IMMUNOLOGICAL RESPONSE TO INFECTION

Purification and partial characterisation of a novel 66-kDa seroreactive protein of *Mycobacterium tuberculosis* H₃₇Rv

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Summary. A seroreactive protein (TB66) was purified from culture filtrate (CF) and cell sonicate (CS) of *Mycobacterium tuberculosis* H₃₇Rv by immobilised metal affinity chromatography (IMAC) on a Ni-nitrilotriacetic acid (NTA) column. The TB66 preparations obtained by IMAC contained predominantly a 66-kDa protein with a pI of *c.* 5.5 as determined by two-dimensional electrophoresis. TB66 was detected in the CF as early as 1 week of growth of H₃₇Rv. The NH₂-terminal amino-acid sequence showed 85 % homology with the N-terminal sequence of bovine serum albumin (BSA) and 80 % homology with human serum albumin. Amino-acid analysis indicated a difference in the amino-acid content of TB66 when compared to BSA, with an abundance of acidic amino acids. A monoclonal antibody (MAb) OD4AG3, raised in this laboratory against an *M. avium* complex (MAC 101) sonicate cross-reacting with H₃₇Rv sonicate, recognised a heat-stable and trypsin-sensitive epitope of this protein. TB66 was also recognised by MAbs IT1 and IT20 which also react with the 14-kDa antigen of the *M. tuberculosis* complex. Antibodies against TB66 were present in the sera of 62 of 64 patients with tuberculosis; sera from normal healthy individuals showed no significant reactivity. TB66 appears to be a predominant secretory protein of *M. tuberculosis* and could play an important role in the pathogenesis of this organism.

Introduction

Great efforts have been made in recent years to purify protein antigens from either mycobacterial cell extracts or culture filtrates.^{1,2} A set of prominent antigens from *Mycobacterium tuberculosis* has been identified by the use of murine monoclonal antibodies (MAbs).³ Secreted antigens are likely to provide the first stimulus *in vivo* for the humoral and cellular responses to mycobacteria and may be valuable in a serological diagnostic test. Several antigens have been identified from culture filtrates of *M. tuberculosis*;⁴ however, their limited specificity and sensitivity are major problems in the development of a definitive serological diagnostic test.^{5,6} For the development of novel vaccines and diagnostic tests for mycobacterial diseases, a major effort has to be invested in the identification and characterisation of specific mycobacterial antigens that are involved in interactions with the host immune system.

In this study, a novel 66-kDa protein from culture filtrate and cell sonicate of *M. tuberculosis* H₃₇Rv was

isolated and partially characterised by single-step immobilised metal affinity chromatography (IMAC). This protein was examined with a MAb raised in this laboratory, with MAbs IT1 and IT20 that are known to be specific for the 14-kDa antigen of the *M. tuberculosis* complex,⁷ and with sera from patients with tuberculosis and from normal healthy individuals.

Materials and methods

Preparation of cell sonicates and culture filtrates

M. tuberculosis H₃₇Rv (obtained from Dr T. Daniel, Case Western Reserve University, Cleveland, OH, USA) was grown in Middlebrook 7H9 Broth (Difco) with and without ADC enrichment (Difco) at 37°C. Cultures were harvested aseptically every week, from week 1 up to week 4, by centrifugation. The culture filtrate (CF) was filter-sterilised by filtration through a 0.45-µm filter and refrigerated until used. The cells were washed and suspended in saline and sonicated in ice in four continuous cycles of 3 min each with a W-375 sonicator (Heat Systems-Ultrasonics,

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Plainview, NY, USA) with a 100-W energy output. The supernate obtained after centrifugation of the sonicated cells was the cell sonicate (CS). Protein content was determined by the BioRad protein assay system (BioRad Laboratories, Richmond, CA, USA).

Sera

Sera from 66 patients with active pulmonary tuberculosis (TB) and from 10 normal healthy individuals (NHS) were used in this study. All test and control sera were obtained from India, identified according to their sources and stored in small volumes at -20°C .

