Hsp70 as a candidate subunit vaccine for paratuberculosis

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ISBN: 978-90-393-5581-7
Printed by Repro Drenthe College
Cover: ‘Us mem’

Hsp70 as a candidate subunit vaccine for paratuberculosis

Hsp70 als kandidaat subunit vaccin
voor paratuberculose
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties in het
openbaar te verdedigen op maandag 27 juni 2011
des middags te 2.30 uur

door

Wiebren Jehannes Santema

geboren op 26 juli 1982 te Kûbaard

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Dit proefschrift werd mogelijk gemaakt met financiële steun van Intervet/Schering-Plough Animal Health, Boxmeer, Nederland

Foar heit en mem

Foar Dieuwke, Feije en Jildau

Contents

Chapter 1

General Introduction

Introduction

Infectious diseases pose enormous threats for human and animal health. Following the discovery of microorganisms as causal agents for many diseases in the late 19th century by Robert Koch and others, knowledge on pathogenesis, treatment and prevention of infectious diseases has improved dramatically. However, in animal production systems infectious diseases still rank high in the list of important diseases. Not only do they limit economic profitability, they also have impact on animal welfare, and some are able to cause human disease (zoonosis). Several diseases can be prevented by vaccination (e.g. Food and Mouth disease, Bovine Viral Diarrhea), but for others, in particular for chronic infections, vaccine development has proven to be difficult.

This thesis focuses on vaccination-based control of bovine paratuberculosis, a chronic mycobacterial infection of the small intestine. Bovine paratuberculosis is a highly prevalent disease affecting ruminants worldwide. It is estimated that over 50% of the European Union (EU) dairy holdings is infected (Nielsen and Toft, 2009). There are concerns that the causative agent is involved in the pathogenesis of human Crohn's disease, a debilitating inflammatory bowel disease (Behr and Kapur, 2008). Therefore, dairy industries have installed national programs to limit human exposure via consumer products, like milk and cheese (Weber et al., 2008). Control programs for paratuberculosis are in need of an effective vaccine (Lu et al., 2010; van Schaik et al., 1996). At the moment there is no vaccine registered for use in cattle in the EU. This thesis evaluates a candidate subunit vaccine, consisting of recombinant Heat shock protein 70, against bovine paratuberculosis.

Paratuberculosis – causal agent

Paratuberculosis is a bacterial disease mainly affecting ruminants worldwide. The causal agent is *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a slow-growing, acid-fast bacterium. It is a member of the Mycobacterium avium complex (MAC), which also includes opportunistic pathogens of humans, as well as innocuous, environmental bacteria (Turenne et al., 2007). Growth of MAP is mycobactin dependent and requires incubation periods of up to 26 weeks before colonies can be observed on solid medium. MAP can survive in the environment for more than a year (Whittington et al., 2004). The complete genome of the reference MAP strain K-10 has been sequenced and is characterized by a relatively high GC-content (69%), and abundance of PP/PPE proteins (putative virulence factors) (Li et al., 2005). Over 4000 genes have been identified, which opens up many opportunities to study the interaction of MAP with its hosts and the environment. Molecular identification of MAP includes the identification of IS900, a specific insertion element for MAP (Vary et al., 1990). Based on genotypic and phenotypic differences, MAP strains can be divided in two major groups, designated type I and type II (Alexander et al., 2009; Stevenson et al., 2002). Type I strains have a host preference for sheep and goats. Type II strains are most commonly identified in cattle, but they have a broad host range. Within a single dairy herd multiple MAP strains can coexist, adding complexity to transmission within farms (van Hulzen et al., 2010).

Paratuberculosis – disease

Young animals, from birth until twelve months of age, are regarded most susceptible to get infected via oral uptake of MAP from a contaminated environment (Windsor and Whittington, 2009). The apparent age resistance afterwards has been linked to changes in intestinal physiology, notably the remission of Peyers patches in the small intestine as port d'entrée for MAP entry during the first year after birth.

Infected animals stay in a subclinical phase for several years, in which the primary locus of infection is mainly limited to the intestinal tissue. In this period infected animals can shed MAP via feces into the environment (Sweeney, 1996; Weber et al., 2010). Some animals progress to the clinical phase, typically at an age between 3 and 5 years, by developing a protein-loosing enteropathy, characterized by intermittent/chronic diarrhea, severe weight loss, and submandibular oedema (Sweeney, 1996). Pathological description shows a chronic granulomatous enteritis, with a thickened intestinal mucosa (Buergelt et al., 1978; Clarke, 1997). Clinical animals ultimately die of severe cachexia.

Genetic variation in susceptibility for paratuberculosis has been described in cattle (Kirkpatrick et al., 2010; Koets et al., 2010; Koets et al., 2000; van Hulzen et al., 2011). Heritability estimates in dairy cattle, ranging from 0.034 to 0.06, are low, but genetic selection of bulls for paratuberculosis resistance may contribute to paratuberculosis control. So far studies of candidate genes focus on those of importance to the interaction between MAP and host. They have identified single nucleotide polymorphisms in multiple genes, like TLR-2, NOD-2, that are associated with resistance or susceptibility to paratuberculosis (Koets et al., 2010; Pinedo et al., 2009; Ruiz-Larranaga et al., 2010).

Paratuberculosis - epidemiology

The main transmission route of paratuberculosis is via oral uptake of MAP by susceptible animals from a contaminated environment. Fecal shedders build up the infectious dose by intermittent shedding of MAP in feces, which may occur already before the age of two years (Weber et al., 2010). The quantity of MAP in feces of infected animals increases when disease progresses (Sweeney, 1996). Recently, it has become clear that MAP can survive and spread in settled dust samples (Eisenberg et al., 2010). However, the impact of transmission by dust has yet to be assessed. Vertical transmission from dam to offspring has also been described, with increasing probability if dams progress to clinical disease (Whittington and Windsor, 2009). Furthermore, studies have shown horizontal transmission between calves (Benedictus et al., 2008; van Roermund et al., 2007). This indicates that infected animals shed quantities of bacteria early in life that are capable of transmission. The consequences of the calf-to-calf transmission route can have considerable impact on paratuberculosis control, as it is common that calves are reared in groups.

Paratuberculosis - diagnosis

Diagnosis of bovine paratuberculosis can be divided in assays that detect the infectious agent or detect a MAP-specific immune response in the host. The assays that identify MAP in fecal samples, by culture or PCR-based, have high specificity (98-100%) and a stage of disease dependent sensitivity. In clinical cases the sensitivity is relatively high (around 70%), because in this phase the bacterium is frequently transmitted via the feces. For subclinical cases, the sensitivity is low (around 25%), because these animals have often an intermittent shedding pattern (Nielsen and Toft, 2008; Whitlock et al., 2000).

Alternatively, diagnosis can be performed with immuno-assays, which depend on the interaction between microorganism and host, leading to a detectable immune response. These can detect either cell-mediated responses (tuberculin skin test, or IFN-gamma release assay) or antibody responses. In bovine paratuberculosis cell-mediated immune responses precede humoral responses during the course of infection (Huda et al., 2004).

Immuno-assays need defined antigens to measure a pathogen-specific response. Crude antigen preparations, made from heat-killed cultured mycobacteria, are most frequently used. The specificity of crude antigens is limited, due to the presence of cross-reactive antigens, complicating diagnosis of bovine paratuberculosis. The specificity of antibody assays has been improved (>98%) by pre-absorption of serum/milk with an extract of an innocuous mycobacterium, *Mycobacterium phlei*, to remove cross-reactive antibodies (Bech-Nielsen et al., 1992). However, the sensitivity of the absorbed ELISA is low for detecting subclinically infected animals (around 15%), and increases if clinically affected animals are tested (around 70%) (Nielsen and Toft, 2008).

Cell-mediated assays are not routinely used for diagnosis of paratuberculosis. They require complex logistics, are labour intensive and expensive. Their performance has not been extensively tested, but there are concerns about their specificity. In animals before 15 months of age the specificity of IFN-gamma assays is compromised due to non-specific release of IFN-gamma by NK cells (Olsen et al., 2005). For cell-mediated assays it is not possible to remove cross-reactive antigens from crude antigens by pre-absorption. Therefore, to support control of paratuberculosis well defined specific antigens are called for to improve performance of both humoral and cellular diagnostic assays.

