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Original article

Bovine IgG1 antibodies against *Mycobacterium avium* subsp. *paratuberculosis* protein p34-cx improve association of bacteria and macrophages

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Abstract – Paratuberculosis, caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), is a chronic granulomatous enteric disease in cattle. Among molecular components of *Map*, protein p34 was identified as specific and immunodominant for bovine B cells. In order to determine if specific antibodies could influence the course of *Map* pathogenesis, the interaction between bacteria and bovine macrophages was studied. Bovine polyclonal antibodies from 3 calves vaccinated with protein p34-cx, 6 calves vaccinated with heat-killed *Map*, 8 naturally infected, and 3 healthy calves -as negative controls- were used. Specific anti-*Map*, -p34-cx and -PPA-3 antibodies were evaluated and isotype characterized. Infected and *Map* vaccinated animals showed similar IgG1 and IgG2 response against *Map* whole bacteria. When p34-cx was used as the antigen, mainly IgG1 and IgG3 were detected in infected and only IgG1 in p34-cx vaccinated animals. Bovine polyclonal antibodies from three animals of each category were isolated and affinity purified through *Map* and p34-cx columns. The effect of these antibodies in association with *Map* and a transformed bovine peritoneal macrophage's cell line (Bov-Mac) as well as activation of NF-κB transcription factor was studied. Our results show that association of *Map* significantly increased in vitro after pretreatment with bovine anti-*Map* or anti-p34-cx antibodies obtained from vaccinated or infected cattle when compared with those of controls. Improved activation of NF-κB was detected in macrophages that ingested *Map* opsonized with either anti-*Map* or anti-p34-cx specific antibodies of infected or vaccinated calves, suggesting that both anti-*Map* and IgG1 anti-p34-cx antibodies support *Map*-macrophage interactions.

***Mycobacterium avium* subsp. *paratuberculosis* / p34 / IgG1 / macrophages / cattle**

1. INTRODUCTION

Paratuberculosis, caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), is chronic granulomatous enteritis in cattle [18]. It is characterized by intermittent diarrhea, weight loss and eventual death. Subclinically and clinically infected cows may shed *Map* through colostrum and milk. Animals become infected at early age through ingestion of contaminated feces and milk, but fetal infection is also possible in dams with advanced disease [38]. After oral infection, *Map* may cross from the lumen of the small intestine into the Peyer's patch area via M cells of the follicles associated epithelium, or enter the small intestinal mucosa in areas with or without Peyer's patches [29, 34].

During the first 2–3 years after the initial infection, host immunity against *Map* is characterized by a cell-mediated immune response that ultimately leads to an intestinal granuloma. A strong cellular immune response is active during early stages, with production of interferon-γ (IFN-γ) by peripheral blood

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mononuclear cells. Animals in the subclinical stage of disease generally shed low numbers of bacteria in their feces and have either none or low antibody titers to *Map*. As infection progresses to a clinical state, bacterial shedding in feces and serum antibody titers increase while IFN-γ production declines. Cows with clinical paratuberculosis showed increased IL-10 and TGF-β cytokine gene expression in ileo compared with subclinically infected animals, which may be indicative of mechanisms causing a shift in T helper (Th) responses from Th1 toward Th2 type [22].

Map has a complex cell wall containing immunogenic proteins, glycopeptides and glycolipids such as lipoarabinomannan, in common with other mycobacterial species. Among molecular components of *Map*, p34 protein was identified as specific and immunodominant for bovine B cells [11]. The amino-terminal hydrophobic region (residues 40-160) is buried within the bacterial envelope, whereas the hydrophilic carboxy-terminus (residues 161-298) is exposed on the cell surface. Using a panel of sera from naturally infected cattle with tuberculosis or paratuberculosis, the 98-amino acid carboxy-terminal region of p34 was postulated as specific for *Map* [11, 15]. However, it was shown that rabbit polyclonal or murine monoclonal antibodies produced against the same p34 fragment cross-reacted with a 38 kDa protein of *Mycobacterium bovis* [10]. 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 in the carboxy-terminal region [30].

The serological response to mycobacterial antigens during paratuberculosis has been extensively studied in order to improve diagnosis. Production of the various immunoglobulin isotypes during the course of the disease was demonstrated by several investigation groups [1, 47]. It was suggested that antibodies against intracellular pathogens present at the time of infection may have an important role in host protection [12]. Recently, the role of antibodies in protection has been reevaluated demonstrating that antibodies are able to modify various aspects of mycobacterial infection to the benefit of the host [16, 44]. Antibodies against *M. tuberculosis* activate macrophages through Fc-dependent endocytosis [26]. However, the presence of antibodies against *Map* does not appear to be an early protective mechanism for infected animals, since B cell numbers (corresponding to lower IgG antibody titers) were significantly reduced in infected lambs compared with controls [6]. In contrast, the percentage of B cells in peripheral blood mononuclear cells was significantly higher in cows with clinical signs of disease compared with control or subclinical animals [40].

