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Identification of *Mycobacterium bovis* antigens by analysis of bovine T-cell responses after infection with a virulent strain

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Abstract

Purification and characterization of individual antigenic proteins are essential for the understanding of the pathogenic mechanisms of mycobacteria and the immune response against them. In the present study, we used anion-exchange chromatography to fractionate cell extracts and culture supernatant proteins from *Mycobacterium bovis* to identify T-cell-stimulating antigens. These fractions were incubated with peripheral blood mononuclear cells (PBMC) from *M. bovis*-infected cattle in lymphoproliferation assays. This procedure does not denature proteins and permits the testing of mixtures of potential antigens that could be later identified. We characterized protein fractions with high stimulation indices from both culture supernatants and cell extracts. Proteins were identified by two-dimensional gel electrophoresis followed by N-terminal sequencing or MALDI-TOF. Culture supernatant fractions containing low molecular weight proteins such as ESAT6 and CFP10 and other proteins (85B, MPB70), and the novel antigens TPX and TRB-B were associated with a high stimulation index. These results reinforce the concept that some low molecular weight proteins such as ESAT6 and CFP10 play an important role in immune responses. Also, Rv3747 and L7/L12 were identified in high stimulation index cell extract fractions. These data show that protein fractions with high lymphoproliferative activity for bovine PBMC can be characterized and antigens which have been already described and new protein antigens can also be identified in these fractions.

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- Antigens
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- Cellular immunity

Introduction

Bovine tuberculosis caused by *Mycobacterium bovis* is an animal health problem throughout the world and also constitutes a major threat to public health. On the American continent there are 420 million heads of cattle, half of which are in countries in which the incidence of bovine tuberculosis is higher than 1%. There are 50,600,000 Argentine heads of cattle, with 5% of the animals and 35% of the farms being infected with bovine tuberculosis (1). Eradication of this zoonotic disease remains an important goal in several

countries. The strategy of test and slaughter has been used widely in an attempt to control dissemination of the disease and is based on the tuberculin skin test as a means for bovine tuberculosis diagnosis. However, implementation of tuberculin tests is cumbersome, requiring a second visit by the veterinarian three days after the tuberculin injection. Some bovine tuberculosis eradication programs have incorporated variants of this test but suboptimal sensitivity and specificity have frequently been reported (2,3). This is considered to be due, in part, to the nature of the poorly characterized antigens used, which are mycobacterial extracts containing components that are not species specific (3). Therefore, more sensitive and specific tests, probably incorporating better defined antigens, are required for efficient detection of this disease.

It is well established that T-cell recognition of mycobacterial antigens is the major immune response to tuberculosis (4-6). Therefore, an effective diagnostic test can be developed if well-characterized T-cell-reactive antigens are identified. This fact has led several research groups to the identification of antigens recognized by immune cells.

One of the main objectives of the present study was to identify the dominant *M. bovis* antigens that are recognized by the bovine cellular immune system. Unlike previous studies, which have focused on secreted antigens, the present study investigated cellular as well as secreted proteins. In addition, we used culture supernatants harvested at various times of the culture to determine if different antigens were released along the growth curve. This was achieved by separating *M. bovis* culture supernatant and whole cell proteins and testing the antigenicity of the resulting fractions by a lymphoproliferation assay (LPA) using peripheral blood mononuclear cells (PBMC) from experimentally infected cattle. This approach allows a fast step of preselection of immunodominant fractions from which it is easy to identify antigenic proteins.

Material and Methods

Bacterial strains, media and preparation of culture supernatant and cell extracts

The *M. bovis* AN5 standard strain was used throughout the study. Cultures were prepared in Middlebrook 7H9 liquid medium containing 0.4% pyruvic acid and glucose. Mycobacterial cultures were incubated at 37ºC and harvested at 24, 38 and 73 days. The supernatants were separated from the cell extract by centrifugation and then filtered through a 0.22-µm Millipore membrane (Bedford, MA, USA) to remove remaining mycobacteria. The proteins were then precipitated with ammonium sulfate (50%) for 18 h at 4ºC. Following centrifugation at 10,000 rpm for 1 h the precipitate was dissolved in a minimal volume of phosphate-buffered saline (PBS) and then dialyzed against PBS for 18 h at 4ºC. After removing the culture supernatant the cell mass was washed and resuspended in PBS. Mycobacteria were killed by heat (20 min at 80ºC) and sonicated on ice five times for 1 min with rest intervals of 2 min. Cell extracts were obtained from a 38-day culture.

