IMMUNOLOGICAL RESPONSE TO INFECTION

Skin reactivity and fibronectin-binding property of TB66 (66-kDa protein of *Mycobacterium tuberculosis*)

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Summary. A 66-kDa 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. TB66 was found to be a fibronectin-binding protein as determined by ELISA and could be purified by affinity chromatography with fibronectin-Sepharose. A similar 66-kDa protein could be isolated also from *M. bovis*, *M. bovis* BCG, *M. africanum* and *M. tuberculosis* H₃₇Ra by IMAC, but not from any other mycobacteria. The NH₂-terminal amino-acid sequence of TB66 from H₃₇Rv and *M. bovis* was identical and showed 85% homology with the N-terminal sequence of bovine serum albumin (BSA). A monoclonal antibody (MAb) OD4AG3 recognised a heat-stable and trypsin-sensitive epitope near the C-terminal end of TB66. This MAb also recognised the 66-kDa protein isolated from the other members of the *M. tuberculosis* complex. In tests of immunogenicity, TB66 elicited a delayed type hypersensitivity reaction in guinea-pigs immunised with either TB66 or with *M. tuberculosis* H37Rv. TB66 also elicited an antibody response in immunised guinea-pigs and stimulated murine macrophages to produce tumour necrosis factor.

Introduction

Mycobacterial diseases continue to be important causes of human morbidity and mortality. It is estimated that tuberculosis is responsible for as many as 26% of all avoidable adult deaths worldwide.¹ The tubercle bacillus contains several immunologically active components, most of which are widely shared among other mycobacterial species.² The development of novel vaccines and diagnostic tests for tuberculosis requires purified and well characterised antigens that are immunologically active and species-specific. However, the complex nature and chemical diversity which characterise the antigens of pathogenic mycobacteria have limited the preparation of pure, specific and standardisable protein antigens by the classical methods of purification from culture filtrates and cell extracts.^{3, 4}

Secreted antigens of *Mycobacterium tuberculosis* may be of particular significance in the development of protective immunity. Some secreted antigens have been shown to bind fibronectin (FN).⁵ FN-binding antigens are prominent components of short-term

culture filtrates (CF) of *M. tuberculosis*.⁶ FN, a large glycoprotein commonly found in plasma and extracellular matrices, has many biological activities. It binds to various macromolecules and participates in cell-surface interactions between eukaryotic cells and micro-organisms.⁷ It has been demonstrated that mycobacteria bind to FN and that BCG CF inhibited attachment of organisms to FN-coated surfaces.⁸ The 30/31-kDa proteins and 57–60-kDa proteins of *M. tuberculosis* have been shown to be FN-binding proteins.⁶ Mycobacteria and mycobacterial proteins are also known to be potent inducers of monocyte-derived tumour necrosis factor (TNF).^{9, 10}

We have identified a novel seroreactive 66-kDa protein (TB66) in cell sonicates (CS) and in short-term CF of *M. tuberculosis*.¹¹ The present study was aimed at further characterising this protein to determine its role *in vivo*.

Materials and methods

Preparation of CS and CF

M. tuberculosis H₃₇Rv, *M. avium* serotypes 4 and 8, *M. kansasii* and *M. fortuitum* (obtained from Dr T. Daniel, Case Western Reserve University, Cleveland,

OH, USA), *M. tuberculosis* H₃₇Ra and *M. bovis* BCG (obtained from Dr J. Crawford, Centers for Disease Control and Prevention, Atlanta, GA, USA), *M. bovis* ATCC 35740 and *M. africanum* ATCC 25420 were grown in Middlebrook 7H9 Broth (Difco Laboratories) without ADC enrichment at 37°C. Three-week-old cultures were harvested aseptically by centrifugation. The CF was filter-sterilised by passing through a 0.45-µm filter and refrigerated before use. 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, Plainview, NY, USA) with a 100-W energy output. The supernate obtained after centrifugation of the sonicated cells was used as the CS. Sonicates of *M. intracellulare*, *M. phlei*, *M. chelonae*, *M. xenopi*, *M. szulgai*, *M. vaccae*, *M. marinum*, *M. smegmatis* and *M. scrofulaceum* were kindly provided by Dr S. Chaparas, Bureau of Biologics, FDA, NIH, Rockville, MD, USA. The sonicate of irradiated purified armadillo-derived *M. leprae* cells (provided by Dr P. J. Brennan, Colorado State University, CO, USA) was prepared as described above. Protein was determined by the BioRad protein assay system (BioRad Laboratories, Richmond, CA, USA).

