Chapter 4

Hsp70 vaccination-induced antibodies recognize B cell epitopes in the cell wall of *Mycobacterium avium* subspecies *paratuberculosis*

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Vaccine 29 (2011) 1364–1373

Abstract

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes a chronic intestinal infection of ruminants and has been associated with the etiology of human Crohn's disease. A MAP Hsp70/DDA subunit vaccine previously showed a significant reduction in fecal shedding of MAP in cattle, concomitant with pronounced antibody production against MAP Hsp70, rather than T cell reactivity. Our hypothesis is that if Hsp70-specific antibodies are able to confer protection, the first requisite would be that the Hsp70 molecule is accessible for antibodies in intact MAP bacteria. In the current study monoclonal antibodies identified MAP Hsp70 B cell epitopes. Two linear epitopes were also recognized by antibodies of vaccinated calves and goats. These epitopes showed to be accessible by antibodies in the bacterial cell wall and in intestinal lesional tissue during natural infection. These results indicate that vaccination-induced antibodies can bind intact bacteria and have the potential to contribute to the protective effect of Hsp70/DDA subunit vaccination against bovine paratuberculosis.

1. Introduction

Paratuberculosis is a highly prevalent chronic mycobacterial infection of the small intestine of ruminants. It causes substantial economic losses at farm level, particularly in cattle [1]. Transmission of the causative organism *Mycobacterium avium* subspecies *paratuberculosis* (MAP) amongst ruminants occurs by excretion via feces into the environment, where it may survive for prolonged periods of time [2]. When the disease progresses towards the clinical stage of infection, MAP can also be present in milk [3]. As a result of the latter it may represent a food safety issue given the possible association between MAP and human Crohn's disease [4].

Currently, a vaccine to control paratuberculosis in cattle is not available, since the whole cell vaccine registered for use in sheep interferes with control programs against bovine tuberculosis. Individual MAP proteins as subunit vaccine candidates may overcome this interference. In bovine paratuberculosis [5,6], similar to other mycobacterial diseases such as tuberculosis and leprosy, heat shock proteins (Hsp) elicit strong cell mediated and antibody responses. Our previous studies indicated that immune responsiveness to recombinant MAP Hsp70 proteins in naturally infected animals was predominantly cell mediated [6,7].

Since protective immunity to intracellular mycobacterial pathogens is thought to be cell-mediated [8], recombinant MAP Hsp70 protein was used as a subunit vaccine in cattle concomitant with experimental infection with MAP. It induced protection as indicated by significantly reduced bacterial shedding [9]. In addition, MAP Hsp70 subunit vaccination did not interfere with current diagnostic methods to diagnose bovine TB [10]. Surprisingly, and in strong contrast with our previous observations in field cases of bovine paratuberculosis, this immunization-challenge study showed limited cell mediated responses against MAP Hsp70 and pronounced MAP Hsp70 specific antibody production in the vaccinated animals [9].

The contribution of antibodies to protection against mycobacterial infections is disputed by some (reviewed in [11,12]), and supported by others (reviewed in [13]). Most of the recent studies on serum therapy of *M. tuberculosis* (MTb) infection report protective effects of antibodies specific for polysaccharide bacterial cell wall antigens such as the polysaccharide lipoarabinomannan (reviewed in [14]). In mice, a monoclonal antibody (IgA) directed against a small surface-expressed mycobacterial heat shock protein (the 16kD α-crystallin homologue) protected against early infection of murine lungs with MTb [15].

We hypothesize that if Hsp70-specific antibodies are able to confer protection, observed in cattle vaccinated with the recombinant MAP Hsp70 subunit vaccine, the first requisite would be that the Hsp70 molecule is accessible for antibodies in intact MAP bacteria. We generated mouse monoclonal antibodies to determine B cell epitopes of the recombinant Hsp70 protein and focused on linear epitopes. Subsequently, epitope-specific antibody responses, induced by vaccination of cattle and goats with recombinant MAP Hsp70, were analyzed to assess whether these antibodies recognized the same linear epitopes. Lastly, the monoclonal antibodies were used to study if these antibodies recognized native MAP Hsp70 protein in lesional tissue in naturally infected animals and if they interact with intact bacteria.

2. Materials & Methods

2.1. Animals and experimental design

2.1.1. Mice

Two Balb/c mice, obtained from Charles River (Someren, the Netherlands), were used for the generation of MAP Hsp70 specific monoclonal antibodies. Animals were kept under standard housing and care conditions at the Central Animal Facilities of Utrecht University (Utrecht, the Netherlands).

2.1.2. Goats

Thirty female goat kids (Saanen breed dairy goats, age 14 ± 3 days at the start of the experiment) were used. The kids were raised using conventional procedures and feeds, and were checked daily for general health. They were randomly assigned to one of the four experimental groups. Goat kids in groups 1 (n=7) and 2 (n=8) (uninfected controls) were housed separately from goat kids in groups 3 (n=7) and 4 (n=8) (MAP infected). Goat kids assigned to groups 2 and 4 were immunized once at the start of the experiment (day 0). The immunization consisted of the administration of 200 µg of recombinant MAP Hsp70 in 1 mL phosphate buffered saline (PBS) containing 10 mg/mL dimethyl dioctadecyl ammonium bromide (DDA) adjuvant (Sigma Aldrich, USA) in the final preparation, subcutaneously in the lower neck region.

Goat kids assigned to groups 3 and 4 were infected orally with 3 oral doses, at days 0, 2 and 4, of $2x10^9$ cfu of MAP strain G195, originally isolated from a goat with clinical signs of paratuberculosis, grown on Middlebrook 7H10 supplemented with OADC and Mycobactin J (a generous gift from D. Bakker, CVI, Lelystad, the Netherlands). The cfu of the infection dose was determined by colony counts of serial dilutions on 7H10 agar plates.

Blood samples were taken from the vena jugularis on a weekly basis for a period of 3 months. Serum was stored at -20 ºC, until further use. Goats were euthanized at the end of the experiment and tissue samples from ileum, jejunum, the ileocecal and a jejunal mesenteric lymph node were analyzed using MAP specific IS900 PCR [16], bacterial culture on mycobactin J supplemented HEY medium (BD Biosciences, Belgium) and histopathology.

2.1.4. Cattle

Sera from cattle subjected to a Hsp70 vaccination–challenge experiment, published previously [9], were used to characterize MAP Hsp70 specific antibody responses. In short, 4 groups of 10 female calves aged 29 ± 9 days, randomly assigned to one of 4 experimental groups, were used in that study. Treatment of the groups was identical to the goat kids described in 2.1.3. Serum samples were taken every 2 weeks for the first 12 months of the experiment and monthly for the remainder 12 months.