The protein content of the supernatants and cell extracts was measured by the bicinchoninic acid method (Pierce, Rockford, IL, USA).

Animals

Four Friesian cattle were selected from a herd known to be free from bovine tuberculosis during the previous five years. These animals were confirmed as bovine tuberculosis negative by testing *in vitro* T-cell responses to mycobacterial antigens. The animals were placed in strict isolation under ventilation with negative pressure. They were infected by intranasal instillation with 10^6 CFU of a wild-type strain of *M. bovis* using previously described methods (6). Two animals of the same age and origin were used as uninfected controls.

Protein purification

Proteins from cell-free supernatants and cell extracts were separated using FPLC anion-exchange column chromatography (Pharmacia, Uppsala, Sweden). Protein, 0.5 to 5.0 mg, was loaded onto a Mono-Q column (Pharmacia) equilibrated with 20 mM Tris buffer, pH 8.0. Proteins bound to the column were eluted using a linear NaCl gradient (0-0.4125 M) while the proteins that bound very strongly were removed with 1 M NaCl. The flow rate was 0.5 ml per min and 84 fractions of 0.5 ml each were collected. Protein elution from the column was monitored by absorbance at 280 nm. All fractions were analyzed by SDS-PAGE (7) and proteins were visualized using a silver stain procedure (8). To simplify the screening, protein fractions from the major peaks of the chromatogram that had similar protein profiles (as determined by SDS-PAGE) were pooled. Pools were dialyzed against PBS, filter-sterilized and protein concentration was measured for use in LPA using PBMC from the experimentally infected cattle.

Lymphoproliferation assay

Blood was collected from four experimentally infected animals 11 months after *M. bovis* inoculation. PBMC were separated by centrifugation over Ficoll-Histopaque (Pharmacia) and viability of the PBMC preparation was determined by Trypan blue exclusion. Microcultures were prepared with 10^6 cells/ml in RPMI 1640, supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 25 µg/ml gentamicin sulfate. The antigenic fractions obtained by FPLC were added to 200-µl cultures (in triplicate) at a standard protein concentration of 4 µg/ml. Total culture supernatant or cell extract was also included as positive control, also at 4 µg/ml protein. Purified protein derivative (PPD) prepared from *M. bovis* was used as a positive antigen control and concanavalin A (4 µg/ml) was used to measure the viability of separated PBMC. PBMC cultures were incubated at 37ºC and in the presence of 5% CO_2. After 4 days, the cultures were pulsed with tritiated thymidine (1 µCi per well) and lymphoproliferation was terminated 18 h later by harvesting the cells onto a filter mat using a Skatron harvester (Skatron, Lier, Norway). Radioactivity was measured using a Betaplate counter (Pharmacia) and the results are reported as stimulation indices (SI) calculated from the ratio of cpm incorporated with antigen to cpm incorporated without antigen (PBS was added in the place of antigen).

Identification of individual proteins in stimulatory fractions

Two-dimensional electrophoresis. The standard method of O'Farrell (9) was used. Briefly, 30 to 50 µg proteins were dialyzed against 40 mM Tris-HCl, pH 9.5, concentrated using a Millipore cartridge with a cutoff of 3 kDa, and solubilized with a buffer consisting of 9.5 M urea, 2% NP40, 5% mercaptoethanol and a mix of ampholytes (0.8%; BioLyte, BioRad Laboratories, Richmond, CA, USA), pH ranges 3-10:5-7 in a 1:4 relationship. Twenty microliters of this mix was loaded onto a first dimension-isoelectrofocusing gel (9.16 M urea, 2% NP40, 4.25% acrylamide-bisacrylamide mix (30:5.4%), 2% of the ampholyte mix mentioned above, 0.02% TEMED, and 0.2% ammonium persulfate). Electrophoresis was performed in 20 mM NaOH (cathode buffer) and 10 mM PO_4H_3 (anode buffer) at room temperature at 200 V for 90 min, followed by 400 V for 12 h and 950 V for 2 h. The second dimension run was performed on 12.5 or 15% SDS-PAGE gel. The proteins were either silver stained or transferred to a PVDF membrane and subsequently stained with Coomassie blue.