NF-κB transcription factor activation was related to protection in mycobacterial infections. In cell nuclei, NF-κB regulates the expression of a series of genes that control differentiation, apoptosis, cell growth, migration or inflammation [4]. NF-κB knock out mice are highly susceptible to *M. tuberculosis* infection and under this condition, Th-1 cytokine (IL-2, IFN-γ, TNF-α) secretion levels are significantly low [46].

In order to determine if bovine anti *Map* or p34-cx specific antibodies could modify the course of paratuberculosis pathogenesis we studied their influence in the interaction between bacteria and bovine macrophages. Bovine polyclonal antibodies from p34-cx or *Map* inactivated whole bacteria vaccinated cattle and *Map* naturally infected cattle were characterized and used to determine the effect on *Map*/macrophage association and on NF-κB activation. Our results show *Map* specific IgG1, IgG2 and IgG3 in infected and *Map* vaccinated animals, p34-cx specific IgG1 and IgG3 in infected but only IgG1 responses in p34-cx vaccinated animals. Association between *Map* and macrophages increased after treatment with anti-*Map* or anti-p34-cx specific antibodies from infected and vaccinated cattle resulting in improvement of NF-κB activation.

2. MATERIALS AND METHODS

2.1. Bacterial strains

Mycobacterium avium subsp. *paratuberculosis* (strain 19698) was used throughout this work. Mycobacteria were grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MD,

USA) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC; Becton Dickinson microbiology system, Sparks, MD, USA), 0.05% Tween 80 and 2 mg mycobactin J/mL (Allied Monitor, Fayette, MO, USA) for 14 days at 37 °C [14]. Bacteria were pelleted by centrifugation at 10 000 × *g* for 30 min and washed three times in pH 7.4, 0.15M phosphate-buffered saline (PBS), dispersed as predominantly single-cell suspensions using a 16 gauge needle and suspended in PBS to a final concentration of 10^9/mL. The number of viable bacteria in the suspension was determined by plate counts on 7H9 agar (CFU). Before each assay, an aliquot was diluted in RPMI-1640 medium (Gibco®, Invitrogen, Carlsbad, CA, USA) and used for in vitro cell infection.

2.2. Cloning, expression and purification of the carboxy-terminal peptide of protein p34

Chromosomal DNA was extracted as previously described [14]. 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, [15]).

The sense (5'-CAGGGATCCAAGTACGACCCCTACGCG-3') and antisense (5'-TGCGAATTCGTCCTCCAGCCGTGTTCAC-3') primers contained BamHI and EcoRI restriction sites, respectively, allowing directional cloning of the amplicon into the polylinker site of expression vector pRSET-A (Invitrogen). After confirming the identity of the insert by DNA sequencing, the recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) pLysS (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 (IPTG) as described elsewhere [32]. The cells were pelleted by centrifugation for 15 min at 6 000 × *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 a His×Bind Resin (Novagen, Madison, WI, USA) following the manufacturer's directions. Recombinant p34-cx was renatured by dialyzing against PBS for 48 h at 4 °C. A further purification step was performed by size exclusion chromatography, using a Sephadex G75-column eluted with PBS. Fractions were collected and analyzed by PAGE and Western blot using serum from *Map* infected cattle. Positive fractions were pooled and concentrated using Centricon-30 (Millipore Corp. Billerich, MA, USA) following the 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. Purified preparations obtained by affinity columns and checked by ELISA tests were used for in vitro experiments.

2.3. Animals

Four groups of animals were used.

– Infected group consisted of eight *Map* naturally infected cows identified by standard fecal culture method, clinical symptoms and macroscopic lesion in necropsy, sera were kindly provided by Dr G. Traveria (University of La Plata, La Plata, Argentina).

– *Map* vaccinated group (*Map* vacc.) consisted of six 8-month-old calves from a free-*Map* establishment, intramuscularly vaccinated with two doses of heat-killed *Map*. Whole bacteria inactivated vaccine was formulated with *Map* heat inactivated emulsified in oil incomplete Freund adjuvant (Sigma Chemical Co., St. Louis, MO, USA). Each dose contained approximately 20 mg of dry microorganisms [24]. No adverse reactions were detected in this group.

– p34-cx vaccinated (p34-cx vacc.) group consisted of three 8-month-old calves from a free-*Map* establishment, subcutaneously vaccinated with three doses of fifteen milligrams (wet weight) of *E. coli* BL21 (DE3) pLysS cells expressing p34-cx lysed as previously described [32], emulsified in Freund's incomplete adjuvant (Sigma Chemical Co.).

– The healthy non-infected calf group consisted of three 8-month-old calves from a free-*Map* establishment and was used as the negative control.

