharide constituents. Thus, innovative approaches to allow the release of the intact proteins from the cell wall without disturbing protein structures or characteristics are required. The present study therefore used non-ionic detergent TX-100, which has been used for the mild solubilisation of a considerable number of membrane proteins, to isolate proteins associated with the cell envelope of *M. tuberculosis* H37Rv. It was possible to isolate large amounts of protein from *M. tuberculosis* and, more importantly, to obtain uniform extraction results in repeated experiments.

Proteins and other components of bacteria are differently localised in the bacterial cells. Disruption of mycobacterial cell walls and membranes usually occurred during the extraction process described above and may have led to the additional solubilisation of hydrophilic components of cytosolic origin. In fact, heat-shock proteins such as the 65-kDa and the 71-kDa fractions were detected in original TSP antigen, and the 65-kDa antigen was not detected in TSP-Aq. However, immunoblotting with MAb CBA1 (65 kDa) showed that a single band was seen in the TSP antigen, while multiple bands were seen in the long-term CF. These results indicate that the TSP antigen is extracted from *M. tuberculosis* without massive degradation of proteins and contains some proteins from the cytoplasmic fraction. Some of the TSP antigen was also present in the CF of long-term cultures. In addition, the proteins shared with the CFs may be considered a cell wall-associated antigen which is gradually released during growth. It is possible that such proteins are associated loosely with cell wall. This possibility was presented by Barnes et al. [5], who suggested that some T-cell antigens in an *M. tuberculosis* cell wall preparation may be secreted as well as cell wall associated. Therefore it is proposed that the TSP antigen is a mixture of numerous proteins associated with the cell envelope and a few secreted or cytoplasmic proteins.

Previous reports observed that the hydrophobic components – such as lipids, carbohydrates, LAM, LM and lipoproteins – segregated into the heavier detergent phase [6,9]. The present studies also showed that irrelevant non-protein molecules from the TSP antigen were removed by phase partitioning with TX-114. In particular, LAM, one of the *M. tuberculosis* cell-wall components, is known to suppress T-cell proliferation. Thus, the TSP-Aq fraction elicited more significant proliferation responses in healthy tuberculin reactors than the unfractionated original TSP.

Several studies have indicated that immune responses to mycobacterial antigens in the human and murine systems do not focus on one or a few dominant antigens, but are the sum of the response to a wide spectrum of antigens including cell wall-associated antigens, secreted antigens and intracellular heat-shock proteins [2,3,16]. The facts that the TSP-Aq fraction shows many protein bands on SDS-PAGE and strong T-cell proliferative responses in healthy tuberculin reactors are in agreement with those of the above-mentioned reports. In contrast, the antibody responses to TSP-Aq fraction in tuberculosis patients showed weak reactivity. Andersen et al. [3] also observed that none of the animals infected with *M. tuberculosis* were found to produce antibodies to antigens in short-term culture filtrate. Taken together, this study suggests the presence of potent T-cell targets in the TSP-Aq fraction.

Preliminary results from this laboratory may suggest that the TSP-Aq fraction, used for in-vitro stimulation, preferentially induces a Th1-like response in healthy tuberculin reactors [17]. It will be of interest, therefore, to explore further the immune responses to the TSP-Aq antigen in tuberculous patients and animal models.

This work was supported by a grant from the Ministry of Health and Welfare of Korea (96-M-2-0012).

**References**