Paratuberculosis – immunology

After oral uptake MAP is able to pass the intestinal wall with a topical preference for the distal part of the small intestine, the ileum. M cells, specialized in uptake of particles, preferentially bind bacteria and transport them to the basolateral side (Momotani et al., 1988). Following release of MAP in the extracellular fluid of the lamina propria, they are taken up by macrophages, which normally process bacteria via enzymatic processes. MAP, however, is specialized in the blockage of phagosome-lysosome fusion, thereby promoting its survival (Kuehnel et al., 2001). Furthermore, they provide anti-apoptotic signals extending the life span of their host cell. MAP interacts with macrophages via different kinds of receptors,

including Toll-like receptors (TLRs) (Ferwerda et al., 2007; Koets et al., 2010). MAP is able to upregulate interleukin (IL)-10, an immunomodulatory cytokine that suppresses killing of MAP by macrophages, as well as Th1-type immune responses, which are most desired to combat intracellular infections, by inhibition of IL-12 (Weiss et al., 2005; Weiss et al., 2002).

Macrophages are important cells of the innate immune system, but also provide signals to induce adaptive immune responses. MAP is able to interfere with antigen presentation, hence subsequent adaptive responses (Weiss and Souza, 2008). Our understanding of the adaptive immune response against mycobacteria in general, and against MAP more specifically, is far from complete. The chronic nature of the infection, its primary locus of infection (the intestinal wall), and the availability of tools to dissect the immune response provide interesting challenges. Much of our understanding is gained from longitudinal responses observed in peripheral blood from infected animals, or obtained by cross-sectional analysis at necropsy. For vaccine development it is critical to find correlates of protection, defined as specific immune responsiveness to a vaccine that is closely related to protection against infection or disease (Plotkin, 2008).

MAP-infected macrophages can activate T cells via antigens presented on major histocompatibility complex (MHC) class I and II molecules, or CD1 molecules. Activated T cells will clonally expand to generate sufficient numbers for an effective immune response. Subsequently, antigen-experienced T cells will differentiate into different memory subsets, called effector memory (Tem) and central memory (Tcm) T cells. Tem are able to directly exert effector functions at the site of inflammation and are relatively short-lived. Tcm are long-lived immune cells, which can respond quickly upon secondary antigen encounter (Sallusto et al., 2010).

Depending on the interaction between MAP and immune cells, T cell responses will be polarized and differentiated into defined subsets, like the classical Th1, Th2, and more recently identified Th17 cells. Mycobacterial infection primarily leads to an immune response characterized by a Th1 type reaction pattern, of which interferon-gamma (IFN-gamma) is the key effector cytokine (Flynn and Chan, 2001; Kaufmann, 2010). IFN-gamma plays an important role in the activation of T cells and macrophages. It is produced by CD4, CD8, and γδ T cells, and natural killer (NK) cells (Bassey and Collins, 1997; Boysen and Storset, 2009; Rogers et al., 2005). Experimental studies in cattle have shown that MAP-specific proliferative T cell responses and IFN-gamma can be detected as first marker of infection in the first year after challenge (Koo et al., 2004; Stabel and Robbe-Austerman, 2011). At the primary locus of infection, the lamina propria where infected macrophages are situated, there is a constant interplay between MAP and the host immune system. In animals that progress to the clinical phase of paratuberculosis immune control is insufficient. It has been shown that the local immune response is altered by a progressive loss of a MAP-specific CD4 T cell population towards an increased proportion of potentially regulatory/anti-inflammatory γδ T cells (Hoek et al., 2009; Koets et al., 2002). The chronic antigenic stimulation at the site of infection can lead to induction of regulatory T cells, which limit the pro-inflammatory response initiated by Th1 T cells (Coussens, 2004; de Almeida et al., 2008).

The B cells respond to their antigen via triggering of the B cell receptor, often in orchestration with T cell help from CD4 T cells in secondary lymphoid tissues (lymph node and spleen). Subsequent proliferation and differentiation gives rise to memory B cells and plasma cells, producing antibodies. Little is known about the development of the B cell response in the course of paratuberculosis. The consequence of the B cell response, indicated by serum antibodies against MAP, is used in diagnostic assays. They can be measured reliably from 2 years of age with the current diagnostic assays. Most of these diagnostic assays measure antibodies directed against crude antigens, like tuberculins. Tuberculins are complex mixtures of soluble protein antigens derived from mycobacteria grown in vitro. The lack of measurable antibody responses may be due to pathogenic mechanisms directing the host pathogen interaction away from antibody-mediated immune responses in the early phases of infection. An alternative explanation may be that antibodies are formed in the early phases of infection. However, the target antigens are not present or scarce in the tuberculin protein mixtures. A recent study by Bannantine et al. indicated that a transient antibody response can be detected at 70 days post-infection against some defined recombinant MAP antigens (notably MAP0862) (Bannantine et al., 2008).

Our understanding of the immune response directed at MAP antigens present at different stages of infection, comparable to e.g. latency antigens in tuberculosis (Lin and Ottenhoff, 2008), will be boosted by increasing availability of recombinant antigens and multiplex antibody detection technologies.

Paratuberculosis – vaccine

The development of paratuberculosis vaccines started in the 1920s with the experiments of Valleé and Rinjard using live MAP. Follow-up studies with whole-cell based vaccines, either live attenuated or inactivated, have collectively shown that occurrence of clinical symptoms and tissue colonization can be reduced, but infection is not prevented in cattle (Larsen et al., 1974; Larsen et al., 1978; Sweeney et al., 2009; Uzonna et al., 2003). Field studies in the Netherlands showed that the rate of infection did not differ between vaccinated and non-vaccinated dairy herds (Kalis et al., 2001; Wentink et al., 1994). One of the major drawbacks of the use of whole-cell based vaccines is the interference with diagnosis of bovine tuberculosis and paratuberculosis (Kohler et al., 2001; Muskens et al., 2002). It is anticipated that regulatory authorities will only license new paratuberculosis vaccines which will not interfere with bovine tuberculosis diagnosis. There are also safety issues regarding the application of an inactivated vaccine, as self-inoculation in humans can cause severe tissue damage for prolonged periods of time (Patterson et al., 1988; Windsor et al., 2005)

One lead for development of new paratuberculosis vaccines is directed at the use of subunits, mostly proteins, potentiated with adjuvants. Several Th1 antigens have been identified by genomic and proteomic analysis: the antigen 85 complex proteins (A, B, and C) and superoxide dismutase (Shin et al., 2005); Hsp65 and Hsp70 (Koets et al., 1999); lipoprotein LprG ((Rigden et al., 2006); PPE family proteins MAP1518 and MAP3184 (Nagata et al., 2005). Only few of these have been tested in cattle, the target species, namely: the antigen 85 complex proteins

together with superoxide dismutase, and heat shock protein 70 (Kathaperumal et al., 2008; Koets et al., 2006).

Heat shock proteins (Hsp) are a family of proteins that are responsible for crucial housekeeping functions, and are molecular chaperones, important for the survival of prokaryotic and eukaryotic cells. Hsp families, grouped based on their molecular weights, are highly conserved throughout evolution. Some microbial Hsp family members have highly conserved mammalian homologues, which results in immunological cross-recognition (Young, 1990). Their role in immune regulation of chronic inflammatory diseases, such as arthritis, is well studied (van Eden et al., 2005).

Despite the high homology between microbial and mammalian Hsp, they are immunodominant antigens in mycobacterial diseases. Several Hsp have been studied in bovine paratuberculosis (Koets et al., 2002; Koets et al., 2001; Koets et al., 1999). For Hsp65 it was shown that it induced antibody production in paratuberculosis infected animals, and limited T cell responses. Hsp70, however, induced mainly T cell proliferation in infected animals, and limited antibodies. Based on the dogma that cell-mediated immune responses are most important against intracellular infections, Hsp70 was selected as candidate vaccine antigen. In a vaccination-challenge experiment in calves immunization with Hsp70, adjuvanted with DDA (dimethyl dioctadecyl ammonium bromide), significantly reduced fecal shedding of MAP during the observation period of two years (Koets et al., 2006). These promising results warranted further studies into the development of an Hsp70 subunit vaccine for paratuberculosis.

Objective and outline of this thesis

At this moment no vaccines against paratuberculosis are registered for use in cattle in the EU. The overall objective of this thesis was to investigate the potential of a candidate paratuberculosis vaccine, consisting of recombinant MAP Hsp70 in DDA as an adjuvant.

An important prerequisite for the registration of a new paratuberculosis vaccine is lack of interference with current immunodiagnostic assays for detection of bovine tuberculosis. In chapter 2 the potential interference of vaccination with Hsp70 on the outcome of the tuberculin skin test and interferon-gamma release assay (Bovigam) was evaluated in tuberculosis-free cattle. Furthermore, the interference of vaccination with antibody-based diagnostic assays for paratuberculosis was assessed.

