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Characterization of antigens recognized by new monoclonal antibodies raised against culture filtrate proteins of *Mycobacterium bovis* bacillus Calmette-Guérin

G. Freer *, W. Florio, B. Dalla Casa, B. Castagna, G. Maisetta, G. Batoni, V. Corsini, S. Senesi, M. Campa

Dipartimento di Biomedicina Sperimentale, Infettiva e Pubblica, Università degli Studi di Pisa, Via S. Zeno 35 39, 56127 Pisa, Italy

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Abstract

Effective protection against *Mycobacterium tuberculosis* may be achieved in experimental animals by immunization with proteins secreted by tuberculous bacilli in the extracellular milieu during growth. In this study, monoclonal antibodies were raised against *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) culture filtrate proteins or live BCG, in an attempt to identify novel mycobacterial secretion antigens: the localization of the antigens recognized by the monoclonal antibodies within the mycobacterial cell was studied and interspecies reactivity was also investigated. The monoclonal antibodies obtained recognized proteins of molecular mass ranging from 5 to 82 kDa, with a prevailing frequency in the 30 kDa region. Three of the monoclonal antibodies recognized proteins present only in culture filtrates, one reacted with a cytoplasmic antigen, while the remaining antibodies recognized components which were mainly associated with the cell wall and the cytoplasmic membrane. The chemical nature and possible identity of the antigens was checked. Three monoclonal antibodies are likely to react with novel mycobacterial antigens of 5, 42 and 82 kDa, respectively.

Keywords: Culture filtrate; Monoclonal antibody; Mycobacterial secretion protein; Subcellular localization

1. Introduction

Proteins released by actively growing tubercle bacilli have been shown to induce protective immunity against tuberculosis in animal models and there is evidence that they are able to elicit cell-mediated immune responses also in man [1–4]. However, only few of these proteins have been isolated and characterized at present, and it is conceivable that there are still important antigens as yet unidentified in short-term culture filtrates (CFs) [5]. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is widely used as a vaccine against tuberculosis, but its efficacy ranges from 0 to 80% in different trials and one reason for this variability seems to depend on the ability of BCG to replicate in the host [6]. The latter limitation could be overcome by the identification of BCG antigens which may be used for the development of a subunit vaccine. Monoclonal antibodies

* Corresponding author. Tel.: +39 (50) 559429; Fax: +39 (50) 559462.

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(mAbs) have provided useful tools to isolate and purify single proteins, to be tested as to their ability to induce protective immunity. In the present study, a panel of mAbs against short-term CFs of BCG was derived from mice and thorough characterization of the antigens recognized was attempted. In order to obtain mAbs with specificity for proteins that were expressed at low levels, screening was carried out by Western blot. The localization of the antigens recognized was determined in different subcellular fractions to assess whether they were of true secretion origin: certain antigens were also part of the cell envelope and were particularly abundant in the cell wall. Interspecies reactivity was also evaluated.

At least three of the mAbs obtained are likely to react with novel mycobacterial antigens and are therefore described in more detail.

2. Materials and methods

2.1. Bacterial strains

M. bovis BCG, strain Pasteur, was supplied by Pasteur Mérieux (Lyon, France). *M. microti* was purchased from ATCC. All other mycobacterial strains were a kind gift of Dr. G. Källenius, Swedish Institute for Infectious Disease Control, Stockholm, Sweden.

2.2. Preparation of short-term CFs

BCG CFs were prepared as described [7]. Briefly, 2×10^9 CFU of BCG were inoculated in 200 ml Sauton's modified medium without Tween 80. Cultures were grown in static conditions for 7–8 days, then they were filtered through a 0.22 μm filter and concentrated 1000 times by ultrafiltration through an Amicon YM3 filter with a cutoff of 3 kDa. Dialysis was performed by further ultrafiltration with two changes of sterile distilled water, then CFs were further concentrated five-fold under vacuum and stored at −20°C until use. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard [8]. Yield ranged between 300 and 500 μg l^{-1}.

