Antigens in culture supernatant of *Mycobacterium tuberculosis*: epitopes defined by monoclonal and human antibodies

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Antigens of *Mycobacterium tuberculosis* found in the supernatant of heat-treated cultures were characterized in order to explore whether antigens from this source could be used for the development of a serological test. Culture supernatants and sonicates of 12, 25 and 39 d cultures were analysed by SDS-PAGE. In culture supernatant, major protein bands of 65, 24, and 12 kDa were visible after Coomassie brilliant blue staining. Using murine monoclonal antibodies in Western blots, a pattern of protein bands distinct from that of the corresponding *M. tuberculosis* sonicates was found in all the culture supernatants. Gel permeation chromatography, in the presence of SDS, was used to separate the major protein bands in the culture supernatant. In ELISA, sera from 20 of 26 patients with tuberculosis reacted with fractions containing mainly 24 kDa or 12 kDa proteins, whereas none of the control sera reacted. In Western blots, each patient serum had its own characteristic banding pattern with culture supernatant, but all the sera from tuberculosis patients and control subjects reacted with protein bands of 65, 61, 58, 30 and 24 kDa. The 12 kDa protein was recognized only by sera from patients with tuberculosis in both Western blots and ELISA. This suggests that different kinds of epitopes on proteins of *M. tuberculosis* are detected by human antibodies in Western blots and ELISA. We assume that epitopes recognized in Western blots by patients with tuberculosis and control subjects are ubiquitous and are also present on normal commensal bacteria. Epitopes recognized by only some patients with tuberculosis in Western blots may be linear and *M. tuberculosis* specific. Epitopes recognized by tuberculosis patients but by none of the control subjects in ELISA may be conformation related and *M. tuberculosis* specific. The major protein bands found in supernatants of heat-treated cultures, 24 and 12 kDa, possess epitopes that may be *M. tuberculosis* specific and are potentially valuable for the development of a serological test.

Introduction

Live *Mycobacterium bovis* BCG vaccine induces higher levels of resistance to tuberculosis than dead bacilli or their cellular components in experimental animals (Collins, 1984). This may be explained by secretion of immunostimulating antigens by the live *M. bovis* BCG in the host tissue. Secreted antigens are likely to provide the first stimulus *in vivo* for the humoral and cellular response to mycobacteria and thus may be more valuable in a serological test than *M. tuberculosis* antigens derived from dead bacilli. Secreted antigens may also be

produced by mycobacteria cultured *in vitro*. Several antigens have been identified from the culture supernatant of *M. bovis* BCG and *M. tuberculosis* (Closs *et al.*, 1980; Wiker *et al.*, 1988; Abou-Zeid *et al.*, 1986, 1988a; Daniel & Anderson, 1978; De Bruyn *et al.*, 1989; Harboe *et al.*, 1986). The culture supernatant proteins of *M. tuberculosis* produce a significantly delayed foot-pad swelling in mice vaccinated with *M. bovis* BCG and a proliferative response in *M. tuberculosis*-sensitive human blood mononuclear cells (Collins *et al.*, 1988).

Limited specificity and sensitivity are still major problems in the development of a serological test for tuberculosis (Ivanyi *et al.*, 1988), although purified proteins from sonicated *M. tuberculosis* and from the culture medium were used (Daniel & Debanne, 1987;

Abbreviations: CBB, Coomassie brilliant blue; mAb, monoclonal antibody.

Harboe *et al.*, 1986; Jackett *et al.*, 1988). Jackett *et al.* (1988) found that sera from different patients recognized different antigens and that the sensitivity of a sero-diagnostic test was increased by combining the results of ELISA using different antigens.

The purpose of the present study was to explore whether a combination of antigens released *in vitro* by *M. tuberculosis* can be used for a serological test for tuberculosis. We characterized the antigens in the culture supernatant and compared them with the antigens detectable in sonicates of *M. tuberculosis*.

Methods

Mycobacteria. The *M. tuberculosis* strain used was isolated in 1983 from sputum of a patient with tuberculosis. The bacteria were cultured on Löwenstein-Jensen medium at 37 °C and subcultured every 2 months.