Bovine serum was obtained from jugular blood collected about 30 days after the last immunization. Complement was inactivated at 56 °C for 30 min and sera were stored at –20 °C until use for immunoglobulin purification.

2.4. Cloning, expression and purification of the constant heavy chain of bovine IgG3 isotype

In order to determine the specific IgG3 bovine isotype, 995 nucleotides coding for CH1 to CH3 domains of gamma 3 were cloned and *E. coli* expressed, using a similar methodology as described for p34-cx protein.

Bovine spleen cells were obtained by perfusion and RNA was obtained using Trizol (Invitrogen

Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA was submitted to reverse transcriptase treatment to obtain cDNA. Briefly, 3 μg RNA, 5 μg of specific primer BoBG3rev (5'-ACC TCG AGA TGG ACT TCT CTT-3'), 2 μL dNTP mix (10 mM) were incubated at 65 °C for 5 min placed on ice for 2 min (RT mix). Then to 18 μL of the RT mix, 5 μL 10X RT buffer, 8 μL MgCl2 (25 mM), DTT (0.1 M), and RNase (2 μL) were added. The mix was incubated at 25 °C for 2 min and 2 μL SuperScript IIRT (Invitrogen Life Technologies) was added. The reaction was performed at 42 °C for 50 min. PCR amplification was carried out at 94 °C for 4 min (1 cycle), 94 °C for 20 s, 55 °C for 30 s, 72 °C for 2 min (30 cycles), and 72 °C for 7 min (1 cycle) using specific primers for the Cγ3 coding sequence (GenBank accession number U63638 and U63639 [31]. The sense (5'- CTC GAG GCC TCC ACC ACA GCC CCG- 3') and antisense (5'- AGG CTT ACT GCA CAC GTG TAG TGG TC-3') primers contained XhoI and HindIII restriction sites, respectively, allowing for directional cloning of the amplicon into the polylinker site of the expression vector pRSET-A (Invitrogen).

The identity of the insert was confirmed by DNA sequencing. Expression and purification of Cγ3r were carried out similarly as described for p34-cx. Two New Zealand White rabbits were immunized with three doses of 1 mg/mL Cγ3 protein using incomplete oil Freund as the adjuvant. The Cγ3r was not recognized by peroxidase labeled sheep anti bovine IgG1 heavy chain specific (O.D. 0.065 ± 0.002) nor peroxidase labeled sheep anti bovine IgG2 heavy chain specific (O.D. 0.065 ± 0.016) (Bethyl Laboratories Inc., Montgomery, TX, USA) but strongly reacted with rabbit anti Cγ3r (O.D. 0.856 ± 0.024) and peroxidase labeled affinity purified goat antibody to bovine IgG (H+L) (KPL) (O.D. 0.198 ± 0.014) by ELISA test. Rabbit serum anti Cγ3r did not cross-react with bovine IgG1 nor IgG2 (O.D. 0.051 ± 0.002, O.D. 0.161 ± 0.004 respectively). Rabbit serum was used as primary antibody in an isotyping ELISA test.

2.5. Characterization of specific antibodies to different *Map* antigens

The ELISA test was used in order to determine antibody responses of sera. Sera were assayed at dilutions ranging from 10^1 to 10^5. The antigens used were the following: whole *Map* bacteria, p34-cx obtained as described above and a commercial antigen obtained from the protoplasmatic extract of *Map* strain 18 *PPA-3* (Allied Monitor, Lafayette, MO, USA). *Map* (5×10^6 CFU/mL), p34-cx (0.5 μg/mL) or *PPA-3* (40 μg/mL) were diluted in 0.1 M $NaHCO_3$ and coated onto microplates (Immulon 2HB Dynex, Chantilly, VA, USA) for 16 h at 4 °C. ELISA test was carried out as described previously [31]. OD were determined by reading at 492 nm in an OpsysMR spectrophotometer (Dynex Technologies, Chantilly, VA, USA).

For specific isotype characterization HRP-labeled antibodies for bovine IgG1 and IgG2, IgM monoclonal antibody from ascitic fluid (clone IL-A30) [43] and IgG3 antibody as described above were used as primary antibody, while peroxidase labeled goat antibody anti-mouse IgG (KPL, Washington DC, USA) or peroxidase labeled goat antibody anti-rabbit IgG (KPL) were used as secondary antibodies, respectively.

2.6. Isolation of cattle immunoglobulins

Immunoglobulins were isolated by ammonium sulfate precipitation (33% saturation), pellets were resuspended and dialyzed against 0.01 M PBS, pH 7. The purity of immunoglobulin preparations was estimated by SDS-PAGE (7%, wt/vol) under reducing conditions [25]. Immunoglobulins were stored at –70 °C until use. Concentration was determined assuming the absorbance at 280 nm of 1 mg/mL solution to be 1.37.