For *M. scrofulaceum*, *M. avium*, *M. kansasii*, *M. vaccae*, *M. marinum*, *M. gordonae*, *M. simiae*, *M. smegmatis* and *M. microti*, CFs were obtained from cultures in Sauton's modified medium without Tween 80 in the late exponential growth phase. Ultrafiltration was performed as above. Crude CF proteins of *M. tuberculosis*, strain $H_{37}Rv$, were kindly provided by Dr. P. Brennan, Colorado State University, Fort Collins, CO, USA.

2.3. Subcellular fractionation of BCG

BCG subcellular fractions were obtained as previously described, with minor modifications [9,10]. Briefly, 7-day-old BCG cells were suspended at 0.5 g of wet bacteria ml^{-1} of lysis buffer (PBS, 0.05% Tween 80, 0.8 mg ml^{-1} phenylmethylsulfonyl fluoride, 156 μg ml^{-1} benzamidine, 30 μg ml^{-1} pepstatin A, 10 mg ml^{-1} iodoacetamide, 50 μg ml^{-1} *Nα-p*-tosyl-L-lysine chloromethyl ketone, 3 mg ml^{-1} EDTA, and 0.26 mg ml^{-1} 1,10-phenanthroline). The suspension was sonicated with a probe sonicator at 1-min-pulser-on, 15-s-pulser-off intervals for a total of 20 min on ice. The whole mycobacterial lysate was centrifuged at $27\,000\times g$ for 30 min. The resulting pellet was washed, resuspended in lysis buffer centrifuged at $100\,000\times g$ for 2 h onto a discontinuous gradient of 60%, 45%, 30% and 15% sucrose in lysis buffer: the 30–45% interphase band was washed in lysis buffer and resuspended in sample buffer for SDS-PAGE (cell wall-enriched fraction) [9]. The supernatant of the $27\,000\times g$ centrifugation was centrifuged at $100\,000\times g$ for 2 h. The pellet was washed and resuspended in lysis buffer (cell membrane-enriched fraction). The $100\,000\times g$ supernatant made up the cytosol-enriched fraction [10].

Fractions were checked for fidelity with antibodies recognizing markers of each fraction, namely HAT5, recognizing the cytoplasmic 65-kDa protein [5,11]; HYT28, specific for a 38-kDa membrane protein [12]; and HYT27, specific for Ag 85, which has been shown to be secreted and associated with the cell wall [7,9,13].

2.4. Monoclonal antibodies

B-cell hybridomas were obtained by standard procedures, with myeloma cell line P3-X63-Ag8.653 as a partner for fusion and splenocytes from mice immunized as follows [14]. BALB/c and C57BL/6 mice

were immunized three times at 3–4-week intervals with 50 μg of CF of BCG in incomplete Freund's adjuvant intraperitoneally. Four days before fusion, they received a boost of 20 μg of CF intravenously (i.v.) in balanced salt solution. C57BL/6 mice were injected i.v. with 2×10^7 CFU of BCG and boosted 2 months later with 2×10^5 CFU i.v. One month after the boost, they were killed.

MAb F116-5 was kindly provided as ascitic fluid by Dr. Arend H.J. Kolk (Royal Tropical Institute, Amsterdam, The Netherlands) [15]. All other control mAbs were kindly provided by Dr P. Andersen (Statens Serum Institut, Denmark) [5,11,12].

2.5. Polyacrylamide electrophoresis (PAGE) and immunoblotting

PAGE was performed according to Laemmli [16]. (1) Denaturing conditions: a 15% (w/v) acrylamide-bisacrylamide gel in 0.37 M Tris-HCl, pH 8.6, 0.1% SDS was used to separate the antigens. Samples were diluted in Laemmli sample buffer and loaded after boiling 5 min. (2) Non-denaturing conditions: the gels were performed as above, except that SDS and 2-mercaptoethanol were not included; samples were loaded without boiling. Each well was loaded with 15 μg of protein. After the run, antigens were electrophoretically transferred onto nitrocellulose paper in 0.7 M glycine, 25 mM Tris, 20% ethanol and the filters were blocked in TBS (20 mM Tris, 0.5 M NaCl, pH 7.5), 5% BSA. The nitrocellulose paper was cut in vertical strips and incubated in mAb supernatants at 1:15 dilution in TBS, 0.5% Tween 20. Secondary peroxidase-labelled goat anti-mouse IgG and IgM sera were used at a 1:1000 dilution. Blots were developed with 3,3′-diaminobenzidine as a substrate.