Preparation of culture supernatant and sonicate. *M. tuberculosis* was first adapted to Sauton medium by transfer from Löwenstein-Jensen medium to a 25 ml capped bottle containing 10 ml Sauton liquid medium (2 mM-MgSO₄, 10 mM-citric acid, 2.5 mM-K₂HPO₄, 30 mM-asparagine, 0.1 mM-ferric ammonium citrate, 830 mM-glycerol, in Milli Q water, final pH 7.2). After incubation at 37 °C for 3 weeks, the bacterial suspension was transferred to a 1 litre flask containing 250 ml Sauton liquid medium and incubated in a horizontal position at 37 °C for 3 weeks (designated preculture).

For preparation of culture supernatant, 30 ml of preculture was inoculated into 500 ml Sauton liquid medium in a 1 litre conical flask, with a cotton plug. This suspension was cultured at 37 °C with shaking at 120 r.p.m., using a gyratory shaker (New Brunswick Scientific). Bacteria were killed by pasteurization of the suspension in a waterbath at 75 °C for 15 min: the temperature of the suspension rose from 20 °C to 71 °C in 10 min and remained at this temperature for 5 min. The suspensions were centrifuged at 16000 *g* for 20 min at 4 °C. The supernatant was centrifuged again under the same conditions. Bacteria in the pellet were washed twice with distilled water, sonicated in distilled water for 1 h at 50% duty cycle with 100 W power, using a Branson sonifier, and then lyophilized. The supernatant was concentrated in a vacuum rotary evaporator. The remaining volume was dialysed for 2 d against distilled water, using a dialysis membrane with a cutoff point of 10 kDa, and subsequently lyophilized.

Gel permeation chromatography. The 12, 25 and 39 d lyophilized culture supernatants of *M. tuberculosis* were solubilized to a concentration of 20 mg dry weight ml⁻¹ in 2.8% (w/v) sodium dodecyl sulphate (SDS) and 0.2 M-sodium phosphate buffer, pH 6.8. The solution was heated for 5 min at 100 °C and centrifuged at 100000 *g*. The supernatant was passed through a 0.45 µm filter and applied in portions of 100 µl to two GF 250 columns (9.4 × 250 mm, Du Pont) used in series. An LKB liquid chromatography system was used. The columns were eluted with 0.1% SDS in 0.2 M-sodium phosphate buffer, pH 6.8, at a flow rate of 0.5 ml min⁻¹.

Protein content. The protein content was determined by a method using bicinchoninic acid (Smith *et al.*, 1985).

SDS-PAGE. This was done as described by Laemmli (1970), on 13% (w/v) slab gels. The antigens were diluted in a sample loading buffer [0.064 M-Tris/HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.002% bromophenol blue, 5% (v/v) mercaptoethanol] and were heated to 100 °C for 5 min. Gels were either stained with Coomassie brilliant

blue (CBB) or used for immunoblotting. For CBB staining, 15 µg protein was used per 4 mm slot and the gels were incubated overnight at room temperature in a solution of 0.05% CBB in 25% (v/v) methanol and 5% (v/v) acetic acid. Gels were destained by shaking in a solution of 25% methanol and 5% acetic acid for 8 h. For immunoblotting, 7.5 µg protein was used per slot. The Pharmacia electrophoresis calibration kit was used with the following molecular mass markers: phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa.

Western blot analysis. Antigens separated by SDS-PAGE were transferred to nitrocellulose and used for immunoblotting as described previously (Kolk *et al.*, 1989). In brief, after electrophoretic transfer, the nitrocellulose sheets were incubated in 0.5% Tween 20 in phosphate-buffered saline (PBS), pH 7.2, to block free protein-binding sites. Mouse monoclonal antibodies (mAbs) or human sera were applied to strips of nitrocellulose membranes or to sheets in narrow lanes using a Miniblotter (Immunitics, Cambridge, Massachusetts, USA). After washing, the sheets were probed with peroxidase-conjugated heavy and light chains (IgG H+L), sheep-anti-mouse or goat-anti-human immunoglobulins (Pasteur Institute, Paris, France) diluted 1:1500 in PBS with 0.5% Tween 20. The reaction was visualized by incubating the sheets with tetramethylbenzidine substrate solution.