*N-terminal sequencing of protein*s. The N-terminal sequencing of selected proteins immobilized on PVDF membranes was

performed by Edman degradation on the first six amino acids (Midwest Analytical, Inc., St. Louis, MO, USA). To identify the proteins the sequence was compared against a database of *M. tuberculosis* proteins (www.sanger.ebi.ac.uk/Projects/M_tuberculosis).

MALDI-TOF. Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry was performed using a Reflex III MALDI-TOF (Brukner Daltonik GmbH, Bremen, Germany) spectrometer on samples prepared as follows. Two-dimensional gels were stained with silver and the protein spots were sliced into small pieces with a stainless-steel scalpel or a vortex mixer and placed in siliconized microcentrifuge tubes. Gel pieces were destained with a ferricyanide-thiosulfate solution and washed in 50% acetonitrile containing 25 mM ammonium bicarbonate, pH 8.0 (three times, 15 min each, 24ºC). Gel slices were dehydrated in 100% acetonitrile for 10 min, the acetonitrile was removed, and the gel slices were dried under vacuum for 30 min. Samples were rehydrated with sequencing-grade trypsin solution (5 µg/ml in 25 mM ammonium bicarbonate, pH 8.0) and incubated overnight at 32ºC. Peptides were extracted with 50% acetonitrile-2% trifluoroacetic acid in distilled water and concentrated with a Speed-Vac. Samples were mixed with the matrix α-cyano-4-hydroxycinnamic acid and analyzed by MALDI-TOF. Mass spectrometry profiles were searched against the National Center for Biotechnology Information database.

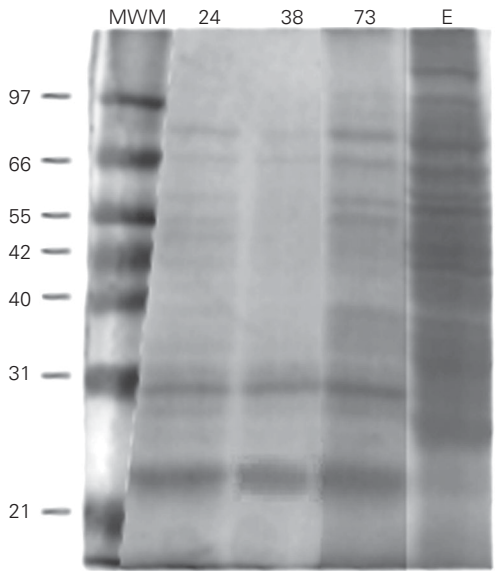

Figure 1. SDS-PAGE of culture supernatants harvested at 24, 38 and 73 days of culture and cell extract (E). Proteins were stained with Coomassie blue and molecular weight markers (MWM) were applied to the left lane. Numbers on the left indicate the molecular mass in kDa.

SDS-PAGE and Western blot. Antigenic fractions were resuspended in loading buffer (2% SDS, 0.125 M Tris-HCl, pH 6.8, 1% 2-mercaptoethanol, 0.02% bromophenol blue, and 10% glycerol), heated for 5 min in boiling water, and loaded onto 12.5% polyacrylamide gels by the method of Laemmli (7). Molecular mass standards (low and high range, BioRad) were run on each gel. Proteins were electrotransferred onto a nitrocellulose sheet (Sleicher and Schuell, Dassel, Germany) by the semidry transfer method (10). The efficiency of transfer was visualized by transient staining with Ponceau red. Membranes were blocked with 5% nonfat milk in TBS (50 mM Tris-HCl, pH 8, and 150 mM NaCl), incubated with the first antibody overnight at 4ºC and, after three washes with TBS, incubated with alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG (Sigma, St. Louis, MO, USA) at 1/1000 dilution for 2 h at 37ºC. After three washes with TBS and one wash with alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$), a color reaction was developed by adding 5-bromo-4-chloro-3-indolylphosphate (BCIP) and toluidine nitroblue tetrazolium (NBT) as substrates. After 20 min the color reaction was stopped by adding water.

