Original Article

Antigen 85A and Mycobacterial DNA-binding protein 1 are targets of IgG in individuals with past tuberculosis

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List of abbreviations:
Acr, alpha-crystallin like protein (also called HspX)
Ag85, Antigen 85 complex proteins (mycolyltransferases)
Ag85A, Antigen 85A
Ag85B, Antigen 85B (also called alpha antigen)
BCG, *Mycobacterium bovis* bacillus Calmette-Guérin
CFP10, 10-kDa culture filtrate protein
DosS, sensor histidine kinase of DosR
DosR, dormancy survival regulator
ELISA, enzyme-linked immunosorbent assay
ESAT6, 6-kDa early secretory antigenic target of *Mycobacterium tuberculosis*
HCs, *M. tuberculosis* uninfected healthy controls
HBHA, heparin-binding hemagglutinin
HrpA, heat-stress-induced ribosome binding protein A (also called Acr2)
LTBI, latent *Mycobacterium tuberculosis* infection
MDP1, mycobacterial DNA-binding protein 1 (also called Mt-HLP, HupB, LBP)
PPD, purified protein derivative
QFT, QuantiFERON-TB
TB, tuberculosis
TST, tuberculin skin test

ABSTRACT

Development of accurate methods for predicting progression of tuberculosis (TB) from the latent state has been recognized as being vitally important in controlling TB, because a majority of cases develop from latent infections. Past TB without medication has a higher risk of progressing than latent *Mycobacterium tuberculosis* infection alone. We evaluated antibody responses against 23 kinds of *M. tuberculosis* proteins in individuals with past TB who had not been medicated. The group showed significantly higher levels of antibodies against Antigen 85A, and mycobacterial DNA-binding protein 1 (MDP1) compared to those with active TB and uninfected controls. Besides, immunohistochemistry revealed colocalization of tubercle bacilli, Antigen 85 and MDP1 inside tuberculous granuloma lesions in an asymptomatic subject, showing that *M. tuberculosis* in the lesion expresses both Antigen 85 and MDP1. Our study suggests the potential usefulness of measuring antibody responses to Antigen 85A and MDP1 for assessing the risk of TB progression.

Key words:

Tuberculosis, humoral response, latent *Mycobacterium tuberculosis* infection, Rv2986c

INTRODUCTION

The World Health Organization (WHO) reports that *M. tuberculosis* latently infects 30% of the world's population, and nearly 8.8 million new cases of TB and 1.1 million deaths occur in 2011 (1).

M. tuberculosis-infected individuals can be classified into symptomatic and asymptomatic infection by the American Thoratic Society. The asymptomatic group is divided into past TB and latent *M. tuberculosis* infection (LTBI). Past TB indicates either history of active TB or abnormal stable radiographic findings of TB. Although LTBI indicates positive of tuberculin skin test (TST) or interferon gamma release assays (IGRAs) and no clinical and no radiographic evidences of active disease. Both past TB and LTBI have no bacteriological evidences of the disease. Individuals with past TB are more than likely to harbor persisting *M. tuberculosis*, because once infection is established, human host immunity alone is hard to eradicate *M. tuberculosis*. Individuals with past TB without current medication have higher risk of TB progression than LTBI alone (2-4). An important issue is the diagnosis of people at risk of TB progression so that appropriate preventative medication can be given. However, recent diagnostic methods, such as TST and IGRAs do not facilitate the assessment of the risk of TB progression (3, 4).

In the diagnosis of symptomatic *M. tuberculosis* infection, as the WHO warns, the currently available commercial kits for TB serodiagnosis are exceptionable, because of the broad and erratic responses among paucibacillary forms of TB. In contrast, antibody responses have the potential to track TB progression from asymptomatic infections, because they are correlated with bacterial burden (5). It is known that during the stable asymptomatic infection or after vaccination of *Mycobacterium bovis* BCG, antibody production is largely negative (6-8). By contrast, antibody titers increased to *M. tuberculosis* antigens before TB progression (9-12).