ELISA. Polystyrene microtitre plates with high binding capacity (Greiner, Nürtingen, FRG) were coated with 0.6 µg protein per well of culture supernatant, 0.8 µg protein per well of sonicated mycobacteria or gel permeation fractions diluted 1:20 in 0.05 M-sodium bicarbonate buffer, pH 9.6, and incubated for 1 h at 37 °C. The percentage of SDS during the coating in ELISA plates was always lower than 0.005%. SDS concentrations higher than 0.005% interfere with protein coating. As a control, wells were coated with 0.1% polyvinylpyrrolidone (PVP, molecular mass 40 kDa) in 0.05 M-sodium bicarbonate buffer. The plates were washed four times with PBS, then incubated for 1 h at 37 °C with 0.1% PVP in PBS, pH 7.2, to block non-specific binding. They were then washed four times with 0.05% Tween 20 in PBS (PBST) and incubated for 1 h at 37 °C with human sera diluted 1:400 in PBST containing 10% (v/v) normal goat serum. The plates were washed four times with PBST and incubated for 1 h at 37 °C with goat-anti-human IgG (H+L) (Pasteur Institute, Paris, France), diluted 1:1000 in PBST containing 10% normal goat serum. After washing four times with PBST, 100 µl substrate solution, prepared by mixing equal volumes of tetramethylbenzidine solution (1.2 mg ml⁻¹ in methanol) and 0.03% H₂O₂ in 50 mM-sodium citrate, 0.1 M-K₂HPO₄, pH 5.0, was added to each well and incubated for 1 h at room temperature. The plates were read at 405 nm in a Titertek Multiscan MCC. Results are given as the 'absorbance index', calculated by expressing the result for the test serum as a fraction of the binding of a high positive reference serum after correcting for reaction in control wells coated only with PVP: absorbance index = [(A₄₀₅ of serum with antigen) - (A₄₀₅ of serum with PVP)] / [(A₄₀₅ of ref. serum with antigen) - (A₄₀₅ of ref. serum with PVP)]. The cutoff value for a positive reaction was defined for each antigen as mean value of the control group plus three times the standard deviation.

Monoclonal antibodies (mAbs). Species-specific and cross-reactive mAbs against *M. tuberculosis* and mAbs against *M. avium*, *M. leprae* and *M. kansasii*, cross-reactive with *M. tuberculosis*, were used (Kolk *et al.*, 1984, 1986, 1989; Van Schooten *et al.*, 1988): see Table 1. SA-12 (Minden *et al.*, 1984) was kindly supplied by Dr P. Minden, Scripps Clinic, La Jolla, California, USA and Dr P. Kelleher, The Woodlands, Texas, USA.

Human sera. (a) *Patients with tuberculosis.* Samples of sera were obtained from 26 Caucasian patients with active pulmonary tuber-

culosis who were either untreated or had been treated for less than 2 months. Patients were selected on the basis of at least one positive culture from sputum or bronchoalveolar lavage. One patient also suffered from acquired immunodeficiency syndrome (AIDS).

(b) *Control group.* Samples of sera were obtained from 20 Caucasian control subjects and one individual from India: four BCG-vaccinated individuals (including the person from India); five skin-test-positive persons, without a history of BCG vaccination or previous tuberculosis; eight skin-test-negative, not BCG-vaccinated, healthy individuals; two patients with *M. avium* infection, diagnosed by positive cultures from the sputum and blood; two patients with leprosy, of whom one had lepromatous leprosy and one borderline lepromatous leprosy.

(c) *Reference serum.* This was a sample of serum from a patient with pulmonary tuberculosis; the ELISA A_{405} value with the antigens used was around 2.6.

Statistical analysis. Data were examined by an analysis of Student's *t*-test for differences between two independent means.

