INFECTION AND IMMUNITY, Mar. 1991, p. 800–807
0019-9567/91/030800-08$02.00/0

Vol. 59, No. 3

Purification and Characterization of Major Antigens from a *Mycobacterium bovis* Culture Filtrate

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Received 16 July 1990/Accepted 30 November 1990

Ten major antigens from *Mycobacterium bovis* culture filtrate of 39, 32, 30, 25, 24, 22 (a and b forms), 19, 15, and 12 kDa have been purified and characterized by classical physicochemical methods. With monoclonal antibodies and/or N-terminal amino acid sequencing data, it was found that the antigens of 32, 30, 24, 22 (a), 19, and 12 kDa are related to *M. bovis* or *M. tuberculosis* antigens P32, MPB59, MPB64, MPB70, 19 kDa, and 12 kDa, respectively. The 39-, 25-, 22 (b)-, and 19-kDa antigens showed concanavalin A-binding properties and were positive in a glycan detection test, suggesting that they are glycoproteins. The 25- and 22 (b)-kDa proteins were found to be glycosylated forms of MPB70.

The tuberculin purified protein derivatives currently used for the diagnosis of tuberculosis in cattle and humans contain a complex mixture of proteins present in culture filtrate material from *Mycobacterium bovis* and *M. tuberculosis*, respectively (8). Some of these proteins are largely specific for the organism of choice, but others share a variety of antigenic determinants common to many species of mycobacteria. This antigenic cross-reactivity leads to false-positive results in intradermal skin tests and serological assays and is a major problem for disease diagnosis.

M. bovis and *M. tuberculosis* are very closely related organisms and are now referred to as members of the *M. tuberculosis* complex of organisms, which also includes bacillus Calmette-Guérin (BCG), *M. microti*, and *M. africanum*. There have been many efforts to purify the dominant antigenic proteins from purified protein derivative preparations, as reviewed by Daniel (8). In recent years, with the advent of monoclonal antibodies, protein sequencing, and DNA cloning techniques, a more detailed analysis of the antigens has been possible (36).

Here we describe the purification and characterization of 10 major antigens from *M. bovis* culture filtrate (CF). These were isolated and characterized as potential diagnostic reagents.

MATERIALS AND METHODS

Mycobacterial cultures. Bulk *M. bovis* AN-5 was grown in a synthetic medium as reported previously (16). The culture supernatant was harvested by aspiration, clarified on a gauze filter, and then sterilized by membrane filtration (0.22-μm pore size). The sterilized supernatant was concentrated 20-fold on a UM10 ultrafiltration membrane (Amicon DC 30) and then washed at constant volume with 10 volumes of phosphate-buffered saline (pH 7.2). Thiomersal (0.01%) was included in all stages of the supernatant processing. To this CF material, phenylmethylsulfonyl fluoride was added to 1.0 mM final concentration, and it was kept frozen at −80°C in appropriate aliquots until further use.

Antigen fractionation. Antigens were precipitated by 75% ammonium sulfate and subjected to either chromatofocusing or anion-exchange chromatography. Chromatofocusing was performed on a PBE-94 column (1.5 by 40 cm; Pharmacia) essentially by a scaled-up version of the procedure described for the purification of the *M. bovis* protein antigen MPB70 (16). Anion-exchange chromatography was performed on a DEAE-Sepharose CL-6B column (2.5 by 30 cm; Pharmacia) equilibrated with 30 mM Tris-HCl (pH 8.8) containing 2% butanol and eluted with a 0 to 200 mM NaCl gradient as described by Harboe et al. (17). In both procedures, the column was linked to a fast protein liquid chromatography system (Pharmacia) to maintain the flow rate at 0.5 ml/min and to generate the gradients. For each run, fractions enriched for particular antigens were pooled, concentrated, and processed as described below.

Columns and buffers. Two gel filtration columns were used, a Biogel P-60 (1.6 by 100 cm) and a Biogel P-100 (0.9 by 50 cm); these were equilibrated with 1% NH_4HCO_3, pH 8.2. A Pharmacia Mono Q HR 5/5 anion-exchange column was equilibrated with 30 mM Tris-HCl (pH 7.8). The proteins, unless otherwise indicated, were eluted with a 0 to 300 mM NaCl gradient in the equilibration buffer. The Pharmacia Mono P 5/20 chromatofocusing column was equilibrated with 0.025 M piperazine (pH 5.5) adjusted with iminodiacetic acid (IMDDA). The proteins were applied to the column in a 1/10 dilution of Polybuffer PB-74 (Pharmacia) adjusted to pH 4.0 with IMDDA and eluted from the column with a pH gradient from 5.5 to 4.0 generated by washing the column with a 1/10 dilution of PB-74–IMDDA at pH 4.0. A Pharmacia concanavalin A (ConA)-Sepharose column was equilibrated with 20 mM Tris-HCl–0.5 M NaCl–1 mM Mn^{2+}–1 mM Ca^{2+} at pH 7.4. The proteins were eluted with 1% α-D-methylglucoside in the equilibration buffer.

Purification of the 39-kDa antigen. Fractions off the DEAE-Sepharose column enriched for the 39-kDa protein were pooled, concentrated, and further fractionated on the P-60 gel filtration column. Fractions containing the antigen were freeze dried and applied to the ConA-Sepharose column. The bound material was eluted with 1% α-D-methylglucoside, dialyzed, freeze dried, and then put on the Mono Q column. The fractions containing the 39-kDa protein were

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pooled and concentrated, and any remaining impurities were removed by gel filtration on the P-100 column.

Purification of the 32-kDa antigen. This antigen was obtained virtually pure from the flowthrough part of the chromatofocusing run. The protein was rather unstable and often precipitated out or denatured in subsequent steps. A more stable form of this antigen was obtained when DEAE-Sepharose fractions containing the 32-kDa protein were concentrated and further purified by gel filtration on the P-60 column. This was followed by anion-exchange chromatography on the Mono Q column as described above. The antigen was purified to homogeneity by gel filtration chromatography on the P-100 column.