2.2. Ethics

Animal experiments were approved by the Ethical committee of Utrecht University, and performed according to its regulations.

2.3. Antigens

The following antigens were used for vaccination and determination of specificity of monoclonal antibodies (mAb): recombinant MAP Hsp 65kD (rMAP Hsp60) and Hsp 70kD (rMAP Hsp70). These antigens were produced as described earlier [6,17].

A recombinant C-terminal deletion mutant protein of the Hsp70 molecule was constructed, comprising the receptor binding part. It consisted of N-terminal amino acids 1-359 of wildtype Hsp70, had a molecular weight of approximately 45kD and was designated RBS70. RBS70 was constructed by restriction endonuclease digestion of the original recombinant MAP Hsp70 pTrcHis expression vector with AflII (NE Biolabs, USA) and HindIII (Gibco-Invitrogen, the Netherlands) using 5 units of each enzyme per µg DNA. The digested fragment was separated from the vector DNA by agarose gel (1%) electrophoresis and isolated from the gel using a QIAEXII kit (Promega, the Netherlands). The vector DNA was blunted by using T4 DNA polymerase (Fermentas, Germany) subsequently purified using a DNA cleaning kit (Zymo Research, USA) , religated using T4 DNA ligase (Quick Ligation kit, NE Biolabs, USA) and purified using the DNA cleaning kit. Finally, chemically competent Top10 bacteria (Invitrogen, the Netherlands) were transformed with the vector DNA using a heat shock protocol provided by the manufacturer. Transformed bacteria were selected and protein expression and purification was performed similar to the procedure described for recombinant MAP Hsp70 [6].

In addition, the following antigens were used: recombinant *M. tuberculosis* Hsp70 (MTb), recombinant *Escherichia coli* (*E. coli*) Hsp70 and bovine Hsc70 purified from bovine brain (generous gifts from Stressgen, Canada). Purified protein derivatives (PPDs) were produced at CVI (Lelystad, the Netherlands) as previously described [18], from MAP strain 3+5/C (PPDP), *M. bovis* (MB) strain AN5 (PPDB), and *M. avium* ssp. *avium* (MAA) strain D4 (PPDA). MAP strain 316F was grown at the CVI (generous gifts from D. Bakker).

To define peptides for the screening of monoclonal antibodies and sera from cattle and goats the following HSP70 Genbank-derived sequences were used: Q00488 (MAP Hsp70); A0QLZ6 (MAA Hsp70); P0A5C0 (MB Hsp70); P0A5B9 (MTb Hsp70); P04475 (*E. coli* Hsp70); NP776975 (*Bos taurus* Hsp70-1A).

A first set of 124 synthetic 14-mer peptides, with an aminoterminal cysteine, a 5 amino acids (aa) shift and an overlap of 9 aa, covering the MAP Hsp70 molecule, was synthesized using the simultaneous multiple peptide synthesis (SMPS) technique described previously [19]. To enable di-sulphate binding of peptides to the solid phase ELISA plate, an amino-terminal cysteine residue was coupled to each peptide during synthesis. For primary screening peptides were pooled in 11 groups of sequential peptides. Positive pools were retested for the single peptide specification.

To enable coupling of peptides to streptavidin coated beads for the Luminex system (see below) a separate set of 14-mer MAP Hsp70 peptides, selected based on the first screening with the 14-mer peptides, was synthesized using SMPS and modified using amino-terminal biotinylation.

A third set of 15-mer peptides consisting of mycobacterial, *Bos taurus* and *E. coli*

homologues to identified MAP Hsp70 linear epitopes was also synthesized using SMPS and modified using amino-terminal biotinylation.

2.4. Generation and screening of monoclonal antibodies

The generation of monoclonal antibodies has been described previously [20]. Briefly, 100 µg of recombinant MAP Hsp70 protein in 80 µL PBS was mixed with 100 µL Specol [21] (Prionics, the Netherlands) to obtain a water in oil emulsion used for i.p. immunization of Balb/c mice. This immunization was repeated 3 weeks later. Another 3 weeks later, four days prior to hybridoma production the mice were boosted i.v. with 50 µg of the antigen in 50 µL PBS. After 4 days spleen cells were fused with mouse myeloma cells (Sp2/0) using polyethyleenglycol (PEG, Merck, Germany). Antigen specific antibody producing hybridoma's were selected by ELISA [22] and subcloned in limiting dilution. The isotype of the monoclonal antibodies was determined using the Mouse Hybridoma Subtyping Kit (Roche, the Netherlands).

2.5. ELISA

In general, 96 well EIA plates (Corning Costar Corp., USA) were coated with 100 µL of antigen diluted in sodium bicarbonate buffer (pH 9.6), for 60 min at 37°C. All subsequent incubations were performed for 30 min at 37°C, and after each incubation step plates were washed 3 times with PBS containing 0.05% Tween 20. Wells were blocked with 200 µL blocking solution (Roche, the Netherlands). All antibody fractions were diluted in blocking solution and peroxidase labelled to appropriate antibodies was used as enzyme.

Finally, plates were washed extensively, and 100 µL ABTS (2,2'-azinobis (3 ethyl) benzthiazolinsulfonic acid (Roche, the Netherlands) substrate buffer was added to each well. The optical density (OD) was measured after 10 minutes at 405 nm on a spectrophotometric Elisa reader (Bio-Rad laboratories, USA). Absorbance values were subsequently analyzed.

2.5.1. Protein ELISA's

The MAP Hsp70 protein, bovine Hsc70 protein, PPDP, PPDA, and PPDB ELISA to measure antibody responses in cattle sera were performed according to methods described previously [6] with minor modifications to detect murine and caprine antibodies as follows. Hybridoma supernatants or sera of immunized/infected goats were used in a predetermined optimal dilution or were serially diluted in blocking buffer as indicated. Secondary antibodies used were polyclonal goat anti-mouse peroxidase (PO) conjugated antibodies (Sigma Aldrich, USA) to detect murine monoclonal antibodies, and rabbit anti-goat IgG-PO (Sigma Aldrich, USA) to detect caprine antibodies.