Results

Separation of culture supernatant by SDS-PAGE

Cultures of *M. tuberculosis* were harvested after 12, 25 and 39 d. After SDS-PAGE and CBB staining (Fig. 1), the 39 d culture supernatant showed major bands at 65, 24 and 12 kDa and faint bands in the 70, 61, 58, 50 and 30 kDa regions. The antigens present in the 39 d culture supernatant were also detectable at day 12 and 25, when the yield of *M. tuberculosis* and protein in the culture supernatant was low. No additional bands were seen in the 39 d culture supernatant. The antigens found in the culture supernatant of *M. tuberculosis* constituted a subset of those present in sonicate of *M. tuberculosis*. Western blot analysis with a panel of 14 murine mAbs against mycobacterial antigens (Table 1) showed that only six of 14 antigenic determinants present in *M. tuberculosis* sonicate were detectable in the culture supernatant. Western blot analysis of the 39 d culture supernatant and the corresponding sonicate with a panel of nine murine mAbs against mycobacteria is shown in Fig. 2. In both culture supernatant and sonicate 65, 38, 34, 24, 21 and 12 kDa protein bands were detectable. In the sonicate, antigens of 42, 33 and 16 kDa were present; these proteins were not detectable in the culture supernatant (Table 1). mAb F116-5 reacted with a 24 kDa protein in *M. tuberculosis* sonicate; in the culture supernatant an additional reaction was seen with a 30 kDa protein (Fig. 2, lane 10). By using mAb F29-47 we identified a 21 kDa antigen both in culture supernatant and in sonicate of *M. tuberculosis* (Fig. 2, lanes 11 and 12). Note that the separate reactions of mAbs F67-18, F67-19 and F23-49 are not shown.

Fig. 1. SDS-PAGE and CBB staining of the 39 d sonicate and culture supernatant of *M. tuberculosis*. Lanes: 1, mycobacterial sonicate; 2, mycobacterial culture supernatant; 3, molecular mass markers.

Separation of proteins in culture supernatant by gel permeation chromatography

The proteins in 12, 25 and 39 d culture supernatants were separated by gel permeation chromatography. In SDS-PAGE no difference was found between these preparations, and since the yield of the 39 d culture supernatant was largest, this culture supernatant was used for further analysis. The gel permeation pattern of the 39 d culture supernatant is shown in Fig. 3. All the column fractions were analysed separately in SDS-PAGE with CBB staining and in Western blots with the same nine mAbs as used in Fig. 2. The results are summarized as horizontal shaded bars below the gel permeation pattern in Fig. 3. Fractions of the first two peaks, representing high-molecular-mass material, and of the last peak, representing low-molecular-mass material, could not be further characterized with the methods used.

Human antibody response in ELISA

Fractions 9 and 14, containing mainly the major protein bands present in the culture supernatant, 24 and 12 kDa, were tested with a panel of sera from 26 patients with tuberculosis and 21 control subjects (Fig. 4). Using as a cutoff value the mean of the control group plus three

Fig. 2. Western blot of sonicate and culture supernatant of *M. tuberculosis* with a panel of mAbs. Reactivity of the mAbs (F67-18, 65 kDa; F29-29, 42 kDa; F67-19, 38 kDa; F126-2, 34 kDa; F67-1, 33 kDa; F116-5, 30 and 24 kDa; F29-47, 21 kDa; F23-49, 16 kDa; and SA-12, 12 kDa) is shown. Odd-numbered lanes, 39 d sonicate; even-numbered lanes, 39 d culture supernatant; lanes 1 and 2, all mAbs; lanes 3 and 4, F29-29; lanes 5 and 6, F126-2; lanes 7 and 8, F67-1; lanes 9 and 10, F116-5; lanes 11 and 12, F29-47; lanes 13 and 14, SA-12. The positions of the molecular mass markers are shown on the left.

Table 1. Reactivity of mAbs in Western blots with sonicate and culture supernatant of *M. tuberculosis*