Purification of 30-kDa antigen. Fractions from the chromatofocusing run enriched in the 30-kDa protein were combined, concentrated, and further purified by gel filtration on the P-60 column. This was followed by an additional chromatofocusing step on the Mono P column with the same buffers as for the initial chromatofocusing step. Minor contaminants were removed by gel filtration chromatography on the P-100 column.

Purification of the 25- and 24-kDa antigens. Fractions from the chromatofocusing or the DEAE-Sepharose columns enriched in the 25- and 24-kDa proteins were pooled, concentrated, dialyzed, and applied to the ConA-Sepharose column. This procedure separated the 25-kDa protein, which bound to the column, from the 24-kDa antigen, which was found in the flowthrough pool. The bound antigens were eluted with 1% α-D-methylglucoside. Both pools were processed in a similar way, first by gel filtration chromatography on the P-60 column and then by chromatofocusing on the Mono P column. Any remaining impurities were removed by gel filtration chromatography on the P-100 column.

Purification of the 22-kDa antigens. Fractions containing the 22-kDa antigens from the chromatofocusing or the DEAE-Sepharose runs were further purified on the ConA-Sepharose column. The majority of the 22-kDa protein (a form) was recovered in the flowthrough pool; however, about 10% of the total 22-kDa protein (b form) bound to the column and was eluted with 1% α-D-methylglucoside. The respective pools were further purified of contaminants by gel filtration on the P-60 and/or P-100 columns.

Purification of the 19-kDa antigen. The first peak obtained from the DEAE-Sepharose column contained mainly the 19-kDa protein with minor contaminants of 22 and 24 kDa. These fractions were combined and applied to the ConA-Sepharose column. The bound antigens, which contained mainly the 19-kDa polypeptide, were eluted with 1% α-D-methylglucoside. The pure 19-kDa antigen was obtained by gel filtration chromatography on the P-100 column.

Purification of the 15-kDa antigen. Fractions from the DEAE run containing the 15-kDa antigen were pooled, concentrated, and further fractionated on the P-60 gel filtration column. The appropriate fractions were then pooled, freeze dried, and subjected to a further anion-exchange step on the Mono Q column. A final gel filtration step on the P-100 column yielded pure 15-kDa protein.

Purification of the 12-kDa antigen. The purification steps were the same as for the 15-kDa antigen. The 12-kDa antigen binds strongly on the Mono Q column and was eluted with a gradient of 200 to 500 mM NaCl.

SDS-PAGE. By using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), reduced antigens were characterized by their electrophoretic mobility on 15% polyacrylamide–SDS gels with a Bio-Rad Protean II mini-gel apparatus as reported previously (16). The molecular mass and purity of each antigen were estimated in this way.

Immunoblotting. Antigens from SDS gels were electrotransferred onto nitrocellulose membranes (0.45-μm pore size; Bio-Rad) with the Bio-Rad Trans Blot SD semi-dry transfer cell. The transfer buffer was that of Towbin et al. (28). The gels were soaked in transfer buffer for a few minutes prior to transfer, with two changes of the buffer. Electrophoresis was carried out at 12 V for 20 min. After transfer, the antigens were immunodetected with the appropriate monoclonal antibodies and developed as described previously (16).

Monoclonal antibodies. Monoclonal antibodies against various *M. tuberculosis* and *M. bovis* antigens were kindly provided by various colleagues. The Wellcome monoclonals TB 78, TB 72, TB 68, and TB 23 (18) were provided by J. Ivanyi (MRC Tuberculosis and Related Infections Unit, Royal Postgraduate Medical School, Hammersmith Hospital, London). HYT 28, HYT 27, and HYT 6 (1, 24) were provided by A. Andersen (Tuberculosis Department, Statens Seruminstitut, Copenhagen, Denmark). The SB 10 monoclonal was raised by Agen Australia and characterized by Wood et al. (32).

Protein estimation. The concentration of proteins was determined by the bicinchoninic acid method (25), with bovine serum albumin as the reference standard. The A_{280} value was also used for pure proteins of known extinction coefficients.

Carbohydrate detection. A glycan detection kit (Boehringer Mannheim) was used for the detection of carbohydrate-containing proteins on nitrocellulose membranes. The hydroxyl groups of the carbohydrates were oxidized to aldehydes with sodium metaperiodate, 10 mM in 100 mM sodium acetate buffer (pH 5.5), for 20 min at room temperature. The membranes were then washed in phosphate-buffered saline and incubated in acetate buffer containing succinyl-ε-amidocaproic acid hydrazine linked to steroid hapten spacer digoxigenin for 1 h. During the incubation, the digoxigenin spacer becomes covalently linked to the aldehydes on the carbohydrate via a hydrazine group. After the incubation, the membranes were washed in Tris-buffered saline (pH 6.5), blocked for 30 min in blocking solution provided in the kit, washed, and incubated with an anti-digoxigenin alkaline phosphatase-conjugated antibody for 1 h. The membranes were then washed and incubated with substrate (5-bromo-4-chloro-3-indolyl-phosphate) until grey-black bands developed.

Monosaccharide composition. Monosaccharides were identified and quantified by gas chromatography-mass spectrometry (GC-MS) as their per-*o*-trimethylsilylmethyl glycosides following methanolysis in 1 M methanolic HCl at 80°C for 16 h (7, 21). To detect phosphorylated sugars in the native glycoproteins, the trimethylsilylated methylglucosides were treated with diazomethane, retrimethylsilylated, and reanalyzed with GC-MS (15). Prior to analysis, samples were spiked with *myo*-inositol to determine the amounts of carbohydrate.

Amino acid sequence. The sequence of the first 15 to 20 amino-terminal amino acids was determined for some of the antigens by automated Edman degradation with the Applied Biosystems 470 A upgraded liquid phase sequenator.