Immunodiagnostic tests, like the skin test, Bovigam and antibody-ELISA, use crude antigen preparations, like tuberculin (PPD). Based on the close genetic relationship of mycobacteria, these crude preparations contain cross-reactive antigens limiting the specificity of immuno-assays. In chapter 3 the protein composition of tuberculins from *Mycobacterium bovis*, *Mycobacterium avium* subspecies *avium*, and *Mycobacterium avium* subspecies *paratuberculosis* was analyzed by proteomics techniques. The proteomes identified were compared to define overlapping proteins and species-specific proteins, with the goal to improve paratuberculosis diagnosis.

Previously it was shown that the dominant immune response after Hsp70/DDA vaccination in cattle was the generation of Hsp70-specific antibodies. To further elucidate aspects of the immune response generated by this vaccine, and to search for correlates of protection, details of the antibody response generated after Hsp70 vaccination were analyzed in chapter 4. Primary immune responses after Hsp70 vaccination at the level of the draining lymph node were studied by efferent lymph vessel cannulation techniques in chapter 5.

For mycobacterial diseases, transmission of infection through early exposure to pathogens is an important obstacle for classical preventive vaccination. This makes this group of pathogens particularly in need of vaccines that work in already exposed individuals. In chapter 6 efficacy of post-exposure Hsp70 vaccination in chronically, naturally paratuberculosis infected cattle was evaluated.

A summarizing discussion is presented in chapter 7.

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Chapter 2

Heat shock protein 70 subunit vaccination against bovine paratuberculosis does not interfere with current immunodiagnostic assays for bovine tuberculosis

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Vaccine 27 (2009) 2312–2319

Abstract

Paratuberculosis or Johne's disease in ruminants is an infectious disease of the small intestine caused by *Mycobacterium avium* spp. *paratuberculosis*, and a global problem of the livestock industry. No therapy is available and the use of a whole bacterin vaccine is limited due to interference with tuberculosis diagnostics.

In earlier studies we showed the protective effect of Hsp70/DDA subunit vaccination against paratuberculosis. In the current study, potential interference of this vaccination with immunodiagnostic procedures to detect bovine tuberculosis and paratuberculosis was studied.

Vaccination of cattle with an Hsp70/DDA subunit vaccine has little side-effects, and does not interfere with current tuberculosis immunodiagnostic assays. Serological assays for paratuberculosis diagnostics can be adapted by inclusion of an Hsp70 pre-absorption step that enables differentiation between vaccinated and infected animals.

In conclusion, the Hsp70/DDA subunit vaccine may contribute to paratuberculosis control strategies, without compromising diagnosis of bovine tuberculosis or paratuberculosis.

1. Introduction

Paratuberculosis or Johne's disease (JD) in ruminants is an infectious disease of the small intestine and a global problem of the livestock industry. The disease is caused by *Mycobacterium avium* spp. *paratuberculosis* (MAP) and leads to substantial economic losses [1]. Young calves acquire the infection in the first months of life through oral uptake of colostrum, milk or feces of infected cows [7]. They either successfully clear the infection, or become subclinically infected for life. The infected animals shed the bacteria in their feces and milk intermittently or continuously from an age of approximately 2 years onwards [8, 9]. After an incubation period of 4 to 5 years, a proportion of the infected animals develops an incurable, progressive form of protein losing enteropathy with chronic diarrhea resulting in death [7, 10, 11]. The presence of the bacteria in the environment and (dairy) food products constitutes a mode of transmission of MAP to humans. As it has also been suggested that MAP is involved in the aetiology of Crohn's disease in humans, both animal and human health aspects justify further research into the immunopathogenesis of paratuberculosis aiming at control (if not eradication) of the disease [3-6].

Disease control and ultimately eradication has been attempted through various strategies such as test and cull, in which infected animals are removed from the herd, calf management, aimed at preventing infection of the most susceptible animals, and to a lesser extent vaccination. As transmission of MAP does not only occur through the oral-fecal route, but also through intrauterine transmission [12-14], some of the preventive measures can not be completely effective in young calves. Integrated approaches including both early detection and vaccination are called for, mostly because no single control method has perfect characteristics. In addition, several modelling studies have shown that, while long-term test and cull strategies are less favourable economically, management strategies and vaccination are economically attractive tools [15].

Several vaccination studies have demonstrated that vaccination of calves in the first month of life with a whole cell vaccine (inactivated) prevents the development of the clinical stage of the disease, and thus reduces economical damage [16]. However, this type of vaccination does not result in elimination of mycobacteria, since subclinically infected animals were detected in approximately the same frequency in vaccinated and non-vaccinated herds [17]. In addition this vaccination strategy interferes with bovine tuberculosis diagnostics [18]. As a consequence, the use of inactivated whole cell vaccines in cattle is limited or even prohibited (e.g. in the Netherlands). Finally, the inactivated whole cell vaccine causes substantial tissue damage at the vaccination site, and misuse of the vaccine in cattle as well as accidental self-inoculation, e.g. by veterinarians, may have serious side-effects [19]. From epidemiological, animal health and human health points of view, these serious drawbacks currently limit the use of vaccination of cattle worldwide, and constitute a major problem in eradication of paratuberculosis [17, 20-24]. Subunit vaccination with one or more well-defined antigens could circumvent some of the disadvantages of the inactivated whole cell vaccine, i.e. it could prevent interference with existing diagnostic approaches for paratuberculosis and tuberculosis, especially when combined with a diagnostic

system lacking the vaccine components. In addition, omitting the use of whole mycobacterial cells in oil adjuvant might reduce extensive tissue damage.

Analysis of the immune responses to recombinant MAP Hsp70 of cattle in various stages of bovine paratuberculosis infection showed that the MAP Hsp70 protein is an antigen inducing predominantly cell-mediated responses [27, 30, 31]. In a recent vaccination-MAP challenge experiment we have used a subunit vaccine composed of the recombinant MAP Hsp70 protein and dimethyl dioctadecyl ammonium bromide (DDA) adjuvant. In this vaccination-challenge study we have shown that immunization with Hsp70 protein prior to experimental infection leads to significant reduction of MAP shedding in feces [32].

An important issue of using evolutionary conserved antigens as vaccine candidates is the possibility that it may interfere with diagnostic procedures for related pathogens. Thus, one important practical consideration for application of a MAP Hsp70 subunit vaccine against bovine paratuberculosis is the potential interference of the subunit vaccine with existing immunodiagnostic procedures for bovine tuberculosis and paratuberculosis.

The aim of the current study was to evaluate potential interference of Hsp70/DDA subunit vaccination with diagnostic procedures for bovine tuberculosis. In addition, influence of the vaccination on current immunodiagnostic tests for paratuberculosis was studied.

2. Materials and methods

2.1 Animals

Thirty healthy, primiparous cows (average age 2.1 year (range 1.8 - 2.4 years)) were used in this study. Fourteen cows were Holstein-Friesian purebred, the other 16 Holstein-Friesian crossbred. The animals were obtained from 10 different tuberculosis-free herds in the Netherlands. They were checked daily for general health and side-effects after vaccination were recorded.

2.2 Ethics

The use of animals in the present study was approved of by the experimental animal committee of Intervet/Schering-Plough Animal Health and conducted according to existing regulations.

2.3 Antigens

Recombinant MAP Hsp70 was produced according to methods described in detail earlier [30]. Purity of the recombinant Hsp70 was checked using SDS-PAGE. For use in in vitro assays Hsp70 preparation was detoxified by polymyxine B chromatography and tested for LPS contamination by Limulus assay (Sigma, St. Louis, USA). The endotoxin content was below the detection limit of the test (<0.125 EU/ml).

Purified protein derivative, prepared from *M. bovis* strain AN5 (PPDB) and *M. a.* spp. *avium* strain D4 (PPDA), according to the OIE manual [33] by the Central Veterinary Institute (Lelystad, the Netherlands), was used for skin testing. The tuberculins used in the Bovigam assay are provided with the kit by the manufacturer. The antigen used in the Pourquier absorbed ELISA has been described as a protoplasmic extract of MAP by the supplier.

2.4 Experimental design

At the start of the experiment (day (D) 0), all animals were tested with the single intradermal comparative cervical tuberculin test (described in Section 2.6). Subsequently, the animals were stratified by age, breed, initial tuberculinisation reaction and in vitro lymphoproliferative reaction to bovine tuberculin (data not shown), and assigned to one of five experimental groups of six animals each.