mAb*	Directed against	Specificity†	Mol. mass (kDa) and reactivity‡ of antigen detected		
			Sonicate (12 or 39 d)	Supernatant	
				(12 d)	(39 d)
F67-18 ¹	<i>M. tuberculosis</i>	CR-B	65	65 w	65 s
F29-29 ²	<i>M. tuberculosis</i>	Mt-C	42	—	—
F67-19 ³	<i>M. tuberculosis</i>	Mt-C	38	38 w	38 s
F86-2	<i>M. bovis</i>	CR-B	34	—	—
F126-2	<i>M. kansasii</i>	CR-B	34	34 w	34 s
F126-5	<i>M. kansasii</i>	CR-B	33	—	—
F67-1 ³	<i>M. tuberculosis</i>	CR-L	33	—	—
F88-7	<i>M. leprae</i>	CR-L	30	—	—
F141-21	<i>M. avium</i>	CR-B	30	—	—
F108-18 ⁴	<i>M. intracellulare</i>	CR-B	26	—	—
F116-5	<i>M. leprae</i>	CR-B	24	24, 30 m	24, 30 s, m
F29-47 ²	<i>M. tuberculosis</i>	CR-B	21	21 m	21 m
F23-49 ²	<i>M. tuberculosis</i>	Mt-C	16	—	—
SA-12 ⁵	<i>M. bovis</i>	CR-L	12	12 s	12 s

* Source/reference: 1, One of several mAbs reacting with the 65 kDa protein – Van Schooten *et al.* (1988); 2, Kolk *et al.* (1984); 3, Kolk *et al.* (1986); 4, Kolk *et al.* (1989); 5, Minden *et al.* (1984); remaining mAbs, unpublished.

† CR-B, broad cross-reactivity, i.e. reacting with the majority of strains tested; Mt-C, *M. tuberculosis* complex, i.e. reacting with *M. tuberculosis* virulent or avirulent strains, *M. africanum*, *M. bovis* and *M. bovis* BCG; CR-L, limited cross-reactivity, i.e. reacting with a restricted number of mycobacterial strains tested.

‡ w, weak reaction; m, moderate reaction; s, strong reaction.

Fig. 3. Fractionation of 39 d culture supernatant of *M. tuberculosis* by gel permeation chromatography. Column, GF 250 (9.4 × 250 mm); elution buffer, 0.2 M-sodium phosphate, 0.1% SDS, pH 6.8; flow rate, 0.5 ml min⁻¹; detector absorption, 275 nm (100% = 0.1 A₂₇₅); sample, 0.05 mg protein of *M. tuberculosis* culture supernatant. Fractions were collected and numbered according to the scale shown. The height of the shaded bars represents the amount of antigens detected in SDS-PAGE. Molecular masses (kDa) are shown to the left of the bars.

Fig. 4. Antibodies to gel permeation fractions in sera from tuberculosis patients and control subjects as determined by ELISA. Sera were diluted 1:400; each point represents one subject. ○, Reaction with gel permeation fraction 9, containing mainly 24 kDa protein; ●, reaction with gel permeation fraction 14, containing mainly 12 kDa protein.

times the standard deviation, sera from 23 patients with tuberculosis were positive with *M. tuberculosis* sonicate (data not shown), 16 sera were positive with the 24 kDa-containing fraction and 14 sera with the 12 kDa-containing fraction (Fig. 4). Six sera reacted with the 24 kDa-containing fraction only and not with the 12 kDa-containing fraction. Four sera reacted with the 12 kDa-

containing fraction only and not with the 24 kDa-containing fraction. Combining the results obtained with the 24 kDa and 12 kDa antigens, 20 of the 26 tuberculosis patients were positive and none of the 21 control subjects. The three sera from tuberculosis patients which were negative with *M. tuberculosis* sonicate did not react with either 24 kDa or 12 kDa antigens.

Fig. 5. Antibodies to *M. tuberculosis* sonicate and culture supernatant in sera from tuberculosis patients and control subjects as determined in Western blots. Lanes 1–17, reaction with sonicate; lanes 24–40, reaction with culture supernatant. Lanes 1–10 and 24–33, sera from 10 tuberculosis patients; lanes 11–12 and 34–35, sera from two BCG-vaccinated skin-test-negative subjects; lanes 13–14 and 36–37, sera from two skin-test-positive subjects; lanes 15–17 and 38–40, sera from three skin-test-negative control subjects.

