B-Cell Epitopes in the Immunodominant p34 Antigen of *Mycobacterium avium* ssp. *paratuberculosis* Recognized by Antibodies from Infected Cattle

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Abstract

Mycobacterium avium ssp. *paratuberculosis* (*M. paratuberculosis*) causes Johne's disease, a chronic and fatal enteritis in ruminants. In the last stage of the disease, antibody titres rise and levels of interferon-γ decrease, suggesting that the host–immune response is switching from a T helper 1 (Th1) to a Th2 profile. In infected cattle, the membrane protein p34 elicits the predominant humoral response against *M. paratuberculosis*. To map the B-cell epitopes of this antigen, affinity-purified bovine antibodies against the carboxy-terminal region of p34 were used to screen a 12-mer phage display library. Several phage clones carrying peptides resembling fragments of p34 were affinity selected. Based on the predicted amino acid sequence, peptides were chemically synthesized, which demonstrated reactivity with serum from naturally infected and p34-vaccinated cattle. Immunization of mice with these peptides elicited an anti-p34 antibody response. Two B-cell epitopes were identified and characterized. Based on the reactivity and the type of immune response elicited, epitope A was determined to be conformational, whereas epitope B was demonstrated to be sequential. Both epitopes were shown to be present in p34 proteins from *M. avium* ssp. *avium* or *M. paratuberculosis* but absent from *M. intracellulare*, the other member of the *M. avium* complex. Furthermore, both epitopes were mapped to regions of p34 that display high variability when compared to homologous proteins from other mycobacterial species of public and animal health importance. We hypothesize that these variable regions of p34 may play a role in the immunobiology of *M. paratuberculosis* infections.

Introduction

Paratuberculosis (Johne's disease) is a severe, chronic gastroenteritis of ruminants, caused by the facultative intracellular pathogen *Mycobacterium avium* ssp. *partuberculosis* (*M. paratuberculosis*) [1]. Neonatal and young animals are at the highest risk of infection, which usually occurs by the fecal–oral route. Once in the gastrointestinal lumen, *M. paratuberculosis* bacilli are endocytosed by M cells present on the mucosal surface and are delivered to the basolateral side of the epithelium by transcytocytosis. Then, bacilli infect the subepithelial and intraepithelial macrophages and mesenteric lymph nodes [2, 3]. Survival within macrophages is a hallmark of *M. paratuberculosis*. Similarly to *M. tuberculosis* [4], *M. paratuberculosis* may subvert the phagolysosomal biogenesis and avoid the acidification of the phagosome [5].

During the first 2–3 years after the initial infection, host immunity against *M. paratuberculosis* is dominated by a cell-mediated immune response that ultimately leads to the appearance of an intestinal granuloma [1]. In this period, the production of interferon-γ (IFN-γ) by peripheral blood mononuclear cells (PBMCs) [6] and IFN-γ gene expression in ileal tissue and cecal lymph nodes [7] increase as compared to healthy animals. In contrast, at this time, antibodies against *M. paratuberculosis* are serologically undetectable. At a later stage concurrent with the onset of clinical manifestations, cell-mediated immunity declines. This is manifested by a significant reduction of IFN-γ gene expression in ileal tissue and cecal lymph nodes [7]. A reduction in the production of IFN-γ by PBMCs is also observed [6], but antimycobacterial antibody titres increase in serum [8–10].

M. paratuberculosis possesses a complex cell-wall containing several immunogenic proteins, glycopeptides and glycolipids such as lipoarabinomannan, shared with other mycobacterial species [11, 12]. Amongst the protein antigens, protein p34 was identified as immunodominant for bovine B cells [13]. The hydrophilicity profile of p34 identified two distinct regions: a strongly hydrophobic aminoterminal region composed of aminoacids 40–160 and a highly hydrophilic domain corresponding to residues 161–298. The aminoterminal hydrophobic region is buried within the bacterial envelope, whereas the hydrophilic carboxyterminal is exposed at the cell surface [14]. In addition, the B-cell epitopes at the C-terminal region were localized by immune electron microscopy to the cell surface [15]. Using a panel of sera from cattle, naturally infected with tuberculosis or paratuberculosis, De Kesel *et al.* [15] concluded that the 98-amino acid carboxyterminal region of p34 is specific for *M. paratuberculosis*. This observation was consistent with the reactivity of serum from rabbits immunized with this polypepetide. However, other studies found that rabbit polyclonal antibodies or murine monoclonal antibodies raised against the same fragment of p34 cross-reacted with a 38 kDa protein of *M. bovis* [16].

Host–immune defenses against intracellular pathogens rely on cell-mediated and antibody-mediated mechanisms [17]. Several studies indicate that antibodies against intracellular pathogens present at the time of infection may have an important role in host protection [18–20]. Antibodies against *M. tuberculosis* activate macrophages through Fc-dependent endocytosis [21]. Monoclonal antibodies against *M. bovis* BCG antigens interfere with the attachment and ingestion of the bacilli by the human bladder transitional cell carcinoma cell line T-24 [22]. For the intracellular fungi *Cryptococcus neoformans*, it has been shown that both the epitopes recognized [23] and the isotype of monoclonal antibodies [24] determine the fate of pathogen within macrophages. Therefore, the identification of epitopes in the *M. paratuberculosis* p34 immunodominant protein, presented in this study, is of importance to determine the role of bovine antibodies in the response against *M. paratuberculosis* infections. In this study, bovine polyclonal antibodies from cattle naturally infected with *M. paratuberculosis* were used to identify two immunodominant B-cell epitopes in p34. These epitopes were only found in p34 proteins from *M. avium* and *M. paratuberculosis*.