RESULTS

Protein antigens were concentrated from the *M. bovis* CF material by Amicon ultrafiltration, followed by ammonium

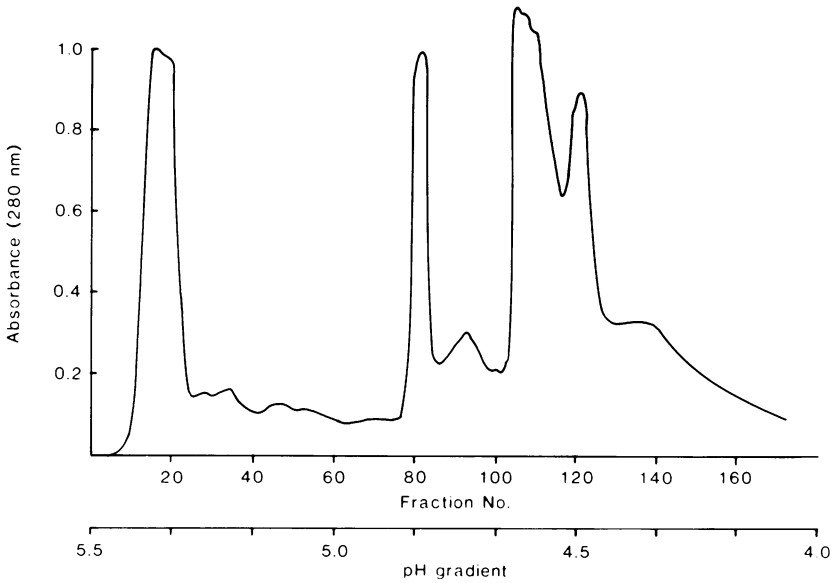

FIG. 1. Separation of *M. bovis* CF antigens by chromatofocusing on a PBE-94 column. Piperazine dialysate (20 ml, 750 A_{280} units) was loaded on a column (1.5 by 40 cm) which was previously equilibrated with dialysis buffer (25 mM piperazine, IMDDA [pH 5.5]). A pH gradient (5.5 to 4.0 pH units) was generated by eluting the column with 800 ml of a 1/10 dilution of Polybuffer PB-74–IMDDA (pH 4.0). Fractions (5 ml) were collected and analyzed for antigen content on SDS gels. Based on this analysis, fractions were pooled as follows: 15 to 25 for 32-kDa antigen and small amounts of the 24-kDa antigen; 26 to 32 for almost pure 32-kDa antigen, 75 to 84 for almost pure 30-kDa antigen; 85 to 100 for 25-, 30-, and 19-kDa antigens and traces of other antigens; 103 to 110 for almost pure 22-kDa antigen; 111 to 115 for 25-, 24-, and 22-kDa antigens; 116 to 120 for 25- and 24-kDa antigens; and 121 to 145 for the 22-kDa and traces of other antigens.

sulfate precipitation. Initially they were fractionated by chromatofocusing or DEAE anion-exchange chromatography. Elution profiles of these steps are depicted in Fig. 1 and 2. Recovery of some antigens was better from the chromatofocusing step, whereas for other proteins the DEAE-Sepharose chromatography step gave better yields. Proteins with molecular masses of 32, 30, and 22 kDa were recovered in an almost pure state from the chromatofocusing column. The 32-kDa protein was recovered in the flowthrough fraction, indicating that it did not bind to the column; however, it often precipitated or denatured in subsequent steps. The recovery of the 39-, 19-, 15-, and 12-kDa proteins was better from the DEAE column, while the 25- and 24-kDa antigens were recovered equally well from both columns. In all cases additional steps were needed for purification.

Fractions enriched for particular antigens from the chromatofocusing or the DEAE runs were pooled. Most antigens occurred in more than one of the pools (Fig. 1 and 2). The pools were concentrated and processed further as described in Materials and Methods for the various antigens. Ten components were purified in this manner, and their molecular masses, as deduced from SDS-PAGE gels (Fig. 3), were 39, 32, 30, 25, 24, 22 (a and b forms), 19, 15, and 12 kDa. Four of these antigens [39, 25, 22(b), and 19 kDa] bound to a ConA-Sepharose column and were eluted with 1% α-D-methylglucoside, indicating that these antigens contain a carbohydrate moiety. The presence of carbohydrate was confirmed when these antigens gave positive reactions with a glycan detection system (Fig. 4). The carbohydrate monomer composition of these antigens is shown in Table 1.

Chromatofocusing was used in an earlier study to purify an *M. bovis* 22-kDa antigen which was shown to be MPB70 (16). The 25-kDa glycoprotein antigen was recognized by a number of monoclonal antibodies which bind to separate epitopes on MPB70 (16), suggesting that the difference in size between the two antigens is due to the associated carbohydrate (Fig. 5b). In the present study it was found that about 10% of the 22-kDa protein (MPB70) has ConA-binding properties. Both the fraction of 22-kDa recovered in the ConA flowthrough (the a form) and the fraction that bound to Con A (the b form) were recognized equally by the same monoclonal antibodies (Fig. 5b). This suggests that a smaller amount of carbohydrate is associated with the 22-kDa MPB70 molecule than with the 25-kDa molecule.

Purified antigens were subjected to SDS gel electrophoresis and transferred onto nitrocellulose membranes, which were then probed with monoclonal antibodies raised against a number of different *M. tuberculosis* antigens. The 19-kDa antigen showed strong reaction with the TB 23 monoclonal (Fig. 5c) and the HYT 6 monoclonal (Fig. 5d). Both of these monoclonals recognize a 19-kDa *M. tuberculosis* antigen (18, 24). In addition, HYT 6 has been shown to react with antigens from a variety of mycobacterial species (1). Therefore, the *M. bovis* 19-kDa protein shares antigenic determinants with antigens from a variety of mycobacterial species. The 32- and the 30-kDa antigens reacted with monoclonal antibody HYT 27 (Fig. 5a). This monoclonal recognized a doublet of 31 to 32 kDa among the *M. tuberculosis* antigens and has also been shown to be widely cross-reactive (1). The 30-kDa protein showed a strong reaction, while the 32-kDa protein reacted only very faintly. When the 15- and the 12-kDa antigens were probed with the TB 68 monoclonal, which reacts with a 14-kDa *M. tuberculosis*/*M. bovis* antigen (18), no reaction was observed with either of them. The 39-kDa antigen was probed with TB 71 and HYT 28 monoclonals, both of which recognize a 38-kDa *M. tuberculosis*/*M. bovis* antigen (18, 24), but neither of them recognized this *M. bovis* antigen.