Control cattle were sham-immunized (G1, non-vaccinated), or immunized in the dewlap with an inactivated whole cell vaccine (Gudair™, CZV, Porriño, Pontevedra, Spain) against paratuberculosis (G5, Gudair MAP) at D84.

Recombinant MAP Hsp70 protein vaccine was formulated as published previously [32]. Immunization consisted of administration of Hsp70/DDA, 200 µg 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 neck. Group 2 animals were subcutaneously vaccinated at D84 (G2 Hsp70 1x s.c.). Group 3 animals were vaccinated subcutaneously at D56, followed by a booster vaccination at D84 (G3 Hsp70 2x s.c.). Group 4 animals were vaccinated intramuscularly at D56, followed by a booster vaccination at D84 (G4 Hsp70 2x i.m.). G2-G4 received an additional booster vaccination with Hsp70/DDA at D189 following the same routes as with the initial vaccination.

The five groups were tested 8 weeks after the first vaccination (D140), and 7 weeks after the Hsp70/DDA boost vaccination (D238) using the single intradermal comparative cervical tuberculin test.

Heparinized blood was collected aseptically from the tail vein of all animals every 4 weeks throughout the experiment, starting at D0 prior to initial tuberculinisation. Additional samples were taken at 7, 14 and 21 days after each tuberculinisation. Serum samples were taken at the same time points and tested for antibody production in response to vaccination and tuberculinisations.

2.5 Diagnosis of paratuberculosis infection

Diagnosis of paratuberculosis infection was performed by fecal culture in the automated TREK-DS paraJEM ESP culture system at the Veterinary Health Service laboratories (Deventer, the Netherlands). Bacterial growth was confirmed to be MAP based by PCR for the specific IS900 insertion sequence [34]. Feces from all animals were cultured once at the end of the experiment.

In addition, serodiagnosis of paratuberculosis was performed for all animals at days 0, 21, 56, 147, 238 and 266 of the experiment by the Veterinary Health Service laboratories (Deventer, the Netherlands) using the Pourquier absorbed ELISA, according to instructions of the manufacturer. The results of the test are calculated using the S/P ratio defined as [OD450 (test sample) - OD450 (negative control serum)]/[OD450 (positive control serum) - OD450 (negative control serum)] x 100. The interpretation of the test was previously modified based on national prevalence studies to the effect that S/P values < 90 were considered negative, S/P values between 90 and 110 were classified dubious, and S/P values > 110 were considered positive.

In a separate experiment, using sera of the animals at D217 of the experiment (4 weeks after the booster vaccination), a second modification to the Pourquier absorbed ELISA was introduced consisting of addition of Hsp70 protein (0, 40 and 80 µg/ml) to the pre-absorption solution provided with the kit, to absorb Hsp70 specific antibodies from the serum.

2.6 Comparative tuberculin skin test

A single intradermal comparative cervical tuberculin test was conducted according to European regulations (EU directive 64/432/EEC) at D0, D140 and D238. The latter two 8 and 7 weeks post-vaccination, respectively. In short, 0.1 ml bovine tuberculin (2000 IU) and 0.1 ml avian tuberculin (2000 IU) were injected intradermally in the neck of each animal. At 72 hours post-injection the skin-fold thickness was measured and corrected for skin-fold thickness measured at time of application.

Reactions to each of the tuberculins were interpreted as follows: a skin test reaction is considered positive when skin thickness increased 4 mm or more, inconclusive when there is an increase of more than 2 mm and less than 4 mm, and negative when the increase is not more than 2 mm. According to the guideline the official interpretation of the intradermal comparative cervical tuberculin skin test is as follows: positive when the positive reaction to bovine PPD is 4 mm larger than the reaction to avian PPD, inconclusive when a positive reaction to bovine PPD was

between 1 and 4 mm greater than the reaction to avian PPD, and negative when there is a negative reaction to bovine PPD, or when a positive or inconclusive reaction to bovine PPD is equal to or less than a positive or inconclusive reaction to avian PPD.

2.7 Hsp70 specific serology

Serological responses to recombinant MAP Hsp70 protein were measured using an ELISA technique as described previously [30] with modifications. Sera were diluted 20 times in blocking buffer and 100 µl samples were measured in duplicate. In addition, in each plate a positive and a negative control sample were added in duplicate. The modifications compared to the original ELISA consisted of the use of biotinylated mouse-anti-bovine IgG (detecting bovine IgG1 and IgG2) (Sigma-Aldrich, USA), followed by three washes. Subsequently peroxidase conjugated streptavidin was used (Dako, Denmark). Following three more washes 100 µl ABTS substrate buffer (Roche Diagnostics, the Netherlands) was used to develop a color reaction which was read in an ELISA reader (Biorad, USA) at 405 nm. Additionally the ELISA assay was run to measure isotype specific antibody responses as described previously [35]. All sera were diluted 20 times in blocking buffer and 100 µl was measured in duplicate. In addition, in each plate a positive and a negative control sample were added in duplicate. Isotype specific secondary antibodies (1 µg/ml of mouse-anti-bovine IgG1, IgG2, IgA and IgM (Cedi-Diagnostics, Lelystad, the Netherlands)) were used followed by 3 washes and incubation with 1 µg/ml peroxidase conjugated polyclonal goat-anti-mouse antibody (Nordic Laboratories, the Netherlands). Finally plates were washed 3 times and 100 µl ABTS substrate buffer (Roche Diagnostics) was used to develop a color reaction which was read in an ELISA reader (Biorad) at 405 nm. ELISA results were expressed as S/P (sample to positive) ratio.

2.8 Antigen specific INF-γ ELISA

Antigen specific INF-γ responses were measured using the whole blood culture Bovigam® assay (Prionics AG, Zurich, Switzerland) according to instructions provided by the manufacturers. In brief, 1.5 ml heparinised whole blood was incubated with bovine and avian tuberculin antigens in a 24 well tissue culture plate for 24 hours in a humidified incubator at 37°C. Nil antigen (PBS) was used to determine spontaneous release of INF-γ in the blood culture. Subsequently the supernatant plasma was collected and stored at -20°C until analysis. In addition, recombinant MAP Hsp70 was used as an antigen at a final concentration of 20 *g/ml. The production of bovine INF-γ was measured using a monoclonal antibody-based sandwich enzyme immunoassay. Results were interpreted according to instructions provided, in addition all results were also expressed as S/P ratio calculated as OD450 (antigen stimulated plasma)/ OD450 (positive control plasma). Positive control plasma is included in the kit.

2.9 Statistical analysis

Data were analyzed with linear mixed effects models or ANOVA using SPSS statistical software (Version 15). A Bonferroni post-hoc test was used to correct for multiple comparisons where applicable. The level of significance was set at $p<0.05$.

3. Results

3.1 Observations on general health status

Based on clinical observations the animals remained healthy during the experiment with the exception of one animal (from group G3) that was culled following a severe lameness that was non-responsive to treatment.

3.2 Side-effects of vaccination

The effect of the multiple vaccinations with Hsp70/DDA subcutaneously in the neck region was a palpable swelling with an approximate diameter of 2-3 cm at 1 week post-immunization. In the majority of cases the swelling was painless and resolved to a small, apparently inert, nodule of less than 1 cm diameter in the course of 3 weeks. Intramuscular vaccinations did not result in a palpable swelling at the site of injection. No generalized effects on animal health of the Hsp70/DDA vaccinations were observed.

Single vaccination with the whole cell Gudair vaccine in the dewlap resulted in an approximate average swelling of 7 cm diameter at 1 week post-immunization. This swelling persisted throughout the experiment.

3.3 Hsp70 specific antibody responses

Serum antibody responses to Hsp70 were readily induced by vaccination with the Hsp70/DDA subunit vaccine as well as with the whole cell MAP vaccine (Gudair). In figure 1 the average IgG responses (S/P ratio) to vaccination are depicted. Three time points prior to vaccination (D0, D21 and D56) were studied. The first tuberculinisation (D0) did not induce Hsp70 specific antibody responses at 3 weeks (D21) and 8 weeks (D56) following the skin test. The three time points post-vaccination showed a clear Hsp70 specific antibody response. Boost vaccination 28 days after the primary vaccination (groups 3 and 4) did not induce a significant increase in antibody responses (data not shown). Route of vaccination (s.c. or i.m.) did not influence antibody titre or isotype distribution of the antibody response (data not shown). ANOVA results showed significant differences pre- and post-immunization time points for all vaccinated groups (G2-G5) ($p<0.05$); no significant differences within groups were observed comparing the 3 time points pre- or post-immunization; no significant differences were observed between different vaccination regimes.