Affinity purification of anti-p34 and anti-Map antibodies – Five milliliters of bovine serum isolated as described above was passed three times through CNBr-activated Sepharose (Sigma Chemical Co.) 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 coupled to 10 mg of purified p34-cx or *Map* whole bacteria. Washing and elution steps were performed as described elsewhere [17] and specificity of the purified serum to p34-cx and *Map* was evaluated by Western blot and ELISA assays.

2.7. Association experiments between macrophages and *Map*

*Map*s were stained with fluorescein isothiocyanate (FITC, Sigma Chemical Co.) as described previously [19]. Briefly, 4×10^7 broth-grown bacteria were suspended in carbonate buffer (0.001% FITC in a 0.2 M Na_2CO_3 - $NaHCO_3$, 150 mM NaCl buffer) pH 9.2 for 30 min. Bacteria were then

washed twice in PBS and resuspended in RPMI 1640 (Gibco®, Invitrogen).

A transformed bovine peritoneal macrophage cell line (Bov-Mac) [36] was cultured in RPMI-1640 (Gibco®, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 5 mg/mL of gentamicine (Gibco®, Invitrogen). Cultured Bov-Mac cells were harvested on ice using a cell scraper. After washing, Bov-Mac cells were resuspended in RPMI 1640 (10^6 cells/mL) without supplements or antibiotics and were infected with *Map* at 10:1 (bacteria:target cell), in the presence or absence of different concentrations of antibodies. The reaction was incubated for 30 min at 37 °C with rotation and stopped with cold PBS and 10 mM EDTA. The cells were analyzed with a flow cytometer with a 488 nm argon ion laser (FACScalibur, Becton Dickinson, San José de California, USA), using CellQuest software (Becton Dickinson). Ten thousand events were recorded. Ingestion of bacteria was indicated by a shift of fluorescence in the FITC channel histogram towards the right (M2). The results were expressed as the percentage of positive cells.

2.8. NF-κB evaluation by Electrophoretic Mobility Shift Assay (EMSA)

Bov-Mac were infected with *Map* alone or pre-incubated with *Map* or *p*34-cx bovine antibodies from the different groups as described above. Control cells were incubated with culture medium only. Positive control cells were incubated with recombinant human interferon (100 U/mL; kindly provided by PC-GEN Argentina) or with lipopolysaccharide (LPS, 10 μg/mL) derived from *E. coli* serotype O26:B6 (Sigma Chemical Co.). NF-κB binding activity was analyzed by EMSA as described previously [2]. Cells were incubated in 400 μL of hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.1% Nonidet P-40) for 15 min on ice and centrifuged at 11 000 $\times g$ for 10 min. Nuclear pellets were resuspended in 80 μL of nuclear hypotonic buffer (20 mM HEPES, 1.5 mM MgCl2, 420 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA, 25% glycerol) and incubated at 4 °C for 15 min, followed by centrifugation at 13 000 $\times g$ for 15 min. The supernatants were stored at –70 °C until further use and nuclear protein concentration was determined by the Bradford assay. Nuclear extracts (4 μg) were preincubated with 0.25 mg/mL poly (dI-dC) (Poly-deoxy-inosinic-deoxy-cytidylic acid GE-Amersham Biosciences) in binding buffer (50 mM Tris–HCl pH 7.5, 250 mM NaCl, 2.5 mM EDTA, 5 mM MgCl2, 2.5 mM DTT, 20% glycerol) for 20 min at room temperature (RT). Lysates were exposed to γ-^{32}P labeled oligonucleotide probes for the consensus binding sites of NF-κB (Promega, Madison, WI, USA) (5'-AGTTGAGGGGACTTTCCCAGGC-3' and 3'-TCAACTCCCCTGAAAGGGTCCG-5') for 30 min at RT. The DNA-protein complexes were separated on a non-denaturating 4% polyacrylamide gel and exposed to an X-ray film for 24 h at –70 °C.

In order to determine the specificity of the band, cold competition experiments were done. Extracts from the different treatments were preincubated with unlabeled NF-κB or Oct-1 (5'-GTCGAATGCAAATCACTAGAA-3' and 3'-ACAGCTTACGTTTAGTGATCTT-5') probes in 100-fold excess and then processed as described above. To determine the components of bovine NF-κB complex a supershift assay was performed. Nuclear protein extracts were preincubated using 1 μL of anti-p50 and anti-p65 specific antibodies, or anti-goat antibodies as isotype control (Santa Cruz Biotechnology Inc.) for 1 h on ice and processed as described above.

2.9. Statistical analysis

For comparisons of antigen-isotype combinations between the different groups of animals, the Kruskal-Wallis test was used for one-way analysis of variance [33]. Data of ingestion experiments were analyzed by use of the Student t test. The level of significance was set at $P < 0.05$.