The amino acid sequence was determined for the first 15 to 20 residues for a number of these antigens (Table 2). Com-

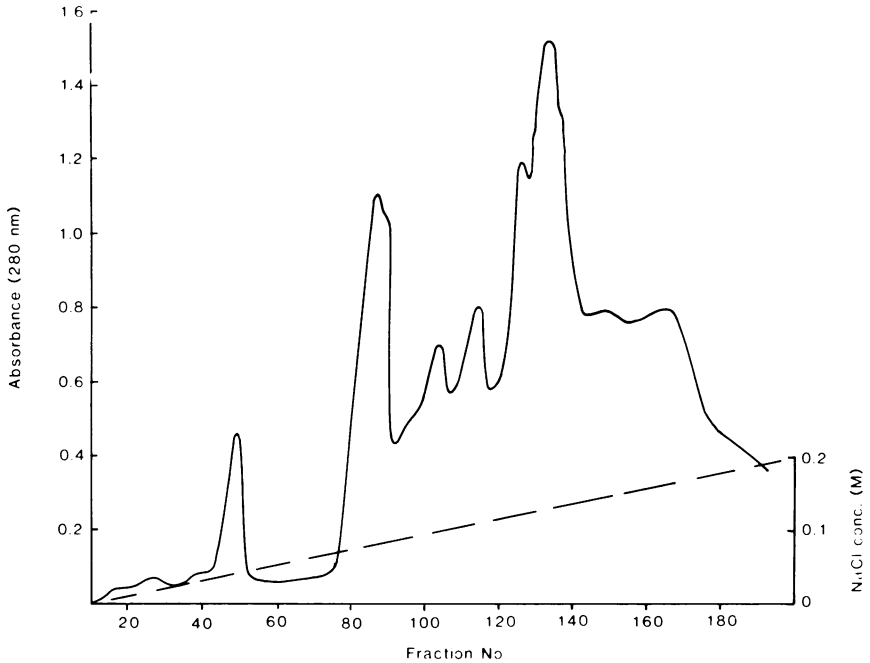

FIG. 2. Separation of *M. bovis* CF antigens by DEAE anion-exchange chromatography. CF antigen dialysate (200 ml, 1,400 A_{280} units) in Tris-HCl (pH 8.8) was applied to a DEAE-Sepharose CL column (2.5 by 30 cm) which was equilibrated with dialysis buffer. The antigens were eluted with 1,600 ml of 30 mM Tris-HCl (pH 8.8) containing 2% butanol and a linear gradient of 0 to 0.2 M NaCl. Fractions (8 ml) were collected and analyzed on SDS gels (result not shown). Based on the gel results, pools were made which were enriched for particular antigens but also contained other antigens as listed in decreasing abundance: fractions 40 to 47 for 19-kDa and traces of 22- and 25-kDa antigens; 78 to 87 for 22-kDa and smaller amounts of 25-, 24-, 32-, and 39-kDa antigens; 88 to 100 for 22-, 24-, 25-, and 30-kDa antigens; 101 to 108 for 25-, 24-, 30-, 19-, and 22-kDa antigens; 109 to 131 for 30-, 24-, 22-, 25-, 15-, and 19-kDa antigens; 132 to 138 for 32-, 30-, 15-, 12-, 25-, and 39-kDa antigens; and 139 to 180 for 12-, 30-, 32-, 25-, 19-, and 39-kDa antigens.

pared with the published sequence for *M. bovis* antigens, the 12-kDa protein had a sequence identical to that of antigen BCG-a described by Minden et al. (22). The 24-kDa protein is almost identical to MPB64 (17). The 30-kDa and the 32-kDa proteins have the same sequence for the first 19 amino acids and correspond to the MPB59 amino-terminal sequence (31).

The amount of these proteins present in CF varied with the age of the cultures. The 30- and 32-kDa antigens were very prominent in young cultures (Fig. 6a), but MPB70 was the most abundant in older cultures (Fig. 6b). Certain proteins, especially the 30- and 32-kDa proteins, appeared to degrade during storage of the CF even in the presence of protease inhibitors. These two proteins are also very unstable when purified and freeze dried.

DISCUSSION

We here describe the purification from CF of 10 *M. bovis* antigens with molecular masses of 39, 32, 30, 25, 24, 22 (a

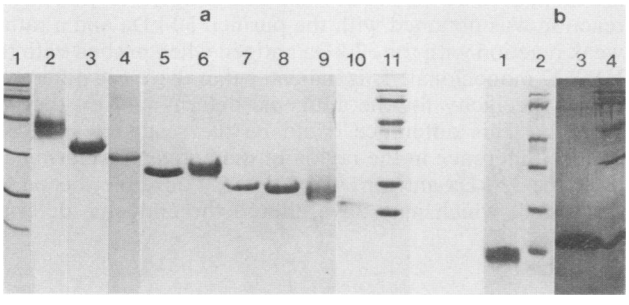

FIG. 3. SDS-PAGE of *M. bovis* CF antigens. From 5 to 10 μg of each antigen was applied. All gels were stained with Coomassie brilliant blue. Lanes b3 and b4 were restained with silver stain (Bio-Rad). Lanes a1, a11, b2, and b4 contain Bio-Rad low-molecular-weight markers (phosphorylase *b* [92,500], bovine serum albumin [66,200], ovalbumin [45,000], carbonic anhydrase [31,000], soybean trypsin inhibitor [21,500], and lysozyme [14,400]). Antigens in lanes: a2, 39 kDa; a3, 32 kDa; a4, 30 kDa; a5, 24 kDa; a6, 25 kDa; a7, 22 (b) kDa; a8, 22 (a) kDa; a9, 19 kDa; a10 and b3, 15 kDa; b1, 12 kDa.

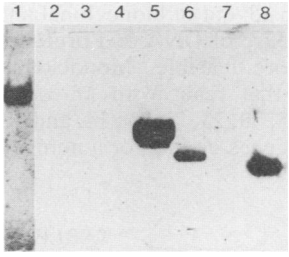

FIG. 4. Detection of carbohydrate on *M. bovis* CF antigens. Antigens (10 μg per slot) were electrophoresed on SDS-PAGE and transferred to nitrocellulose membranes. A glycan detection system (Boehringer Mannheim) was used to probe for carbohydrate (see text). Antigens with carbohydrate showed as dark grey-black bands. Antigens in lanes: 1, 39 kDa; 2, 32 kDa; 3, 30 kDa; 4, 24 kDa; 5, 25 kDa; 6, 22a kDa; 7, 22a kDa; 8, 19 kDa. The 15- and 12-kDa antigens were also probed but did not show any reaction (not shown).