2.6. Isotype characterization

The isotype of the mAbs was determined by Western blot: strips of nitrocellulose, prepared as above, were incubated with mAb supernatants diluted 1:15. A second-stage goat antibody recognizing mouse IgG1, IgG2a, IgG2b, IgG3 or IgM (mouse monoclonal antibody isotyping kit, Sigma) at a 1:500 dilution was then incubated for 1 h. After washing, a third-stage rabbit anti-goat peroxidase-labelled polyclonal serum was incubated for 1 h. Blots were developed as above.

2.7. Determination of the chemical nature of the antigens and epitopes recognized by the mAbs

Samples of BCG CF (15 μg) were diluted in 100 μl PBS containing 1 mg ml^{-1} trypsin or 1 mg ml^{-1} papain and incubated at 37°C for 2 h. To determine if the epitopes reacting with the mAbs were glycidic, 15 μg of BCG CF were diluted in 100 μl of 100 mM Na-acetate pH 4.5, 10 mM $NaIO_4$ and incubated at 37°C for 2 h. They were then subjected to SDS-PAGE and abrogation of recognition by the mAbs was evaluated by Western blotting [17].

To evaluate if lipid substituents were present the protein recognized by mAb L8D8, 80 μl BCG CF, 1 mg ml^{-1}, were extracted with 1% Triton X-114, as described [18].

2.8. Protein immunoaffinity purification

Sepharose-protein A (Sigma Chemical Co, St. Louis, MO, USA) was covalently bound to mAbs, produced as culture supernatants, by use of dimethylpimelimidate, following standard procedures [14]. Twelve-day-old BCG CF was added to the gel slurry at 1 mg ml^{-1} and incubated 16 h. After washing the gel with 10 mM phosphate buffer (pH 7.0), the antigens bound to the gel were eluted with 100 mM Na_3PO_4 (pH 12.5). The eluate was precipitated with 90% ammonium sulfate and analyzed by Western blotting.

3. Results

3.1. Antigen recognition and isotype of mAbs in Western blotting

The main features of the mAbs obtained in this study are summarized in Table 1. The supernatants of growing hybridomas were screened by Western blot after electrophoresis both in denaturing and in non-denaturing conditions. The six most interesting mAbs are shown, obtained from immune splenocytes of BALB/c mice: mAb 1G2 only recognized the native antigen; mAb AO3A4 only reacted with the de-

natured antigen, while the others recognized both denatured and native forms.

MAbs obtained from infected mice were only screened against denatured proteins and they all recognized products with a MW around 30 kDa. Because they recognized several bands and/or were highly cross-reactive and little species-specific, they were not analyzed in detail.

MAbs from mice immunized with CFs were of the IgG1 subclass, except for 2H2 and WB8A11, which were IgG2a, and L6G10, which was IgM. This is very similar to the results reported by Worsaae et al. [19]. All mAbs obtained from BCG-infected mice were of the IgM isotype.

3.2. Chemical nature of the antigens recognized by mAbs

The antigens recognized by the mAbs shown in Table 1 were also analyzed to assess whether they were sensitive to trypsin/papain digestion. These treatments abolished recognition by all mAbs studied, therefore the mAbs obtained all recognized polypeptides (data not shown). An exception was represented by the antigen recognized by L8D8, which was therefore analyzed in more detail (Fig. 1). As can be seen, although trypsin/papain did not abolish antigen recognition by L8D8, other less specific proteases did destroy the antigen. DNase/RNase digestion did not affect recognition of CF by L8D8. The L8D8 antigen was recovered in the aqueous phase after Triton X-114 extraction, demonstrating that no lipid substituent was present on the protein.

