Production and Characterization of Monoclonal Antibodies Specific for *Mycobacterium bovis*

By P. R. WOOD,^{1*} J. RIPPER,¹ A. J. RADFORD,¹ P. G. BUNDESEN,²
D. B. RYLATT,² L. E. COTTIS,² M. JOHN² AND P. PLACKETT¹

¹CSIRO Division of Animal Health, Animal Health Research Laboratory, Private Bag No. 1,
Parkville, Vic. 3052, Australia

²Agen Biomedical Ltd, Acacia Ridge, Qld. 4110, Australia

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A series of monoclonal antibodies (MAbs), specific for *Mycobacterium bovis* and BCG strains, were tested extensively for cross-reactivity to a wide range of mycobacterial species using ELISA, Western blotting and dot-blot analysis. The MAbs bound specifically to *M. bovis* and BCG and showed limited cross-reactivity with some strains of *M. tuberculosis*. All these MAbs recognized a 22 kDa protein previously termed MPB70, and by competitive ELISA analysis appeared to detect at least three *M. bovis*-specific determinants on the MPB70 molecule.

INTRODUCTION

The development of reliable serological tests for the diagnosis of tuberculosis in humans and cattle has been complicated by the large degree of cross-reactivity between antigens of *Mycobacterium tuberculosis*, *M. bovis* and a wide range of other mycobacteria (Krambovitis, 1987; Grange, 1984). The production of monoclonal antibodies (MAbs) specific for these two species has therefore been of interest for the isolation and characterization of species-specific antigens for use in serological assays. Several groups (Engers *et al.*, 1985; Ivanyi *et al.*, 1985) have produced MAbs specific for the *M. tuberculosis* complex of organisms (*M. tuberculosis*, *M. bovis*, BCG and *M. africanum*). Only Morris *et al.* (1985) have reported a MAb that recognizes an epitope present on *M. bovis* which is not present on BCG, *M. tuberculosis* or a large range of other mycobacterial species.

We have produced MAbs specific for determinants of *M. bovis*. These have been used to characterize specific antigens for incorporation into serological and cellular assays for the diagnosis of bovine tuberculosis.

METHODS

Mycobacterial antigens for Western blotting. The strains of mycobacteria used were obtained from the following sources in Australia: MAIS (*M. avium*–*intracellulare*–*scrofulaceum*) complex, *M. kansasii*, *M. terrae*, *M. gordonae* and *M. fortuitum* from the Department of Health, Brisbane; *M. phlei* and *M. paratuberculosis* from the Veterinary Research Institute, Parkville; *M. bovis* strain AN5, BCG, and *M. tuberculosis* strains PN, C and DT from the Commonwealth Serum Laboratories, Parkville; *M. tuberculosis* strain H37Rv from the Fairfield Hospital, Fairfield; and *Norcardia asteroides* from the School of Veterinary Science, University of Melbourne. The two remaining strains (PMC203, PMC235) were from our own collection. *M. leprae* antigens were supplied by Dr R. Garcia, University of Sydney. Mycobacteria were grown on modified Middlebrook 7H11 medium (Gallagher & Horwill, 1977) in Petri dishes at 37 °C in an atmosphere of 9% CO₂-in-air for 7 weeks. Then 2 ml normal saline (0.15 M-NaCl) was added to the surface of each of two plates and bacteria gently scraped off using an angled

Abbreviations: ABTS, 2,2'-azino-di-(3-ethylbenzthiazoline sulphonic acid), HRP, horseradish peroxidase; MAb, monoclonal antibody; MAIS, *Mycobacterium avium*–*intracellulare*–*scrofulaceum*.

pipette. The bacterial suspensions were adjusted to approximately the same turbidity (10^9 bacterial ml^{-1}) and heat-killed in a 70°C waterbath for 2 h. They were then centrifuged at 4000 g for 15 min and the pellets resuspended in 10 times their volume of saline. Sodium dodecyl sulphate (SDS) was added to each suspension to give a final concentration of 4% (w/v) SDS. Samples were then boiled at 100°C for 10 min to produce SDS-extracted antigens.