Antisera and antibodies. Anti-ESAT6 monoclonal antibody was kindly provided by Ida Rosenkrands and Peter Andersen (Statens Serum Institute, Copenhagen, Denmark). Anti-MPB70 monoclonal antibody (4C3/17) was purchased from CSL (Victoria, Australia). Polyclonal antisera recognizing 85B were kindly provided by T. Fifis (Animal Health Research Laboratories, Victoria, Australia).

Results

Fractionation of culture supernatants and cell extracts by anion-exchange chromatography

Culture supernatants, harvested at three different culture times (24, 38 and 73 days), and cell extracts were fractionated by FPLC anion-exchange chromatography with a linear gradient of 0-0.42 M NaCl. As indicated in Figure 1, nonfractionated supernatant and extracts were complex mixtures of several individual proteins. In the culture supernatant fractions, proteins of <10, 20, 22, 24, 31, 32, 40, 43, 45, 50, 65 and 75 kDa were observed. In cell extracts, heavily stained bands were seen at 10, 14, 16, 20, 22, 24, 31, 40, 66 and 71 kDa.

When culture supernatants and cell extract preparations were separated by anion-exchange chromatography, four (24-day culture supernatant) to three (73-day culture supernatant and cell extract) major peaks were observed in the gradient zone. A chromatogram from a 73-day culture supernatant is shown in Figure 2 as an example. Protein profiles for each fraction were determined by SDS-PAGE. Figure 3 shows the fractions from a 38-day culture supernatant.

Fractions having a similar protein profile, from each chromatogram peak, were pooled and dialyzed and their concentration was measured and used for LPA using PBMC from four animals experimentally infected with *M. bovis*. Two uninoculated animals of the same age, sex and source were used as uninfected controls.

T-cell responses to chromatographic fractions

The FPLC profile of the 24-day culture supernatant contained five peaks. Pools from these regions were prepared, representing fractions 11-17, 20-24, 25-28, 32-36, and 81-84 (Table 1). Some of these fractions showed an SI equal to or higher than that of PPD. The mean reactivity of pools was 25-28 > 20-24 = 32-36 > 81-84 > 11-17, except for animal 4 where pool 81-84 was the more reactive. SDS-PAGE analysis of the most stimulatory pool (25-28) showed proteins of <10 kDa and a less intense band of 20 kDa. Fraction 20-24 had intense bands of 20, 25, 32 and 41 kDa. The 32-36 pool had a <10-kDa protein, while the 81-84 pool had a 20-kDa protein and the lowest stimulatory pool (11-17) showed bands of 20, 31 and 32 kDa and additional weaker bands.

Four peaks were observed during the sepa-

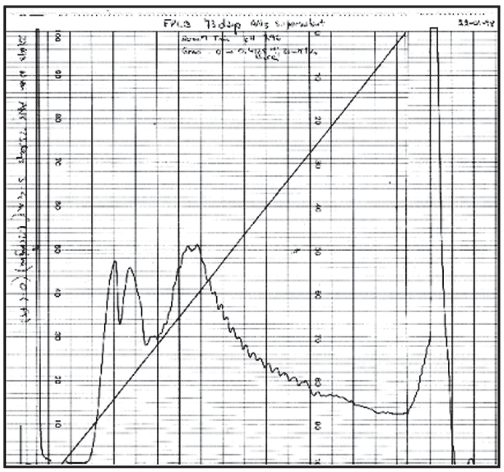

Figure 2. A representative anion-exchange chromatogram of a 73-day culture supernatant. Ordinate: absorbance at 280 nm. Abscissa: fraction numbers.