Table 2. Reaction of sera with 24 kDa and 12 kDa protein fractions in Western blots and ELISA

Sera	No. of sera tested	No. of positive sera with			
		24 kDa protein in		12 kDa protein in	
		Western blots	ELISA	Western blots	ELISA
Tuberculosis patients	26	26	16	9*	14†
<i>M. avium</i> infection	2	2	–	–	–
Leprosy	2	2	–	–	–
Skin test negative, BCG vaccinated	4	4	–	–	–
Skin test positive	5	5	–	–	–
Skin test negative	8	8	–	–	–

* Seven sera were positive in both Western blots and ELISA with the 12 kDa protein.

† Seven sera reacted with 12 kDa protein in ELISA and not in Western blots.

Human antibody response in Western blots

All the sera from patients with tuberculosis and from control subjects were tested in Western blots, with sonicate and culture supernatant of *M. tuberculosis*; a

representative example is given in Fig. 5. Although each patient serum had its own characteristic banding pattern, sera from both patients and controls reacted with protein bands around 65, 61, 58, 30 and 24 kDa in culture supernatant. These bands were not found with

anti-human Ig peroxidase conjugate alone, indicating that they were not caused by a non-specific reaction. Overloading is unlikely, as the 30 kDa protein was present in much smaller amounts than the 12 kDa protein. No great difference in the intensity of reaction in Western blots was observed between sera from patients with tuberculosis who were positive in ELISA with *M. tuberculosis* sonicate, sera from patients with tuberculosis who were negative in ELISA, and sera from control subjects. However, some differences between patients with tuberculosis and control subjects were found in the area below 20 kDa: compare lanes 24–33 with lanes 34–40.

All the sera were tested in Western blots with the 24 kDa- and 12 kDa-containing gel permeation fractions and the results were compared with the ELISA results obtained with the same protein fractions (Table 2). All the sera from patients with tuberculosis and from control subjects were positive in Western blots with the isolated 24 kDa protein, whereas in ELISA, 16 of 26 sera from patients with tuberculosis but none of the controls were positive. Nine of the sera from patients with tuberculosis reacted in Western blots with the 12 kDa protein; of these sera, seven also reacted in ELISA.

Discussion

Antigens detectable in culture supernatants by CBB staining and Western blot analysis were shown to be a selection of antigens found in sonicates of *M. tuberculosis*. Two antigens, 24 and 12 kDa, present in sonicates, were clearly more prominent in culture supernatants. Some antigens, 42, 33 and 16 kDa, present in sonicates of *M. tuberculosis* were not detectable in culture supernatants. All antigens detectable by Western blotting in the culture supernatant were already present after 12 d culture.

Previous studies (Abou-Zeid *et al.*, 1988a; Collins *et al.*, 1988; Daniel & Anderson, 1978; De Bruyn *et al.*, 1989; Harboe *et al.*, 1986) have identified major protein bands in the culture supernatant of *M. tuberculosis*. Abou-Zeid *et al.* (1988a) reported that in resting cultures of *M. tuberculosis* a protein of 23 kDa (in non-denaturing conditions) represented about 20% of the secreted proteins and consisted of 13 kDa subunits in denaturing conditions. The 12 kDa protein (MPB57; Harboe *et al.*, 1986) which we detected as one of the major protein bands in our culture supernatant is identical to the 12 kDa minor protein band found by Abou-Zeid *et al.* (1988a) in resting cultures of 3 and 21 d since it also reacted with mAb SA-12. Minden *et al.* (1984) showed that mAb SA-12 reacted with a 10 kDa antigen; they used cytochrome *c* (12 kDa) as molecular mass marker.

This marker appears just above the 14.4 kDa of α -lactalbumin in our gels, explaining the discrepancy. Recently, the genes encoding this protein in *M. bovis* BCG and *M. tuberculosis* have been cloned and sequenced (Yamaguchi *et al.*, 1988; Baird *et al.*, 1989). The protein sequence was homologous to two prokaryote heat-shock proteins but lacked a signal peptide sequence, suggesting that it might be originally an intracellular protein, released into the medium by lysis during cultivation (Yamaguchi *et al.*, 1988). It remains to be elucidated if secretion without a signal peptide is possible since many investigators have found this antigen in culture fluid, even in 3 d cultures of *M. tuberculosis* (Abou-Zeid *et al.*, 1988a).