Culture-filtrate antigen and sonicated antigen. Mycobacteria were grown in Dubos broth medium (DIFCO) as static cultures at 37°C for 10 weeks in an atmosphere of 9% CO_2 -in-air. One hundred ml of medium was inoculated with 1 ml of a suspension of bacteria in normal saline. The opacity of these suspensions was equal to or denser than Brown's opacity tube 8. Cultures were then centrifuged at 4000 g for 15 min, after which the supernate was removed and filtered sequentially through $0.45\text{ }\mu\text{m}$ and $0.22\text{ }\mu\text{m}$ cellulose acetate filters, for the preparation of culture-filtrate antigen. Filtered supernates were concentrated 10-fold with Aquacide II (Calbiochem) and dialysed against distilled water overnight. The total protein content was estimated by measuring UV absorbance at 280 nm. For preparation of sonicated antigen the cell pellet ($0.2\text{--}0.5\text{ g}$) was washed in saline, resuspended in 10 times its volume of saline and heat-killed at 70°C for 2 h. The cells were then sonicated for 3 min (Vibra, cell sonicator, $8 \times 30\text{ s}$ bursts of 15 W) and the suspension centrifuged at 13000 g for 10 min to pellet cellular debris. The supernate prepared in this manner is referred to as sonicated antigen.

Preparation of anti-*M. bovis* MAbs. Balb/c mice (6–8 weeks old) were injected subcutaneously on three occasions at 2 week intervals with 0.1 ml of a suspension of aluminium hydroxide (Herbert, 1978) in phosphate-buffered saline (0.1 M -sodium phosphate, 0.15 M -NaCl; PBS) pH 7.2 containing a sonicate of UV-killed *M. bovis* ($10\text{ }\mu\text{g}$ protein ml^{-1}). For production of UV-killed material, 5 ml bacterial suspension (2 mg ml^{-1} , wet weight) was placed in a 90 mm Petri dish 16 cm from a UV source (15 W) and exposed for 60 min. Samples of the bacterial suspension were then plated to check for sterility, and the remainder was concentrated to 10 mg ml^{-1} and sonicated as described above.

Five days after the last injection the spleens of the mice were removed for fusion with NS-1 myeloma cells. Hybridoma production, the MAb screening assay, isotyping and production of ascites fluid were done by the methods described by Bundesen *et al.* (1985), with *M. bovis* sonicate as the screening antigen. Selected hybridomas, chosen on the basis of the screening assay, were subcloned by limiting dilution and a number of monoclonal anti-*M. bovis* antibody-secreting cell lines were selected for expansion into mass culture and production of ascitic fluids. All assays of the MAbs were done using ascites fluid preparations.

ELISA. The ELISA method used was adapted from that described by Grange *et al.* (1980). Polystyrene microtitre trays (Disposable Products) were coated with $0.5\text{ }\mu\text{g}$ per well of *M. bovis* culture filtrate or mycobacterial sonicate antigen in PBS (pH 7.2) overnight at 4°C . Trays were then washed four times in PBS with 0.05% Tween 20 (PBST), blocked with 2.5% (w/v) non-fat powdered milk in PBS (Blotto) and incubated with MAbs diluted to their optimum concentration in PBST. Trays were incubated for 90 min at room temperature, washed and reincubated with an anti-mouse IgG labelled with horseradish peroxidase (HRP; Sigma). After 90 min the trays were washed and incubated with ABTS substrate [2,2'-azino-di-(3-ethylbenzthiazoline sulphonic acid)] for 30 min. Results were read at 405 nm in a Titertek Multiskan.

Competitive ELISA. The MAbs were labelled with HRP as previously described (Rylatt *et al.*, 1985). All HRP-conjugated MAbs were titrated on *M. bovis* AN5 culture-filtrate antigen, and used in subsequent ELISAs at their optimal concentration. After coating with *M. bovis* antigen ($0.5\text{ }\mu\text{g}$ per well in $50\text{ }\mu\text{l}$), trays were blocked with 2.5% Blotto for 90 min. Serial dilutions of unlabelled MAbs were then added in duplicate and incubated for 90 min at room temperature followed by $50\text{ }\mu\text{l}$ of a HRP-labelled MAb. After a further 2 h incubation the trays were washed and incubated for 30 min with substrate (ABTS). Results were calculated as a percentage of the A_{405} obtained with the HRP-labelled MAb bound at its optimum concentration.

Dot-blotting. Sonicated antigen preparations were diluted to $10\text{ }\mu\text{g ml}^{-1}$ and $100\text{ }\mu\text{l}$ samples applied to each well of a dot-blotting manifold loaded with nitrocellulose (Bio-Rad, $0.45\text{ }\mu\text{m}$ pore size). Samples were left to bind overnight at 4°C , after which the filters were rinsed once in PBS and then blocked for 90 min at room temperature with Blotto. After being washed four times in PBST the filters were probed for 90 min at room temperature with MAb diluted in PBST to its optimum concentration as ascertained by probing *M. bovis* antigen bound to nitrocellulose (Hawkes *et al.*, 1982). Following a further four washes in PBST, filters were incubated with anti-mouse HRP conjugate, washed again, and developed in 4-chloro-1-naphthol substrate (0.4 mg ml^{-1} in 100 mM -Tris/HCl pH 7.4, containing 0.03% H_2O_2).