2.5.2. The mycobacterial whole cell ELISA

The mycobacterial whole cell ELISA was a modification to the protein ELISA. In brief, 96-well plates (Corning Costar Corp., USA) were coated with 100 µL of washed bacteria (both MAP and MAA; 1×10^8 cfu/mL), diluted in sodium bicarbonate buffer pH 9.6 for 60 min at room temperature, while shaking at 300 rpm on a electronic MTS shaker (IKA Werke, Germany). All subsequent incubations were performed for 30 min shaking at room temperature. After each incubation step, plates were

washed three times with PBS containing 0.01% Tween 20. The secondary antibody was goat anti-Mouse (GAM)-PO (Roche, the Netherlands) 1:2000.

2.5.3. Peptide ELISA

Peptide ELISA was used for the initial epitope mapping of the monoclonal antibodies generated against MAP Hsp70. The peptide ELISA using cys-linked peptides has been described previously [23]. The different cys-linked peptides were diluted in 0.1 M Tris-HCl, pH 8.0 at a concentration of 15 µg/mL, and 100 µL was added at each well.

2.6. Flowcytometric analysis of monoclonal antibodies binding to mycobacteria.

To study whether monoclonal antibodies bind to intact bacteria, indicative of the presence of MAP Hsp70 in the bacterial cell wall, suspensions of MAA strain D4 and MAP strain 316F (generous gifts from D. Bakker, CVI) were prepared from log phase liquid cultures. Suspensions of MAA and MAP (both 1010 bacteria/mL in PBS) were diluted 1:100, washed three times by centrifugation (1 min at 14.000 RPM in an Eppendorf centrifuge (Eppendorf, Germany)) and resuspended in PBS. These suspensions were diluted 1:100 in PBS supplemented with 1% BSA and 0.01% sodium azide (both from Sigma Aldrich, USA) and divided in volumes of 100 µL. The Hsp70 specific monoclonal antibodies were added in a concentration of 5 µg/mL. After incubation for 25 min at room temperature (RT) and three washes with PBS supplemented with 1% BSA and 0.01% sodium azide (FACS buffer), FITC-labelled Goat anti-mouse antibodies (Becton-Dickinson, USA) were added and incubated for 25 min at RT. After three more washes, 10.000 bacterial cells were used for analysis by FACScan (Becton-Dickinson, USA).

2.7. Luminex multiplex immunoassay

Multiplex peptide specific antibody measurements were performed using biotinylated peptides linked to avidin coated fluorescent microspheres (LumAv, Luminex, USA) on a Luminex 100 platform according to instructions provided by the manufacturer (Luminex). A total of 2.5 x 10^5 beads (100 µL) per uniquely labelled beadset were washed twice with PBS, and subsequently incubated with 10 µmol biotinylated peptide for 10 minutes at 20°C. After two washes with PBS, the beads were resuspended in their original volume (100 µL) using PBS supplemented with 1% bovine serum albumine (Sigma Aldrich, USA) and 0.01% sodium azide, and stored in the dark at 4°C until further use. For multiplex analysis 20 µL of resuspended coated beads of each of up to 20 unique beadsets were pooled in an eppendorf container. To the final volume of beads, the same volume of PBS was added, and mixed.

In a round bottom 96 well microtiter plate, 10 µL of the mixed beads was added per well. Subsequently, 100 µL of goat or calf serum per well was added. This mixture was incubated for 30 min at 4°C and subsequently washed once with PBS supplemented with 1% bovine serum albumine (Sigma Aldrich, USA) and 0.01% sodium azide. Next, bead-bound antibodies were labelled with 50 µL 1:5000 diluted protein-A-RPE (Prozyme, USA). This mixture was incubated for 30 min at 4°C at which point 100 µL PBS supplemented with 1% bovine serum albumine (Sigma Aldrich, USA) and 0.01% sodium azide was added. The 96 well plate was placed in the Luminex 100 analyzer and per sample the amount of PE derived

fluorescence was measured for each of the 20 unique beadsets by acquisition of data of 100 beads per set and expressed as mean fluorescence intensity (MFI) as a measure for antibody bound to the peptide coupled to the designated beads.

2.8. Immunohistochemistry

Selected recombinant Hsp70 specific monoclonal antibodies recognizing linear epitopes were used in immunohistology to study whether these epitopes were detectable in wildtype MAP, present in infected lesional tissue. Tissues samples from archived formalin fixed, paraffin embedded tissues were used from cattle diagnosed with paratuberculosis and uninfected control animals. Microbiological and immunological characterization of these cattle samples has been published previously [7].

Tissue specimens were processed by routine methods for microscopic examination using a Haematoxylin and Eosin (H&E) and Ziehl Neelsen (ZN) stains. For immunohistology tissue sections were dewaxed in xylene and rehydrated through graded alcohols for 2 min each step till distilled water. They were then pre-treated with Citrate buffer pH 6.0 in microwave 700 Watt for 10 min. Endogenous peroxidase activity was suppressed by 1% H2O2 in methanol for 30 min. This was followed by treatment with 10% normal horse serum (NHS) 1:10 in PBS for 15 min for removal of non-specific reactivity and by incubation with primary antibody (4°C overnight). The secondary antibody (biotin labelled horse anti-mouse 1:125, Dako, Denmark) was applied for 30 min at room temperature. The two solutions A and B of the ABC kit were diluted 25 times in PBS, mixed and the ABC reagent was stored for 30 min. until further use. Then the slides were incubated for 30 min with ABC-complex at room temperature. Conjugate binding was detected by adding the substrate chromogen (3.3-diaminobenzidine, DAB) and color was allowed to develop for 10 minutes. Finally, tissue sections were washed with distilled water, counter-stained with haematoxylin, rinsed, dehydrated and mounted.

2.9 Statistical analysis

Data were analyzed using SPSS v15 software. Student t-test or ANOVA were used as indicated. Level of statistical significance was set at $p<0.05$.

3. Results

3.1 Characterization of rMAP Hsp70 specific monoclonal antibodies

Eight hybridoma supernatants reacted with rMAP Hsp70. None of these 8 supernatants reacted with rMAP Hsp60 or PPD-A control antigens, 3 supernatants recognized their epitope in PPDP (KoKo.B03, KoKo.B05, KoKo.B06) (Figure 1A).

Furthermore, these 8 culture supernatants were screened for reactivity with rHsp70 from MTb, *E. coli* and purified bovine Hsc70 to identify cross-reactivity. Four supernatants reacted only with MAP Hsp70 (KoKo.B01, KoKo.B02, KoKo.B05, KoKo.B06), three supernatants also recognized recombinant Hsp70 from MTb (KoKo.B03, KoKo.B04, KoKo.B08), 3 supernatants recognized bovine Hsc70 (KoKo. B04, KoKo.B07, KoKo.B08) and only one supernatant recognized recombinant Hsp70 from *E. coli* (KoKo.B03) (Figure 1B).