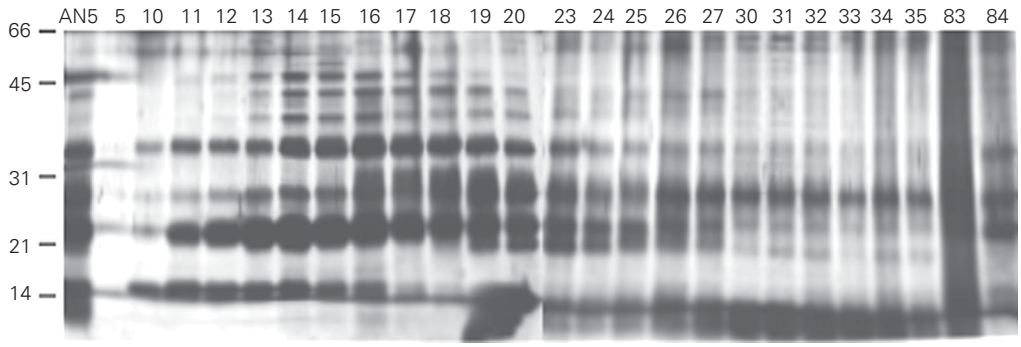

Figure 3. SDS-PAGE analysis of the anion-exchange chromatography elution profile of 38-day AN5 culture supernatants. The fraction number is indicated above each lane. AN5 = culture supernatants before chromatography. Note the relatively large amounts of low MW proteins in fractions 30 to 35. Numbers on the left indicate the molecular mass in kDa.

ration of the 38-day culture supernatant. Pools were prepared from fractions 17-18, 25-26, 30-34, and 83 (Table 1). The reactivity order of the pools was 30-34 > 83 > 25-26 > 17-18, except for animal 1 where 83 was the more reactive pool. SDS-PAGE analysis (Figure 3) of the low stimulatory pool (17-18) showed prominent bands of 16, 20 and 32 kDa, as well as additional bands of 24, 40, 43 and 45 kDa. Pool 25-26 had proteins of 14, 16, 20, 24, 31, and 44 kDa (doublet), and less intense low molecular weight proteins. The most stimulatory pool (30-34) showed an intense <10-kDa band (Figure 3) and a 24-kDa protein, with minor bands of 43 and 45 kDa as well as other bands. Fraction 83 had proteins of 14 and 16 kDa (Table 1).

Table 1. Stimulation indices obtained in a lymphoproliferation assay.

Antigen	Stimulation index					
	Infected animals				Noninfected animals	
	1	2	3	4	88	99
Culture supernatant - 24 days						
PBS	1 (158)	1 (63)	1(67)	1 (103)	1 (234)	1 (170)
Con A	13	49	97	200	352	277
PPD B	13	23	86	217	1.71	2.02
Pool 11-17	5	5	21	17	1.06	1.70
Pool 20-24	25	57	27	294	1.37	2.00
Pool 25-28	28	68	24	303	1.06	1.96
Pool 32-36	26	56	26	177	1.77	1.52
Pool 81-84	9	27	9	345	1.26	2.29
Culture supernatant - 38 days						
PBS	1 (433)	1 (330)	1 (477)	1 (389)	1 (234)	1 (170)
Con A	126	234	148	119	352	277
PPD B	109	55	71	64	1.71	2.02
TP	123	50	49	48	1.70	1.74
Pool 17-18	32	12	12	89	1.10	1.04
Pool 25-26	80	45	21	73	0.70	1.24
Pool 30-34	64	91	14	136	1.07	1.32
Pool 83	118	67	nd	108	2.23	3.29
Culture supernatant - 73 days						
PBS	1 (433)	1 (330)	1 (477)	1 (389)	1 (234)	1 (170)
Con A	126	234	148	119	352	277
PPD B	109	55	71	64	1.71	2.02
TP	120	59	40	105	2.70	1.04
Pool 14-16	110	55	28	75	1.42	1.24
Pool 17-18	107	62	31	50	2.08	1.72
Pool 22-23	107	93	30	57	0.98	1.52
Pool 25-28	98	73	31	97	1.09	1.92
Pool 29-31	97	68	32	113	2.10	1.46
Pool 81	96	54	15	103	1.76	2.97
Cell extract						
PBS	1 (98)	1 (68)	1 (567)	1 (200)	nd	1 (325)
Con A	542	980	540	607	nd	250
PPD B	109	55	71	64	nd	2.30
Pool 6-14	113	25	34	16	nd	3.09
Pool 20-24	225	93	234	408	nd	2.40
Pool 25-29	623	178	76	52	nd	2.47
Pool 30-32	660	186	98	57	nd	3.25
Pool 33-40	606	161	102	46	nd	3.42
Pool 82-84	457	30	43	11	nd	1.26

All assays used a protein concentration of 4 µg/ml. Values given within parentheses in cpm are for PBS control. Con A = concanavalin A; nd = not determined; PPD B = purified protein derivative B; TP = non-fractionated proteins.