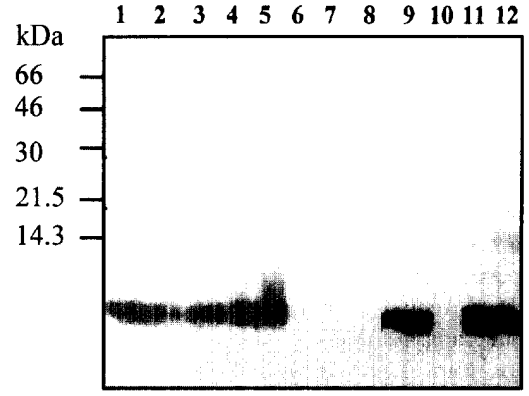

Fig. 1. Chemical nature of the antigen recognized by mAb L8D8. Samples (15 μg) were electrophoresed on a 15% SDS-PAGE gel after treating with: (1) no treatment; (2) $NaIO_4$ pH 4; (3) $NaIO_4$ pH 7; (4) trypsin, 1 mg ml^{-1}; (5) papain, 1 mg ml^{-1}; (6) protease XXIIV, 1 mg ml^{-1}; (7) protease XIV, 1 mg ml^{-1}; (8) proteinase K, 1 mg ml^{-1}; (9) Triton X-114, acqueous phase; (10) Triton X-114, detergent phase; (11) DNase, 1 mg ml^{-1}; (12) RNase, 1 mg ml^{-1}. After transfer onto nitrocellulose, the filter was probed with mAb L8D8. Bars on the left represent MW markers.

In addition, treatment of CF with $NaIO_4$, known to oxidize sugar residues to aldehydes, did not abrogate recognition by the mAb, showing that no glycidic substituent was involved in mAb-antigen binding [17]. This treatment only abolished recognition by mAb WB8A11, indicating that at least one of the antigens, the 82-kDa protein, is glycosylated (Fig. 2). Recognition by all other mAbs was not affected by $NaIO_4$ treatment.

3.3. Subcellular localization of the antigens

To assess whether the mAbs obtained recognized true secretion proteins or constituents of the bacterial cell, cellular localization of the antigens recognized by the mAbs was investigated by Western blot of four different subcellular fractions: CF, cytosol-, cell membrane- and cell wall-enriched. The various preparations were resolved by PAGE and transferred

Table 1
MAb features

MAb	Form of antigen recognized[a]	MW (kDa) of antigen[b]	Isotype[b]	Trypsin/papain digestion
2H2	den/non-den	23	IgG2a	S
AO1F12	den/non-den	32	IgG1	S
3E11	den/non-den	30	IgG1	S
WB8A11	den/non-den	80	IgG2a	S
1G2	non-den	30	IgG1	n.d.
AO3A4	den	19	IgG1	S
L8D8	den/non den	5	IgG1	R
L6G10	den/non den	42	IgM	S
C1B6	den	26	IgM	n.d.
C1G4	den	22+34–38	IgM	n.d.
C3F3	den	34	IgM	n.d.
C2F3	den	35–38+48	IgM	n.d.
C3C6	den	22+33	IgM	n.d.

[a]MAbs from C57BL/6 mice were only analyzed by SDS-PAGE and immunoblotting.
[b]Determined by SDS-PAGE and immunoblotting with CFs of BCG.
S: sensitive; R: resistant; n.d.: not determined.

onto nitrocellulose filters. The fidelity of the fractions was checked as described in Section 2 [7]. The mAbs from infected mice showed a high degree of interspecies reactivity in these experiments, recognizing several bands in CF from almost all mycobacterial species tested (data not shown).

Fig. 3A,F,G shows the results with antibodies AO1F12, L6G10 and L8D8, respectively, recognizing antigens present exclusively in CFs. MAb AO3A4 (Fig. 3D) recognized a 17-kDa cytoplasmic protein. All other mAbs recognized components which were present not only in CFs, but also in the cell wall and/or membrane: mAb WB8A11 (Fig. 3B) recognized a main band of 82 kDa, which was detectable also in the cell wall and, to a lesser extent, the membrane fractions. A band of 45 kDa was present in the cytosol fraction, while another band of 35 kDa was only present in CFs. MAb 3E11 (Fig. 3C) recognized two bands of 24 and 30 kDa present to a greater extent in the cell wall fraction, but detectable in all fractions analyzed. Antibody 2H2 (Fig. 3E) recognized a band of 23 kDa present also in the cell wall and membrane fractions.