MAbs

The murine MAb OD4AG3 was raised initially in this laboratory against the soluble sonicate of *M. avium* complex (MAC 101). During the screening of the culture supernates obtained from positive MAb-producing clones, one clone (OD4AG3) was found to produce a MAb that cross-reacted with H₃₇Rv sonicate. On Western blotting, this MAb was found to react with an *M. tuberculosis* protein with a mol. wt of c. 66 kDa. MAbs IT1⁷ and IT20⁸ which react with the 14-kDa antigen of *M. tuberculosis*, and MAbs IT13 and HAT3 which react with the 65-kDa and 70-kDa antigens of *M. tuberculosis*, respectively,^{8,9} were obtained from the WHO-IMMTUB monoclonal bank (courtesy of Dr T. Shinnick, Centers for Disease Control and Prevention, Atlanta, GA, USA).

Immobilised metal affinity chromatography

Proteins were purified with a nickel nitrilotriacetic acid (Ni-NTA) resin¹⁰ under non-denaturing and denaturing conditions with two buffer systems designated I and II respectively.

Non-denaturing conditions. Briefly, columns containing 1 ml of Ni-NTA resin (Qiagen, Chatsworth, CA, USA) were equilibrated with buffer A (0.5 M sodium phosphate buffer containing 0.5 M NaCl, pH 8.0). The CS and CF (2.5 ml each in buffer A) obtained from cultures harvested at weekly intervals were then loaded on to the columns. The column was washed with buffer A until absorbance (A_{280}) = 0 and bound proteins were eluted with buffer B (10 mM potassium phosphate buffer containing 0.75 M NH₄Cl, pH 6.6).

Denaturing conditions. Ni-NTA resin was equilibrated with 10 ml of buffer A (6 M guanidine hydrochloride, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 8.0). The CS and CF in buffer A were then loaded on to the columns. The samples were washed with 10 ml of buffer A, 10 ml of buffer B (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 8.0) and then with buffer C (buffer B, pH 7.0) until A_{280} = 0. The bound proteins were eluted with buffer D (buffer B, pH 5.9).

In both cases, samples (2 ml) were collected and those showing $A_{280} > 0$ were analysed by 12% SDS-PAGE (Mini-Protein II ready gels, BioRad) followed by staining with Coomassie Brilliant Blue R-250 (CBB). Fractions containing the 66-kDa protein (typically the first two to three samples) were pooled, dialysed and used for further studies.

Two-dimensional chromatography

The 66-kDa protein preparation (10 μl containing c. 5 μg of protein), obtained by IMAC of CS and CF, respectively, with buffer system I, was mixed with 10 μl of lysis buffer (urea 11.14 g, 4 ml of Nonidet P-40 10%, 0.2 ml of ampholytes at pH 3–10, 0.8 ml of ampholytes at pH 5–8, 1 ml of 2-mercaptoethanol and H₂O to 20 ml) and incubated at room temperature for 30 min. A sample was applied to immunoelectrophoresis tube gels (1 mm, pH range 3–10) and electrophoresis was performed at 400 V overnight. The gels were allowed to equilibrate in SDS reducing sample buffer and placed on top of an acrylamide 10% gel. Protein standards (2D SDS-PAGE markers, BioRad) were used to estimate the pI and the mol. wt of the protein in the sample. After electrophoresis, the gels were silver stained.

Amino-acid analysis and amino-terminal sequencing

The 66-kDa protein separated by two-dimensional (2-D) electrophoresis was transferred electrophoretically to polyvinylidene difluoride membranes (0.2 μm , BioRad) with the Transblot apparatus at a constant current of 270 mA for 90 min. The transfers were stained for 1 min in Coomassie Blue, briefly destained in methanol 40% and acetic acid 10% in water, washed in distilled water and air-dried. Protein was cut from the membrane, and amino-acid analysis and amino-terminal sequencing were performed by Dr J. Pohl, Emory University, Atlanta, GA, USA. Searches of the Gen Bank (release 72.0) and SWISS-PROT (release 22.0) data bases were conducted by FASTA software to determine sequence homology.

Western blotting

The 66-kDa proteins obtained by IMAC were subjected to 12% SDS-PAGE and transferred electrophoretically from the gel to nitrocellulose membranes (0.2- μm pore size, BioRad) by the method of Towbin *et al.*¹¹ with a Trans-blot apparatus (BioRad) and LKB 2002 Power supply. MAbs IT1 and OD4AG3 were used as primary antibodies. Peroxidase-conjugated rabbit anti-mouse polyvalent immunoglobulin (BioRad) diluted 1 in 1000 was used as the secondary antibody. The reaction was visualised with 4-chloro-1-naphthol and H₂O₂ as the substrate and was stopped by washing the membrane in distilled water. Trypsin-sensitive epitopes were identified by incubating the blotted antigen with trypsin 100 $\mu\text{g}/\text{ml}$.