Materials and methods

Cloning, expression and purification of the carboxyterminal peptide of protein p34. M. paratuberculosis-strain ATCC 19698 was grown at 37 °C in appropriately supplemented Middlebrook 7H9 broth as described previously [25]. When cultures reached an OD_{600} of 0.5–0.9, cells were harvested by centrifugation at 6000 × *g* for 15 min, washed and resuspended in phosphate-buffered saline (PBS). Chromosomal DNA was extracted as previously described [26]. Template DNA was used for polymerase chain reaction (PCR) amplification of the 132-amino acid carboxyterminal fragment of protein p34 (p34-cx), encoded by nucleotides 1239 to 1638 (GenBank accession number X68102 [25]). The sense (5′-CAGGGATCCAAGTACGACCC CTACGCG-3′) and antisense (5′-TGCGAATTCGTCCTCCAGCCGTGTT-CAC-3′) oligonucleotide primers contained a *Bam*HI and *Eco*RI restriction sites, respectively, allowing for directional cloning of the amplicon into the polylinker site of the expression vector pRSET-A (Invitrogen, Carlsbad, CA, USA). Several transformants were selected, and the identity of the insert was confirmed by DNA sequencing.

The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) p*LysS* (Stratagene, La Jolla, CA, USA). Expression of p34-cx was carried out for 18 h in cells stimulated with 1.0 mM isopropyl β-D-thiogalactoside as described elsewhere [27]. Cells were pelleted by centrifugation for 15 min at 6000 *g* and lysed in lysis buffer (6 M urea, 5 mM imidazol, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) for 2 h at room temperature. The p34-cx polypeptide was partially purified using an His*Bind Resin (Novagen, Madison, WI, USA) following the manufacturer's directions. Recombinant p34-cx was renatured by dialyzing against ×1 PBS for 48 h at 4 °C. A further purification step was performed by size exclusion chromatography using a Sephadex G75-column eluted with buffer PBS. Fractions were collected and analysed by PAGE and Western blots using serum from *M. paratuberculosis*-infected cattle. Positive fractions were pooled and concentrated using Centricon-30 following directions provided by the manufacturer. The combination of nickel affinity and size exclusion chromatography led to a highly purified product as evidenced by PAGE analysis.

Vaccination with p34-cx. E. coli BL21 (DE3) pLysS cells expressing p34-cx were grown to mid-log phase in SOB medium, harvested and lysed as previously described [27]. Fifteen milligrams (wet weight) of the resulting lysate containing p34-cx was emulsified in Freund's incomplete adjuvant (Sigma, St. Louis, MO, USA) and used to immunize two 8-month calves by the subcutaneous route at days 0, 7, 14, 46, 52 and 60. Serum samples were collected at day 70.

Collection of serum samples from infected cattle. Sera from five *M. paratuberculosis*-naturally-infected calves, six *M. bovis*-naturally-infected calves with serum reactivity against PPD, and 10 uninfected cows were obtained from the School of Veterinary Sciences of the University of Buenos Aires. Sera from an additional six *M. paratuberculosis*-naturally-infected cattle were kindly provided by Dr G. Traveria (Universidad de La Plata, La Plata, Argentina). The infection status of all animals was verified by bacterial culture tests.

Affinity purification of anti-p34 antibodies. Five millilitres of serum obtained from a naturally infected cow was diluted 1 : 10 in 10 mM Tris (pH 7.5) and passed three times through CNBr-activated Sepharose (Sigma) coupled to 30 mg of an *E. coli* lysate. This adsorption step was performed to remove antibodies against *E. coli* antigens. Non-binding antibodies were recovered and passed three times through CNBr-activated Sepharose (Sigma) coupled to 10 mg of purified p34-cx. Washing and elution were performed as described elsewhere [28], and the specificity of the purified serum to p34-cx was evaluated by Western blots and enzyme-linked immunosorbent assay (ELISA).

Selection of phages displayed peptides using anti-p34 antibodies. Phages displaying peptides of interest were selected from a random 12-mer library expressing more than 10^8 peptides using anti-p34 affinity-purified antibodies as described previously in our laboratories [29]. Bound phages were eluted with 100 mM Tris (pH 11.5). Sepharose particles were centrifuged briefly, the eluate was transferred to another tube and the pH was adjusted to 7.0 with 1 M Tris (pH 7.5). An aliquot of the eluted phage particles was titrated in *E. coli* ER2738, and the remaining phage library was amplified for 4.5 h in *E. coli* ER2738. This enriched library was subjected to two further cycles of enrichment, followed by precipitation with 3.33% PEG 8000 and 0.42 M NaCl for 16 h at 4 °C. The phage library was screened two more times with anti-p34 antibodies, and suitable candidates were plaque purified, propagated and sequenced as described [29].

Peptide synthesis. Peptides were synthesized using standard solid phase technology and 9-fluorenylmethoxy carbonyl chemistry at the Protein Core Facility of the Center for Biotechnology, University of Nebraska. The sequences of the peptides used in this paper were: peptide p5 (LYRPSDSSLAGP), peptide p8 (NVESQPGGQPNT) and irrelevant peptide p7 (QRAYLELPPWPPC).

ELISA. For peptide ELISAs, reacti-bind, maleimide-activated plates (Pierce, Rockford, IL, USA) were coated with 2.5 μg of synthetic peptides and blocked with 0.5 mg/well of L-cysteine hydrochloride (Sigma) as described elsewhere [29]. For conventional ELISAs, Immulon 2HB plates (Dynex, Chantilly, VA, USA) were coated with 50 μg of either p34-cx or *M. paratuberculosis* cytoplasmatic antigen (Allied Monitor, Lafayete, MO, USA) in 0.1 M $NaHCO_3$. For whole-cell ELISAs, Immulon 2HB plates were coated with 50 μl of 5×10^6 CFU/ml of UV-killed *M. paratuberculosis* or *M. avium* or *M. intracellulare* or *M. smegmatis* suspended in 0.1 M $NaHCO_3$. Blocking was performed with PBS−0.05% Tween-20–10% nonfat milk for 2 h at room temperature. In all cases, the plates were washed extensively after blocking with ×1 PBS containing 0.05% Tween-20. Sera from control and immunized cattle were added at a 1 : 60 dilution and incubated for 1 h at room temperature. Plates were washed with ×1 PBS containing 0.05% Tween-20, and peroxidase-conjugated secondary antibody was added for 30 min at room temperature at a 1 : 500 dilution. Plates were washed and incubated with ABTS substrate (KPL, Gaithersburg, MD, USA) for 15 min at room temperature, and the OD (405 nm) was read in a OpsysMR spectrophotometer (Dynex Technologies, Chantilly, VA, USA).