TABLE 1. Monosaccharide composition of *M. bovis* glycoproteins

Monosaccharide	% (wt/wt) of total[a] in antigen:			
	39 kDa	25 kDa	22 (b) kDa	19 kDa
Arabinose	12	32	Tr	55
Xylose	1	Tr	9	1
Mannose	87	68	90	42
Galactose	Tr	Tr	Tr	2
N-Acetylglucosamine	Tr	—[b]	—	—

[a] Average of duplicate analyses. The carbohydrate content (percent of glycoprotein mass) determined by adding a known amount of the internal standard *myo*-inositol was 15, 4, and 0.2% for the 39-, 25-, and 22 (b)-kDa antigens, respectively (not determined for the 19-kDa antigen).

[b] —, None detected.

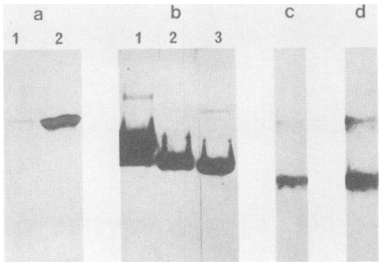

FIG. 5. Immunoblotting of *M. bovis* CF antigens with monoclonal antibodies. The 32-kDa (lane a1) and 30-kDa (lane a2) antigens were probed with monoclonal HYT 27. The 25-, 22 (b)-, and 22 (a)-kDa antigens (lanes b1, b2, and b3, respectively) were probed with monoclonal SB 10. The 19-kDa antigen was probed with monoclonal TB 23 (lane c) and monoclonal HYT 6 (lane d).

and b forms), 19, 15, and 12 kDa. Their molecular characteristics, yield, and relationship to previously studied antigens are summarized in Table 3. For the initial fractionation, chromatofocusing and DEAE anion-exchange were used. The 32-, 30-, and 22-kDa proteins were obtained virtually pure by chromatofocusing, while almost pure 19-kDa protein was recovered from the DEAE chromatography. In most cases additional steps were required for the final purification of the antigens. Most antigens occur in more than one peak, which indicates that they exist in several isoelectric point (pI) forms. The existence of mycobacterial proteins in more than a single pI form was reported previously for MPB70 (16, 17) and other antigens (6).

Four of the antigens, namely those of 39, 25, 22 (b form), and 19 kDa, were found to contain a carbohydrate moiety, suggesting that they are glycoproteins. The association of carbohydrates with mycobacterial proteins has also been reported previously (9, 14, 35). The carbohydrate composition of three of the four antigens consists largely of the monosaccharides mannose and arabinose. These two monosaccharides are also the main units of mycobacterial polysaccharides (8). The monosaccharide composition of the two glycosylated MPB70 species of 25 and 22 (b) kDa is quite different (Table 1). The presence of xylose in the 22(b)-kDa antigen indicates that different carbohydrate moieties are associated with each species.

Many studies have been done on mycobacterial antigens and their use as diagnostic reagents and/or vaccines for human tuberculosis. Cross-referencing of antigens reported in such studies in the past was rather difficult due to variations in the characterization techniques used by various groups. With the advent of monoclonal antibody technology and the greater ease of DNA and protein sequencing, comparisons are more feasible. Monoclonal antibodies have been produced that react with *M. tuberculosis*/*M. bovis* proteins of 70, 65, 38, 23, 22, 19, 14, and 12 kDa (1, 5, 18, 19, 24, 32, 37), and genes coding for a number of these proteins have been sequenced (2–4, 20, 23, 26, 27, 33, 34). In addition, some of these antigens have been purified, mainly by affinity chromatography on monoclonal antibody columns or by recombinant gene expression (4–6, 9, 19, 20, 22, 26, 33, 37) but also with conventional physicochemical methods (12, 13, 16, 17, 30, 31). In this study a thorough attempt was made to cross-reference our antigens with those described in other studies by using monoclonal antibodies where available and sequence data if known.

From the amino acid sequence data (Table 2), we established that the 12-kDa antigen is the same as the antigen BCG-a described by Minden et al. (22) and recently sequenced form BCG and *M. tuberculosis* DNA libraries (3, 34). The 24-kDa protein had an identical amino-terminal sequence, with the exception of residue 6, to that of MPB64 (17). The 30- and 32-kDa antigens had almost identical amino-terminal sequences that corresponded to the MPB59 amino-terminal sequence (31). Antigens MPB59 and P32 were reported to have an identical amino-terminal sequence (12). The 32-kDa protein, characterized in this study, is probably the same as the P32 antigen, whereas the 30-kDa protein is the same as the MPB59 antigen. The P32 and MPB59 antigens are also referred to as antigens 85A and 85B, respectively, in Harboe's crossed immunoelectrophoresis system (30).

Monoclonal antibody HYT 27 has previously been shown to bind to a doublet in the 30- to 32-kDa region (24), which are thought to be the MPB59 and P32 antigens (29). A strong reaction was obtained with the purified 30-kDa and a rather weak reaction with the 32-kDa antigen when probed with the HYT 27 monoclonal. This indicates that there is a difference in the specificity for the antigenic determinant on the two proteins. This difference could be the result of some sequence difference in the region of the antigenic determinant or of the 32-kDa antigen, which is very unstable, becoming denatured, which may have altered the antigenic determi-

TABLE 2. N-terminal amino acid sequences of *M. bovis* antigens

Antigen	Amino acid no.																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
32 kDa	Phe	Ser	Arg/Pro	Pro	Gly/Leu	Leu	Pro	Val	Glu	Tyr	Leu	Gln	Val	Pro	Ser	Pro	Ser	Met	Gly
30 kDa	Phe	Ser	Arg	Pro	Gly	Leu	Pro	Val	Glu	Tyr	Leu	Gln	Val	Pro	Ser	Pro	Ser	Met	Gly
24 kDa	Ala	Pro	Lys	Thr	Tyr	Lys/Leu	Glu	Glu	Leu	Lys	Gly	Thr	Asp	Thr	Gly	Gln			
12 kDa	Ala	Lys	Val	Asn	Ile	Lys	Pro	Leu	Glu	Asp	Lys	Ile	Leu	Val	Glu	Ala	Asn	Glu	Ala