Comparison of binding of the 8 MAP Hsp70 specific monoclonal antibodies in ELISA to the recombinant deletion mutant protein RBS70 (containing the N-terminal amino acids 1-359 of wild type MAP Hsp70) indicated that KoKo.B01, KoKo.B02 and KoKo.B06 recognize an epitope at the C-terminus of Hsp70, which is not present in RBS70. The other five antibodies recognized epitopes in the N-terminal RBS70 mutant molecule (Figure 1C).

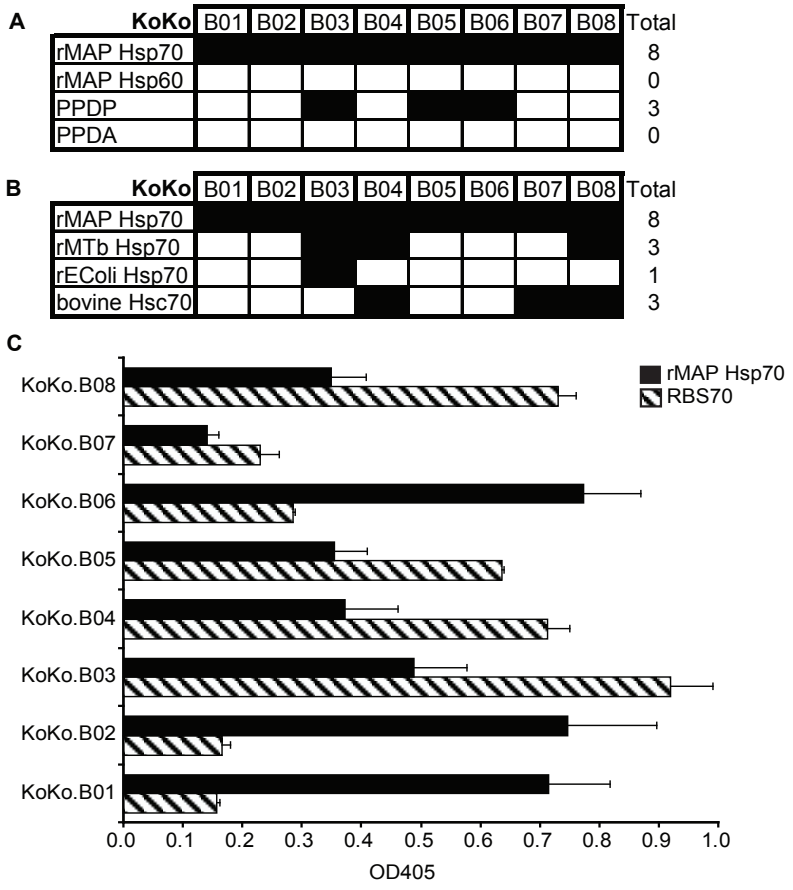

Figure 1 Characterization of rMAP Hsp70 specific hybridoma's. Panel A: reactivity of hybridoma supernatants to rMAP Hsp70 protein, a recombinant control protein (rMAP Hsp60 protein), and Johnin (PPDP) and avian tuberculin (PPDA). Panel B: reactivity of hybridoma supernatants to several Hsp70 proteins, recombinant mycobacterial Hsp70 from MAP and *M. tuberculosis* (MTb), recombinant Hsp70 from *E. coli* and purified bovine Hsc70. Top rows of representative table indicate the clone name (KoKo.B01–B08). Cells that have been filled (black square) indicate ELISA responses in which OD405nm> (average OD405nm of background control samples + 3× SD) in two separate experiments. Panel C shows the binding of antibodies (KoKo.Bo1–B08) to the recombinant MAP Hsp70 protein (black bars) and the recombinant deletion mutant protein RBS70 (hatched bars) (containing the N-terminal amino acids 1–359 of wildtype Hsp70). Results are presented as the average OD405 + SD of 3 independent experiments.

3.2 MAP HSP70 contains multiple linear B cell epitopes

All 8 antibodies reacting with recombinant MAP Hsp70 were tested for recognition of synthetic MAP Hsp70 peptides to identify linear epitopes. In a primary screening, three antibodies (KoKo.B01, KoKo.B02 and KoKo.B03) displayed reactivity to specific pools of MAP Hsp70 peptides (data not shown). The other five monoclonal antibodies did not recognize linear peptide epitopes. Subsequent, fine mapping of the epitopes using the single peptides of the pools in a solid phase ELISA confirmed that KoKo.B01, KoKo.B02, KoKo.B03 recognized linear epitopes in MAP Hsp70. The antibodies KoKo.B01 (IgG1 isotype) and KoKo.B02 (IgG2b isotype) recognized the aminoacid sequence P595-603 (PDGAAAGGG) (Figure 2A+B), located in the C-terminal part of MAP Hsp70. The third antibody, KoKo.B03 (IgG2a isotype), recognized a conserved epitope in the N-terminus of the MAP Hsp70 protein with the apparent core region sequence P111-124 (ITDAVITVPAYFND) (Figure 2C).