3. RESULTS

3.1. Antibody responses in *Map*-infected and *Map* or p34-cx vaccinated animals

Various dilutions of sera (10^{-1} to 10^{-3}) were used to detect *Map* specific antibodies in infected and vaccinated groups of calves. Means of O.D. from the animals of each group obtained by ELISA assay at 10^{-3} dilution are shown in Figure 1. Sera from infected animals were positive, however higher O.D. values (1.207 ± 0.161) were obtained against *Map* whole bacteria in p34-cx vaccinated bovine sera. Despite individual differences, infected and p34-cx vaccinated cattle showed higher reactivity against *Map* and p34-cx than those

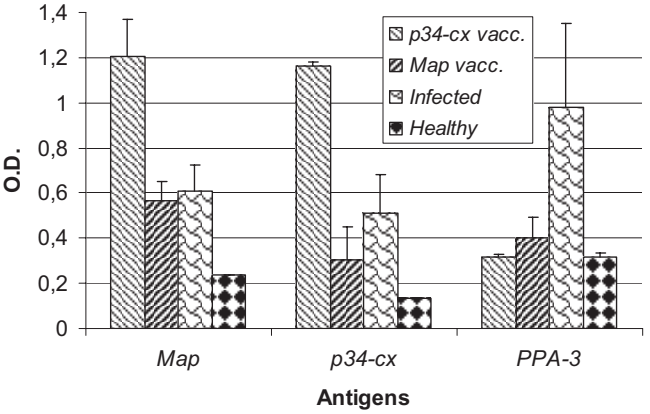

Figure 1. Antibody response of vaccinated and infected cattle. ELISA assay for each antigen was developed. *Map* (5×10^6 CFU/mL), p34-cx (0.5 μg/mL), *PPA-3* (40 μg/mL) were diluted in 0.1 M $NaHCO_3$ and coated onto plates, 1/1000 diluted bovine sera were added; after washing, they were incubated with peroxidase-labeled anti-bovine Ig. The results are expressed as optical density at 492 nm (O.D.) and correspond to p34-cx vaccinated ($n = 3$), *Map* vaccinated ($n = 6$), naturally infected ($n = 8$) and healthy non-vaccinated ($n = 3$) animals.

vaccinated with the whole bacteria. As expected, antibodies from infected animals recognized commercial antigen (*PPA-3*).

3.2. Isotype characterization from *Map*-infected and *Map* or p34-cx vaccinated animals

Isotype characterization of antibodies against p34-cx, *Map* and *PPA-3* was carried out in bovine sera from all groups analyzed, as shown in Table I. When antibodies specific to whole bacteria were analyzed, significantly increased *Map*-specific IgG1 and IgG2 antibodies were found in sera of p34-cx and *Map* vaccinated as well as in infected animals, compared to healthy controls. Low signal *Map* specific IgG3 responses were found in *Map*-infected or vaccinated animals. IgM specific to *Map* was detected only in sera from p34-cx vaccinated animals.

When p34-cx antigen was used, significant increase in IgG1 was observed in the p34-cx vaccinated and *Map*-infected groups when compared to *Map* vaccinated and negative control groups. No significant differences were found in p34-cx specific IgG2 in all three groups while low signal p34-cx specific IgG3 responses were found only in *Map*-infected animals. IgM responses against p34-cx were low and similar in all groups of animals.

When *PPA-3* was used, specific IgG1 was significantly high in *Map*-infected animals, while IgG2 increased only in *Map* vaccinated and *Map*-infected animals. *PPA-3* specific IgG3 could not be detected in any of the groups while IgM against *PPA-3* was low and similar in all groups of animals.

3.3. Association between *Map* and macrophages

Map-macrophage association was evaluated by flow cytometry. Total antibodies from either infected or vaccinated animals showed increased association evaluated by flow cytometry (Tab. II). After treatment with p34-cx or *Map* total or affinity-purified antibodies the level of association between bacteria and cells increased compared with Igs from bovine negative sera. Calves vaccinated with p34-cx showed similar results for p34-cx specific (82.94 ± 4.48) or *Map* specific antibodies (82.58 ± 0.79) while antibodies from *Map* vaccinated cattle showed lower percentage of association. However anti-*Map* specific antibodies (66.47 ± 1.64) showed higher values than anti- p34-cx specific antibodies (53.53 ± 1.98). Affinity-purified antibodies

Table I. Bovine sera isotyping.