Fig. 1. SDS-PAGE 12% of IMAC fractions of *H₃₇Rv*: **a** and **b**, CS and CF from *H₃₇Rv* grown in 7H9 containing ADC (buffer system I); **c** and **d**, CS and CF from *H₃₇Rv* grown in 7H9 without ADC (buffer system I); **e** and **f**, CS and CF from *H₃₇Rv* grown in 7H9 containing ADC (buffer system II). Lane **1**, mol. wt markers (top to bottom, kDa: phosphorylase b, 97; bovine serum albumin, 66; ovalbumin, 43; carbonic anhydrase, 31; soybean trypsin inhibitor, 21.5; lysozyme, 14.4).

at 37°C for 2 h before assessment of its immunoreactivity.

Indirect ELISA

The ELISA was performed in polystyrene 96-well flat bottomed microtitration plates (Falcon, Becton-Dickinson Labware, NJ, USA) as described previously.¹² Briefly, TB66 (0.5 µg of protein obtained by IMAC of *H₃₇Rv* CF) was used as antigen for coating the wells. Non-fat dried milk 10% was used as the blocking agent. To test the reactivity of TB66 with MAbs that recognise *M. tuberculosis* antigens, MAbs IT1, IT20, IT13 and HAT3 were used as primary antibodies. MAb OD4AG3 was also used as the primary antibody and peroxidase-conjugated rabbit anti-mouse polyvalent immunoglobulin (BioRad) was used as secondary antibody. For seroreactivity studies, sera were used at a dilution of 1 in 800 and peroxidase-conjugated rabbit anti-human polyvalent immunoglobulin (BioRad) diluted 1 in 1000 was used as secondary antibody. The reaction was visualised with *o*-phenylene diamine dihydrochloride and H₂O₂ as the substrate. The extinction (E) was measured in a Titertek Multiskan reader at 492 nm. Each serum sample was tested in duplicate and mean E was recorded. The cut-off value for a positive reaction was defined for each serum sample as the mean value of the control group (NHS) plus twice the standard deviation. This criterion was adopted to ensure the exclusion of doubtful or false positive results in the test. The results were analysed by analysis of variance; levels of significant difference were determined by Newman-Keuls multiple range test.

Results

Purification and characterisation of TB66 from *H₃₇Rv*

Ni-NTA chromatography (with buffer system I) of CS and CF of *H₃₇Rv* grown in 7H9 broth with and without ADC yielded a fraction that contained predominantly a protein with a mol. wt of 66 kDa (TB66) by SDS-PAGE and Coomassie Blue Staining (fig. 1). Addition of ADC enrichment to 7H9 broth enhanced the growth of *H₃₇Rv* leading to a greater yield of TB66 in both CS and CF, compared to that obtained when grown without ADC. Buffer system II also yielded TB66 from CS and CF of *H₃₇Rv* grown in 7H9 containing ADC (fig. 1). However, with buffer system II, no detectable protein could be obtained when CS or CF of culture grown without ADC was subjected to IMAC. TB66 appears in the CF and CS as early as the first week of growth, the concentration in CF being higher than that in CS. Sterile 7H9 broth containing ADC enrichment showed no binding to Ni-NTA. Heating of the CS and CF at 80°C for 10 min did not alter the binding of TB66 to Ni-NTA. Addition of CuSO₄ to the CF led to the precipitation of TB66. 2-D electrophoresis of TB66 from CS and CF showed a predominant spot with a mol. wt of 66 kDa and pI c. 5.5 in both cases. Fig. 2 shows the 2-D electrophoresis of IMAC fraction of *H₃₇Rv* CS.

Amino-acid analysis

The amino-acid composition of TB66 from CF and CS grown in 7H9 with and without ADC was found to be similar. The table shows the amino-acid analysis of TB66 in comparison to bovine serum albumin (BSA).