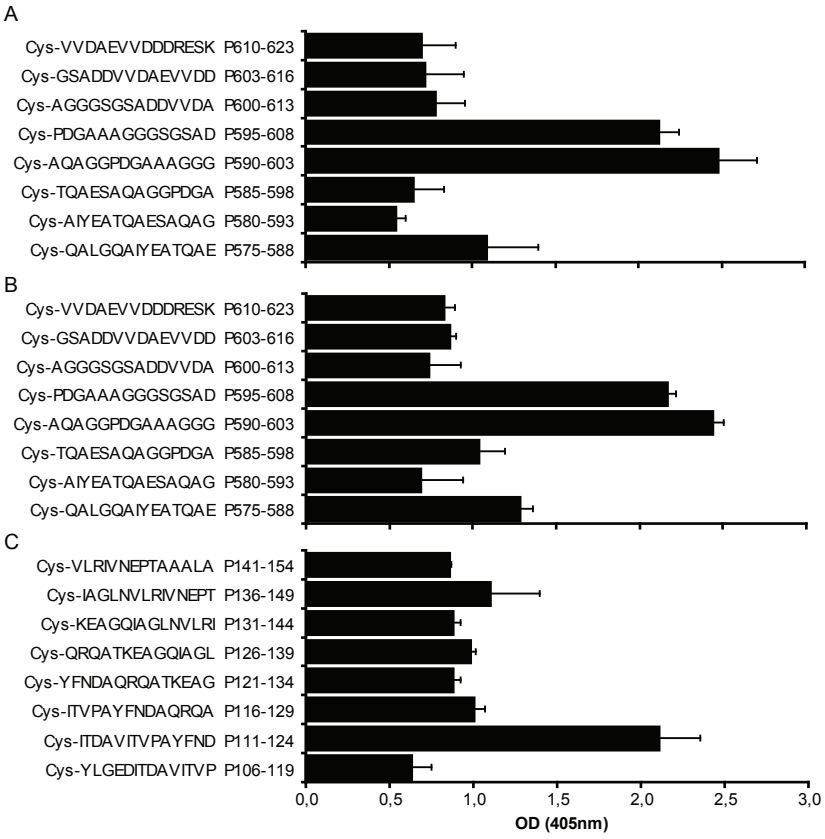

Figure 2 rMAP Hsp70 reactive monoclonal antibodies identify two linear B cell epitopes. Antibodies showing reactivity with recombinant MAP Hsp70 were tested for reactivity with synthetic MAP Hsp70 peptides. Antibody reactivity was tested on individual peptides of a positive 14-mer peptide pool, with a 5 amino acid (aa) shift and an overlap of 9 aa. (Panel A) KoKo.B01 (panel B) KoKo.B02, and (panel C) KoKo.B03 test results are presented as the average optical density at 405nm (OD405) + SD.

The specificity of the monoclonal antibodies KoKo.B01-03 in relation to homologous Hsp70 proteins was tested by Luminex multiplex immunoassay. The data indicated that KoKo.B01 (not shown) and KoKo.B02 recognize an epitope which is present and identical in Hsp70 from MAP and MAA, but absent in Hsp70 from MB, MTb, and E. coli and bovine Hsc70 (Figure 3A). Finally, the data regarding KoKo.B03 indicate that conserved mycobacterial homologues (MB, MTb) are equally well recognized, while recognition of the *E. coli* homologue is at approximately 50% of that of the MAP epitope, while recognition of the bovine homologue is near background levels (Figure 3B).

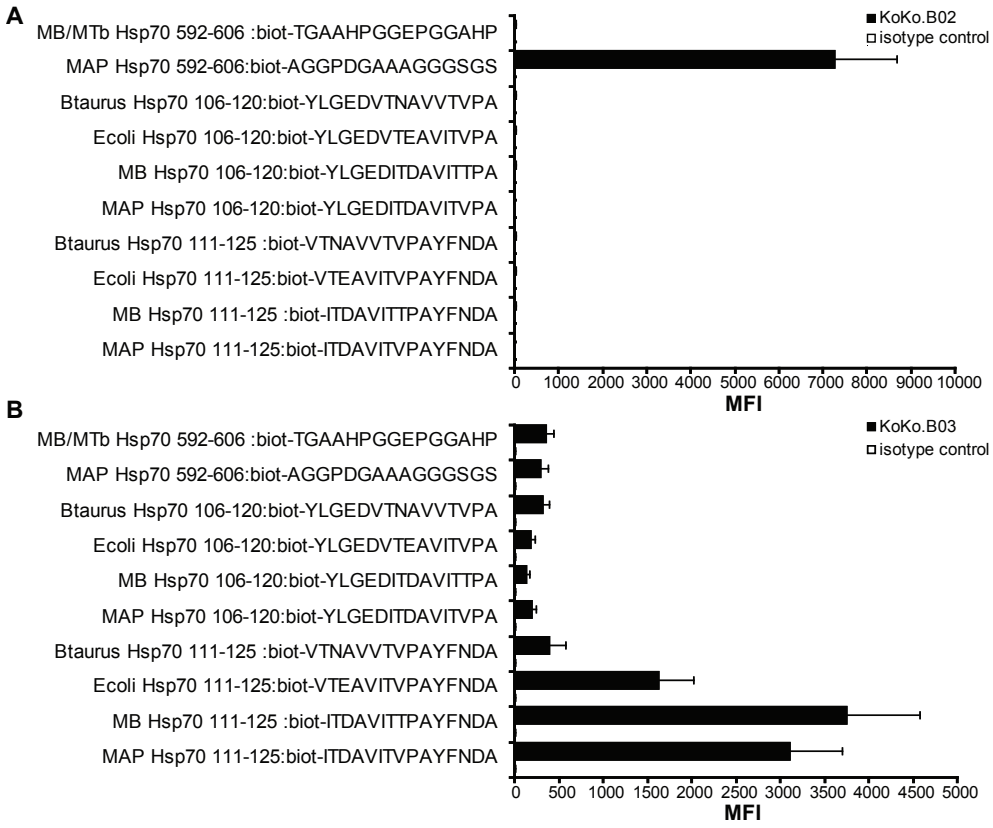

Figure 3 Specificity of KoKo.B02 and KoKo.B03 mAb's binding linear epitopes of MAP Hsp70. Specificity of (Panel A) KoKo.B02 (IgG2b) mAb and (Panel B) KoKo.B03 (IgG2a) mAb was tested on a set of 15-mer biotinylated peptides bound to avidin coated fluorescent beads (LumAv), containing relevant MAP Hsp70 peptides as well as (myco)bacterial homologues present in MB, MTb, *E. coli* and the *Bos taurus* Hsc70. Test results are presented as the mean fluorescent intensity (MFI) + SD and compared with matched isotype controls.

3.3 Recognition of linear B cell epitopes of MAP Hsp70 by cattle and goats sera

In cattle, Hsp70 specific antibody responses were detected 3 weeks post vaccination [9] (data not shown). In goats, Hsp70 specific antibody responses were detected 4 weeks post vaccination, remained stable between 4 and 12 weeks post vaccination and were not influenced by exposure to MAP (Figure 4A). The MAP Hsp70 antibody responses in unvaccinated goats remained at background levels during 12 weeks irrespective of exposure to MAP. Similar kinetics were observed using the ELISA with the RBS70 molecule (data not shown). Sera obtained at 3 (cattle) and 4 (goats) weeks post vaccination were analyzed for the presence of Hsp70 specific antibodies directed at the protein regions identified by the mouse monoclonal antibodies by incubation of the sera with 7 different Hsp70 peptides. Recognition patterns of P111-124, and 6 peptides comprising the less conserved C-terminus of Hsp70 are shown in Figure 4B. These indicated that in vaccinated goats the dominant responses are directed against the peptides P111-124, P605-618, and P610-623.

Vaccination with simultaneous exposure to MAP does not alter responses to P111-124, and P605-618. Lower responses are detected for P610-623, in MAP exposed groups as compared to those after vaccination alone. Similar differences were observed at later time points (data not shown).