Bovine groups	Antigens			
	Map			
	IgG1	IgG2	IgG3	IgM
p34-cx vacc. (n = 3)	2.02(± 0.09)*	1.94 (± 0.16)*	0.11 (± 0.00)	0.57 (± 0.07)*
Map vacc. (n = 6)	1.10 (± 0.24)*	1.15 (± 0.36)*	0.18 (± 0.06)*	0.47 (± 0.12)
Infected (n = 8)	1.10 (± 0.36)*	1.41 (± 0.30)*	0.21 (± 0.12)*	0.49 (± 0.13)
Healthy (n = 3)	0.44 (± 0.01)	0.65 (± 0.00)	0.11 (± 0.08)	0.40 (± 0.05)
	p34-cx			
	IgG1	IgG2	IgG3	IgM
p34-cx vacc. (n = 3)	0.33 (± 0.13)*	0.16 (± 0.05)	0.27 (± 0.06)	0.18 (± 0.01)
Map vacc. (n = 6)	0.12 (± 0.02)	0.12 (± 0.02)	0.22 (± 0.03)	0.16 (± 0.02)
Infected (n = 8)	0.23 (± 0.02)*	0.16 (± 0.04)	0.26 (± 0.04)*	0.16 (± 0.04)
Healthy (n = 3)	0.10 (± 0.00)	0.13 (± 0.03)	0.10 (± 0.01)	0.16 (± 0.01)
	PPA-3			
	IgG1	IgG2	IgG3	IgM
p34-cx vacc. (n = 3)	0.47 (± 0.13)*	0.29 (± 0.19)	0.18 (± 0.02)	0.29 (± 0.09)
Map vacc. (n = 6)	0.58 (± 0.23)*	0.43 (± 0.16)*	0.23 (± 0.03)	0.32 (± 0.07)
Infected (n = 8)	1.03 (± 0.66)*	0.71 (± 0.45)*	0.24 (± 0.04)	0.33 (± 0.20)
Healthy (n = 3)	0.20 (± 0.00)	0.19 (± 0.01)	0.24 (± 0.03)	0.25 (± 0.00)

For each antigen and isotype, an ELISA assay was developed. *Map* (5×10^6 CFU/mL), p34-cx (0.5 μg/mL), *PPA-3* (40 μg/mL) were diluted in 0.1 M $NaHCO_3$ and coated onto plates, 1/100 diluted bovine sera were added, after washing they were incubated with anti-bovine isotypes. The results were expressed as means of optical density from bovine sera diluted 1:100 in each group ± S.D.

* Significantly different from the responses of healthy non-vaccinated cattle ($P \leqslant 0.05$) determined by Kruskal Wallis.

Table II. Improvement of *Map*-macrophage association.

Bovine group	Total antibodies	Purified antibodies	
		p34-cx	*Map*
p34-cx vaccinated	60.11 (± 5.13)* (3)	82.94 (± 4.48)* (3)	82.58 (± 0.79)* (3)
Map vaccinated	55.66 (± 6.65)* (6)	53.53 (± 1.92)* (3)	66.47 (± 1.64)* (3)
Infected	61.89 (± 4.98)* (8)	71.63 (± 1.98)* (3)	72.60 (± 0.61)* (3)
Healthy non-vacc. control	41.12 (± 3.05) (3)	41.36 (± 1.87) (3)	46.41 (± 0.98) (3)

Association of *Map* opsonized with specific p34-cx or *Map* antibodies from vaccinated or infected cattle evaluated by FACS with FICT-labeled *Map*.

The results are expressed as mean values of M2 % area (± S.D.). (n) Number of sera evaluated.

* Significantly different from the responses of healthy non-vaccinated cattle ($P \leqslant 0.05$) determined by Student t-test.

from infected cattle increased association similarly. Significant differences were detected with p34-cx and *Map* purified antibody treatments for all the groups studied.

3.4. Activation of NF-κB in bovine macrophages

To establish whether antibodies-opsonized *Map* affect cell activation, EMSA was used to measure the NF-κB transcription factor. The results using concentrations from 25 to 100 μg/mL of antibodies are shown in Figure 2A, showing that activation was dependent on the antibody concentration. The best signals were detected at 100 μg/mL, thus this concentration was selected for further experiments. The specificity of NF-κB activation was confirmed by competition studies with unlabeled oligonucleotides. No signal could be