Immunization of mice. Peptides (150 mg) were coupled to maleimidobenzoyl-*N*-hydroxysuccimide ester-activated Blue Carrier protein (Pierce) and emulsified in Freund's incomplete adjuvant (Sigma). Specific pathogen-free BALB/*c* mice (Jackson Laboratories, Bar Harbor, ME, USA) were inoculated intraperitoneally two times at 2-week intervals. Antibody responses were measured using ELISA and Western blots. The animals were maintained throughout the experiments in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Western blotting. M. paratuberculosis cell-wall extracts (CWEs) were obtained by disruption of the bacterial envelope by sonication, as previously described by us [30]. For Western blotting procedures, 5–10 μg of either p34-cx or CWE were boiled for 5 min in sample buffer [0.12 M Tris buffer (pH 9.8), 20%(v/v) glycerol, 0.015 M SDS and 0.4 mM 2-mercaptoethanol] and loaded onto 12% polyacrilamide gels. Proteins were transferred to nitrocellulose membranes at 0.25 A for 18 h, using a Mini-PROTEAN II electrophoretic cell (Bio-Rad Life Science Group, Hercules, CA, USA). Membranes were blocked with PBS−0.05% Tween-20–10% nonfat milk and probed with sera from immunized mice or cattle. Peroxidase-conjugated antimouse or antibovine antibodies (KPL) were used as secondary antibodies, and binding was detected with BCIP/NBT phosphatase substrate (KPL).

Flow cytometry. Mid-log phase cultures of *M. paratuberculosis* and *M. smegmatis* were harvested by centrifugation at $6000 \times g$ for 15 min. A single cell suspension was prepared as previously described [31], and each culture was adjusted to a final OD_{600} of 0.5. Live bacteria were incubated for 1 h with murine antibodies against whole *M. paratuberculosis*, peptide p5, peptide p8 or mouse preimmune serum followed by 1 h incubation with fluorescein isothiocyanate-conjugated goat antimouse antibodies (KPL). The immunofluorescence of individual cells was analysed in a FACSvantage® cell sorter (Becton Dickinson, San Jose, CA, USA) at a wavelength of 530 ± 15 nm. A total of 10,000 cells were analysed per sample reading. Histograms of fluorescence frequency distribution were generated by plotting the number of cells (*y*-axis) versus the $\log_{10}$ fluorescence intensity (*x*-axis) with the LYSIS II data analysis program (Becton Dickinson). The size distribution of cells with or without added serum was uniform when examined by forward-scatter and side-scatter parameters. There was no difference in light scattering when stained and unstained cells were compared, indicating that the average particle size for both populations was the same.

Sequencing of the genes encoding protein p34 of M. paratuberculosis and M. avium. The oligonucleotide primers (Integrated DNA Technologies Inc., Coralville, IA, USA) for PCR amplification of the gene encoding the *M. paratuberculosis* protein p34 were P34FOR (5′-AGC AAC AGG AGG AGC CAT G-3′) and P34REV (5′-GTT GTC CTC CAC CCG TGT CAC-3′). PCR amplifications were performed in a 50-μl reaction volume containing 0.25 μ *Taq* DNA polymerase (Fisher Scientific Co, Suwanee, GA, USA), 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate and 10% dimethyl sulfoxide. DNA samples from strains K-10, S-8, K-39, Lorraine, K-48 *M. avium* strains TMC 724, MAC 104 and MAC 109 were used as templates [30]. Thermocycling was performed using a Perkin-Elmer Gene Amp 9600 thermocycler (Roche Molecular Systems, Basel, Switzerland) using the following conditions: one cycle of 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, with a final elongation step at 72 °C for 7 min. The amplified products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and subjected to DNA sequencing by the fluorescent dideoxy chain terminator method. These sequences were aligned using the PILEUP program (Wisconsin Sequence Analysis Package, Genetics Computer Group) with the previously published sequence of the 34-kDa protein from *M. leprae* (GenBank accession number AAC69251 [32]), *M. tuberculosis* strain $H37R_V$ (GenBank accession number P71556), *M. bovis* AF2122/97 and *M. smegmatis* MC2155 (obtained from the ongoing sequence projects at http://www.tigr.org).

Results

Identification of two epitopes in the carboxyterminal fragment of protein p34

To study B-cell epitopes in the immunodominant region of protein p34, a subgenomic fragment-encoding amino-acid 167–298 (p34-cx) was expressed in *E. coli* as described in *Materials and methods*. The recombinant p-34cx fragment was used to immunize calves. The reactivity of sera from p34-cx-vaccinated and *M. paratuberculosis*-naturally-infected cattle was evaluated with different antigens derived from *M. paratuberculosis*. Table 1 shows that sera from both naturally infected and vaccinated animals recognized by p34-cx. In contrast, *M. paratuberculosis* cytoplasmatic antigen and whole *M. paratuberculosis* were only recognized by serum from *M. paratuberculosis*-infected cattle. The lack of reactivity of the serum from the p34-vaccinated cow against the cytoplasmic antigen is in agreement with the exclusive location of p34 in the cell envelope of *M. paratuberculosis* [15]. The reactivity against the cytoplasmatic antigen exhibited by the serum obtained from the naturally infected cow is likely to be directed against immunogenic antigens different from p34. These antigens may be cross-reactive to other nonpathogenic mycobacteria, as serum from the negative control also shows some reactivity against *M.paratuberculosis*. The presence of anti-p34 antibodies was confirmed by Western blot analysis using CWE from *M. paratuberculosis*. Antibodies from *M. paratuberculosis*-infected and p34cx-vaccinated cattle recognized the presence of p34 in CWE (Fig. 1). However, serum from the p34-cx-vaccinated cattle did not recognize the p34 protein on the surface of live whole *M. paratuberculosis* bacilli, antigen that was readily recognized by serum from infected cattle (Table 1). This lack of reactivity of serum from the vaccinated cow may be due to denaturation of p34-cx in the vaccine preparation. Thus, antibodies elicited in the vaccinated cow only recognized sequential epitopes such as those that can be determined by Western blot analysis. In contrast, the serum from the infected cow possesses antibodies against both sequential and conformational epitopes that can be determined by both Western blot and whole *M. paratuberculosis* ELISA (Table 1) (Fig. 1).