Monoclonal antibodies

Murine monoclonal antibodies (MAbs) were raised in this laboratory against the soluble sonicate of *M. avium* complex (MAC101). During the screening of the culture supernates obtained from the clones, one clone (OD4AG3) was found to produce a MAb that cross-reacted with H₃₇Rv sonicate in ELISA. On Western blotting, this MAb was found to react with an *M. tuberculosis* protein with an M_r of c. 66 kDa.

Immobilised metal affinity chromatography (IMAC)

Proteins were purified with a nickel nitrilotriacetic acid (Ni-NTA) resin¹² under non-denaturing conditions as described previously.¹¹ Briefly, columns containing c. 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 (in buffer A) were then loaded on to the column. The column was washed with buffer A until absorbance at 280 nm (A₂₈₀) was 0, and bound proteins were eluted with buffer B (10 mM potassium phosphate buffer containing 0.75 M NH₄Cl, pH 6.6). Samples (2 ml) were collected and those showing A₂₈₀ > 0 were analysed by SDS-PAGE with acrylamide 12% gels (Mini-Protean II ready gels, BioRad) followed by Coomassie Brilliant Blue R-250 (CBB) staining. Fractions containing the 66-kDa protein (typically the first two to three samples) were pooled, dialysed and used for further studies.

Fibronectin-Sepharose chromatography

The CF of strain H₃₇Rv was subjected to FN-Sepharose chromatography. Plasma FN (Sigma) was

bound to gelatin-Sepharose (Sigma) by the method of Vuento.¹³ CF from strain H₃₇Rv was then applied to the column equilibrated with 0.05 M Tris-HCl, pH 7.5, and material bound to FN was eluted with 1 M NaCl or with 20 mM EDTA in Tris buffer; 2-ml fractions were collected and assayed for the presence of protein by SDS-PAGE.

Amino terminal sequencing

The 66-kDa protein obtained from CF of strain H₃₇Rv and *M. bovis* was transferred electrophoretically from an SDS-PAGE gel 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, destained briefly in methanol 40% and acetic acid 10% in water, washed in distilled water, and air-dried. Protein was cut from the membrane and amino terminal sequencing was performed by Dr J. Pohl, Emory University, Atlanta, GA, USA. Searches of the Gen Bank (release 72.0) and SWISS-PROT (release 22.0) databases were conducted by FASTA software to determine sequence homology.

Western blotting

The 66-kDa proteins obtained by IMAC were subjected to SDS-PAGE 12% and transferred electrophoretically from the gel to nitrocellulose membranes (0.2 µm pore size, BioRad) by the method of Towbin *et al.*¹⁴ with a Trans-blot apparatus (BioRad) and LKB 2002 Power supply. MAb OD4AG3 was used as the primary antibody. 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.

Dot immunobinding assay

The assay was performed as described previously.¹⁵ Briefly, TB66 obtained from strain H₃₇Rv CF and CS was spotted on nitrocellulose and air-dried. The subsequent immunoassay was performed as described above. MAb OD4AG3 was used as the primary antibody and the effect of trypsin and carboxypeptidase on the reactivity of TB66 was determined by incubating the protein with each enzyme before assessment of its immunoreactivity.

Detection of FN-binding property of TB66

The ability of TB66 to bind FN was determined in two ways by ELISA. (1) TB66 (1 µg/well) was added to wells of a microtitration plate (Falcon, Becton-Dickinson Labware, NJ, USA) coated with FN

(1 µg/well). Bound protein was detected by MAb OD4AG3. (2) FN was added to wells coated with TB66 (1 µg/well) and bound FN was detected with affinity purified rabbit anti-FN polyclonal antibody (Sigma). Controls included TB66 added to bovine serum albumin (BSA)-coated wells and FN-coated wells without TB66. The reaction was visualised with the appropriate peroxidase conjugated secondary antibody, and *o*-phenylene diamine dihydrochloride and H₂O₂ as the substrate. The extinction was measured in a Titertek Multiskan reader at 492 nm.