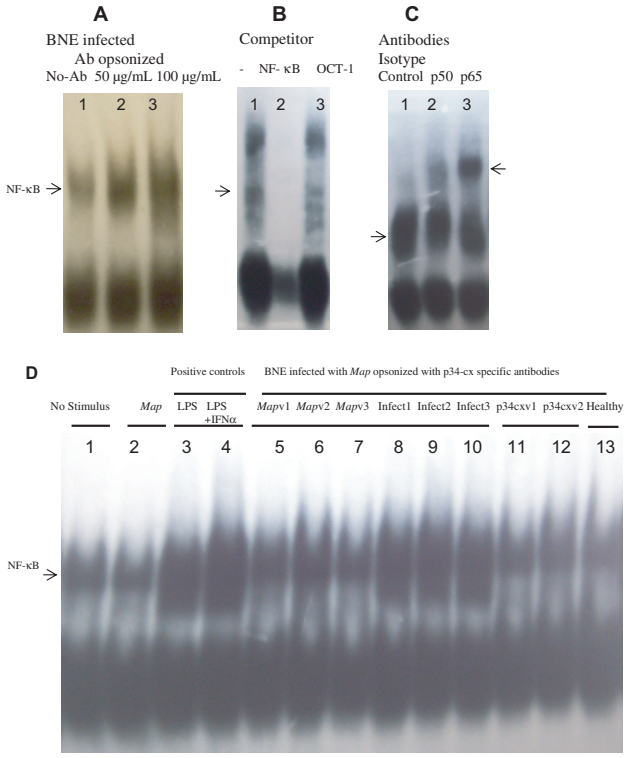

Figure 2. EMSA analysis of nuclear NF-κB activity and modulation by p34-cx or *Map* specific antibodies. **(A)** *Map* infected Bov-Mac cells with or without *Map* specific antibodies. Line 1: Bov-Mac nuclear extract (BNE) infected with *Map*; Line 2: BNE from infected *Map* opsonized with *Map* specific antibodies from infected animals 50 μg/mL; Line 3: BNE from infected *Map* opsonized with *Map* specific antibodies from infected animals 100 μg/mL. The arrow indicates specific NF-κB signal. **(B)** Cold competition with specific (NF-κB) or nonspecific (OCT-1) binding site oligonucleotides showing the specificity of the protein/DNA binding complex in Bov-Mac. Line 1: BNE from cells infected with *Map* opsonized with *Map* specific antibodies (100 μg/mL) from infected animals; Line 2: BNE from cells infected with *Map* opsonized with *Map* specific antibodies pre-incubated with specific cold NF-κB as competitor; Line 3: BNE from cells infected with *Map* opsonized with *Map* specific antibodies pre-incubated with specific cold OCT-1 as the competitor. The arrow indicates specific NF-κB signal. **(C)** Supershift assays with antibodies (Ab) to the p50 and p65 subunits and normal goat serum (isotype control) demonstrating subunit composition of NF-κB in Bov-Mac. Line 1: BNE from infected *Map* opsonized with *Map* specific antibodies from infected animals 100 μg/mL pre-incubated with irrelevant isotype control antibodies; Line 2: BNE from infected *Map* opsonized with *Map* specific antibodies from infected animals 100 μg/mL pre-incubated with anti p50 subunit of NF-κB complex; Line 3: BNE from infected *Map* opsonized with *Map* specific antibodies from infected animals 100 μg/mL pre-incubated with anti p65 subunit of NF-κB complex. The arrows indicate a shift of the specific NF-κB signal by the protein complex. **(D)** Improved activation of *Map* opsonized with p34-cx specific antibodies from different bovine groups. A representative from three independent experiments is shown. Line 1: Bov-Mac nuclear extract (BNE) without stimulus; Line 2: BNE infected with *Map*; Lines 3 and 4: BNE positive controls, stimulated with LPS and IFNrhu alfa; Lines 5 to 7: BNE infected with *Map* opsonized with p34-cx specific antibodies (100 μg/mL) from *Map* vaccinated animals; Lines 8 to 10: BNE infected with *Map* opsonized with p34-cx specific antibodies (100 μg/mL) from *Map* infected animals; Lines 11 to 12: BNE infected with *Map* opsonized with p34-cx specific antibodies (100 μg/mL) from p34-cx vaccinated animals; Line 13: BNE infected with *Map* opsonized with immunoglobulins (100 μg/mL) from healthy non-vaccinated animals.

detected when unlabelled NF-κB was used in pre-incubation, while when OCT-1 was used the specific NF-κB component showed no change (Fig. 2B).

The composition of NF-κB active complex was analyzed using a supershift assay showing that the band was super shifted by both anti-p50 and anti-p65 antibodies, indicating that this complex in bovine cells contained both subunits (Fig. 2C).

Specific antibodies from infected or vaccinated cattle were used to opsonize the bacteria prior to macrophage infection. Antibodies against p34-cx and *Map* from infected and vaccinated cattle increased macrophage NF-κB activation at 30 min post-infection compared with those from healthy controls. The results of a representative experiment with p34-cx purified antibodies are shown in Figure 2D. Purified anti-*Map* antibodies showed similar results (data not shown).

In order to confirm that the differences detected in activation were independent on endotoxin contamination level, LPS was analyzed in antibody samples with Limulus Amebocyte lysate Pyrotell®. The values obtained in all samples were similar (2 to 8 units).

4. DISCUSSION

Immunity to most mycobacterial infections is dependent on cell-mediated immune responses; antibodies have little or no predictive value and are detected late during the infection [9]. In calves, experimental tonsilar crypt infection was reported to induce humoral immune responses after 134 days post-infection [41], while maternal colostrums or milk are the source of *Map* specific antibodies during early infection. However, calves from infected cows have a large probability of being infected and the role of such antibodies still remains unknown.