Like Abou-Zeid and coworkers, we failed to detect a reaction in culture supernatant with mAb F23-49, directed against a 16 kDa protein (known as the 14 kDa protein in the workshop on mAbs: Engers *et al.*, 1986), although it was present in large amounts in sonicate.

The 23 kDa protein (MPB64, Harboe *et al.*, 1986) and the 27 kDa protein (Abou-Zeid *et al.*, 1988a) found in high concentrations in the culture supernatant of *M. tuberculosis* probably correspond to our 24 kDa protein. The gene encoding this protein in *M. bovis* BCG has been cloned and sequenced (Yamaguchi *et al.*, 1989). The gene has a signal sequence, indicating that the protein is a candidate for a secreted antigen.

Our mAb F67-19 (which shows the same reaction pattern as mAbs HYT28 and TB-71) reacted with a 38 kDa protein. The same reaction was found by Abou-Zeid *et al.* (1988a), using mAb TB-71. The gene encoding this protein in *M. tuberculosis* has been cloned and sequenced (Andersen & Hansen, 1989). It has a signal sequence. Daniel & Anderson (1978) found a 28–35 kDa protein (antigen 5) as a major protein band in culture filtrate; this antigen is the same as antigen 78 in *M. tuberculosis* (Wiker *et al.*, 1988) and the 38 kDa protein we found.

De Bruyn and coworkers detected a 32 kDa antigen as a major component in culture filtrate, corresponding to antigen BCG 85A (De Bruyn *et al.*, 1989; Wiker *et al.*, 1986; Closs *et al.*, 1980). The gene encoding this protein in *M. tuberculosis* has been cloned and sequenced (Borremans *et al.*, 1989). Again, the gene has a signal sequence. With mAb F116-5 we found a 30 kDa antigen in culture supernatant only, not in the corresponding sonicate (Fig. 2, Table 1). This mAb reacted with this 30 kDa protein and with the 24 kDa protein, giving a reaction similar to that of the antigen BCG 85B with anti-BCG 85 serum (Abou-Zeid *et al.*, 1988b). Further investigations are needed to see whether the 30 kDa protein we found in the culture supernatant of *M. tuberculosis* corresponds to BCG 85B or BCG 85A. Antibodies in serum react with one 30 kDa protein from

culture filtrate, and with a protein of slightly higher molecular mass in sonicates (Fig. 5).

We found a 21 kDa antigen in culture supernatant and sonicate reacting with our mAb F29-47. This antigen is known as the 19 kDa protein in the workshop paper on mAbs (Engers *et al.*, 1986). It has not been reported previously in culture supernatant. The gene encoding this protein in *M. tuberculosis* has been cloned and sequenced, and shown to have a signal sequence (Ashbridge *et al.*, 1989). We are further characterizing this antigen and the frequency with which human antibodies against it appear.

The use of polyclonal rabbit serum against *M. tuberculosis* did not increase the number of protein bands identified in culture supernatant by Western blotting (data not shown).

There is a clear discrepancy concerning which antigens are detected in the culture supernatant described in the various reports on this subject. Differences in amount and composition of proteins in culture supernatant are almost inevitable given the wide range of culture conditions used: pellicle growth in Proskauer and Beck medium (Daniel & Anderson, 1978); growth on the surface of Sauton medium (De Bruyn *et al.*, 1989; Harboe *et al.*, 1986); cultivation in roller bottles and addition of 0.05% Tween 80 to Sauton liquid medium (Collins *et al.*, 1988); cultivation in Eagle's and Sauton medium in tissue culture plates (Abou-Zeid *et al.*, 1988a); and shaking culture in Sauton liquid medium (this study). De Bruyn *et al.* (1989) found a 15-fold increase in the yield of the 65 kDa stress-protein (Young *et al.*, 1988) in culture filtrate when the medium was zinc depleted, whereas others did not find this protein in supernatant of *M. tuberculosis* cultured in the same medium. Using the culture conditions described here, we found a 65 kDa protein. The lack of a signal peptide in the corresponding gene (Thole *et al.*, 1987) argues against secretion of this protein. Apparently shaking of the culture is, like zinc depletion, a stress condition leading to an increased synthesis of the 65 kDa protein. Some form of lysis could then easily lead to the presence of the 65 kDa protein in the culture supernatant. If bacterial lysis is the explanation it remains to be elucidated why the proteins in culture supernatant constituted a subset of those present in sonicate.