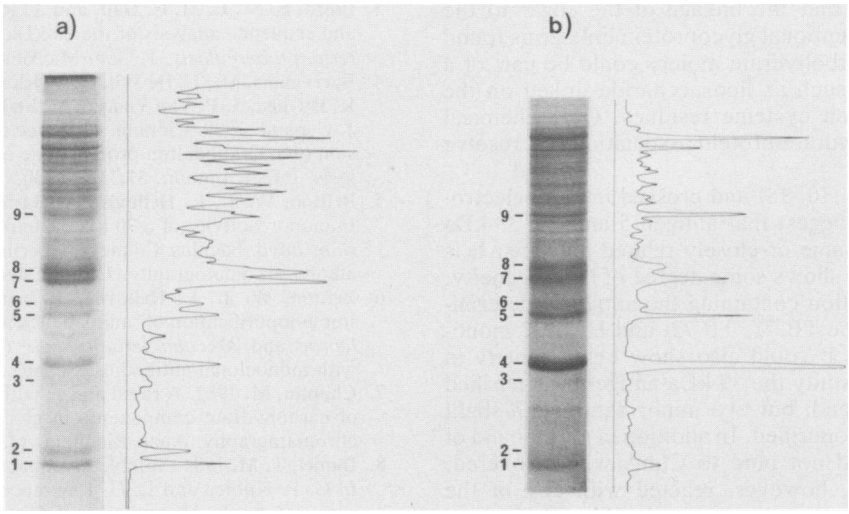

FIG. 6. SDS-PAGE and gel scan of unfractionated *M. bovis* CF antigens. A 40-μg amount of CF was loaded in each well. The gels were stained with Coomassie brilliant blue and scanned on a Quick Scan R&D (Helena Laboratories Aust. P/L). Antigens: 1, 12 kDa; 2, 15 kDa; 3, 19 kDa; 4, 22 kDa (MPB70); 5, 24 kDa (MPB64); 6, 25 kDa; 7, 30 kDa; 8, 32 kDa; 9, 39 kDa. (a) CF antigens isolated from 6-week-old cultures. Among the most abundant antigens are the 30- and 32-kDa proteins (bands 7 and 8). (b) CF antigens isolated from 12-week-old cultures. MPB70 is the most abundant antigen (band 4).

nant. The genes for both the MPB59 and P32 antigens have now been cloned and fully sequenced and show 72.8% homology in amino acid sequence (4). Thus, although the two proteins are largely homologous, there is enough sequence difference for antigenic variation between the two.

Monoclonal antibodies TB 23 and HYT 6 gave a strong reaction with the 19-kDa protein (Fig. 5). These monoclonal antibodies were raised to an *M. tuberculosis* 19-kDa antigen and cross-reacted with antigens from a variety of species outside the *M. tuberculosis* complex (1, 18); therefore, the *M. bovis* 19-kDa antigen must contain a cross-reactive determinant. We found that the 19-kDa antigen is associated with carbohydrate. Andersen et al. (1) also found that the *M. tuberculosis* 19-kDa protein has ConA-binding affinity.

The 39-kDa antigen did not react with the TB 71, TB 72, and HYT 28 monoclonal antibodies, and therefore this antigen is different from the reported 38-kDa protein of the *M. tuberculosis* complex (1, 18). It has been shown that these monoclonals recognize a minor component in *M. bovis* CF (18). We also observed this reaction in unfractionated CFs. However, this component was not purified by us and is most likely present only in very small amounts. The 39-kDa protein we purified has ConA-binding properties and gives positive results with the glycan detection system (Fig. 4). In that respect, the 39-kDa antigen is similar to antigen 5 reported in various publications by Daniel et al. (9–11). Recently, Daniel (8a) explained the carbohydrate association of antigen 5 as nondescript carbohydrate contamination rather than glycosylation. Apart from antigen 5, glycosylated mycobacterial proteins have been reported in other studies (14, 35).

The existence of glycoproteins in bacteria is a controversial topic (14). We strongly favor the view that the carbohydrates are covalently linked to the proteins because they were found to be associated with the protein throughout several different purification steps and they did not dissociate even in the presence of SDS and dissociating agents such as guanidine and urea (result with dissociating agents not shown). Furthermore, the carbohydrate monomer composition is different for each antigen and constant from preparation to preparation. The percent carbohydrate content in each of the glycosylated MPB70 species [22(b) and 25 kDa] approximately accounts for the molecular size difference observed between them and the nonglycosylated form

TABLE 3. Summary of antigens purified in this study and their relationship to previously published mycobacterial antigens

Mol mass (kDa)	Yield[a] (mg)	Carbohydrate association	Reaction with monoclonal antibodies	Sequence homology	Identity with published antigens	Reference(s)
39	1.8	Yes			Antigen 5?	9–11, 29
32	22.3	No	HYT 27 (weak)	P32, MPB59	P32, 85A	1, 4, 24, 30, 31
30	25.0	No	HYT 27 (strong)	MPB59, P32	MPB59, 85B	1, 24, 30, 31
25	6.7	Yes	SB 10		MPB70	16
24	3.9	No		MPB64	MPB64	17, 33
22 (a)	38.5	No	SB 10	MPB70	MPB70	16, 17, 23, 26, 32
22 (b)	3.3	Yes	SB 10		MPB70	
19	2.1	Yes	TB 23, HYT 6		19-kDa antigen	1, 2, 18, 24
15	0.8	No				
12	22.8	No		12-kDa antigen	BCG-a, 12-kDa antigen, MPB57	3, 22, 34

[a] Yield = milligrams per gram of protein in the 75% ammonium sulfate cut of CF.

[22(a)]. It is possible that the linkage of the sugar to the protein is not the conventional glycoprotein linkage as found in eukaryotes. The carbohydrate moiety could be part of a heteropolysaccharide such as liposaccharide linked on the protein by acylation on cysteine residues. Only chemical analysis of the carbohydrate-protein association will resolve this question.