DTH reaction

Three groups, each consisting of three male Hartley strain guinea-pigs weighing 400 g each, were used for the study. Each animal from groups I and II was given two subcutaneous injections, 3 weeks apart, of H₃₇Rv sonicate containing 1 mg of protein and 200 µg TB66 (from H₃₇Rv CF), respectively, in incomplete Freund's adjuvant (IFA). Animals in group III formed the control group and were given IFA only. Three weeks after the second injection, all the animals were tested with 0.1-ml intradermal injections of H₃₇Rv sonicate (5 µg), TB66 of H₃₇Rv (1 and 5 µg) and PPD. PPD (5 tuberculin units corresponding to protein 0.4 µg/0.1 ml) was kindly provided by Dr J. Crawford (CDC, Atlanta, USA). The reactions (diameter of erythema and induration) were read at 18, 24, 48 and 72 h. The animals were bled by cardiac puncture after 72 h and the serum from each animal was tested by ELISA for the presence of antibodies to TB66. Subsequently, sera from each group were pooled and the antibody titre was determined. The titre was defined as the last serum dilution that gave a positive reaction with TB66.

TNF assay

Supernates containing TNF activity were generated by adding TB66 to 16-mm diameter tissue-culture wells (Falcon) containing 10⁶ mouse macrophages of J774 (American Type Culture Collection [ATCC], Rockville, MD, USA) in 1 ml of RPMI 1640 medium supplemented with 2 mM L-glutamine, penicillin 100 units/ml and streptomycin 100 µg/ml. The macrophages were incubated with TB66 (5 µg) at 37°C in air with CO₂ 5% and harvested at 22–24 h. Control wells contained macrophages incubated without TB66. In some wells, TNF activity was generated by stimulation of macrophages with bacterial LPS (10 µg, Sigma). TB66 was also pre-incubated for 1 h at 37°C with MAb OD4AG3 before addition to macrophages, to determine whether this MAb neutralised TB66 and prevented it from stimulating the macrophages. As a control, MAb OD4AF1 (which does not recognise TB66) was also pre-incubated with TB66 and then added to the macrophages. The supernates were collected after sedimentation of the cells and were stored at –20°C until the time of assay. TNF activity was assessed by cytotoxicity for murine L-929 fib-

roblasts (ATCC) by the method described by Flick and Gifford¹⁶ with some modifications. Supernates from experimental and control wells were added to confluent monolayers of L-929 fibroblasts obtained by incubation of cells at a density of 2.5 × 10⁴ cells/0.1 ml/well (flat-bottomed tissue culture treated plates; Corning, NY, USA) in fetal bovine serum 20% and modified Eagle's medium for 24–48 h at 37°C. Actinomycin D (1 µg/ml) was added to each well and the cells were incubated for 20 h at 37°C. Cell lysis was determined by staining viable cells with 50 µl of neutral red dye (0.1% w/v) in phosphate-buffered saline (PBS) for 1 h at 37°C. The stained cells were washed with PBS and lysed with ethanol 50%–0.1 M monobasic sodium phosphate. Absorbance of neutral red was measured at 570 nm with a Titertek Multiskan reader. Measurements were the mean of eight replicates. The cytotoxicity of TB66 towards the fibroblasts, if any, was determined by incubating the protein with the fibroblasts and performing the assay as above. Cytotoxicity was defined as a reduction in the neutral red uptake by the fibroblasts which was observed as a decrease in absorbance of neutral red.

Results

Purification of TB66

Ni-NTA chromatography. IMAC of CS and CF of strains H₃₇Rv, H₃₇Ra, *M. bovis*, BCG and *M. africanum* yielded fractions that contained predominantly a protein with an M_r of 66 kDa. Fig. 1 a–e shows the Ni-NTA eluates obtained from the CF of these organisms. No binding to Ni-NTA was observed with any other mycobacterial CS or CF tested. Sterile 7H9 broth showed no binding to Ni-NTA.