Identification of specific antibodies in asymptomatic *M. tuberculosis* infection is an important step in realizing the diagnosis of the risk of TB progression. It is conceivable that heterogeneous populations of tubercle bacilli at the dormancy and actively

multiplying phases are included within granulomatous lesions at the pre-recurrence status. In this study, we assessed antibody responses to 23 kinds of major *M. tuberculosis* proteins including expressed from growing and dormant bacilli (13-19), in individuals with past TB, who were not receiving current TB chemotherapy. The aim of this study was to explore biomarker-targeted antibody responses in those who have risk of TB progression.

MATERIALS AND METHODS

Study populations

The following individuals were enrolled (Table 1). 1) Healthy control (HC) group: 17 students (aged 20-24 years, males/females=9/8) at Osaka City University Medical School (Osaka, Japan). They were negative for TB by chest x-ray and immune-based assessment (TST and IGRAs), and were suspected of having no risks of *M. tuberculosis* infection, such as HIV infection, close contact, and chest x-ray findings. 2) Active TB group: 15 individuals (aged 35-71 years, M/F=13/2) diagnosed with active TB based on microbiologic examinations using either a positive culture for *M. tuberculosis* or a positive DNA amplification test specific for *M. tuberculosis* (TRC Test; TRCRapid-160, Tosoh, Tokyo, Japan) from sputum specimens. A positive IGRAs was obtained for all cases in this group. 3) Past TB group: 15 patients who had a definitive past history of pulmonary TB more than 5 years previously. Their bacteriologic examinations were negative in the sputum culture and nucleic acid amplification *M. tuberculosis* tests. Their chest X-ray showed sclerotic lesions and stable cavities. Since no infiltrating shadow was found around these cavities, cavitary lesions indicated a radiographic diagnosis of TB. In this group, 33% of individuals were IGRAs positive. Subjects were excluded from this study when disease due to nontuberculous mycobacteria was confirmed by repeated cultures and satisfied the American Thoracic Society guidelines (20). The serum specimens were assayed without knowledge of the patients' clinical characteristics. The studies in human subjects were approved by the research and ethical committees of the

National Toneyama Hospital and Osaka City University Graduate School of Medicine, and informed consent was obtained from all subjects.

Materials and reagents

pET-21b and Bugbuster HT were obtained from Novagen (Darmstadt, Germany). *Escherichia coli* BL21 (DE3) cells were purchased from Toyobo (Osaka, Japan). Lowenstein-Jensen (LB) medium and carbenicillin were from Sigma (St. Louis, MO, USA). Isopropyl-1-thio-beta-$_D$-galactopyranoside (IPTG), and Ni-NTA agarose was obtained from Qiagen (MD, USA). Skimmed milk was from Morinaga (Tokyo, Japan). Horseradish peroxidase (HRP)-conjugated anti-human IgG, IgA, or IgM antibodies and Envision kits were purchased from Dako (CA, USA). SureBlue reserve™ TMB microwell peroxidase substrate was from KPL, Inc. (Gaithersburg, MD). Monoclonal anti- alpha-crystalline like protein (Acr) antibody was from HyTest Ltd. (Turku, Finland).

Recombinant protein preparation

A pET-21b-based expression vector containing the full coding sequence for heparin-binding hemagglutinin (HBHA, Rv0475) was generated by a polymerase chain reaction (PCR)-based approach and maintained in *E. coli* BL21 (DE3) cells. The DNA sequence encoding HBHA was amplified from *M. tuberculosis* H37Rv DNA using appropriate primers and cloned into the *Nde*I and *Hind*III sites of pET-21b. The vectors expressing Acr, heat stress-induced ribosome binding protein A (HrpA, Rv0251c), early secreted antigenic target-6 (ESAT-6, Rv3875), culture filtrate protein10 kDa (CFP10, Rv3874), Antigen 85A (Ag85A, Rv3804c), and Antigen 85B (Ag85B, Rv1886c) were produced similarly by a PCR-based approach, with a bacterial chromosome. Each PCR product containing coding regions was designed to allow the expression of a C-terminal, 6× histidine (His)-tagged variants of the recombinant proteins following ligation into pET-21b. After construction, expression vectors were confirmed by DNA sequencing. Recombinant *M. tuberculosis* proteins were purified by utilizing Ni-NTA column (1 ml bed volume) according to the manufacture's instruction.