3.4 Diagnosis of paratuberculosis

At days 0, 21, 56, 147, 238 and 266 of the experiment the paratuberculosis seroprevalence was determined using a commercially available absorbed ELISA. Results, as depicted in table 1, indicated that at D0 all animals were seronegative. At D21, 3 weeks after the first skin test, 2 animals tested dubious and one animal tested positive. At D56 all animals tested negative again. At D266, at the end of the experiment, one seropositive animal was present in G1 (non-vaccinated). In the groups treated s.c. (G2 and G3) 3 out of 6 and 3 out of 5 animals tested positive respectively, in addition in both groups 1 animal tested dubious. In the group G4 (Hsp70 2x i.m.) 1 dubious and 1 seropositive animal was found. Animals in G5 (Gudair MAP), vaccinated with the whole cell vaccine, were all seropositive at D266.

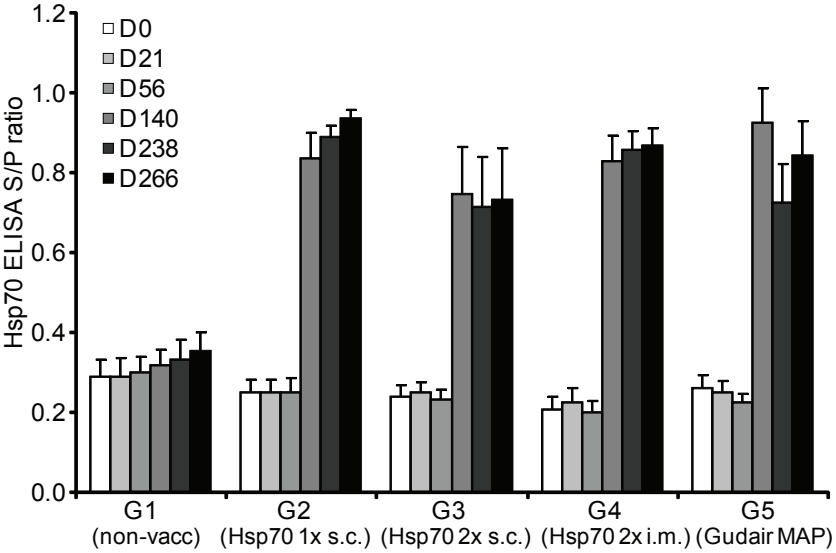

Figure 1 Hsp70 specific IgG antibody responses were measured in ELISA in sera of non-vaccinated (G1), Hsp70 1× s.c. (G2), Hsp70 2× s.c. (G3), Hsp70 2× i.m. (G4) and Gudair MAP (G5) vaccinated animals. Serum was diluted 20 times. Results are expressed as sample to positive (S/P) ratio. Responses are summarized per group (average S/P ratio of N= 6 animals per group + SEM) on days (D) 0, 21, 56, 140, 238 and 266 of the experiment. Immunization regimes started on D56. ANOVA results showed significant difference pre- and post-immunization time points for all vaccinated groups (G2–G5) ($p < 0.05$); no significant differences within groups were observed comparing the 3 time points pre- or post-immunization; no significant differences were observed between different vaccination regimes.

Table 1 Antibody responses were measured in the Pourquier absorbed ELISA in sera of non-vaccinated (G1), Hsp70 1× s.c. (G2), Hsp70 2× s.c. (G3), Hsp70 2× i.m. (G4) and Gudair MAP (G5) vaccinated animals. Responses are summarized per group (N= 6 animals per group) on days (D) 0, 21, 56, 147, 238 and 260 of the experiment. Immunization regimes started on D56. Responses with a S/P ratio below 90 are designated as negative, between 90 and 110 as dubious (±), and higher than 110 as positive (+) in the diagnostic assay. Analysis of variance using the S/P ratio data of the assay indicated that at days 0, 21, and 56 there were no significant differences between the groups. At post-vaccination time points G5 (Gudair MAP) S/P ratio's were significantly different from all other groups ($p < 0.005$, for all comparisons). The other groups did not differ significantly from each other.

Group	Animals	Pre-vaccination						Post-vaccination					
		D0		D21		D56		D147		D238		D266	
		+	±	+	±	+	±	+	±	+	±	+	±
G1 (non vaccinated)	6	0	0	0	0	0	0	0	0	1	0	1	0
G2 (Hsp70 1x s.c.)	6	0	0	0	0	0	0	1	0	4	0	3	1
G3 (Hsp70 2x s.c.)	5[a]	0	0	0	1	0	0	1	1	3	1	3	1
G4 (Hsp70 2x i.m.)	6	0	0	0	1	0	0	3	0	1	1	1	1
G5 (Gudair MAP)	6	0	0	1	0	0	0	6	0	6	0	6	0

[a] In G3 one animal was culled and excluded from analysis.

Analysis of variance using the S/P ratio data of the assay indicated that at days 0, 21, and 56 there were no significant differences between the groups. At D266 G5 (Gudair MAP) S/P ratio's were significantly different from all other groups ($p<0.005$, for all comparisons). The other groups did not differ significantly from each other.

To test if the trend towards a higher number of Pourquier absorbed ELISA seroconverters in the Hsp70/DDA vaccinated groups 2, 3 and 4 was induced by the vaccination procedure, additional serum pre-absorption with Hsp70 was performed. As is shown in figure 2, pre-absortion of the D217 sera with Hsp70 removes reactivity from the sera of Hsp70 vaccinated animals. The one animal that seroconverted in the non-vaccinated group (G1) remained positive in the Pourquier ELISA regardless of the addition of Hsp70 to the pre-absorption step (data not shown). The effect on the sera of whole cell vaccinated animals (G5 Gudair MAP) was limited, with an average drop in S/P ratio from 263 in unabsorbed sera to 257 in the 40 µg/ml Hsp70 pre-absorption, to 228 in the 80 µg/ml Hsp70 pre-absorption.

The animals were all fecal culture negative for MAP at the end of the experiment.

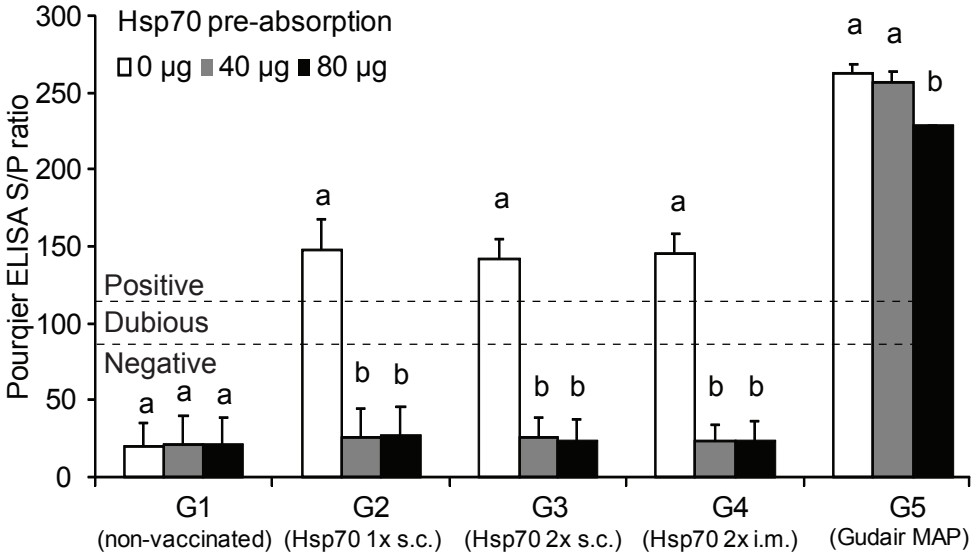

Figure 2 Antibody responses were measured in the Pourquier absorbed ELISA in sera of non-vaccinated (G1), Hsp70 1× s.c. (G2), Hsp70 2× s.c. (G3), Hsp70 2× i.m. (G4) and Gudair MAP (G5) vaccinated animals. Results are expressed as sample to positive (S/P) ratio; responses below 90 are designated as negative, between 90 and 110 as dubious, and higher than 110 as positive in the diagnostic assay. Responses are summarized per group (average S/P ratio of N= 6 animals per group + SEM) using sera from D217. Open bars show results from the unmodified absorbed ELISA, grey bars and black bar show results when 40 µg/ml Hsp70 and 80 µg/ml Hsp70was used in the *M. phlei* pre-absorption step, respectively. Different letters indicate statistical differences ($p < 0.05$) between bars within groups.