Fig. 2. Two-dimensional electrophoresis of IMAC fraction of $H_{37}Rv$ CS on SDS-PAGE 10%. The pIs and the mol. wts (kDa) of the markers are indicated on the top and on the left respectively. Egg white conalbumin, 76 and pI 6.0–6.6; bovine albumin, 66.2 and pI 5.4–5.6; bovine muscle actin, 43 and pI 5.0; glyceraldehyde 3-phosphate dehydrogenase, 36 and pI 8.4; carbonic anhydrase, 31 and pI 6.0; trypsin inhibitor, 21.5 and pI 4.5; myoglobin, 17.5 and pI 7.0). Arrow, 66 kDa protein.

Table. Amino-acid composition of TB66* and BSA*

Amino acid	Residues/molecule of TB66	Residues/molecule of BSA
Lysine	24.2	43.0
Histidine	10.8	12.8
Arginine	23.5	18.6
Aspartic acid	46.1	41.9
Glutamic acid	82.3	75.0
Serine	62.3	24.0
Threonine	18.1	16.3
Proline	15.4	19.8
Glycine	115.0	80.3
Alanine	38.4	58.2
Cysteine	2.3	10.2
Valine	20.0	35.5
Methionine	5.8	1.2
Isoleucine	17.0	17.5
Leucine	41.5	58.2
Tyrosine	13.8	15.1
Phenylalanine	16.2	17.5
Tryptophan	ND	ND
Total residues	552.7	545.1

ND, not detected.

* Based on a mol. wt of 66 kDa.

Fig. 4. Immunoblot of IMAC fractions of $H_{37}Rv$: a and b, CS and CF fractions treated with MAb IT1; c and d, CF and CS fractions treated with MAb OD4AG3; e, f, g and h, CS and CF fractions incubated with trypsin before treatment with MABs IT1 and OD4AG3. The positions and mol. wts of the markers are shown on the right.

The acidic amino acids were more abundant than the basic amino acids. The ratio of acidic to basic amino acids was 2.2. Tryptophan could not be detected by the method used for analysis. Aromatic amino acids represented 5.4% of the total residues. The ratio of non-polar to polar amino acids was 0.39.

Amino-terminal amino-acid sequence

The N-terminal amino-acid sequence of TB66 (from CS and CF) from $H_{37}Rv$ grown in 7H9 with and without ADC was found to be identical and is shown in fig. 3. It was found to have 85% identity with the N-terminal sequence of BSA and 80% identity with human serum albumin (fig. 3).

Immunoreactivity of TB66

TB66 showed a very strong reaction with MABs OD4AG3 and IT1 in immunoblotting (fig. 4). No reaction was observed with MABs IT1 and OD4AG3 when the protein was incubated with trypsin (fig. 4). In ELISA, the reactivity obtained with OD4AG3 was higher than that with IT1 or IT20; no reactivity was observed with MABs HAT3 and IT13. Heating of

	1																			20
TB66	D	K	H	K	S	E	I	A	H	R	F	K	D	L	G	E	X	P	F	K
a	D	T	H	K	S	E	I	A	H	R	F	K	D	L	G	E	E	H	F	K
	1																			20
	1																			20
TB66	D	K	H	K	S	E	I	A	H	R	F	K	D	L	G	E	X	P	F	K
b	D	A	H	K	S	E	V	A	H	R	F	K	D	L	G	E	E	N	F	K
	1																			20

Fig. 3. N-terminal sequence comparison of TB66 of $M. tuberculosis H_{37}Rv$ and other proteins. The amino-acid sequence of TB66 is aligned with that of: a, BSA; b, human serum albumin. Single letter symbols are used and the numbers refer to the position of the amino acid.

TB66 did not destroy its reactivity with the MAbs. TB sera showed 94% positivity (62 of 66) with TB66; there was no reactivity with NHS (none of 10 positive). The mean E value for TB sera was 0.85 (SD 0.32), that of NHS was 0.25 (SD 0.061).