Enzyme-linked immunosorbent assay (ELISA)

The levels of IgG, IgA, and IgM antibodies were determined using recombinant proteins by ELISA. 96-well microplates (Sumilon Type H, LMS Co. Ltd, Japan) were coated with each recombinant antigen in bicarbonate buffer, pH 9.6 (0.5 μg/well) overnight at 4°C. The plates were blocked with PBS containing 0.05% Tween 20 and 5% skimmed milk for 12 hours at 4°C and washed four times with PBS containing 0.05% Tween 20. Plates were washed and then human serum samples diluted 1:100 in PBS containing 0.05% Tween 20 and 0.5% skimmed milk were incubated for 12 hours at 4°C. After washing the wells, HRP-conjugated anti-human IgG, IgA, or IgM antibodies was added at a 1:5000 dilution. After 1 hour incubation at 37°C, the plates were washed four times before 100 μl of SureBlue reserve-TMB was added to each well. The reaction was stopped after 3 minutes by adding 50 μl of 0.1 M HCl and the plates were read at 450 nm using a Multiskan (Thermo Fisher Scientific K.K., Yokohama, Japan).

Histopathology of granulomatous lesions

Immunohistochemical staining of paraffin-embedded lung sections was performed using the DAKO EnVision system and lung sections were stained with monoclonal anti-Acr (1:500), polyclonal anti- mycobacterial DNA-binding protein 1 (MDP1) (1:500) and polyclonal anti-Ag85 (1:1000) according to the manufacturer's instructions.

Statistical analyses

The optical density (OD) differences between study groups were determined using the Mann–Whitney U test. The receiver operating characteristic (ROC) curve analysis and the area under the curve (AUC) with respective 95% confidence intervals (CI) for each antigen were calculated with the GraphPad Prism version 5.0 (GraphPad Software, Inc. San Diego, CA, USA). In all analysis, P values < 0.05 were considered statistically significant.

RESULTS

The levels of serum antibodies against ESAT-6 and CFP10

We examined the levels of serum antibodies against ESAT-6 and CFP10, which are produced from *M. tuberculosis* during the growth phase, by ELISA. We found that the levels of IgG-class antibodies to ESAT-6 and CFP10 in the active TB group were significantly higher than those in the HC group ($p<0.05$, Fig. 1). In addition the level of IgG against CFP10 in the active TB group was significantly higher from those of the past TB group ($p<0.05$).

The levels of antibodies against 16 proteins encoded by the Dormancy survival regulator (DosR, Rv3133c) region of *M. tuberculosis*

In order to clarify antibody responses to latency-associated antigens of *M. tuberculosis*, we next examined the levels of serum antibodies against 16 proteins encoded by the DosR regulon, namely Rv0574, Rv0079, Rv1998, Rv2005, Rv2029, Rv2030, Rv2031c (Acr), Rv2032, Rv2623, Rv2624, Rv2628, Rv2629, Rv3127, Rv3129, Rv3132 (DosS), and Rv3134. With the exception of Acr and DosS, levels of IgG antibodies to DosR regulon-encoded proteins were low or absent in the three groups (Supporting information Fig. 1, Figs. 2A and 2B). The levels of IgG antibody to Acr were significantly higher in the active TB and past TB groups, compared with the HC group. However there was no difference in IgG levels to either Acr or DosS between the past and active TB group. In contrast to IgG antibodies, IgM and IgA antibody responses to the proteins were below measurable limits in all the tested individuals (data not shown).