The T-cell-proliferative response was similar for all six pools analyzed (14-16, 17-18, 22-23, 25-28, 29-31, 81) from the 73-day culture supernatant (Table 1). SDS-PAGE analysis showed that all of them, except fraction 81, contained protein bands <10 kDa, with little difference in the protein profile of all these pools. Major bands were seen at <10, 14.5, 20, 22, 24, 25, 32, 40, 44 and 46 kDa.

Using whole cell extract, six peaks were observed. Pools were prepared from fractions 6-14, 20-24, 25-29, 30-32, 33-40 and 82-84. Many fractions showed an SI equal to PPD or higher. The reactivity order was roughly 30-32 > 25-29 > 33-40 > 20-24 > 82-84 > 6-14 for animals 1 and 2, and 20-24 > 30-32 > 33-40 > 25-29 > 82-84 = 6-14 for animals 3 and 4. SDS-PAGE analysis of fraction 30-32 showed proteins of <10, 19, 25, 40 and 70 kDa. Fraction 20-24 contained proteins of <10, 12, 14, 19, 25, 30, 40 and 70 kDa. Fraction 6-14 contained proteins of <10, 19, 25, 30, 40, 45, 66 and 70 kDa. Finally, fraction 82-84 showed several heavily stained and smeared bands.

When PBMC from healthy, noninfected animals were stimulated with pools of the fraction, very low SI were observed (Table 1, animals 88 and 99).

To assess the functionality of the methodology (PBMC preparation and LPA) throughout the experiment, we analyzed the indices of concanavalin A stimulation for all

four animals, which were found to be highly uniform. Variations in LPA from animal to animal and within individual animals throughout the experiments were determined by analyzing LPA results when PPD was used as antigen.

Identification of proteins in antigenic fractions

Six proteins were identified in fractions 25-28 from 24-day culture supernatants and in fractions 30-34 from 38-day culture supernatants. They were CFP10 (11 kDa), ESAT6 (9 kDa), TRB-B (36 kDa), 85B (32 kDa), TPX (17 kDa), and MPB70 (20 kDa) (Table 2). N-terminal sequences of 85B, MPB70 and TRB-B are compatible with signal sequences. Specific antisera were used to confirm the identification of MPB70 and 85B by Western blot (data not shown). ESAT6 was identified only by Western blot (data not shown).

Several cell extract fractions from 38-day cultures showed stimulatory properties. We concentrated on one of them (fraction 30-32) that showed high T-cell reactivity. Since the N-terminus seemed to be blocked in most proteins, we used MALDI-TOF for protein identification. Two proteins were identified: Rv3747 (13 kDa) and L7/L12 (13 kDa) (Table 2). Proteins corresponding to three other spots could not be identified.

Discussion

In this study we used a novel approach to detect T-cell-stimulating antigens from *M. bovis*, i.e., fractionation of antigens using anion-exchange chromotagraphy. Other investigators have used anion-exchange columns to purify antigens already identified in *M. bovis* (11). Compared to protein separation by SDS-PAGE, the advantage of the present methodology is that antigens are maintained in the native state. In addition, we screened antigens from cell extracts as well as culture supernatant proteins. Finally, we harvested culture supernatant proteins at various times instead of using a single sample to test whether the bacteria might secrete different proteins during the growth phases.

Since cellular responses are the major immune mechanism in tuberculosis, T-cell reactivity toward well-characterized mycobacterial antigens has been extensively studied (5,12,13). However, the direct screening of antigens with immune cells is technically much more complicated compared to antigen-antibody interactions. Several approaches have been used previously to test soluble antigens prepared from a nitrocellulose membrane or "T-cell Western blot" (14-17). Mustafa et al. (18) performed direct screening of an *M. tuberculosis* expression library with PBMC, and identified a protein reacting with T cells but not with antibodies.