Based on these results, affinity-purified anti-p34 antibodies from the *M paratuberculosis*-infected cow were used to identify the epitopes recognized by them. The specificity of these antibodies was verified by ELISA, using p34-cx and whole *M. paratuberculosis* as antigens (data not shown) and Western blot analysis (Fig. 1, lane d). These antibodies were then used to screen a 12-mer phage display library. Fifty phage clones isolated from three different rounds of selection were amplified and sequenced.

Table 1 Reactivity of bovine sera with lysate of *Escherichia coli* expressing p34-cx, cytoplasmic extract of *Mycobacterium paratuberculosis* and whole *M. paratuberculosis**

	Antigen		
Bovine†	p34-cx	Cytoplasmic	Whole *M. paratuberculosis*
Infected (number 8)	0.453 (±0.035)	0.482 (±0.025)	0.478 (±0.010)
p34cx vaccinated (number 2)	1.411 (±0.021)	0.032 (±0.007)	0.110 (±0.009)
Negative (number 22)	0.090 (±0.011)	0.008 (±0.005)	0.237 (±0.004)

*Results are expressed as the mean ± SD (samples run in triplicate) of the optical density read at 405 nm.
†Identification number of individual animals is shown in parenthesis.

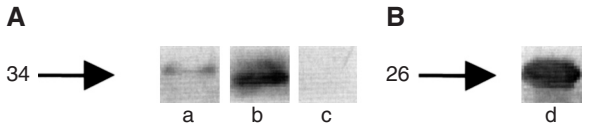

Figure 1 Western blot analysis of antibodies from *Mycobacterium paratuberculosis*-infected and p34-cx-vaccinated cattle. Panel A, reactivity of sera from the p34-cx-vaccinated calf (lane a), *M. paratuberculosis*-infected cow (lane b) and uninfected cow (lane c) against a cell-wall extract of *M. paratuberculosis*. Panel B, reactivity of p34cx-affinity-purified antibodies (lane d) against a cell extract from recombinant *Escherichia coli* expressing p34-cx. Numbers on the left indicate molecular masses of p34 (34 kDa) and p34-cx (26 kDa).

BLAST analysis of the deduced peptide sequences revealed partial identity with protein p34, as it is frequently found by this approach [29]. The sequences of selected peptides that showed a similarity of at least 40% with the sequence of p34 are shown in Fig. 2. Clones 326 and 210 expressed peptides sharing a YxxxxSSxxGP consensus motif. A fragment of this motif was also present in phage clone 34 (SSxxGP). In addition, phage clones 24 and 26 carried peptides with a TTxxGP motif. These two motifs are likely to represent the same epitope, as both threonine and serine are small, polar amino acids containing a hydroxyl group in the side chain. Thus, the sequence YxxxxSSxxGP spanning residues 285–295 of protein p34 may represent a putative conformational epitope, which we designated as epitope A.

Phage clone 22 carried a peptide containing a QPGGQP motif, which has 100% identity with p34 at residues 195–200. Although this was the only phage clone that aligned with p34-cx in this region, it was selected for further investigation because of its high identity with p34. This region of p34 was considered as a second putative sequential epitope that was designated as epitope B.

Epitopes A and B are located in highly hydrophilic and flexible areas of this protein (data not shown), as determined by the Kyte & Doolittle and Bhaskaran algorithms (http://www.expasy.ch). High values for these two parameters are generally expected to be present in immunodominant epitopes [33].

Reactivity of field sera to peptides resembling epitopes A and B

To confirm the antigenicity of the two putative epitopes identified, the peptides carried on phages 326 and 22 were chemically synthesized as peptides p5 and p8, respectively. These peptides were chosen, because they were representative of the two putative epitopes described above. These synthetic peptides were then used as antigens in ELISAs to determine their reactivity with sera from two calves vaccinated with denatured p34-cx, 11 cattle naturally infected with *M. paratuberculosis*, six cows naturally infected with *M. bovis* and 10 healthy cows (Fig. 3). Sera from the two p34-cx-vaccinated calves strongly recognized p34-cx and peptide p8 but did not recognize peptide p5. Sera from six *M. paratuberculosis*-infected animals recognized p34-cx and both peptides p5 and p8. Samples from three *M. paratuberculosis*-infected animals recognized p34-cx and either peptide p5 or p8. One *M. paratuberculosis*-infected animal recognized only p34-cx but none of the peptides, suggesting that p34 possesses additional epitopes. There was a *M. paratuberculosis*-infected animal that did not recognize either p34 or peptides p5 and p8. None of the *M. bovis*-infected animals or the uninfected controls recognized p34 or the synthetic peptides ($P < 0.01$) (Dunnett's test). These data indicate that natural infection with *M. paratuberculosis* often results in the production of antibodies against epitopes A and B.