The levels of antibodies to non-DosR regulon proteins

We next examined the IgG level to immunogenic *M. tuberculosis* proteins other than ESAT-6, CFP10, and DosR-regulon proteins. Although the IgG level to HBHA and HrpA in the past TB group was higher than in the active TB group, we could not find a

difference between the past TB and HC groups (data not shown). In contrast, the levels of IgG antibody against Ag85 and MDP1 were elevated in the past TB group compared to those in the HC and active TB groups ($p<0.01$, Fig. 2C-2E).

Sensitivity of IgG response to TB status

Based on these data, we performed ROC analyses on data from the past TB and HC groups (Fig. 3 and Table 2). Acr, Ag85A, and MDP1 produced ROC curves acceptable for diagnostics with AUC of 0.992 (95% CI: 0.972-1.013, $p<0.0001$), 0.965 (95% CI: 0.910-1.019, $p<0.0001$), and 0.973 (95% CI: 0.925-1.024, $p<0.0001$). Moreover, the AUC for Ag85A and MDP1 were 0.809 (95% CI 0.642-0.976, $p=0.0040$) and 0.858 (95% CI: 0.720-0.995, $p=0.0008$) in a ROC analysis between the past TB and active TB groups.

Tables 3 summarizes the sensitivity to the antigens in each group. These data showed that IgG responses to CFP10 and Acr were more often in the active TB group than the past TB or HC groups. The sensitivities of using CFP10 and Acr to active TB were 53.3%, and 60.0%, respectively. By contrast, the sensitivity of IgG responses to Ag85A (86.7%) and MDP1 (60.0%) was in the past TB group, when compared to active the TB and HC groups.

Colocalization of Ag85 and MDP1 with M. tuberculosis in human pulmonary granulomatous lesions

In order to confirm expression of Ag85 and MDP1 from persistent *M. tuberculosis*, we histopathologically examined the lung. We used the biopsied specimen from a patient who was negative for sputum bacteriology (both smear and culture) but microbiologic examinations of the biopsied sample showed *M. tuberculosis* infection. There was a solitary coin lesion in the chest X-ray, clinically diagnosed as a tuberculoma. We stained the biopsy section with the Hematoxylin-Eosin (H-E) (Fig. 4A), Ziehl-Neelsen (Fig. 4B, C), and antibodies to control (Fig. 4D), Ag85 (Figs. 4E), and MDP1 (Fig. 4F). The H-E stain showed typical granuloma formation with caseation in the center (Fig. 4A). Ziehl-

Neelsen technique revealed acid fast bacilli located in the center of the granuloma (Fig. 4B and C). The positive lesion with antibodies against Ag85 and MDP1 stained the area positive for Ziehl-Neelsen. Thus, this result demonstrated for the first time the colocalization of Ag85, MDP1, and persistent *M. tuberculosis* bacilli.

DISCUSSION

Asymptomatic *M. tuberculosis* infection represents a large pathogen pool cause reactivation. Therefore developing a diagnosis for those at risk of TB progression is an important challenge for successful control of TB. Although applying serodiagnosis for active TB is controversial, several lines of evidence suggest it has the potential to track disease progression. For example, several studies reported elevated antibody levels in sputum smear-positive population comparing to that in smear-negative population (6, 21) and increased antibody production when disease progresses in both macaques (5) and HIV-infected human cohorts (9-12). Since antibody titers and targets are different from each other (22, 23), identification of several antigens targeted by antibodies in people who have risk of TB progression is the first critical process. Because people possessing radiographic evidence of past or spontaneously cured TB have a higher risk of disease progression than LTBI alone, we selected subjects with past TB in this study and examined the level of antibodies against *M. tuberculosis* proteins.