Heating of a mycobacterial culture (to kill the mycobacteria) may also influence the pattern of secreted antigens (Harboe, 1981). We found that heating to 71 °C did not apparently change the yield and composition of proteins in the culture supernatant (data not shown). The varying yield and composition may also depend on the different strains of *M. tuberculosis* used and different culture times (Abou-Zeid *et al.*, 1988a; De Bruyn *et al.*, 1989). Apparently, culture conditions strongly influence

the amount and composition of proteins present in the culture supernatant of *M. tuberculosis*.

Gel permeation chromatography fractions containing the major 24 kDa and 12 kDa antigens from culture supernatant were tested with sera from patients with tuberculosis and control subjects. The results indicate that these antigens contain epitopes, detectable in ELISA, which may be specific for tuberculosis and therefore valuable for the development of a serological test.

A comparison of the reaction of sera with mycobacterial antigens in Western blots and ELISA showed some surprising differences (Table 2). In ELISA, 20 of 26 sera from patients with tuberculosis and none of the sera from control subjects reacted with 24 or 12 kDa antigens (Fig. 4). However, in Western blots, all the sera, from patients with tuberculosis as well as from control subjects, reacted with antigens in the culture supernatant of 65, 61, 58, 30 and 24 kDa (Fig. 5). The 65 kDa protein has epitopes in common with other bacteria (Thole *et al.*, 1987). mAb F116-5 reacts in Western blots with the 30 and 24 kDa mycobacterial antigens in culture supernatant. This 24 kDa protein has an epitope in common with a 12 kDa protein from *Escherichia coli* (Verstijnen *et al.*, 1989). We assume that the epitopes recognized on mycobacterial antigens in Western blots by sera from both control subjects and patients with tuberculosis are ubiquitous epitopes, also present on normal commensal bacteria. It remains to be investigated if the sera of tuberculosis patients do recognize epitopes on the 61, 58 and 30 kDa proteins in ELISA which are not recognized by control sera. The 12 kDa protein is an example of an antigen recognized only by sera from tuberculosis patients in Western blots and ELISA (Table 2). The finding that not all sera are positive in both assays suggests that different epitopes are recognized in Western blots and ELISA.

The difference between the results obtained with the 24 kDa antigen in ELISA and Western blots is unexpected. For both techniques the antigens were boiled in the presence of SDS, so one would expect the same epitopes to be present. Contamination of the 24 kDa-containing gel permeation fraction with other antigens is unlikely, since the same ELISA results were obtained when the 24 kDa antigen was isolated by preparative SDS-PAGE (data not shown). Other work done by our group has shown that, besides mAbs reacting in both Western blots and ELISA, there are mAbs reacting in Western blots but not in ELISA with the same denatured (with SDS) antigen. This indicates that the epitopes exposed in Western blots differ from those exposed in ELISA, even when denatured antigens are used for both techniques (data not shown). We suggest that in ELISA the 24 kDa protein is partially rearranged in such a way that only sera from patients with tuberculosis recognize

the exposed epitopes, whereas the epitopes exposed in Western blots are recognized by both control and patient sera. The epitopes recognized on the 12 kDa protein in Western blots and ELISA by sera from patients with tuberculosis seem to be specific for tuberculosis, since only patients with tuberculosis have these antibodies.

All our antigens were treated with SDS prior to separation. It remains to be investigated if separation of the antigens without SDS treatment will change the results of the ELISA with sera from patients with tuberculosis and from control subjects. In this respect it is interesting that the 38 kDa protein, isolated under native conditions by Jackett *et al.* (1988), gave such promising results in ELISA, whereas we found with this antigen separated under denaturing conditions (preparative PAGE), that sera from only a few patients were positive in ELISA (data not shown). Experiments are planned to investigate if isolation of *M. tuberculosis* proteins under non-denaturing conditions will result in an increased diagnostic specificity of the ELISA, and if recombinant proteins are useful in an ELISA for the detection of tuberculosis.

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