Epitopes A and B are immunogenic in mice

To investigate whether epitopes A and B are immunogenic, mice were immunized with synthetic peptides p5 and p8. Sera from these mice recognized p34-cx, whole *M. paratuberculosis* and whole *M. avium* ssp. *avium* by ELISA. Immunization of mice with whole *M. paratuberculosis* also elicited antibodies against p34 and peptides p5 and p8 (Table 2). Western blot analysis under denaturing conditions, using CWE from *M. paratuberculosis* as antigen, revealed that antipeptide p8 serum recognized denatured p34. However, antipeptide p5 serum yielded negative results (Fig. 4). This data confirms that epitope A is conformational, whereas epitope B is sequential. Furthermore, it also demonstrates that both peptides can elicit anti-p34 antibodies in mice.

Epitope B

P-34 ^{167}KYDPYAQYGQYGQYGQYGQQPYYGQPGG**QPGGQP**GGQQHSPQGYGSQYGGYGQGGAPTGGFGAQPSP^{234}

P8:NVESQPGGQPNT(22)

Epitope A

P-34 ^{235}QSGPQQSAQQQGPSTPPTGFPSFSPPPNVGGGSDSGSATANYSEQAGGQQS**YGQEPSSPSGP**TPA ^{298}

QYTDHHSSLLGP (326)
P5:LYRPSDSSLAGP (210)
MNVTLSSSLDGP (34)
NGNRSPTTLMGP (24)
DRLLTTLSGPAQ (26)

Figure 2 Deduced amino acid sequences of peptides carried by selected phage clones. The sequences of the peptides are shown aligned with the published sequence of protein p34 of *Mycobacterium paratuberculosis* (GenBank accession number CAA48222) using the standard single letter amino acid code. The phage clones are identified by the numbers in parenthesis. Putative epitopes A (panel B) and B (panel A) are bolded. Amino acid residues on phage-displayed peptides that are identical to the amino acids of the corresponding epitopes at the same positions are underlined.

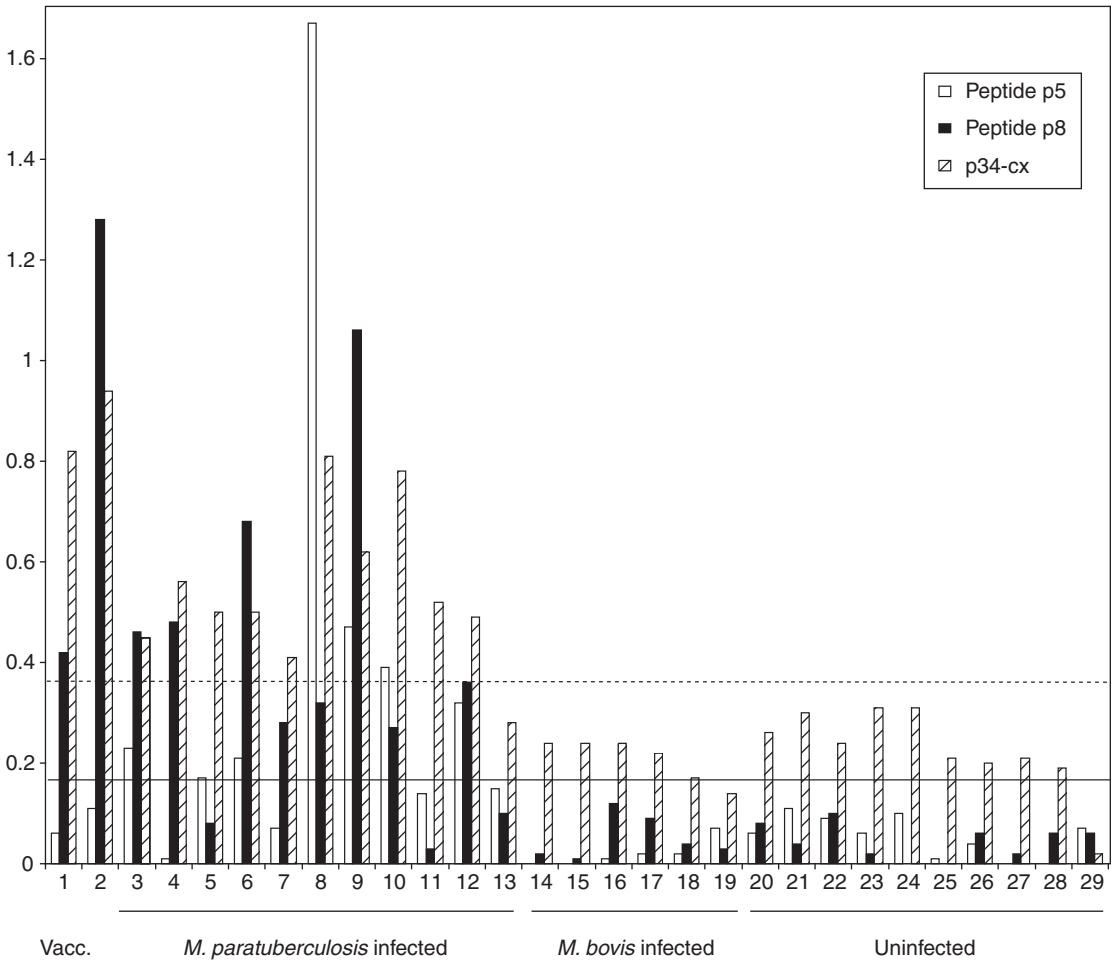

Figure 3 Recognition of peptides p5 (epitope A), p8 (epitope B) or p34-cx by sera from p34-cx-vaccinated, *Mycobacterium paratuberculosis*-infected, *M. bovis*-infected and healthy uninfected cattle. Numbers below the bars identify individual animals. Results correspond to the average ± SD of samples run in triplicate. Cut-off OD values for antigens p34-cx (dotted line) and peptides p5 and p8 (solid line) are shown.