3.5 Diagnosis of tuberculosis

3.5.1 Tuberculin skin testing

As depicted in figure 3, two animals (1 from G1 and 1 from G5) reacted inconclusive to PPDA (with 3 mm skin thickness increase) at the beginning of the experiment (D0). None of the animals reacted positive in the comparative skin test.

The second skin test (D140) was conducted 8 weeks following the vaccinations in G2-G5 (D84). At this point animals from G5 (Gudair MAP) had significantly higher increase of skin thickness compared to the other groups ($p<0.05$) for both tuberculins, and all animals of G5 had skin reactions of > 8 mm. Test results in the other groups (G1-G4) did not differ significantly from each other. These groups had 1-3 reactors with skin thickness increase of 2-9 mm to PPDA and PPDB. One animal from G2 (Hsp70 1x s.c.) had a positive comparative skin test at this time point.

The third skin test (D238) was conducted 7 weeks following the Hsp70/DDA booster vaccinations (D189) in groups G2, G3 and G4. In G5 (Gudair MAP) all animals had positive skin reactions to PPDA (4-27 mm skin thickness increase), 5 out of 6 animals reacted positive to PPDB (4-15 mm skin thickness increase) and the remaining animal reacted inconclusive to PPDB. One animal also had a positive comparative skin test. As a group, G5 reactions were significantly different from those of the 4 other treatment groups. The remaining groups had 1-4 reactors with skin thickness increase of 3-15 mm for PPDA and 1-2 reactors with skin thickness increase of 3-8 mm for PPDB; none of these animals had a positive comparative skin test. Responses between G1-G4 did not differ significantly for both tuberculins.

3.5.2 Bovigam IFN-γ ELISA

Bovigam IFN-γ ELISA was used to study whether the vaccination strategies compromised the diagnostic outcome of the Bovigam test, used to identify M. bovis infected animals. In table 2 the results are presented as the number of animals per group that have a positive test outcome, when applying the standard interpretation of the test (OD PPDB–OD PPDA> 0.100). After the first skin test (D0) there were reactors in all groups, except G3. Post-vaccination reactors were found in all groups. However, the number of Hsp70 vaccinated animals that reacted positive was always lower or equal to the number of reactors in MAP vaccinated animals (G5). When excluding animals that reacted prior to vaccination, analysis showed that at D140 reactors were only found in G5 (Gudair MAP), where 3 out of 5 remaining animals were positive. At D238 (182 days following the single Gudair vaccination) 2 animals still reacted positive. In contrast, Hsp70 vaccinated animals did not become positive in the Bovigam assay when pre-vaccination reactors were excluded at D140. At D238, when animals in G2-G4 had been vaccinated up to 3 times, 1 cow in G3 and 1 cow in G4 reacted positive.

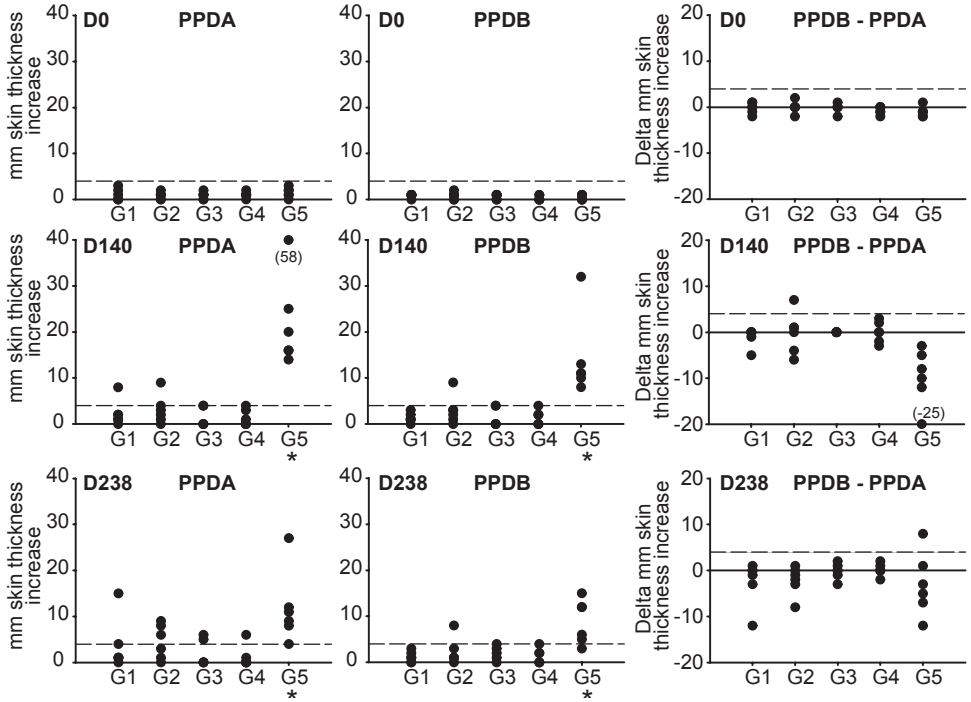

Figure 3 Increase in skin thickness after the intradermal comparative cervical tuberculin test at days (D) 0, 140 and 238 in non-vaccinated (G1), Hsp70 1× s.c. (G2), Hsp70 2× s.c. (G3), Hsp70 2× i.m. (G4) and Gudair MAP (G5) vaccinated animals is shown. Results are expressed as mm skin thickness increase for each tuberculin and mm size differentials (PPDB–PPDA) over a 72 h period and depicted for all individual animals grouped by treatment. In each plot a horizontal reference line is included at 4 mm to show the threshold above which an animal has a positive reaction. G5 responses to PPDA and PPDB at days 140 and 238 were significantly different from all other groups ($p < 0.05$), as indicated with an asterisk. No other significant differences between groups were observed.

Table 2 Results of the Bovigam IFN-γ assay for non-vaccinated (G1), Hsp70 1× s.c. (G2), Hsp70 2×s.c. (G3), Hsp70 2×i.m. (G4) and Gudair MAP (G5) vaccinated animals. The animals were tested four times (7, 14, 21 and 28 days) after each skin test. Responses are summarized per group (N= 6 animals per group) and per skin test. The results are expressed as the number of animals that have a positive outcome (+) in the test, when applying the standard interpretation (OD PPDB–OD PPDA) > 0.100.

Group	Animals	Pre-vaccination	Post-vaccination			
		IDT D0	IDT D140		IDT D238	
		+	+	+[a]	+	+[a]
G1 (non-vaccinated)	6	2	0	0	2	0
G2 (Hsp70 1x s.c.)	6	2	2	0	1	0
G3 (Hsp70 2x s.c.)	5[b]	1	0	0	1	1
G4 (Hsp70 2x i.m.)	6	1	1	0	2	1
G5 (Gudair MAP)	6	1	3	3	2	2

[a] The number of animals that reacted positive, when excluding animals that reacted prior to vaccination.

[b] In G3 one animal was culled and excluded from analysis.

Based on the fact that also non-vaccinated animals started responding in the Bovigam, the effect of skin testing on antigen specific IFN-γ responses was studied in more detail. As is depicted in figure 4A none of the animals showed spontaneous release of IFN-γ at the start and the end of the experiment. However all animals had a high release of IFN-γ after stimulation with PPDA and PPDB 7 days after the first skin test which dropped sharply in the first month thereafter (figure 4C and D). No response was observed when Hsp70 was used as antigen at the start of the experiment (Figure 4B). At the end of the experiment the antigen specific induction of IFN-γ release by skin testing was less pronounced, however all animals showed production of IFN-γ in the in vitro assays of D232 – D260. The IFN-γ production was significantly higher in the G5 (Gudair MAP) group when stimulated with PPDA and PPDB antigens as compared to the other groups ($p<0.05$). The other groups did not differ significantly. When cells were stimulated with Hsp70 the G1 (non-vaccinated) and G5 (Gudair MAP) groups were not significantly different. In comparison with the three Hsp70 vaccinated groups (G2, G3 and G4) significantly higher IFN-γ responses were observed in G5 (Gudair MAP).