Thus, at least three mAbs are likely to react with true secretion products, present only in CFs.

3.4. Interspecies reactivity

All mAbs recognizing the denatured form of antigen were also analyzed for their ability to recognize CF proteins of different mycobacterial species by Western blot. Table 2 summarizes the results obtained for mAbs from mice immunized with CF, except L8D8, which is shown in Fig. 4. In general, mAbs from infected mice showed a higher degree of interspecies reactivity compared to the others (data not shown). As shown in Table 2, one mAb, AO3A4, was specific only for the *M. tuberculosis* complex, WB8A11 reacted with products of the latter and, to a lower extent, also of *M. avium*/*M. intracellulare* and mAb 2H2 recognized the product of the *M. tuberculosis* and *M. avium*/*M. intracellulare* complexes and of *M. kansasii*. MAbs L6G10, 3E11, AO1F12 reacted with proteins present in CF of a great number of species.

As can be seen in Fig. 4, mAb L8D8 recognized an antigen present in the CFs of the *M. tuberculosis* complex and, in addition, of *M. marinum*, *M. kansasii* and *M. gordonae*, in the latter with a slightly higher MW (8–10 kDa).

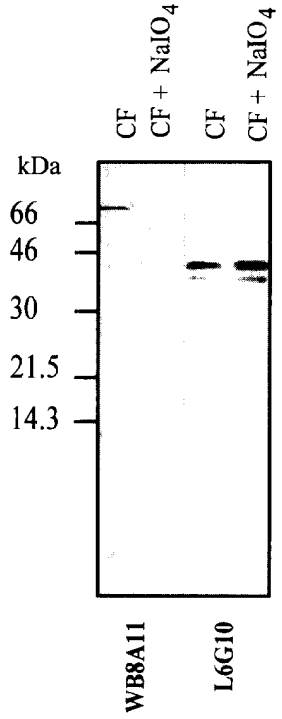

Fig. 2. Chemical nature of the epitopes recognized by mAbs L6G10 and WB8A11. Samples (15 μg) were electrophoresed on a 15% SDS-PAGE gel after no treatment (CF) or treatment with $NaIO_4$. After transfer onto nitrocellulose, the filters were probed with mAbs WB8A11 or L6G10. Bars on the left represent MW markers.

3.5. Identification of antigens recognized by selected mAbs

Certain mAbs were selected for further study on the basis of their limited interspecies reactivity, their likely secretion origin or the MW of the antigen recognized: the antigens recognized by the mAbs were purified by affinity chromatography using sepharose-*Staphylococcus aureus* protein A as a solid phase matrix, in order to further characterize and possibly identify the antigens.

AO3A4: The purpose was to define whether the purified 17-kDa antigen was the same as the one recognized by other mAbs, HBT11 and HYT6, reacting with proteins of 17–19 kDa [19,20]. As can be