Table 2 Reactivity of murine sera with lysate of *Escherichia coli* expressing p34-cx, peptide p5, peptide p8 or whole micobacteria*

	Antigen					
		Peptide‡		Whole mycobacteria		
Sera from immunized mice†	P34-cx	P5 (epitope A)	P8 (epitope B)	*Mycobacterium paratuberculosis*	*M. avium*	*M. intracellulare*
Whole *M. paratuberculosis* 1	1.667 (±0.011)	1.309 (±0.060)	0.664 (±0.012)	0.448 (±0.025)	0.497 (±0.031)	0.353 (±0.013)
Whole *M. paratuberculosis* 2	1.714 (±0.057)	0.271 (±0.028)	0.819 (±0.031)	0.652 (±0.019)	0.720 (±0.015)	ND
Peptide p8 1	0.531 (±0.081)	0.064 (±0.009)	0.119 (±0.081)	0.654 (±0.044)	0.442 (±0.052)	0.166 (±0.040)
Peptide p8 2	0.641 (±0.021)	0.077 (±0.035)	0.130 (±0.040)	0.820 (±0.029)	0.650 (±0.002)	ND
Peptide p5 1	0.355 (±0.055)	0.199 (±0.041)	0.059 (±0.012)	0.610 (±0.044)	0.559 (±0.013)	0.193 (±0.021)
Peptide p5 2	0.400 (±0.069)	0.233 (±0.078)	0.074 (±0.007)	0.600 (±0.052)	0.425 (±0.009)	ND
Unvaccinated	0.152 (±0.044)	-0.009 (±0.000)	0.009 (±0.008)	0.250 (±0.009)	0.225 (±0.010)	0.203 (±0.002)

ND, not determined.
*Results are expressed as the mean ± SD (samples run in triplicate) of the corrected optical density (OD) read at 405 nm.
†Sera of mice vaccinated with whole *M. paratuberculosis*, peptide p5 (LYRPSDSSLAGP) or peptide p8 (NVESQPGGQPNT).
‡Results are expressed as the corrected OD 405 nm (OD 405 nm for tested peptide, OD 405 nm for irrelevant peptide p7).

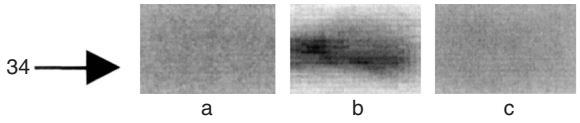

Figure 4 Western blot analysis of murine antipeptide antibodies. The reactivity of murine preimmune (lane a), antipeptide p8 (lane b) or antipeptide p5 (lane c) sera against *Mycobacterium paratuberculosis* cell-wall extracts is displayed. The molecular mass of p34 (34 kDa) is indicated to the left.

Epitopes A and B are exposed on the surface of *M. paratuberculosis*

Flow cytometry was used to determine the reactivity of sera from mice immunized with either peptide p5 or p8 or with UV-killed *M. paratuberculosis* against whole mycobacteria. Mouse anti-*M. paratuberculosis* serum showed high fluorescence signals against *M. paratuberculosis* and *M. smegmatis* (Fig. 5A,B) due to the presence of cross-reactive antigens on the surface of these two bacteria. Sera from mice immunized with either peptide p5 or p8 showed high fluorescence signals against *M. paratuberculosis* (Figs 4A and 5A) but not against *M. smegmatis*. This result indicates that epitopes A and B are expressed on the surface of *M. paratuberculosis* and that they do not cross-react with *M. smegmatis*.

Variability of p34 protein epitopes in mycobacteria

To determine the degree of variability of p34 homologous proteins within the *M. avium* species, genomic DNA obtained from five *M. paratuberculosis*, three *M. avium* and one *M. intracellulare* mc^2 76 isolates was PCR amplified and sequenced using primers specific for the p34 gene. The translated sequences obtained from all five *M. paratuberculosis* isolates were identical to the three previously published sequences for protein p34 (*M. avium* ssp. *paratuberculosis* strain ATCC 19698 accession number AF411607, *M. avium* ssp. *paratuberculosis* strain ATCC 19698 accession number X68102 and *M. paratuberculosis*, strain unidentified, accession number AF334164). The translated sequences from all three *M. avium* isolates were identical to the *M. paratuberculosis* sequences except for an in-frame insertion of four amino acids in the *M. avium* sequence at position 630 of the ORF encoding