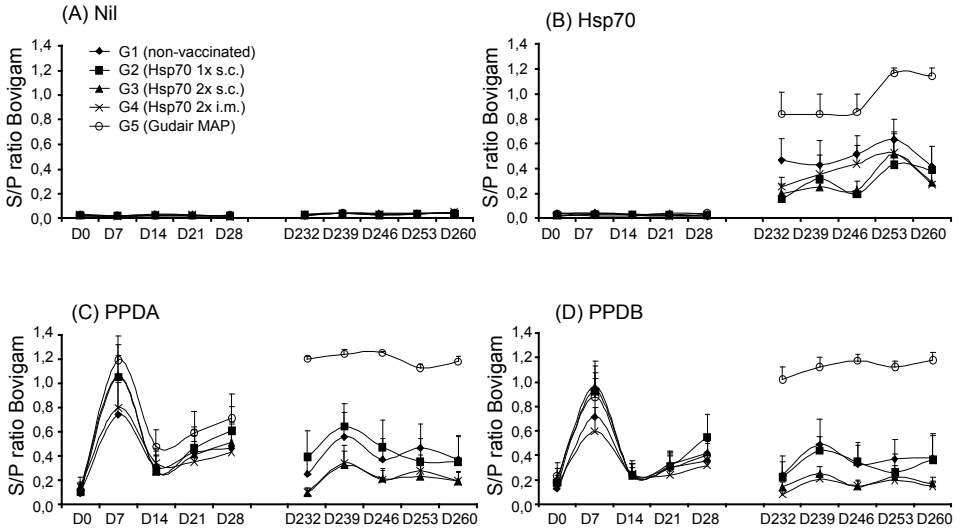

Figure 4 Antigen specific IFN- responses in whole blood cultures of non-vaccinated (G1), Hsp70 1× s.c. (G2), Hsp70 2× s.c. (G3), Hsp70 2× i.m. (G4) and Gudair MAP (G5) vaccinated animals measured in Bovigam ELISA. Results are expressed as sample to positive (S/P) ratio. Responses are summarized per group (average S/P ratio of N= 6 animals per group + SEM) on day of tuberculinisation at the beginning and the end of the experiment (D0 and D232), and on days 7, 14, 21 and 28 following each skin test. Responses shown are those of: panel A (nil) unstimulated cells, panel B (Hsp70) cells stimulated with Hsp70 protein, panel C (PPDA) cells stimulated with PPDA, panel D (PPDB) cells stimulated with PPDB. Following the tuberculinisation on D0 there are no differences between groups within treatment. On D232 and ensuing days G5 is significantly different from the other groups ($p < 0.05$) regarding the Hsp70, PPDA and PPDB antigen stimulations, other groups show no significant differences.

4. Discussion

Repeated vaccination of tuberculosis-free cattle with the recombinant MAP Hsp70/DDA subunit vaccine against paratuberculosis did not lead to a significant induction of false positive reactors in the intradermal skin test for bovine tuberculosis and the Bovigam antigen specific IFN-γ assay. The whole cell MAP vaccine leads to significantly higher induction of positive skin test reactors for both tuberculins as compared to the Hsp70/DDA subunit vaccine. Some skin reactors were found in the Hsp70/DDA vaccination groups, however the number of false positive reactors was not significantly different from false positive responders in the non-vaccinated group. When evaluated as a comparative skin test, one positive animal was detected in G2 (Hsp70 1x s.c.) at D140 and one positive animal in the whole cell MAP vaccinated group (G5, Gudair) at the final time point (D238) of the study. At D238 none of the animals vaccinated 2 or 3 times with the Hsp70/DDA vaccine had a positive comparative skin test. Triple testing of cattle within a 9-month time frame with the comparative skin test as performed in the current study may be causal to the occurrence of false positive reactors. The induction of false positive skin tests following repeated skin tests was described earlier in a study using young calves. In that study minor (1-4 mm) reactions were noted following 3 skin tests applied with 8-week intervals [36]. In our study we used older cattle which may have been exposed to atypical mycobacteria at an earlier time and hence may have been sensitized. The animal in the control group (G1), which showed a 15 mm skin reaction to PPDA at the end of the experiment, also had an 8 mm reaction to PPDA at D140. This animal was positive in the Bovigam for PPDA consistently also, suggestive for prior sensitization with atypical mycobacteria. The IFN-γ response primarily to PPDA and to a lesser extend PPDB, but not the recombinant Hsp70 protein, in the Bovigam assay 7 days following the first skin test may be indicative for such sensitization prior to the experiment. Boosting of anamnestic immune responses by skin test tuberculins has been described, and is for instance one of the schemes for use for the Bovigam assay, as a confirmatory test, following a positive skin test, indicative of tuberculosis infection [37]. Similarly antibody responses may be boosted using tuberculins and this effect is also markedly increased in infected animals as compared to vaccinated animals and may therefore be of diagnostic value [38].

The results suggest that specificity of the Bovigam IFN-γ ELISA is not compromised by the Hsp70/DDA vaccination. IFN-γ production in Hsp70/DDA vaccinated groups was never significantly higher as compared to the non-vaccinated control group, in contrast to G5 (Gudair MAP). Furthermore, the results from the IFN-γ ELISA in the current study confirm earlier observations with IFN-γ ELISPOT that the Hsp70/DDA vaccine does not induce a dominant Th1 response in the absence of MAP infection. This study also confirmed induction of predominantly IgG1 antibody responses by the Hsp70/DDA vaccine [32, 35].

With respect to the effect of the Hsp70/DDA vaccine on the current immunodiagnostic assays for paratuberculosis, all animals did seroconvert as measured with Pourquier absorbed ELISA 4 weeks after vaccination. The addition of Hsp70 antigen to the standard serum *M. phlei* pre-absorption step removed Hsp70/DDA vaccination-induced antibodies and restored specificity of the assay, i.e. Hsp70 absorbed sera

of non-infected Hsp70/DDA vaccinated animals were negative in the Pourquier ELISA. In contrast sera of the animals of G5 (Gudair MAP) that all seroconverted after vaccination, remained positive in the Pourquier ELISA after pre-absorption with Hsp70. This effect highlights a clear advantage of subunit vaccination with defined antigens as it allows for rapid and simple adaptation of diagnostic assays to enable the distinction between vaccinated and infected animals. The effect of additional pre-absorption with Hsp70 on the sensitivity of the Pourquier ELISA was not studied in detail in the present study. However it is known from previous studies that the production of MAP Hsp70 specific antibodies in animals naturally infected with MAP is very limited [30]. The observation that the S/P values of the animals in the non-vaccinated group remained stably low despite Hsp70 pre-absorption adds to the hypothesis that the effects are anticipated to be minor, although further tests are obviously needed to confirm this.

Finally, the serology results of this study also indicate that the antigenic preparation used in the Pourquier ELISA, MAP protoplasmic protein antigen, contains substantial amounts of native MAP Hsp70 protein. As tuberculins are essentially also complex soluble protein mixtures from heat killed mycobacteria, it is very likely that these also contain Hsp70 protein. The *M. avium* spp. *avium* Hsp70 protein and the *M. bovis* Hsp70 protein are 99.8% and 94.5% identical to the MAP Hsp70 protein respectively. For the *M. bovis* Hsp70 protein most of the differences with the MAP Hsp70 protein are concentrated in the C-terminus of the protein, with the last 25 amino acids sharing very little homology with the C-terminus region of MAP Hsp70. Most of the *M. bovis* Hsp70 protein and especially the N-terminus are nearly identical to the MAP Hsp70 protein. Although to our knowledge no comprehensive studies of the qualitative and quantitative protein composition of tuberculins have been published, it is likely that these preparations will also contain Hsp70 protein. Nonetheless the MAP Hsp70/DDA vaccine does not sensitize for the tuberculin skin test with PPDA and PPDB, likely containing highly homologous Hsp70 protein, indicating again that the quality of immune responses generated during natural infection are substantially different to those generated by the Hsp70/DDA subunit vaccine. Taking into account the IFN-γ and lymphoproliferation data from this and earlier studies [27, 32] these data again point towards predominant induction of Th2 driven antibody responses by the Hsp70/DDA subunit vaccine rather than a Th1 type of response.

Conclusion

In conclusion we demonstrated that vaccination of cattle with an Hsp70/DDA vaccine, that has previously shown to reduce shedding of MAP in the feces significantly [32], has little direct and long term side-effects, and does not interfere with current tuberculosis diagnostics. Serological assays for paratuberculosis diagnostics can easily be adapted by additional pre-absortion to enable differentiation between vaccinated and infected animals.

Hsp70/DDA subunit vaccine may contribute to the paratuberculosis eradication strategies, without compromising diagnosis of bovine tuberculosis or paratuberculosis.

Acknowledgements

The authors express their thanks to Katja Moors-Pollaart for the production of recombinant Hsp70 protein.