Titers and isotype profile of *Map* specific antibodies in sera of infected cattle were described as highly dependent on antigen used for evaluation [23]. Our results indicate that infected cattle recognized all the evaluated antigens. This pattern of recognition is consistent with p34 immunodominance in *Map*-infected cattle as previously described [15]. We also demonstrated that antibodies elicited by infection cross-react with p34-cx, suggesting that structural conformation was conserved in the recombinant protein despite the addition of amino acids during the expression procedure, as previously reported [5]. The specific p34-cx response detected was higher than those reported [11]; such differences may be explained either by the presence of immunodominant epitopes in this carboxy-terminal fragment or because the structure of p34-cx mimics, in a best way, the native conformation of the antigen. In addition, antibody titers against *Map* and p34 in sera from infected cattle were highly variable among animals. Individual variations were described in field animals and in sera of *Map* infected cattle whose results were generally analyzed individually [37, 40].

Inactivated *Map* vaccine induced antibody to *Map* showing that after heat treatment the antigenic structure was conserved in the bacteria. Besides, the low antibody responses to p34-cx detected in these sera might be attributed to partial p34 denaturizing by heat treatment.

Isotypes of bovine antibodies and their allelic forms have been described but limited information about its function and cellular receptors was reported [20]. Th2 response, directed towards IgG1 antibody production is critical for blocking bacterial colonization and toxins at sites of tissue infection. Our results showed solely IgG1 response only in p34-cx vaccinated cattle reacting against p34-cx. This may be caused by the particular structure of this protein, the number of doses used or by the adjuvant effect of the *E. coli* extract. In fact, this Th2 response had been previously described as characteristic to protein antigens in cattle [7]. Recently, Chaiyotwittayakun et al. [8] evaluated a J5 *E. coli* bacterin and described that at least six doses of vaccine were needed to detect an increment of specific IgG2 in cattle. We were not able to establish a predominant isotype response in *Map* whole bacteria vaccinated cattle with two doses of vaccine.

Levels of IgG1 and IgG2 specific to *Map* and *PPA-3* were similar in infected and

vaccinated animals in accordance with those reported previously [23, 47]. Low levels of IgG1 detected in p34-cx vaccinated animals by *PPA-3* ELISA may be due to the cross reactivity among mycobacteria and antibodies against mycobacteria in healthy cattle [3] which may influence the test specificity, as previously described by others [28, 35, 47].

Despite the T independent profile of the lipids in the mycobacteria membrane, only in few animals was specific IgM detected, according to other authors who detected IgM specific to whole bacteria in three of five infected bovines studied [40].

It was reported that *Mycobacterium* spp. can be ingested in the presence or absence of serum and it has been suggested that different entry pathways could affect bacilli survival into the macrophages [13, 39]. Besides, other authors described that complete immune sera and normal bovine sera were similar in the ability to promote macrophage uptake of *Map* in vitro [19].

We selected *Map* whole bacteria and p34-cx protein as antigen to study the effect of specific antibodies in macrophage infection in vitro. Our results demonstrate that anti-p34-cx or *Map* specific antibodies from infected or vaccinated cattle increased association of *Map* with an SV40-transformed bovine peritoneal macrophage cell line (Bov-Mac) in vitro, regardless of the functional alterations recently described for this cell line by Woo et al. [45]. It is remarkable that p34-cx vaccinated cattle produced only IgG1 antibodies suggesting that this isotype may be responsible for the association between *Map* and macrophages, in accordance with the biological properties described for bovine IgG1 [27].

Our results show that IgG1 specific *Map* or p34-cx antibodies from infected or vaccinated cattle clearly increased and overcame constitutively NF-κB activation in Bo-Mac. Activation of NF-κB in macrophages induces the production of proinflammatory cytokines IL-2, IL-6 [3] which may be involved in granuloma formation. Besides, mycobacterial specific IgG1 antibody responses and production of high levels of IFN-γ (Th0 profile) were associated with increased pathogenesis in bovine tuberculosis [42]. Thus, we suggest an active role of bovine IgG1 in paratuberculosis infection through bacteria-macrophage association.

Mycobacterium specific IgG3 responses were detected in infected animals. To our knowledge, this is the first work reporting this isotype in bovine paratuberculosis.

Protective immunity against mycobacterial disease is poorly understood. Inactivated vaccines for paratuberculosis are not completely effective in preventing the disease, but they decrease environmental elimination of the bacteria and prevent symptoms of the disease [21, 24]. Our results may explain some effect of antibodies generated by vaccination due to the biological properties of the IgG1 involved in the immune response.

We conclude that humoral immunity response elicited by *Map* infection, inactivated whole bacteria vaccine and p34-cx vaccine subunit promotes the association between the *Map* and target cell, improving NF-κB activation. These results suggest that the presence of IgG1 in the early phases of the disease may prevent spreading of the bacteria activating macrophages that participate in granuloma formation. Further studies on the destiny of *Map* inside macrophages after association are necessary in order to determine the utility of p34-cx antibodies in protection against paratuberculosis infection.

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