In calves (Figure 4C) the dominant responses in vaccinates are directed against the peptides P111-124, P590-603, P600-613, and P610-623. Simultaneous exposure to MAP does not alter responses to P111-124; lower responses are detected to P590-603; and P600-613 is recognized preferentially by vaccinated and MAP exposed calves. Finally, P610-623 is recognized by Hsp70 vaccinated calves only. Similar data were obtained with sera from calves at later time points post vaccination (data not shown).

Vaccinated goats and calves recognized the same epitopes as KoKo.B01-03.

3.4 MAP Hsp70 linear B cell epitopes are recognized in the cell wall of MAP

Based on comparable recognition of the identified linear epitopes in Map Hsp70 by antibodies from cattle, goats and mice, and to circumvent problems associated with polyclonal sera, the mouse monoclonal antibodies (KoKo.B01-03) were used to study interactions with MAP in whole cell ELISA. Both described epitopes (P111-124 and P595-603) were recognized in the cell wall of Map. Despite high sequence similarities of MAP and MAA Hsp70 protein (99.8% similarity, the only difference being Q198H), reactions with intact MAA were significantly lower in ELISA ($p<0.001$) compared to reactions with intact MAP (Figure 5A/B). A low reaction was observed with MB.

Similar data were obtained for KoKo.B01 and KoKo.B03 using a flowcytometric approach to address the binding of antibodies to intact living mycobacteria, an example of which is shown in Figure 5C.

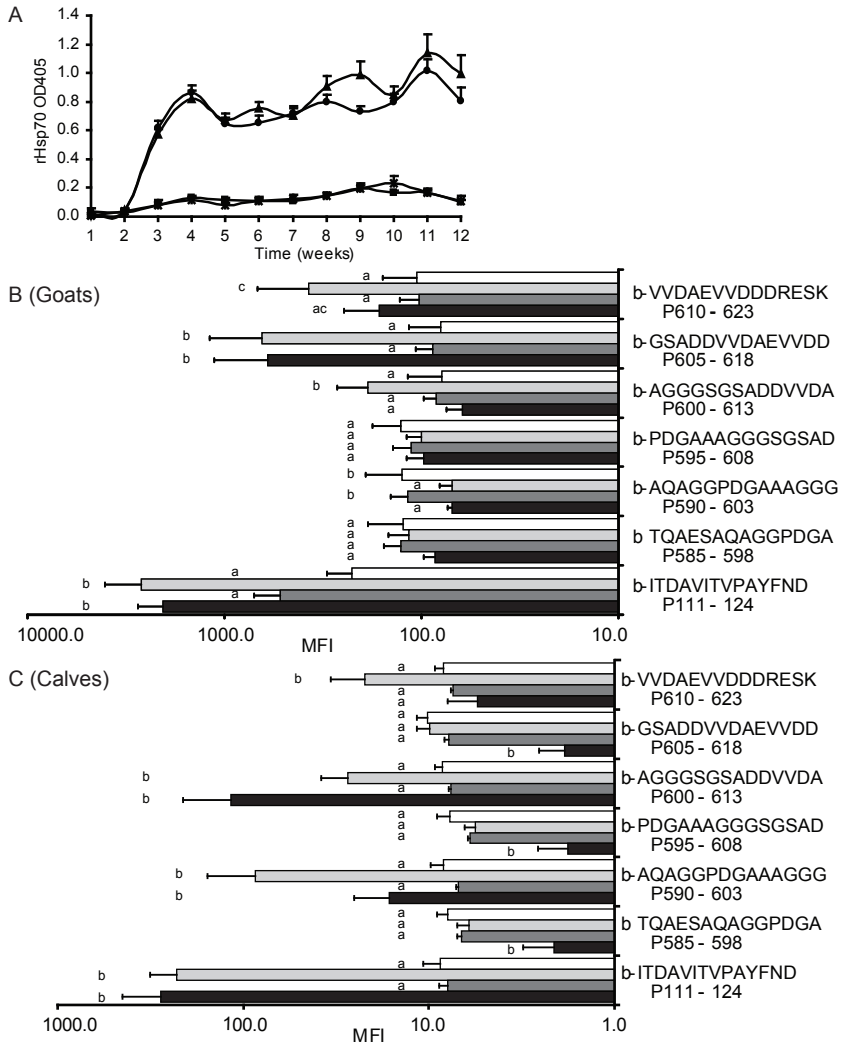

Figure 4 rMAP Hsp70 vaccinated calves and goats recognize linear B cell epitopes of MAP Hsp70. Panel A shows rMAP Hsp70-specific serum antibody responses in vaccinated goats and controls. Goats were immunized with 200g of rMAP Hsp70 in 1mL phosphate buffered saline (PBS) containing 10mg/mL DDA adjuvant (group 2 (triangle) n = 7 and group 4 (circle) n = 8) at time point week 1. Animal in group 3 (X) and group 4 (circle) were experimentally infected with MAP during the first week with 3 doses of MAP one day apart. Animals in group 1 (square) n = 7 were neither immunized nor infected. Sera were prediluted 1:20, results are expressed as the average OD405 of animals in the group + SEM. Panel B (goats) and panel C (calves) shows a multiplex Luminex analysis of serum antibody responses to a set of 14-mer biotinylated MAP Hsp70 peptides. Animals were immunized with 200g of recombinant MAP Hsp70 in 1mL phosphate buffered saline (PBS) containing 10mg/mL dimethyl dioctadecyl ammonium bromide (DDA) adjuvant (group 2 (light grey bars) and group 4 (black bars)) at time point 0. Animal in group 3 (dark grey bars) and group 4 were experimentally infected with MAP. Animals in group 1 (white bars) n = 7 were neither immunized nor infected. Sera were prediluted 1:2, results are expressed as the mean fluorescence intensity (MFI) of animals in the group + SEM. Bars related to responses to a peptide that do not share letters are significantly different at $p < 0.05$ according to ANOVA.