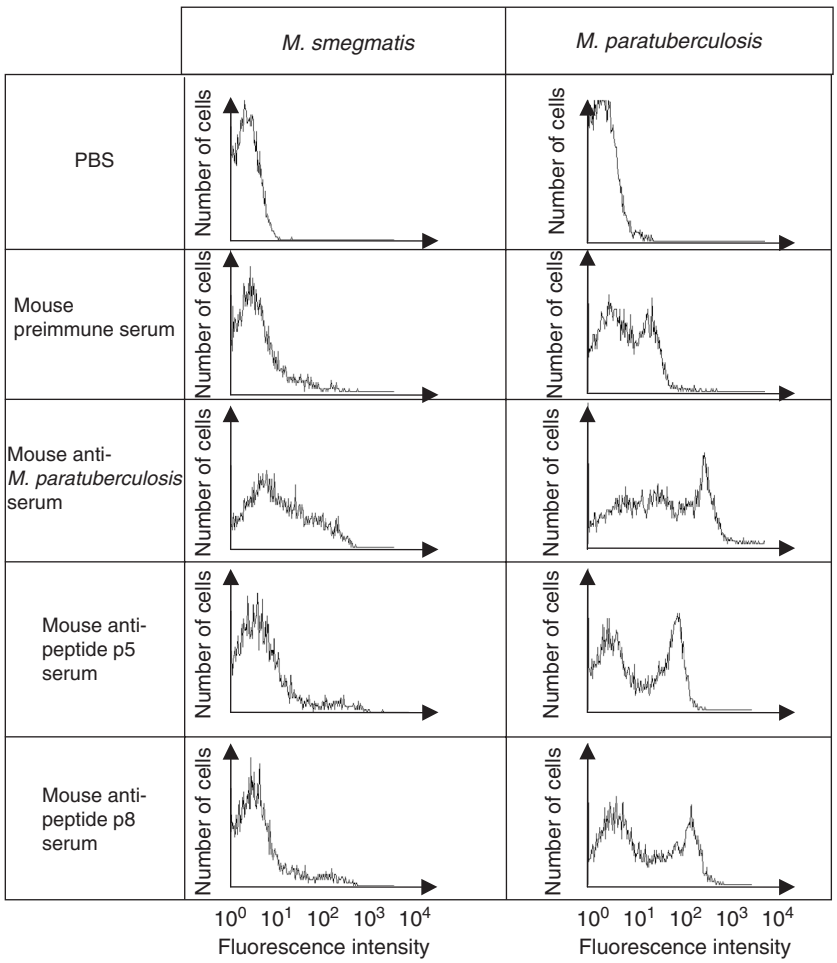

Figure 5 FACS analysis of mycobacterial cells stained with mice immune sera. Profiles of cell suspensions of *Mycobacterium smegmatis* (A) or *M. paratuberculosis* (B) labeled with the indicated serum. Each panel represents the analysis of 10,000 cells by fluorescence emission. *y*-axis, the number of cells; *x*-axis, fluorescence intensity.

for p34 (Fig. 6), which lies outside the regions of epitopes A and B.

A comparison of the amino acid sequences of p34 homologues from other mycobacterial p34 proteins, including *M. smegmatis*, *M bovis*, *M. tuberculosis* and *M. leprae* demonstrated a high degree of variability at the carboxyterminal regions (Fig. 6). The sequences corresponding to epitopes A and B were only identical in *M. paratuberculosis* and *M. avium*. These data explain the reactivity found in mouse antipeptide sera against *M avium* ssp. *avium and M. paratuberculosis* (Table 2).

Discussion

In this study, we used a phage combinatorial library to identify B-cell epitopes in the immunodominant C-terminus of *M. paratuberculosis* protein p34. Using affinity-purified anti-p34 antibodies obtained from a *M. paratuberculosis*-infected cow, we identified a conformational and a sequential epitope within the 130-amino acid C-terminal immunodominant domain (Fig. 2). Sinthetic peptide p5 (LYRPSDSSLAGP), synthesized based on the deduced sequence of one of the selected phage peptides, mimics conformational epitope A. Two independent lines of evidence revealed the conformational nature of this epitope: mice vaccinated with peptide p5 recognized p34 and whole *M. paratuberculosis* by ELISA but not by Western blot (Table 2) (Fig. 1) and a denatured p34-cx vaccine preparation was unable to induce antipeptide p5 antibodies in immunized cattle. This epitope includes residues Y^{284}, S^{290}, S^{291}, G^{294} and P^{295} of protein p34 and matches the consensus motif YxxxxSSxxGP found in the peptides displayed by other phages selected by our approach. This array of mimotopes suggests that other amino acid residues of p34 may contribute to the epitope-binding site recognized by bovine antibodies.

Our study also revealed the presence of another epitope within p34-cx. Epitope B was mapped to residues Q^{195}PGGQP^{200}. Peptide p8 (NVESQPGGQPNT), synthesized based on the deduced amino acid sequence of phage clone 22, mimics this epitope. In contrast to p5, peptide p8 is recognized by antibodies from both

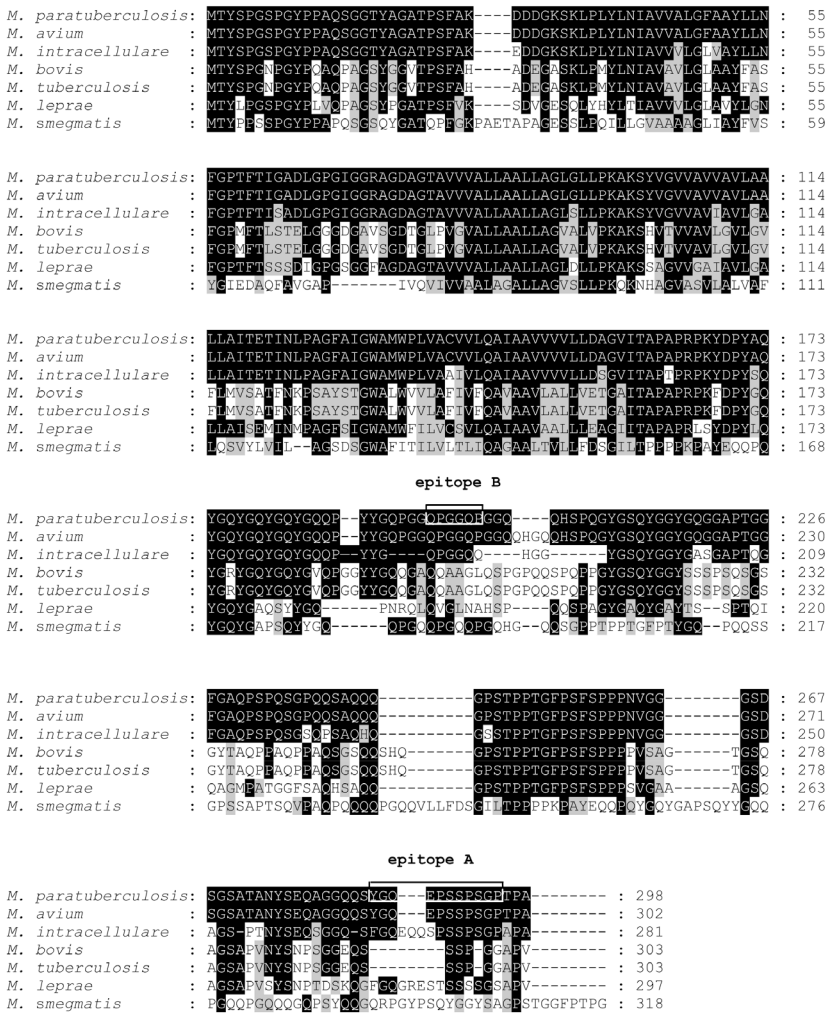

Figure 6 Comparison of the deduced amino acid sequences of 34 kDa proteins from mycobacteria. The diagram displays the pileup alignment of homologous proteins from *Mycobacterium paratuberculosis, M. avium, M. intracellulare strin* mc^2 76, *M. bovis* strain AF2122/97, *M. tuberculosis* strain H37Rv, *M. leprae* and *M. smegmatis* mc^{2}155. Black boxes indicate complete identity, and shaded boxes indicate conservative amino-acids substitutions. Epitopes A and B are boxed in the sequence of *M. paratuberculosis*.