Species distribution (10, 18) and crossed immunoelectrophoresis results (29) suggest that antigen 5 and the 38-kDa protein might be the same or closely related antigens. It is possible that antigen 5 shows some degree of heterogeneity, with only a small fraction containing the antigenic determinants recognized by the TB 71, TB 72, and HYT 28 monoclonal antibodies, and it could also show heterogeneity in glycosylation. In this study the 39-kDa antigen was purified mainly as one main band, but two minor bands with slight size differences were copurified. In addition, a minor band of the same size that did not bind to ConA was recovered. None of these bands, however, reacted with any of the monoclonal antibodies that recognize the *M. tuberculosis* 38-kDa protein.

Monoclonal antibody TB 68 was used to probe the 15-kDa antigen, but no reaction was observed with purified antigen; however, when CF was probed with the monoclonal, a reaction was observed at about the same position as the 15-kDa antigen. The *M. tuberculosis* 14-kDa antigen that is recognized by TB 68 has been cloned (19), but its sequence is not yet known. It appears that the antigen we purified is a different protein migrating in approximately the same position as the 14-kDa antigen.

Most of the purified antigens were quite unstable during storage. They were normally stored freeze-dried in a desiccator below 0°C. The most unstable antigens were the 30- to 32-kDa group, followed by the 15- and 19-kDa proteins. The most stable antigens were the 22 (a)-kDa (nonglycosylated MPB70) and the 25- and 22 (b)-kDa (glycosylated MPB70 molecules) proteins, followed by the 12-kDa protein. Perhaps the fragility of these antigens also affects their occurrence in the culture media both during cell growth and after the cultures were harvested. This would explain the higher amounts of these proteins in young cultures and the predominance of MPB70 in older cultures.

In this report we describe the purification and characterization of the main antigens of *M. bovis* CF. Their use in serological and cellular response studies could provide the basis for improved diagnosis and/or further vaccine development for either the bovine or human disease.

ACKNOWLEDGMENTS

This work was supported by grants from the National Brucellosis and Tuberculosis Eradication Campaign, the Australian Meat and Livestock Research and Development Corporation, and the Commonwealth Government Special Research Center Grant.

We are grateful to Z. Felton for excellent technical assistance and to P. Strike (CSIRO Division of Biomolecular Engineering) for the amino acid sequence. John Cox from the Commonwealth Serum Laboratory generously provided the bulk of *M. bovis* AN-5 culture supernatant for these studies.

REFERENCES

1. **Andersen, A. B., Z.-L. Yuan, K. Haslov, B. Vergmann, and J. Bennedsen.** 1986. Interspecies reactivity of five monoclonal antibodies to *Mycobacterium tuberculosis* as examined by immunoblotting and enzyme-linked immunosorbent assay. J. Clin. Microbiol. **23**:446–451.
2. **Ashbridge, K. R., R. J. Booth, J. D. Watson, and R. B. Lathigra.** 1989. Nucleotide sequence of the 19-kDa antigen gene from *Mycobacterium tuberculosis*. Nucleic Acids Res. **17**:1249.
3. **Baird, P. N., L. M. C. Hall, and A. R. M. Coates.** 1989. Cloning and sequence analysis of the 10-kDa antigen gene of *Mycobacterium tuberculosis*. J. Gen. Microbiol. **135**:931–939.
4. **Borremans, M., L. De Wit, G. Volckaert, J. Ooms, J. De Bruyn, K. Huygen, J.-P. Van Vooren, M. Stelandre, R. Verhofstadt, and J. Content.** 1989. Cloning, sequence determination, and expression of a 32-kilodalton-protein gene of *Mycobacterium tuberculosis*. Infect. Immun. **57**:3123–3130.
5. **Britton, W. J., L. Hellqvist, A. Basten, and A. S. Inglis.** 1986. Immunoreactivity of a 70 kD protein purified from *Mycobacterium bovis* bacillus Calmette-Guerin by monoclonal antibody affinity chromatography. J. Exp. Med. **164**:695–708.
6. **Britton, W. J., L. Hellqvist, J. Ivanyi, and A. Basten.** 1987. Immunopurification of radiolabelled antigens of *Mycobacterium leprae* and *Mycobacterium bovis* (bacillus Calmette-Guerin) with monoclonal antibodies. Scand. J. Immunol. **26**:149–159.
7. **Chaplin, M.** 1982. A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas-liquid chromatography. Anal. Biochem. **123**:336–341.
8. **Daniel, T. M.** 1984. Soluble mycobacterial antigens, p. 417–465. *In* G. P. Kubica and L. G. Lawrence (ed.), The mycobacteria: a source book. Microbiology Series vol. 15. Marcel Dekker, New York.

8a. **Daniel, T. M.** 1989. Letter to the editor. Am. Rev. Respir. Dis. **139**:1566–1567.