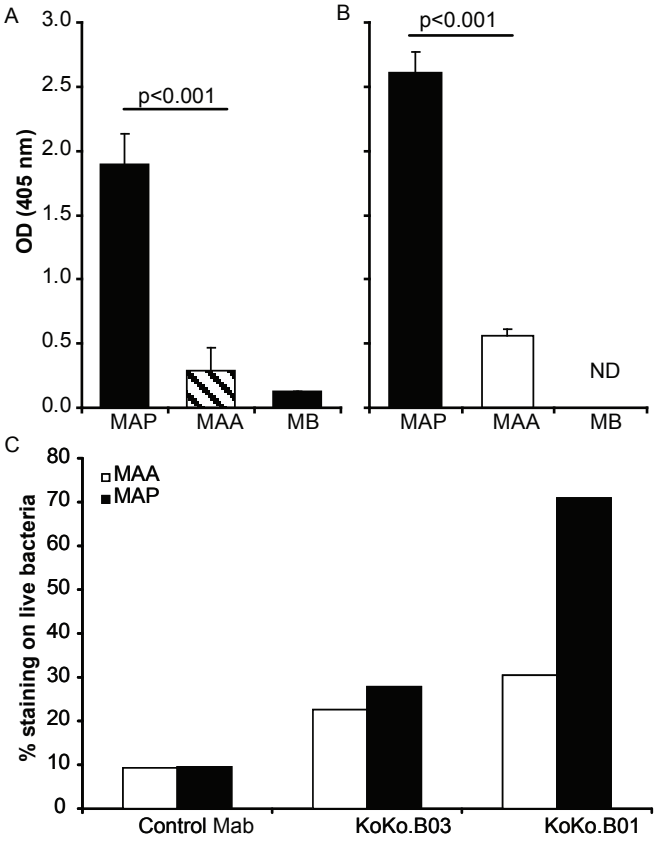

Figure 5 MAP Hsp70 linear B cell epitopes are recognized in the cell wall of MAP. Panel A, whole mycobacterial cell ELISA testing binding of antibody KoKo.Bo3 to *M. avium* spp. *paratuberculosis* (MAP), *M. avium* spp. *avium* (MAA), and *M. bovis* (MB) and panel B, testing of antibody KoKo.Bo1 (MB not determined). Results are presented as the average OD405 + SD, p-values reflect comparison of MAP and MAA, $p < 0.001$. Panel C shows flow cytometric analysis of MAP Hsp70 specific antibodies KoKo.B01 and B03 after binding to live bacteria. White bars are results obtained using MAA strain D4, solid black bars are results using MAP strain 316F. Results are expressed as the percentage of propidium iodine negative bacteria which display positive staining with the FITC conjugated Goat-anti-Mouse second step antibody. Isotype control mAb for KoKo.Bo3 (IgG2a) and KoKo.B01 (IgG1) were included to investigate background staining. For each condition staining of 10,000 live bacteria was analyzed.

3.5 MAP Hsp70 is present in lesional tissue from paratuberculosis infected animals

The KoKo.B02 and KoKo.B03 antibodies recognizing two different linear epitopes of MAP Hsp70, also recognized by sera of immunised goats and cattle, were tested for recognition of these epitopes in immunohistochemical analysis of formalin fixed, paraffin embedded bovine tissue. Both antibodies recognized the bacteria in situ in tissue sections (N=3, independent animals), indicating that the epitope, and therefore the Hsp70 protein, is expressed by MAP in intestinal lesions. Figure 6 shows immunohistochemical staining of MAP infected intestinal tissue with KoKo. B02; an isotype control antibody was used at equal concentrations and showed no staining.

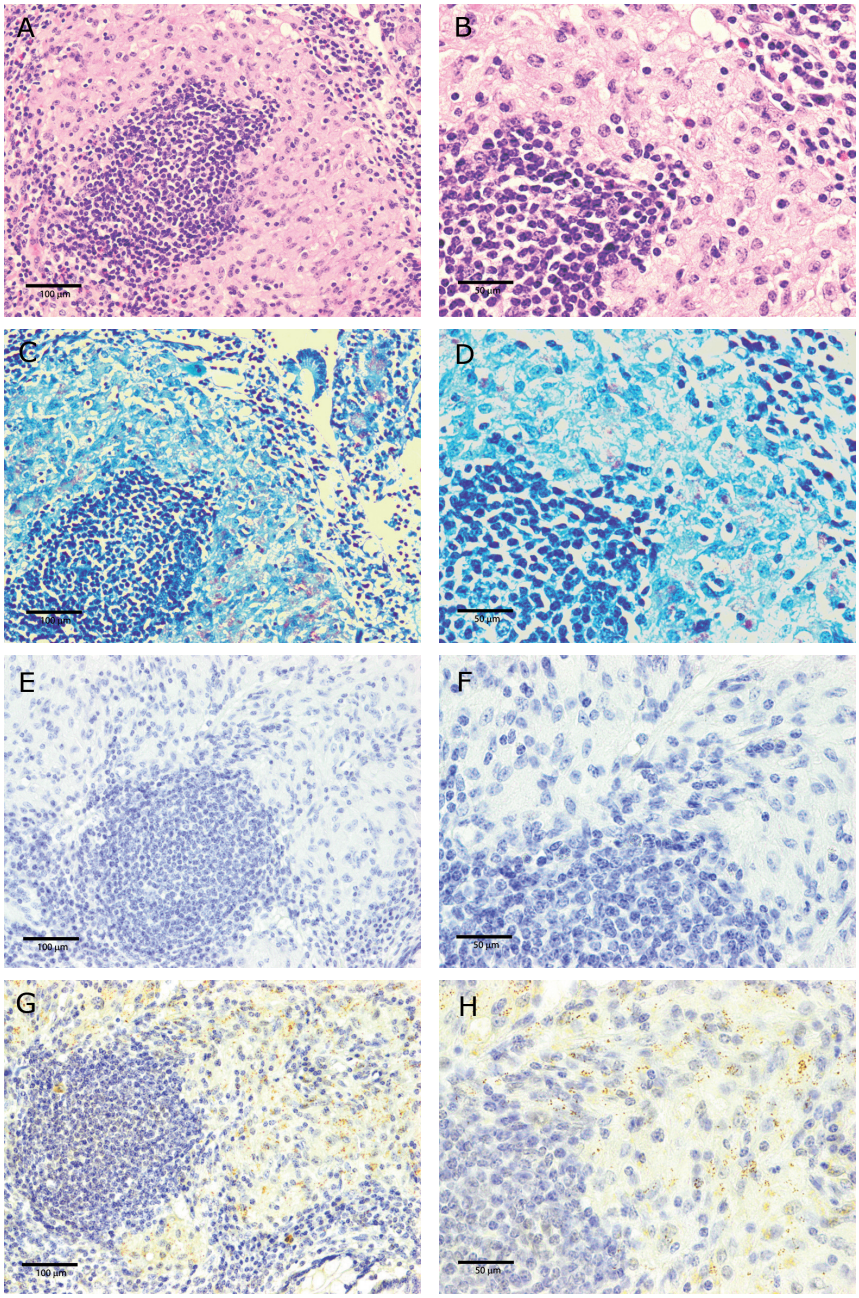

Figure 6 Staining of sequential lesional tissue sections from the ileum of a cow diagnosed with paratuberculosis by MAP Hsp70 specific monoclonal antibody KoKo.Bo2. Sequential sections of this paratuberculosis diagnosed cow, showing clinical signs, were processed using routine HE staining (panels A and B), Ziehl–Neelsen staining for acid fast bacteria (panels C and D), immunohistochemical staining with an isotype control antibody (panels E and F) and stained for MAP Hsp70 with KoKo.B02 (panels G and H). The left panels (A, C, E and G) are 200× magnifications and the right panels (B, D, F and H) are 400× magnifications of a section of the left panels.