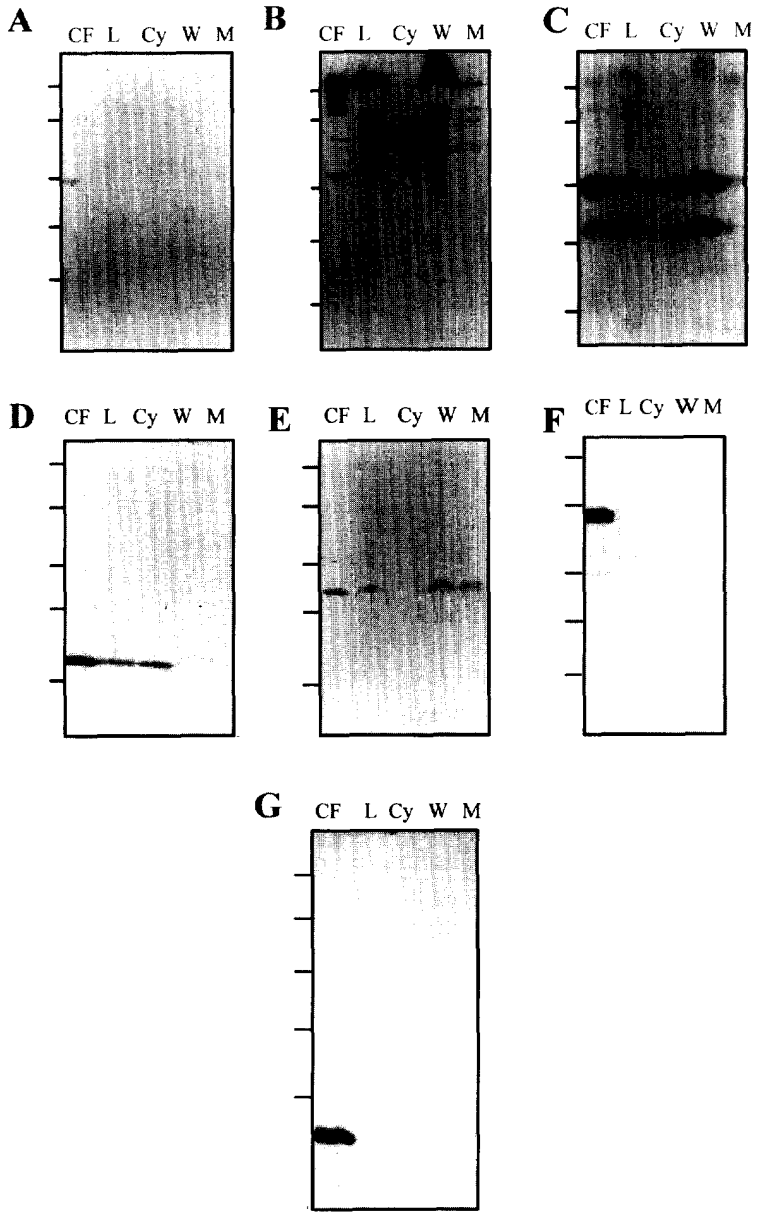

Fig. 3. Immunoblotting analysis of subcellular BCG fractions with mAbs. Samples (15 μg) were electrophoresed on a 15% SDS-PAGE gel and transferred onto nitrocellulose filters. Lanes: CF: CF day 7; L: BCG lysate; Cy: cytoplasm; W: cell wall; M: cell membrane. Each filter was incubated with different mAb supernatants diluted 1:10. (A) AO1F12. (B) WB8A11. (C) 3E11. (D) AO3A4. (E) 2H2. (F) L6G10. (G) L8D8. Bars on the left represent MW markers. From top to bottom (kDa): 66, 46, 30, 21.5 and 14.3.

seen in Fig. 5, the antigen purified by AO3A4 is also recognized by HBT11, an antibody known to react also with a cytoplasmic protein only produced by mycobacteria of the *M. tuberculosis* complex, that has been cloned [20]. Thus, AO3A4 does not identify a novel antigen.

2H2: The purpose was to define whether the purified 23-kDa protein was superoxide dismutase, rec-

Table 2
MAb interspecies reactivity[a]

Species	MAb					
	2H2	3E11	AO1F12	AO3A4	WB8A11	L6G10
M. tuberculosis	+	+	+	+	+	+
M. bovis BCG Pasteur	+	+	+	+	+	+
M. bovis BCG Tokyo	+	+/−	+	+	+	+
M. microti	+	+	+	+	+	+
M. avium S112/87	+/−	+	+	−	+	−
M. intracellulare	+/−	+	+	−	+	−
M. kansasii	+/−	+	+	−	−	+
M. vaccae	−	+/−	+	−	−	−
M. scrofulaceum	−	+	+	−	−	−
M. marinum	−	+/−	−	−	−	+
M. gordonae	−	+	+	−	−	+
M. simiae	−	−	−	−	−	−
M. xenopi	−	−	−	−	−	−
M. smegmatis	−	−	−	−	−	−

[a]Evaluated by immunoblotting with CFs from the mycobacterial species.

ognized by mAb F116-5 [15,21]. No recognition of the purified antigen by this mAb could be seen. However, the purity of the antigen was too low to allow amino-terminal sequencing, therefore further steps are still required to ascertain its identity.