Humoral antibody (IgG) responses of infected people to the major pathogenic proteins of *M. tuberculosis*, ESAT-6 and CFP10 that are produced at the growth phase, were seen in 20.0 and 53.3% of active TB group but only in 6.7 and 33.3% of the past TB group (Fig. 1 and Table 3). Indeed, only 33.3% of the past TB group were positive for IGRAs using these antigens. This suggested that both humoral and cell-mediated immune responses to ESAT-6 and CFP10 are low in the past TB group, demonstrating the difficulty of diagnosis of asymptomatic infection, which has potential to reactivate.

The DosR is considered as a key regulator of the adaptation of *M. tuberculosis* under hypoxic conditions (24-26). It has been reported that DosR regulon-encoded proteins are preferentially recognized by T cells of asymptomatic subjects with LTBI (14, 27). With

the exception of Acr and DosS, our present study has shown that the levels of antibodies were low among past TB group. Possible explanations for the present data are low antigenicity or expression level of DosR regulon-encoded proteins except for Acr and DosS. Another possible explanation is the location of DosR regulon encoded proteins, because antigens encoded by the DosR regulon are not secreted, and therefore humoral immune responses to such intracellular antigens cannot be evoked.

In this study, we have identified two candidate antigens, Ag85A and MDP1, for diagnosing the risk of TB progression. Ag85A is belonging to the Ag85 complex, consisting of 3 members of proteins designated Ag85A, Ag85B, and Ag85C. Ag85 genes are encoded by three genes located at different sites in the *M. tuberculosis* genome and show cross-reactivity as well as homology at amino acid levels (18). Amino acid homology between Ag85A and Ag85B is 82 %. The Ag85 complex has mycolyltransferase enzymatic activity, which translocates mycolic acids of trehalose 6-monomycolate (TMM) to free sugars, such as trehalose (28). Recent studies show that dynamic remodeling of the mycolic acid-containing glycolipids in the cell wall occurs *in vivo*, a process mediated by the broad substrate specificity of Ag85 (29, 30). Importantly, it has been reported that immune responses to glycerol monomycolate (GroMM) specifically occur in people with LTBI or BCG-vaccinated individuals but not active TB patients (31). Thus, Ag85-dependent exchange of TMM to GroMM is likely to occur in LTBI and BCG-vaccinated sites. Collectively these reports suggest that the detection of immune responses to Ag85 in this study is not surprising, and this protein is presumably useful to detect asymptomatic *M. tuberculosis* infection with the risk of TB progression.

MDP1 has pleiotropic functions and an essential protein for the survival of *M. tuberculosis* where it influences various biological functions of mycobacteria, from the growth phase to the hypoxic dormant state (32, 33). Our present data suggest the possibility of enhanced expression of this protein in latent rather than in the active disease state (Figs. 2 and 3). MDP1 has a suppressive activity on mycobacterial multiplication by controlling gene expression (33, 34) and also has ferritin-like activity controlling iron homeostasis (35). Iron is essential for the survival and multiplication of *M. tuberculosis* inside macrophages, whereas the host utilizes iron to generate bactericidal reactive oxygen intermediates. MDP1 stores iron inside bacteria and prevents the iron-dependent

generation of oxygen radicals (35). Such multifunctional activities of this protein should be involved in downshifting the growth rate and the long-term persistence of *M. tuberculosis* in the latent state, therefore resulting in obvious IgG production in the past TB group in the present study.

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DISCLOSURE

The authors declare no financial or commercial conflict of interest.

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FIGURE LEGENDS

Fig. 1. The level of IgG to ESAT-6 and CFP10 of *M. tuberculosis*

ELISA optical densities of recombinant antigens, ESAT6 and CFP10, indicating the antibody levels in sera from the HC (n=17), active TB (n=15), and past TB (n=15) groups. All results are expressed as individual data (OD) and geometric mean ± SD.

Fig. 2. The level of IgG to proteins encoded by the DosR regulon and other mycobacterial proteins

ELISA optical densities of DosR regulon-encoded recombinant antigens, Rv2031 (Acr) (A) and Rv2132 (DosS) (B), and DosR regulon-encoded recombinant antigens, MDP1 (C), Ag85A (D), and Ag85B (E) indicating the antibody levels in sera from the HC (n=17), active TB (n=15), and past TB (n=15) groups.