4. Discussion

This study indicates that the Hsp70 protein is accessible to antibodies both on intact MAP bacteria in suspension as well as on MAP incorporated in lesional tissue of cows infected with MAP. The presence of the Hsp70 protein in the cell wall appears more pronounced in MAP as compared to MAA, despite high sequence similarities of MAP and MAA Hsp70 protein. In a previous study we showed that vaccination of cattle with recombinant MAP Hsp70 significantly reduced bacterial shedding [9]. This reduction coincided unexpectedly with a clear Hsp70 antibody response and a limited cell mediated response. This suggests that induction of Hsp70 antibodies could contribute to effective immune responses against Map in vivo. Similar to the smaller 16kD α-crystallin heat shock protein with respect to MTb [15], Hsp70 appears to be present in the intact cell wall of MAP, as evidenced by a recent study identifying cell wall proteins using a proteomics approach [24]. Furthermore it has been shown that local application of specific monoclonal antibodies to the 16kD α-crystallin confers protection to early stage tuberculous infection in a murine model of tuberculosis [15]. Thus, likewise, antibodies specific for Hsp70 may contribute to protective immunity in mycobacterial infections, which other studies have also indicated (reviewed in [14]).

We characterized MAP Hsp70 B cell epitopes recognized by murine monoclonal antibodies as well as sera from Hsp70 vaccinated goat and cattle. Our synthetic peptide approach resulted in definition of two linear epitopes. One of them (recognized by KoKo.B03) is located in the conserved N-terminus of the native protein, while the other (recognized by KoKo.B01 and KoKo.B02) is located in the less evolutionary conserved C-terminal region of the protein. Five more monoclonal antibodies most likely recognized conformational epitopes, of which four are located in the N-terminus of MAP Hsp70. Although we were not able to fine-map these epitopes, this finding shows that Hsp70 contains multiple targets for antibody interactions. Immunization of mice with whole-cell extracts of MAP also led to the generation of monoclonal antibodies specific for Hsp70 (MAP3840), indicating that this protein is immunogenic and abundantly present in MAP [25].

The intact protein, as well as the dominant linear epitopes were recognized by antibodies of cattle vaccinated with recombinant Hsp70 protein. Whether or not these calves were experimentally infected with MAP did not alter the antibody response to these epitopes. Similar results were obtained with goat kids. Both in goats and calves, the experimental exposure to MAP concurrent with vaccination did not substantially influence the major B cell responses to vaccination with Hsp70. In the C-terminus of MAP Hsp70 other linear epitopes were also recognized, indicating that in vaccinated calves and goats multiple targets are recognized.

For diagnostic purposes the combined use of antibodies specific for the C-terminal and N-terminal epitopes of Hsp70 offers possibilities as an alternative to Ziehl-Neelsen staining, increasing specificity for detection of mycobacteria in diagnostic specimen. The known specificity of the monoclonal antibodies KoKo.B01-03 allows differentiation between MAP/MAA Hsp70 and pathogenic MTb complex species and *M. leprae* (MLE) Hsp70. In addition, outside the genus mycobacterium, these mAb can distinguish the presence of MAP/MAA Hsp70 from Hsp70 of other prokaryotic

origin, without cross-reaction with eukaryotic (host) 70 kD heat shock proteins.

This and previous studies show that in naturally acquired paratuberculosis or experimental infection very little Hsp70 specific antibody is formed, while the Hsp70 protein does induce a cell mediated response [5,6,9]. Pathogen derived Hsp70 may be present in debris of dead mycobacteria and apoptotic bodies from infected host cells, and thus taken up and processed by antigen presenting cells. In the context of local mycobacterial infection, especially in early stages of paratuberculosis, adaptive immune responses have a Th1 signature and responses to various antigens may be skewed in this direction under these conditions [26].

In contrast however, following vaccination with MAP Hsp70 formulated with DDA adjuvant a dominant antibody response is mounted against the protein. We have recently shown that epitopes from MAP Hsp70 activate bovine T helper cells, including IFNγ producing CD4+ Th1 T cells in a MHC class II restricted manner in MAP Hsp70 vaccinated cattle [27]. However following a short measurable induction of cell mediated immunity to Hsp70, we have very little evidence of a substantial prolonged period of activation of Hsp70 specific cell mediated immunity after Hsp70/DDA vaccination [9,10,28].

In general, the (local) skewing of immune responses following infection is the result of host pathogen interaction. Since MAP infects and manipulates antigen presenting cells the adaptive response induced by infection may therefore not give rise to the optimal protective response [29,30]. Especially in paratuberculosis the Th1 directed responses in early stages of infection are easily detected [31]; however most animals do not recover from infection but become chronically infected, pointing towards insufficient protective immunity. An early adequate antibody response to surface exposed antigens, not readily induced by natural contact with intact mycobacteria, may therefore be an additional feature of protective immunity in addition to cell mediated responses as a result of Hsp70/DDA subunit vaccination.

In conclusion, this study demonstrates that at least two dominant linear B cell epitopes are present in the Hsp70 molecule. These epitopes are present in the bacterial cell wall of MAP and accessible to antibodies. It may be argued that vaccination-induced antibodies, apparently not produced during MAP infection as such, indeed bind intact bacteria and possibly alter their cellular fate following uptake by macrophages and other antigen presenting cells. Next to IFNγ producing Th1 cells, these Hsp70-specific antibodies may play a role in the protective effect shown after Hsp70/DDA subunit vaccination against bovine paratuberculosis [9].

Acknowledgements

The authors express their thanks to Bart Hoogstraten, Katalin Farkas, Mano Loeb, Ton Ultee, and Ronald Molenbeek for expert technical assistance. Furthermore the authors thank Ruurd van der Zee, Mayken Grosfeld† and Alida Noordzij for generating synthetic peptides. This study was supported by a grant from the Technology Foundation (STW) of the Dutch Research Council (NWO), grant number STW-UDG5589.

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