FN-Sepharose chromatography. The experimental conditions used allowed the binding of FN to gelatin-Sepharose for subsequent use in the isolation of FN-binding proteins. CF of strain H₃₇Rv bound to FN-Sepharose and the bound material could be eluted with NaCl as well as EDTA. The eluates showed the presence of a 66-kDa protein by SDS-PAGE (fig. 1 f and g). CF did not bind to gelatin-Sepharose which was not coupled to FN. Also, NaCl or EDTA did not wash away the FN that was bound to the gelatin-Sepharose column.

Characterisation of TB66

The N-terminal amino-acid sequence of TB66 (from CS and CF) from strain H₃₇Rv and from *M. bovis* was found to be identical. The sequence was determined to be ASP-LYS/THR-HIS-LYS-SER-GLU-ILE-ALA-HIS-ARG-PHE-LYS-ASP-LEU-GLY-GLU-XXX-PRO-PHE-LYS and it was found to have 85% identity with the N-terminal sequence of BSA. Binding of FN to TB66 (coated to the wells of a polystyrene plate) could be detected in ELISA with anti-FN antibody

Fig. 1. SDS-PAGE (12%) of fractions obtained by IMAC of: **a**, *M. bovis* BCG; **b**, $H_{37}Ra$; **c**, *M. bovis*; **d**, $H_{37}Rv$; *M. africanum*; and those obtained by **(f)** NaCl or **(g)** EDTA elution of FN-Sepharose bound material of $H_{37}Rv$ CF. Lane **1**, mol. wt (kDa) markers (top to bottom: phosphorylase b, 97·4; bovine serum albumin, 66·2; ovalbumin, 43; carbonic anhydrase, 31; soybean trypsin inhibitor, 21·5; lysozyme, 14·4).

Fig. 2. Immunoblot analysis of IMAC fractions of: **a**, *M. bovis* BCG; **b**, $H_{37}Ra$; **c**, *M. africanum*; **d**, *M. bovis*; **e**, $H_{37}Rv$ and of **(f)** NaCl and **(g)** EDTA eluates obtained by FN-Sepharose chromatography. MA b OD4AG3 was used as the primary antibody. The positions and mol. wts of the pre-stained markers are shown on the left.

(OD₄₉₂ c. 0·6). Also, TB66 bound to FN-coated wells could be detected with MA b OD4AG3 (OD₄₉₂ c. 0·7).

Immunoreactivity of TB66

TB66 obtained by IMAC of all the members of *M. tuberculosis* complex showed a very strong reaction with MA b OD4AG3 in immunoblotting (fig. 2a–e). TB66 obtained by FN-Sepharose chromatography of strain $H_{37}Rv$ CF also showed the presence of OD4AG3 reactivity (fig. 2f and g). No reaction was

observed in the dot immunobinding assay with MA b OD4AG3 when TB66 was incubated with trypsin (fig. 3). Treatment of TB66 with a mixture of carboxypeptidases A and B for 15 min produced a protein of c. 60 kDa which was not recognised by MA b OD4AG3 in the dot immunobinding assay (fig. 3).

DTH reaction

The results are summarised in the table as the average diameter of erythema observed in all animals

Fig. 3. Dot immunobinding assay of TB66 with MAb OD4AG3: a, b, TB66 from H37Rv CS and CF; c, d, TB66 from CS and CF after trypsin treatment; e, f, TB66 from CS and CF after carboxypeptidase treatment.

Table. DTH reaction in guinea-pigs at 24 h

Average diameter (mm) of erythema with Sensitising antigen	Eliciting antigen			
	TB66		H ₃₇ Rv CS	PPD
	1 µg	5 µg		
H ₃₇ Rv sonicate	5	11	18	5
TB66	6	10	15	5
None (control)	NR	NR	NR	NR

NR, no reaction.

from a single group. TB66 at 5 µg as well as 1 µg concentration was effective in eliciting DTH in animals sensitised with H₃₇Rv sonicate or with TB66 itself. Maximum reactions were observed at 24 h. No reaction was observed in any animal from the control group. The reactions observed with 5 µg of TB66 were greater than those observed with PPD. Sera obtained from guinea-pigs sensitised with H₃₇Rv sonicate as well as TB66 showed the presence of antibodies to TB66. The titre in both cases was 4000, as determined by ELISA.