WB8A11: The antigen purified by WB8A11 yielded an 82-kDa band in SDS-PAGE and Coomassie staining (not shown). It was then checked whether two other mAbs, CBA4 and HBT1, both

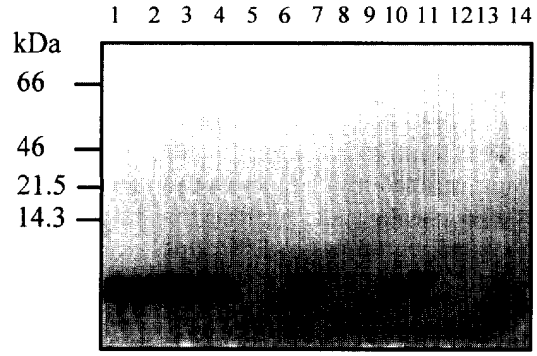

Fig. 4. Interspecies reactivity of mAb L8D8. CF (15 μg) from the following species were electrophoresed on a 15% SDS-PAGE gel and transferred onto nitrocellulose filters: (1) *M. bovis* BCG Pasteur; (2) *M. bovis* BCG Tokyo; (3) *M. tuberculosis*; (4) *M. microti*; (5) *M. avium*; (6) *M. intracellulare*; (7) *M. kansasii*; (8) *M. vaccae*; (9) *M. scrofulaceum*; (10) *M. marinum*; (11) *M. gordonae*; (12) *M. simiae*; (13) *M. xenopi*; (14) *M. smegmatis*. The filter was probed with mAb L8D8. Bars on the left represent MW markers.

specific for proteins of a similar MW, were able to react with this 82-kDa antigen [20]. As can be seen in Fig. 5, no recognition could be seen.

AO1F12, 3E11, 1G2: The antigens purified by these mAbs all belonged to the Ag 85 complex, as shown by the fact that they were all recognized by mAb HYT 27 [11]. Mabs AO1F12 and 3E11 were also assayed in Western blot against recombinant MPT59, the major component of Ag 85 (data not shown). As for 1G2, only reacting with the native form of the antigen, the purification of the latter was carried out to determine its MW in SDS-PAGE, and the purified protein yielded a band of 30 kDa as judged by SDS-PAGE and Coomassie blue staining (not shown).

L8D8: This mAb seems to react with a previously undescribed antigen, since no other mycobacterial protein of a similar MW has been described, except for the ESAT-6 antigen, which is absent in BCG [22]. The antigen recognized by L8D8 is being sequenced at its amino-terminus to ascertain whether it is indeed new.

L6G10: This mAb was chosen because of the subcellular localization of its 42-kDa antigen, which is only present in CF, and not in other subcellular fractions. The purification of this antigen is still in the process of being performed by chemical methods, since immunoaffinity purification did not yield any results, and is aimed at establishing whether this

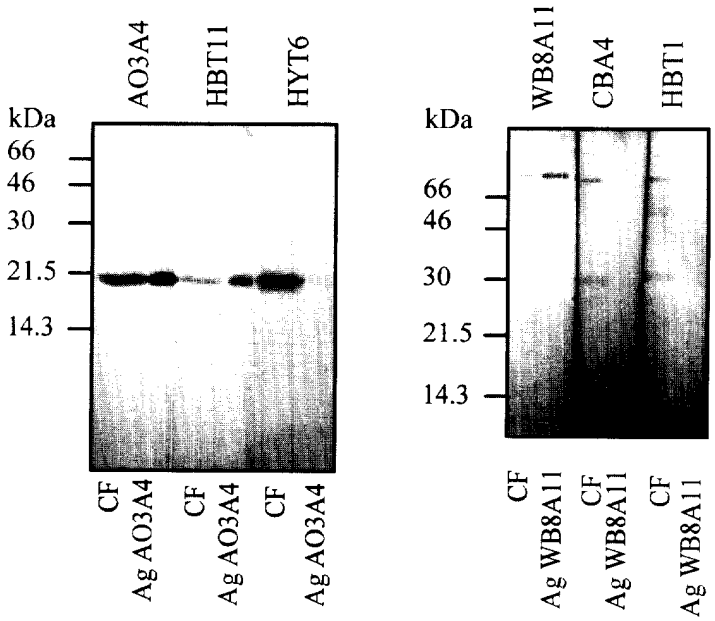

Fig. 5. Recognition of antigens purified with mAbs AO3A4 and WB8A11 by control mAbs. CF (15 μg) and antigens purified with AO3A4 (AgAO3A4) and WB8A11 (AgWB8A11) were electrophoresed on a 15% SDS-PAGE gel and transferred onto nitrocellulose. Filters were probed with the mAbs reported on top of each anel. Bars on the left represent MW markers.

antigen is the equivalent of other described antigens of a similar MW [23].