Electroelution from SDS-PAGE gels was first used by Gulle et al. (19) to obtain soluble antigens. Using a similar technique, Andersen and Heron (20) identified *M. tuberculosis* antigens related to memory response. Gulle et al. (21) screened BCG cellular and secreted proteins for T-cell stimulant fractions using PBMC from cattle immunized with either viable or gamma-irradiated BCG. In contrast, in the present study, a virulent strain was used to infect cattle and to prepare proteins.

We identified protein in fractions prepared from both culture supernatants and cell extracts which, when tested by LPA, showed high SI. The selected fraction con-

Table 2. Identification of *Mycobacterium bovis* in antigenic fractions.

Antigen	N-terminal sequence	Position in ORF	Identification by MALDI-TOF	Confirmation by Western blot
CFP10	AEMKTD	2	–	–
85B	FSRPGL	41	–	+
MPB70	GDLVGP	31	+	+
ESAT6	–	–	+	+
TPX	AQITLR	2	–	–
TRB-B	TELTGA	16	–	–
L7/L12	–	–	+	–
Rv3747	–	–	+	–

tained several protein bands, some of them shared with other fractions while others were not. This allowed us to deduce which bands confer reactivity to the fraction. In 24- and 38-day culture supernatants, fractions associated with a high SI contained low molecular weight proteins (<10 kDa) together with other proteins ranging from 20 to 35 kDa. In an attempt to identify individual antigens in antigenic fractions using a combination of methods, we determined that these stimulant fractions consisted of 85B, TRB-B, MPB70, TPX, CFP10 and ESAT6. 85B (22), ESAT6 (6,23) and MPB70 (24-26) are well known T-cell-stimulating antigens, while the antigenicity of CFP10 has only recently been demonstrated (23,25,27). These results support the view that low molecular weight proteins such as ESAT6 (6) play an important role in bovine immune responses to *M. bovis*. TRB-B and TPX are novel candidate antigens. While there are no previous references concerning TRB-B, TPX has been already identified as a protein by Rosenkrands et al. (28) and Weldingh et al. (29), who named it CFP20. Only two proteins, Rv3747 and L7/L12, could be identified in cell extracts. Rv3747 is a small protein with unknown function and L7/L12 is a ribosomal protein previously described as a major component of PPD (30). To fulfill one of our objectives of identifying *M. bovis* immunodominant antigens, the proteins described here should be made by recombinant methods and the antigenicity evaluated in infected cattle. These studies are underway in our laboratories.

Differences in the immunodominance of certain fractions were less marked in protein fractions derived from culture supernatants obtained at later times. An explanation may be the appearance in the culture supernatant of multiple antigens (proteins, complex lipids) released from the cells due to cell lysis. These antigens may mask the antigenicity of secreted low molecular weight proteins. Another explanation based on a lower content of low molecular weight proteins in late culture supernatants is less probable because we did not observe a decrease of low molecular weight proteins in late culture supernatants in SDS-Tricine-PAGE gels (data not shown). Subsequent close examination of gels indicated that the low molecular weight fraction is composed of several proteins, as demonstrated by SDS-Tricine-PAGE gels (data not shown). Our results differ from those of Diaz et al. (31) who worked with *M. bovis* AN5 culture supernatant proteins separated by isoelectrofocusing and screened with PBMC from naturally infected animals. These investigators did not report low molecular weight proteins in stimulating fractions, a fact possibly explained by apparent differences in SDS-PAGE conditions.

Importantly, we observed a strong stimulation using cell extract proteins. Most researchers agree that the main protective antigens are secreted proteins. However, non-secreted, cell extract proteins may also be useful antigens for diagnosis, as we have reported in another study (32). It was difficult to identify a single pool from whole cell extracts which was clearly dominant. Gulle et al. (21) also showed strong antigenic responses from lysed cell proteins. As reported in other studies (6,11,21), the T-cell response presented considerable individual variability.

With the methodology described here we identified protein fractions that stimulate lymphoproliferation of bovine PBMC. Although one of the most stimulatory pools was highly enriched with low molecular weight protein, there were other stimulatory fractions such as those derived from cell extracts which contained higher molecular weight proteins. These fractions contain a complex mixture of proteins and must be further separated to identify individual T-cell-reactive antigens.

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