TNF assay

Addition of 50 µl of supernate obtained from TB66-treated macrophages to L-929 cells produced a 50% reduction in neutral red uptake by the fibroblasts when compared to the uptake by untreated fibroblasts. No cytotoxicity to the L-929 cells was observed when TB66 and supernate from untreated macrophages were added to the fibroblasts. Supernate from LPS-treated macrophages produced a 75% reduction in the A₅₇₀. A concentration as low as 1 µg of TB66 stimulated TNF production by macrophages. TB66 pre-incubated with MAb OD4AG3 did not stimulate TNF production (no reduction in neutral red uptake), but protein incubated with MAb OD4AF1 caused a 45% reduction in the A₅₇₀.

Discussion

There is considerable interest in the identification of immunodominant and potentially protective protein

antigens of *M. tuberculosis* because of the current increase in tuberculosis world wide. Earlier, we reported the isolation of a 66-kDa protein (TB66) from *M. tuberculosis* in a single step by IMAC.¹¹ This seroreactive protein was found to be distinct from the well-characterised immunodominant proteins of *M. tuberculosis*. A heat-stable and protein epitope of this protein was recognised by MAb OD4AG3 raised in this laboratory. OD4AG3-reactivity was abolished in the 60-kDa protein which was produced after carboxypeptidase treatment of TB66, suggesting that the epitope lies within a 60 amino acid region at the C-terminal end of TB66. No such protein could be isolated from mycobacteria other than the members of the *M. tuberculosis* complex. Even MAC101, against which MAb OD4AG3 was raised, did not bind to NiNTA, suggesting that MAC101 possess an OD4AG3-reactive protein distinct from TB66, which thus appears to be an *M. tuberculosis*-complex-specific protein.

TB66 was found to bind FN and could be purified by FN-Sepharose chromatography. Neither the salt nor the EDTA eluates obtained by FN-Sepharose chromatography contained the well-known FN-binding antigens of *M. tuberculosis*—i.e., the 30–32-kDa or the 57–60-kDa proteins.⁶ It is possible that, under the conditions used here for chromatography, these proteins do not bind to FN, or are bound to FN-Sepharose but could not be eluted with NaCl or EDTA. Abou-Zeid *et al.*⁶ have reported their unsuccessful attempt to isolate the FN-binding antigens by FN-Sepharose, because of the strong binding of these antigens to FN. MAb OD4AG3 was found to bind strongly the synthetic 37 amino acid FN-binding

peptide D3 (Sigma)¹⁷ in ELISA (OD₄₉₂ c. 0.6). Binding of MAb OD4AG3 did not inhibit the FN-binding capacity of D3 or TB66, suggesting that the epitope recognised by this antibody is not involved in the FN-binding function. The OD4AG3-binding region of peptide D3 probably lies within the N1–N20 region of D3 because the FN-binding site has been shown to be within the 21–33 amino acid region.¹⁷

Mycobacterial preparations such as CF, PPD and antigen 5 are known to stimulate monocytes and alveolar macrophages to produce TNF.⁹ CF proteins of 150, 92, 60, 58, 46 and 20 kDa have been reported to have TNF stimulating activity.^{10, 18} This is the first report that a 66-kDa protein of *M. tuberculosis* stimulates TNF production by macrophages *in vitro*. It remains to be determined whether, and how, TNF may be relevant to the immunopathogenesis of tuberculosis. The direct stimulatory effects of mycobacterial constituents may provide the basis for the apparent restricted specificity of suppression by monocytes in tuberculosis.

TB66 at a 1- μ g concentration produced DTH reactions in TB66- and H₃₇Rv-sensitised guinea-pigs.

Also, 400 μ g of TB66/animal was sufficient to induce a good DTH reaction in guinea-pigs tested with TB66, H₃₇Rv sonicate or PPD. A good antibody response to TB66 was elicited in all the guinea-pigs sensitised with TB66 or H₃₇Rv sonicate. Antibodies to TB66 have been detected in sera from patients with tuberculosis,¹¹ indicating the involvement of TB66 in the human antibody response to *M. tuberculosis*. The FN-binding property of TB66 could be important in the pathogenesis of *M. tuberculosis*. However, even avirulent members of the *M. tuberculosis* complex produce this protein, ruling out the possibility that TB66 is associated with virulence. The antigenicity of TB66 might be very beneficial in the development of a vaccine. It could also have potential as a reagent for DTH testing.

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