Discussion

A 66-kDa protein (TB66) was purified by a single step by IMAC from CS and CF of *M. tuberculosis* H₃₇Rv grown in Middlebrook 7H9 broth with and without ADC enrichment. This protein contains epitope(s) common to the 14-kDa *M. tuberculosis* antigen, is distinct from the 65 kDa or 70 kDa antigen of *M. tuberculosis*, and has not been previously described. A 68-kDa extracellular protein identified as hsp71 from *M. tuberculosis* grown in 7H9 without ADC,¹³ and a 65–68-kDa protein from *M. tuberculosis* CS and CF which was not recognised by MAb IT20 have been described.¹⁴ A purified 67-kDa protein from CF of *M. tuberculosis* has been reported to have high specificity for detecting *M. tuberculosis* infection.¹⁵ A 68-kDa protein from *M. tuberculosis* CS and CF distinct from 65-kDa hsp has been identified by Western blotting with human MAbs.¹⁶ A 66-kDa protein that produced DTH in guinea-pigs has been isolated from *M. tuberculosis* by MAbs.¹⁷ However, the identity of these proteins with TB66 has not been established at the present time. TB66 did not react with any of the known MAbs that recognise the dominant antigens of *M. leprae*.¹⁸ Preliminary studies in this laboratory also indicate the presence of the 66-kDa protein in *M. bovis*, *M. africanum* and *M. tuberculosis* strain Erdman, but not in other mycobacterial strains, including *M. leprae*, *M. avium*, *M. chelonae*, *M. phlei*, *M. marinum*, *M. kansasii*, *M. fortuitum*, *M. xenopei* and *M. intracellulare*. Thus, TB66 appears to be a novel protein belonging to the *M. tuberculosis* complex and bearing some similarity with the amino-acid region 41–144 (recognised by MAb IT1) of the 14-kDa antigen of *M. tuberculosis*.¹⁹ However, it is distinct from the 14-kDa antigen as the N-terminal amino-acid sequence bears no homology with the established sequence of the 14-kDa antigen.¹⁹

Ni-NTA is known for its ability to bind proteins containing surface-exposed neighbouring histidine residues.¹⁰ TB66 showed strong binding to Ni-NTA and could be eluted with a high concentration of NH₄Cl, as reported earlier for the purification of superoxide dismutase (SOD) from a protozoan parasite by IMAC with a Cu-Superose column.²⁰ Proteins containing multiple surface-accessible histidine residues can be precipitated by CuSO₄.²¹ TB66 could be precipitated from CF by CuSO₄, which further confirms the presence of surface-exposed histidine

residues in the protein. In view of this fact, CF and TB66 were treated with iodoacetate, an alkylating agent that leads to carboxymethylation of imidazole of histidine. This strategy was followed by Maisano *et al.*²² who showed a decreased adsorption of modified protein on IMAC column. Not surprisingly, a significant reduction of binding of iodoacetate-treated CF and TB66 to Ni-NTA was observed.

TB66 appears to be a cell-associated as well as a secreted protein. It does not seem to be a product of autolysis as it is found in the CF within 1 week of growth. TB66 from CS as well as CF had identical mol. wts and an identical N-terminal amino-acid sequence.

MAb OD4AG3 recognised a heat-stable protein epitope of TB66. This MAb also reacted with BSA in ELISA. Furthermore, in view of the fact that the N-terminal of TB66 showed homology with BSA during the search for sequence homology with known proteins, amino-acid compositions of the two proteins were compared. They were found to be different, indicating that TB66 was distinct from BSA. Thorns and Morris²³ have reported a strong reaction between five MAbs produced against *M. bovis* sonicated extract and bovine albumin, and concluded that mycobacteria contain epitopes that are present in host tissue antigens. TB66 was also produced by H₃₇Rv grown in media lacking ADC enrichment such as 7H9 broth without ADC and Sauton medium, indicating that TB66 does not arise from the ADC additive and is distinct from BSA.

With Ni-NTA resin and guanidine-urea buffer, Kong *et al.*²⁴ have purified a recombinant 60-kDa protein of *M. tuberculosis* that displayed 61% amino-acid sequence identity with the 65-kDa antigen. However, we were unable to isolate the 60-kDa antigen from H₃₇Rv with Ni-NTA. TB66 is distinct from the 65-kDa antigen in that it is not recognised by MAb IT13 and its N-terminal amino-acid sequence shows no homology with the 65-kDa antigen.²⁵

TB66 obviously contains antigenic determinants recognised by sera from patients with tuberculosis. The presence of antibodies to TB66 might be a marker of infection by *M. tuberculosis*. However, the specificity and sensitivity need to be determined with a larger number of sera from patients with different stages of tuberculosis, other mycobacterial infections and from normal healthy individuals.

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