4. Discussion

BCG is an attenuated *M. bovis* strain widely used as a vaccine against tuberculosis. Its efficacy as a human vaccine ranges from 0 to 80% in different trials and one reason for this variability seems to depend on the ability of BCG to replicate in the host and, therefore, to produce sufficient quantities of antigens able to elicit a protective response. Because tuberculosis remains a worldwide health problem, much effort is being devoted to the identification of individual mycobacterial antigens that may be used to develop improved diagnostic methods for tuberculosis and/or a subunit vaccine alternative to BCG. MAbs have been widely used as tools to identify and isolate mycobacterial proteins and/or their genes in different studies.

In the present paper, a panel of monoclonal antibodies was obtained from BALB/c and C57BL/6 mice immunized in two different ways, with CF in IFA or by infection with BCG, in the attempt to diversify antigen recognition as much as possible. Unfortunately, mAbs obtained from infected mice were highly cross-reactive, probably because whole bacilli, at the dose used, may exert a somewhat suppressing effect on somatic maturation of B cells, as previously suggested for *M. leprae* [15]. This hypothesis was also suggested by the fact that all mAbs obtained from infected mice were of the IgM isotype. However, it must also be kept in mind that the strain of mice was different: an alternative explanation for the latter phenomenon might be that C57BL/6 mice are less efficient in isotype switching.

In an attempt to distinguish true secretion proteins from the others present in CFs, the subcellular localization of the antigens recognized by those mAbs reacting with the denatured form of the protein was examined. All subcellular fractions used for such experiments were checked with marker antibodies which showed that they were indeed enriched in extracellular, soluble cytoplasmic, cell wall and membrane products. Of seven antibodies analyzed in detail, five reacted with truly secretory proteins: mAbs L8D8 and L6G10 react with as yet unidentified

products, while AO1F12, 3E11 and 1G2 recognized components of a well-known secretion antigen, which can remain attached to mycobacterial surface and has been described as an immunodominant antigen for antibody reactivity in mice [13,23].

The antigen described in the greatest detail is the one recognized by L8D8, a protein of 5 kDa present in CF of a limited number of mycobacteria. This antigen is most likely to be a secretory protein, being found only in CF and not in other fractions. Its identity with a recently described antigen, ESAT-6, a 6-kDa secretory protein, can be excluded because the latter is absent in BCG [22]. Current work is aimed at amino-terminal sequencing of the L8D8 antigen.

MAb WB8A11 recognized a sugar epitope of a protein of 82 kDa, which was highly represented in the cell wall of mycobacteria belonging to the *M. tuberculosis* complex and also in *M. avium*. Because it is not recognized by two mAbs reacting with products of a similar MW, further investigation is required to assess whether this antigen is new. Amino-terminal sequencing of the latter protein will provide such information.

MAb 2H2 reacted with a 23-kDa antigen; at present, it can only be excluded that this is the BCG homologue of the *M. tuberculosis* superoxide dismutase, a 23-kDa secretion antigen which has been characterized in detail, since the purified antigen does not react with a mAb against superoxide dismutase [21].

One of the mAbs, AO3A4, showed the most limited specificity, being specific only for mycobacteria belonging to the *M. tuberculosis* complex. This mAb reacts with the previously described antigen, recognized by mAb HBT 11.

To conclude, at least two mAbs, L8D8 and WB8A11, are likely to describe novel mycobacterial antigens. The aim of future work is to assess the identity of these antigens, in order to produce the proteins in a recombinant form. This will allow to study the antigens as to their immunogenicity in animal models.

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