SDS-PAGE and Western blotting. Samples of mycobacteria grown on 7H11 medium were solubilized in Tris/HCl buffer pH 6.8 containing 2% (w/v) SDS, 5% (v/v) glycerol and 0.002% (w/v) bromophenol blue by heating at 100°C for 5 min. Vertical slab SDS-PAGE was done as described by Laemmli (1970). Proteins were transferred overnight by electroblotting to nitrocellulose (Towbin *et al.*, 1979), blocked with Blotto for 60 min, then probed for 2 h with MAbs diluted in PBST to an appropriate concentration as previously determined in the dot-blot system. The blots were incubated with HRP-conjugated affinity-purified rabbit anti-mouse IgG, followed by the HRP substrate (4-chloro-1-naphthol) until the colour product became visible.

Table 1. *MAbs specific for M. bovis*

Original no.	Code	Isotype
1A3/157	SB1	IgG1
4C6/70	SB2	IgG1
2D6/8	SB3	IgG1
2D6/150	SB4	IgG2a
2B5/39	SB5	IgM
2C5/18	SB6	IgG1
4B5/121	SB7	IgG2a
2C3/126	SB8	IgG1
3D3/14	SB9	IgA
4C3/17	SB10	IgG2a

Table 2. *Specificity of MAbs SB1 to SB10 for antigens from various mycobacterial species*

The MAbs were tested on antigens from the organisms listed, by ELISA, dot-blot and Western blot analysis. The antigen preparations used were: ELISA, culture filtrate or sonicated; dot-blot, sonicated; and Western blot, SDS-extracted. All results were repeated on at least two or three occasions.

Source of antigens	ELISA	Dot-blot	Western blot
MAIS 2	—	—	—
5	—	—	—
6	—	—	—
8	ND	—	—
9	—	—	—
11	ND	—	—
18	—	—	—
<i>M. phlei</i>	—	—	—
<i>M. kansasii</i>	—	—	—
<i>M. fortuitum</i>	—	—	—
<i>M. terrae</i>	—	—	—
<i>M. gordonae</i>	—	—	—
<i>M. paratuberculosis</i>	—	—	—
<i>Nocardia asteroides</i>	ND	—	—
<i>M. bovis</i> (AN-5)	+	+	+
BCG (CSL)	+	+	+
PMC203 (<i>M. bovis</i> field isolate)	+	+	+
PMC235 (<i>M. bovis</i> field isolate)	+	+	+
<i>M. tuberculosis</i> H37Rv	—	±	—
<i>M. tuberculosis</i> PN	—	—	—
<i>M. tuberculosis</i> C	—	—	—
<i>M. tuberculosis</i> DT	—	—	—
<i>M. leprae</i>	ND	—	—

ND, not done.

RESULTS AND DISCUSSION

Hybridomas secreting antibody to *M. bovis* were tested for reactivity to a limited range of MAIS complex organisms. Following the initial screening in an ELISA assay and cloning, 10 MAbs reacting only with *M. bovis* antigen were selected for detailed analysis.

The code and isotype of these MAbs are listed in Table 1 (the original designations are included as the MAbs have been distributed to several laboratories under these codes). The specificity of the selected MAbs for a wide range of mycobacterial species was examined in three different assays (ELISA, dot-blot analysis and Western blotting) using culture-filtrate, sonicated or SDS-extracted antigen preparations. All 10 of these MAbs appeared to be specific for wild-type *M. bovis* strains and *M. bovis* BCG (Table 2). Cross-reactivity was seen with one isolate of *M. tuberculosis* in the dot-blot system. Results of the dot-blot given in Table 2 are for sonicated antigen, but culture-filtrate preparations of the mycobacteria were also probed on dot-blot with

Fig. 1. (a) SDS-PAGE stained with Coomassie Brilliant Blue, and (b) Western transfer probed with MAb SB10, of sonicated antigen preparations. Lane 1, Bio-Rad low-MW markers; lane 2, *M. phlei*; lane 3, *M. bovis*; lane 4, *M. tuberculosis* H37Rv.