9. **Daniel, T. M., and P. A. Anderson.** 1978. The isolation by immunoabsorbent affinity chromatography and physicochemical characterization of *Mycobacterium tuberculosis* antigen 5. Am. Rev. Respir. Dis. **117**:533–539.
10. **Daniel, T. M., J. J. Ellner, L. S. Todd, D. W. McCoy, V. D. N. Payne, P. A. Anderson, and F. T Bhe.** 1979. Immunobiology and species distribution of *Mycobacterium tuberculosis* antigen 5. Infect. Immun. **24**:77–82.
11. **Daniel, T. M., N. J. Gonchoroff, J. A. Katzmann, and G. R. Olds.** 1984. Specificity of *Mycobacterium tuberculosis* antigen 5 determined with mouse monoclonal antibodies. Infect. Immun. **45**:52–55.
12. **De Bruyn, J., K. Huygen, R. Bosmans, M. Fauville, R. Lippens, J. P. Van Vooren, P. Falmagne, M. Weckx, H. G. Wiker, M. Harboe, and M. Turneer.** 1987. Purification, characterization and identification of 32 kDa protein antigen of *Mycobacterium bovis* BCG. Microb. Pathog. **2**:351–366.
13. **De Bruyn, J., R. Bosmans, M. Turneer, M. Weckx, J. Nyabenda, J.-P. Van Vooren, P. Falmagne, H. G. Wiker, and M. Harboe.** 1987. Purification, partial characterization, and identification of a skin-reactive protein antigen of *Mycobacterium bovis* BCG. Infect. Immun. **55**:245–252.
14. **Espitia, C., and R. Mancilla.** 1989. Identification, isolation and partial characterization of *Mycobacterium tuberculosis* glycoprotein antigens. Clin. Exp. Immunol. **77**:378–383.
15. **Ferguson, M. A. J., S. W. Homans, R. A. Dwek, and T. W. Rademacher.** 1988. Glycosyl-phosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. Science **239**:753–759.
16. **Fifis, T., P. Plackett, L. A. Corner, and P. R. Wood.** 1989. Purification of a major *Mycobacterium bovis* antigen for the diagnosis of bovine tuberculosis. Scand. J. Immunol. **29**:91–101.
17. **Harboe, M., S. Nagai, M. E. Patarroyo, M. L. Torres, C. Ramirez, and N. Cruz.** 1986. Properties of proteins MPB64, MPB70, and MPB80 of *Mycobacterium bovis* BCG. Infect. Immun. **52**:293–302.
18. **Ivanyi, J., J. A. Morris, and M. Keen.** 1985. Studies with monoclonal antibodies to mycobacteria, p. 59–90. *In* A. J. L. Macario and E. C. Macario (ed.), Monoclonal antibodies against bacteria, vol. I. Academic Press, Inc., New York.
19. **Kingston, A. E., P. R. Salgame, N. A. Mitchison, and M. J. Colston.** 1987. Immunological activity of a 14-kilodalton recombinant protein of *Mycobacterium tuberculosis* H37Rv. Infect. Immun. **55**:3149–3154.
20. **Matsuo, K., R. Yamaguchi, A. Yamazaki, H. Tasaka, and T. Yamada.** 1988. Cloning and expression of the *Mycobacterium bovis* BCG gene for extracellular α antigen. J. Bacteriol. **170**: 3847–3854.

21. **McConville, M. J., and A. Bacic.** 1989. A family of glycoinositol phospholipids from *Leishmania major*: isolation, characterization, and antigenicity. J. Biol. Chem. **264**:757–766.
22. **Minden, P., P. J. Kelleher, J. H. Freed, L. D. Nielsen, P. J. Brennan, L. McPheron, and J. K. McClatchy.** 1984. Immunological evaluation of a component isolated from *Mycobacterium bovis* BCG with a monoclonal antibody to *M. bovis* BCG. Infect. Immun. **46**:519–525.
23. **Radford, A. J., B. J. Duffield, and P. Plackett.** 1988. Cloning of a species-specific antigen of *Mycobacterium bovis*. Infect. Immun. **56**:921–925.
24. **Schou, C., Z. L. Yuan, A. B. Andersen, and J. Bennedsen.** 1985. Production and partial characterization of monoclonal hybridoma antibodies to *Mycobacterium tuberculosis*. Acta Pathol. Microbiol. Immunol. Scand. Sect. C **93**:265–272.
25. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**:76–85.
26. **Terasaka, K., R. Yamaguchi, K. Matsuo, A. Yamazaki, S. Nagai, and T. Yamada.** 1989. Complete nucleotide sequence of immunogenic protein MPB70 from *Mycobacterium bovis* BCG. FEMS Microbiol. Lett. **58**:273–276.
27. **Thole, J. E. R., W. J. Keulen, J. De Bruyn, A. H. J. Kolk, D. G. Groothuis, L. G. Berwald, R. H. Tiesjema, and J. D. A. van Embden.** 1987. Characterization, sequence determination, and immunogenicity of a 64-kilodalton protein of *Mycobacterium bovis* BCG expressed in *Escherichia coli* K-12. Infect. Immun. **55**:1466–1475.
28. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
29. **Wiker, H. G., M. Harboe, J. Bennedsen, and O. Closs.** 1988. The antigens of *Mycobacterium tuberculosis* H37Rv, studied by crossed immunoelectrophoresis. Comparison with a reference system for *Mycobacterium bovis*, BCG. Scand. J. Immunol. **27**:223–239.
30. **Wiker, H. G., M. Harboe, and T. E. Lea.** 1986. Purification and characterization of two protein antigens from the heterogenous BCG85 complex in *Mycobacterium bovis* BCG. Int. Arch. Allergy Appl. Immunol. **81**:298–306.
31. **Wiker, H. G., M. Harboe, S. Nagai, M. E. Patarroyo, C. Ramirez, and N. Cruz.** 1986. MPB59, a widely cross-reacting protein of *Mycobacterium bovis* BCG. Int. Arch. Allergy Appl. Immunol. **81**:307–314.
32. **Wood, P. R., J. Ripper, A. J. Radford, P. G. Bundesen, D. B. Rylatt, L. E. Cottis, M. John, and P. Plackett.** 1988. Production and characterization of monoclonal antibodies specific for *Mycobacterium bovis*. J. Gen. Microbiol. **134**:2599–2604.
33. **Yamaguchi, R., K. Matsuo, A. Yamazaki, C. Abe, S. Nagai, K. Terasaka, and T. Yamada.** 1989. Cloning and characterization of the gene for immunogenic protein MPB64 of *Mycobacterium bovis* BCG. Infect. Immun. **57**:283–288.
34. **Yamaguchi, R., K. Matsuo, A. Yamazaki, S. Nagai, K. Terasaka, and T. Yamada.** 1988. Immunogenic protein MPB57 from *Mycobacterium bovis* BCG: molecular cloning, nucleotide sequence and expression. FEBS Lett. **240**:115–117.
35. **Yano, O., T. Fukuda, H. Abe, and T. Sudo.** 1984. Purification and characterization of a tuberculin-active substance from *Mycobacterium bovis* BCG. J. Biochem. (Tokyo) **96**:523–531.
36. **Young, D. B.** 1988. Structure of mycobacterial antigens. Br. Med. Bull. **44**:562–583.
37. **Young, D., L. Kent, A. Rees, J. Lamb, and J. Ivanyi.** 1986. Immunological activity of a 38-kilodalton protein purified from *Mycobacterium tuberculosis*. Infect. Immun. **54**:177–183.