M. paratuberculosis-infected and p34-cx-vaccinated cattle. In addition, sera from mice immunized with peptide p8 recognized p34-cx and whole *M. paratuberculosis*, both by ELISA and Western blot (Table 2) (Fig. 4), confirming that epitope B is sequential. Both epitopes A and B are localized on the surface of *M. paratuberculosis* as shown by whole-cell ELISA (Table 2) and by immunofluorescence (Fig. 5). These results are in agreement with those of others that found that the carboxyterminal region of p34 is exposed on the surface of *M. paratuberculosis* [15]. Thus, anti-p34 antibodies recognizing these epitopes bind to the surface of the bacterium.

Antibody titres against epitopes A and B in sera from infected cattle were highly variable. For example, sera from some of the infected animals showed high reactivity against peptide p5, and low reactivity against peptide p8, whereas the opposite pattern of reactivity was also found. This variability is expected, when epitopes are used as antigens to test sera from field animals [34, 35]. Moreover, for *M. paratuberculosis* antigens, this variable type of response has also been observed in infected cattle [36]. Nevertheless, all but one of the infected cattle displayed reactivity against one or both epitopes. This pattern of recognition is consistent with their immunodominance in *M. paratuberculosis*-infected cattle.

Antibodies from cattle infected with *M. bovis* do not recognize either peptide p5 or p8 (Fig. 3). In addition, mouse antipeptide p5 and p8 antibodies recognize whole *M. avium* ssp. *avium* and *M. avium paratuberculosis* but not whole *M. intracellulare* by ELISA (Table 2). These results are consistent with the divergence observed in the regions of epitopes A and B by comparing the sequences of *M. paratuberculosis* with other mycobacteria (Fig. 6). Thus, epitopes A and B are shared by the subspecies *M. avium* ssp. *avium* and *M. avium paratuberculosis* and are absent in the *M. avium*-related *M. intracellulare* and the veterinary pathogen *M. bovis*. The region comprising the 98-amino acid C-terminal of protein p34 was previously described as specific for *M. paratuberculosis* [15]. In addition, this polypeptide comprising the C-terminal of protein p34 was used to differentiate *M. paratuberculosis* from *M. bovis*-infected cattle in an ELISA test [37]. However, another study showed that anti-p34 rabbit polyclonal antibodies also reacted with the p34 homologue in *M. bovis* BCG [16]. The larger 132-amino acid fragment of p34, used in our study, allowed the identification of the sequential epitope B, which lies just beyond the region spanned by the C-terminal 98-amino acid polypeptide used by De Kesel *et al*. In addition, we demonstrated that epitope A, which is present in this 98-amino acid fragment, is conformational and is lost when the polypeptide is denatured. Thus, the negative results obtained by De Kesel *et al*., using rabbit anti-*M. avium* ssp. *avium* serum, may be due to the denaturation of the protein fragment used in that study, thus, abolishing the reactivity of epitope A. In contrast, the positive reactivity against rabbit anti-*M. paratuberculosis* serum observed by these authors seems to indicate the presence of additional sequential epitopes in the 98-amino acid fragment. The cross-reaction observed by Coetsier *et al*. with the *M. bovis* 38 kDa protein may be attributed to different sequential epitopes present in regions of high homology in the 98-amino acid C-terminal region of p34 (Fig. 6). Although these epitopes are not immunogenic in naturally infected cattle, they may become immunogenic in mice vaccinated with denatured protein fragments. In our study, we demonstrate that *M. bovis*-naturally-infected cattle do not produce antibodies against any of the immunodominant epitopes A and B. In contrast, *M. paratuberculosis*-naturally-infected cattle recognize these epitopes which are shared only by *M. avium* ssp. *avium* and *M. paratuberculosis* (Table 2) (Fig. 6).

Identification of immunodominant epitopes may provide significant information to elucidate a possible role of the humoral response in protection against intracellular pathogens [19]. For example, only some epitopes elicited a protective response in mice infected with *C. neoformans* [23]. Moreover, the protective response was also dependent of the gamma sub-isotype of murine antibodies used in passive transfer experiments [24]. This protective effect may be due to the differential effect of these epitopes to induce opsonization [23]. Alternatively, epitope density may influence the ability of the Fc region to activate complement [38]. These events could influence the level of macrophage activation, leading to either pathogen survival or elimination. In addition, endocytosis of antibody-coated bacteria can induce mouse macrophages to switch an immune response from a Th1 profile to a Th2 profile after an infection has taken place [39]. Moreover, the variability of the protein regions comprising epitopes A and B across the various species of mycobacteria suggests a significant role of p34 in the immunobiology of infection. Therefore, it is possible that antibodies against epitopes A and B, in the context of specific bovine subisotypes, could influence the course of *M. paratuberculosis* pathogenesis.

In summary, in this study, we identified two B-cell epitopes, one sequential and another conformational, within the *M. paratuberculosis* p34 protein C-terminal immunodominant domain. These epitopes were also found in the homologous protein from *M. avium* ssp. *avium* but not in the p34 protein from *M. bovis*. Moreover, both epitopes were recognized by sera from *M. paratuberculosis*-infected cows but not by *M. bovis*-infected cattle, a finding of diagnostic significance. Current efforts in our laboratories are directed towards the production of mouse bovine chimeric antibodies against epitope A and B as well as finding new B-cell epitopes in p34 to evaluate the role of the humoral immune response in the course of *M. paratuberculosis* infections in cattle.

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