Table 3. Competitive analysis of MAbs recognizing *M. bovis* culture-filtrate antigen

The inhibition of binding of HRP-labelled MAb by unlabelled MAb was scored as positive for >50% inhibition, plus/minus for 30–50% inhibition and negative for <30% inhibition. All results were repeated on at least two or three occasions.

Unlabelled MAb	HRP-labelled MAb:									
	SB1	SB2	SB3	SB4	SB5	SB6	SB7	SB8	SB9	SB10
SB1	+	+	+	–	–	–	–	–	–	–
SB2	+	+	+	+	–	–	–	–	–	–
SB3	+	+	+	+	–	–	–	–	–	–
SB4	–	+	+	+	–	–	–	–	–	–
SB5	–	–	–	–	+	+	+	–	–	–
SB6	–	–	–	–	+	+	+	+	–	±
SB7	–	–	–	–	±	+	+	±	–	–
SB8	–	–	–	–	–	±	+	+	–	–
SB9	–	–	–	–	–	–	–	–	+	+
SB10	–	–	–	–	–	±	–	–	+	+

the SB MAbs. *M. bovis* culture filtrates contained large amounts of the recognized antigen, but the other mycobacteria produced no antigen in the culture filtrate detectable with the MAbs. However, not all the mycobacteria tested grew well on the Dubos medium required for production of culture filtrate, and consequently sonicate or SDS-extracted antigen was also used to examine the SB series of MAbs for cross-reactivity.

In the Western blot analysis of SDS-extracted or sonicated *M. bovis* antigen all the SB MAbs bound to a 22 kDa protein (Fig. 1). However, when Western blots were performed with some sonicate or Ribi press lysate antigen preparations from *M. bovis*, multiple bands of higher molecular mass were often detected with these MAbs (T. Fifis, personal communication).

Competitive ELISA analysis between enzyme-labelled and unlabelled antibodies on culture-filtrate antigen of *M. bovis* suggested that these MAbs recognize at least three distinct antigenic determinants on a 22 kDa protein produced by *M. bovis* (Table 3). The individual MAbs within the three groupings may not necessarily recognize exactly the same epitope, as individual MAbs within a group were not equally efficient in blocking binding of other MAbs within that group.

A large range of MABs specific for the *M. tuberculosis* complex of organisms have now been described (Engers *et al.*, 1985), and used widely to isolate and characterize *M. tuberculosis*-complex-specific antigens (Young, 1985). These MABs recognize three major proteins (14, 38 and 65 kDa) which are distinct from the protein detected by the SB series of MABs described in this paper, and none of them discriminate between *M. tuberculosis* and *M. bovis* strains.

The 22 kDa protein recognized by the SB MABs is most likely that described by Nagai *et al.* (1981), and termed MPB70. Both proteins have similar molecular masses, are present in high concentration in culture filtrate (in some strains more than 10% of the total protein secreted), often occur in higher molecular mass forms, and appear to be limited to the *M. tuberculosis* complex of organisms. The binding of MABs to multiple bands in mycobacterial extracts, as seen with the SB MABs (T. Fifis, personal communication), has also been reported by others (Young *et al.*, 1987).

Harboe *et al.* (1986) detected some cross-reactivity between MPB70 and *Nocardia asteroides*; however, the SB series of MABs recognize epitopes on MPB70 that are not present in *N. asteroides*. The finding that MPB70 is detectable in *M. tuberculosis* and some BCG strains at only very low concentrations (Harboe & Nagai, 1984) would explain the low level of cross-reactivity of the SB MAB to *M. tuberculosis* strains. This would also explain why none of the many MABs produced to the *M. tuberculosis* complex of organisms appear to recognize MPB70, as the strains used for immunization were all low-level producers of this protein (Minden *et al.*, 1984; Kolk *et al.*, 1984).

The MABs reported in this paper recognize antigenic determinants that are specific for *M. bovis* and BCG. Competitive binding and Western blot studies of these MABs with various *M. bovis* culture-filtrate antigen preparations suggest that they detect at least three different epitopes on a 22 kDa protein. These MABs are also distinct from the *M. bovis*-specific MABs (MB-5) described by Morris *et al.* (1985), which identified a 30 kDa protein that is not present in BCG or culture-filtrate preparations from *M. bovis*. We have recently reported the isolation and sequencing of the MPB70 gene from a λ gt11 expression library of *M. bovis* (Radford *et al.*, 1988). The characterization of the species-specific epitopes on this protein will hopefully provide antigens for use in highly specific diagnostic tests for bovine tuberculosis.

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