Fig. 3. Receiver-operator characteristics (ROC) curve of the IgG levels for *M. tuberculosis* antigens

The ROC curves for accuracies of single analyses to differentiate between past TB and HC group IgG levels.

Fig. 4. Histological analysis of human pulmonary past tuberculoma

(A) Hematoxylin and eosin stain. (B-C) Ziehl-Neelsen, acid-fast, stain. Immunohistochemistry staining with (D) control antibody, (E) Ag85 antibody, and (F) MDP1 antibody.

Table 1 Characteristics of the study population

	Healthy control (HC)	Active TB	Past TB
Number of participants	17	15	15
Age, mean (years)±SD	21.82±1.13	255.93±11.25	69.40±12.73
Age rang (years)	20-24	35-71	42-91
Male/female ratio	9/8	13/2	9/6
IGRA positive (%)	0	100	33.33

Table 2 Individual analyses against *M. tuberculosis* antigen results as past TB or HC

Antigens	Level			ROC analysis		
	HC (n=17)	Active TB (n=15)	Past TB (n=15)	AUC	95% CI	*p*-value
ESAT-6	0.137±0.094	0.297±0.325	0.210±0.065	0.782	0.62-0.95	0.0066
CFP10	0.077±0.096	0.662±0.836	0.151±0.305	0.702	0.52-0.88	0.0519
Acr	0.147±0.058	0.989±1.419	0.333±0.145	0.992	0.97-1.01	<0.0001
DosS	0.090±0.052	0.136±0.072	0.144±0.058	0.773	0.61-0.94	0.0087
Ag85A	0.984±0.570	1.899±0.928	2.918±0.839	0.965	0.91-1.02	<0.0001
Ag85B	0.836±0.437	1.110±0.630	1.611±0.497	0.886	0.77-1.00	0.0002
HBHA	0.890±0.207	0.485±0.388	0.923±0.347	0.526	0.31-0.74	0.8061
HrpA	0.260±0.304	0.172±0.122	0.341±0.205	0.651	0.46-0.85	0.1460
MDP1	0.112±0.145	0.269±0.141	0.509±0.238	0.973	0.92-1.02	<0.0001

Average±SD levels in HC, active TB, and past TB measured in ability to discriminate between IgG antibody levels of each subject

A ROC analysis was performed using the established past TB and HC groups as comparator group

Table 3 Sensitivity of antibodies against *M. tuberculosis* antigen in each group

Antigens	HC (n=17)	Active TB (n=15)	Past TB (n=15)
ESAT-6	0.0% (1)	20.0% (3)	6.7% (1)
CFP10	6.7% (1)	53.3% (8)	33.3% (5)
Acr	0.0% (1)	60.0% (9)	60.0% (9)
DosS	0.0% (1)	6.7% (1)	13.3% (2)
Ag85A	6.7% (1)	33.3% (5)	86.7% (13)
Ag85B	0.0% (1)	13.3% (2)	33.3% (5)
HBHA	6.7% (1)	6.7% (1)	20.0% (3)
HrpA	0.0% (1)	0.0% (1)	0.0% (1)
MDP1	0.0% (1)	13.3% (2)	60.0% (9)

Cut-off value was set as average±2SD of IgG levels in HC group
Sensitivity (%) and number of subjects

FIGURE LEGENDS FOR SUPPORTING INFORMATION

Supplemental Fig. 1. The level of IgG to proteins encoded by 16 DosR regulon proteins

ELISA optical densities of DosR regulon-encoded recombinant antigens, Rv0574, Rv0079, Rv1998, Rv2005, Rv2029, Rv2030, Rv2031, Rv2032, Rv2623, Rv2624, Rv2628, Rv2629, Rv3127, Rv3129, Rv3132, Rv3134 indicating the antibody levels in sera from the HC (n=17), active TB (n=15), and past TB (n=15) groups.