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Chapter 3

Searching for proteins of *Mycobacterium avium* subspecies *paratuberculosis* with diagnostic potential by comparative qualitative proteomic analysis of mycobacterial tuberculins

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Veterinary Microbiology 138 (2009) 191–196

Abstract

Accurate immunodiagnosis of bovine paratuberculosis is among others hampered by the lack of specific antigens. One of the most frequently used antigen preparations is purified protein derivative (PPD), also known as tuberculin. This crude extract has limitations when used in diagnostic assays due to the presence of cross-reactive antigens. The aim of the current study was to systematically analyze the qualitative protein composition of PPD of the major mycobacterial pathogens.

One-dimensional gel electrophoresis followed by tandem mass spectrometry analysis of PPD from *Mycobacterium avium* subspecies *paratuberculosis* (MAP), *Mycobacterium avium* subspecies *avium* (MAA) and *Mycobacterium bovis* (MB) identified 156, 95 and 132 proteins, respectively. Comparative sequence analysis led to the selection of a MAP-specific protein (MAP1718c), and finally heterologous expression in Escherichia coli of this and other diagnostic candidate proteins (MAP3515c and MAP1138c (LprG)) enabled evaluation of their immunogenicity. Lymphocyte proliferation responses did not indicate substantial diagnostic potential of the antigens tested. In contrast serum antibody levels for MAP1138c in paratuberculosis infected cows (N=20) were significantly higher ($p<0.05$) than in control animals (N=20), despite the conserved nature of this protein.

In conclusion, this study showed that a combination of proteomics and genomics, starting from complex protein mixtures, present in tuberculins, can reveal novel proteins aiding the development of immunodiagnostics for mycobacterial diseases.

1. Introduction

Accurate immunodiagnosis of bovine paratuberculosis, a chronic incurable infection of the small intestine caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is among others hampered by the lack of specific antigens (Harris and Barletta, 2001). Purified protein derivative (PPD), also known as tuberculin, produced from heat-processed mycobacterial culture filtrates, is one of the antigen preparations most used in mycobacterial immunodiagnostics, like skin tests in animals and humans, IFNγ assays, and in serum antibody ELISA (Monaghan et al., 1994). The PPD protein mixture likely contains many individual proteins. To limit false positive reactions to antigens shared with other pathogens or innocuous environmental micro-organisms, MAP-specific antigens are needed. Aided by the completion of the prototype mycobacterial genomes of MAP, MAA and *Mycobacterium bovis* (MB) / *Mycobacterium tuberculosis* (MT) we performed a qualitative, comparative proteomic analysis of commercially produced PPDs from MAP, MAA and MB, and evaluated the immunogenicity of three selected MAP proteins.

2. Material and methods

2.1 Proteomics

The purified protein derivatives, produced according to the OIE manual for diagnostic tests from MAP strain 3+5/C (PPDP), MAA strain D4 (PPDA) and *M. bovis* strain AN5 (PPDB), were contracted from two producers, CVI-Lelystad (Lelystad, the Netherlands) and CZ Veterinaria (Pontevedra, Spain). Hundred micrograms of each PPD was separated by one-dimensional gel electrophoresis in a 13% (w/v) SDS-PAGE gel, followed by in-gel digestion with trypsin. Proteins were reduced with 6.5mM DTT (Roche Diagnostics) followed by alkylation with 54mM iodoacetamide (Sigma-Aldrich, St. Louis, USA) for 1 h, and then digested with trypsin at an enzyme: substrate ratio of 1:50 (w/w). Nano-LC-MS/MS was performed as previously described (van Balkom et al., 2005). Peptides were identified using Mascot software (MatrixScience, Version 1.9) using the following settings: trypsin was used as an enzyme with one miscleavage allowed, peptide tolerance was set to 1.2 Da; carbamidomethyl and oxidation of methionine were set as fixed and variable modifications, respectively. Scaffold (Version Scaffold_2_00_05, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide and protein identifications were accepted if they could be established at greater than 90.0% and 95.0% probability as specified by the Peptide/Protein Prophet algorithms, respectively. Protein identifications were accepted if they contained at least two identified peptides. The Geneplot program (available at http://www.ncbi.nlm.nih.gov/) was used to identify protein homologues between the sequenced genomes of M. avium spp. paratuberculosis strain K-10, M. avium 104 and M. bovis AF2122/97. TBLASTN was used to calculate the homology on protein level.

2.2 Recombinant proteins

Primers were designed from the coding sequence of the MAP strain K10 genome (GenBank: AE016958) using Primer software (Version 1.0). Each forward primer contained the 5' extension CACC to facilitate directional cloning into the pET100/D TOPO vector (Invitrogen, USA). The following primers were used to clone the genes of interest:

MAP1718cF, 5'CACCGTGGCGTTCTCACACATTG3';

MAP1718cR, 5'CTAGCTCTTCCAGACCACCTTG3';

MAP3515cF, 5'CACCATGTATTCCGAGTCGAAG3';

MAP3515cR, 5'TCATGTGCGGCAGCTGATTTCC3';

MAP1138cF,5'CACCATGCTGGGTATGCAGACCCG3';

MAP1138cR, 5'TCACTTGGTGACCTGGACCTGCTC3'.

Genomic DNA from MAP strain G195 and MAA strain D4 was used as template for the PCR reactions. Amplification was performed on an I-Cycler (BioRad, USA) with Pfu Turbo Cx Hotstart DNA Polymerase (Stratagene, USA). The selected genes were cloned into the pET100/D TOPO vector (Invitrogen, USA), containing an N-terminal His-tag. Expression was performed in BL21 Star (DE3) *E. coli*

cells (Invitrogen, USA) using 1.0 mM isopropyl ß-D-thiogalactoside induction. Recombinant proteins were affinity-purified on a Ni-column, using Chelating Sepharose Fast Flow (Amersham Bioscience, USA). Purity was checked on 13% (w/v) SDS-PAGE gels and confirmed by Western blot analysis using an anti-His monoclonal antibody (Amersham Bioscience, USA). The purified recombinant proteins were dialyzed against PBS, concentrated using a Vivaspin 10,000 MWCO PES membrane (VivaScience, Germany) and quantified by the BCA kit (Pierce, USA). All procedures were performed according to instructions provided by the manufacturers.

2.3 Immunoassays

Sera were derived from Holstein-Friesian cattle on farms which participated in paratuberculosis field surveys in the Netherlands. The sera were classified based on results from repeated fecal culture and serological responses measured in the absorbed ELISA for paratuberculosis diagnosis (Pourquier, France) and stratified in three groups: frequent shedders (N=20), intermediate shedders (N=20) and control animals (N=20). Serological responses to the recombinant proteins were measured using an ELISA technique as described previously (Koets et al., 2001) with minor modifications. Plates were coated with 1 µg protein per well, and IgG1 antibody responses were measured. For the proliferation assays, blood samples were used from Holstein-Friesian cattle on dairy farms participating in control programs in the Netherlands. Animals were classified according to their clinical history and laboratory tests as described above. Control animals (N=14) originated from a single dairy farm with no known history of paratuberculosis, participating in the Dutch paratuberculosis control programs and having obtained the highest herd status based on repeated testing. The infected animals (N=12) originated from different dairy farms and were classified as MAP infected based on repeated positive fecal culture results. Lymphocyte stimulation tests (LSTs) were performed as described previously (Koets et al., 1999), with the antigens at a concentration of 10 µg/ml. To correct for differences in background proliferation in wells stimulated with culture medium alone, data were expressed as Δ–cpm (cpm in antigen stimulated wells minus cpm in medium control wells). Data were analyzed using SPSS 15 software. ELISA data were normalized by log transformation and ANOVA was used for comparison of groups. Results of the proliferation assays were compared using a nonparametric Mann-Whitney U test on Δ-cpm values. The level of significance was set at $p<0.05$.

3. Results

3.1 Protein identification and selection

Analysis of the three Lelystad PPDs by tandem mass spectrometry led to the generation of proteomes for PPDP, PPDA and PPDB, in which 156, 95 and 132 proteins were identified, respectively. Comparison of the generated proteomes by matching protein homologues showed that a large proportion (44-73%) of proteins were shared between the PPDs (Figure 1A). In PPDA the total number of proteins identified was markedly less than in both other PPDs, however the ratio of shared proteins with the other PPDs was the highest (73%). In total 25 proteins were shared by PPDP, PPDA and PPDB produced by one manufacturer (CVI-Lelystad). To determine the overlap in proteome within a specific PPD, we next analyzed three PPDA's from different manufacturers (two independent batches from CZ Veterinaria and one from CVI-Lelystad). The comparison of the proteomes generated for the PPDA's is depicted in figure 1B. The ratio of shared proteins ranged from 67 to 81%. In total 39 proteins were shared between all PPDA preparations, representing 35–43% of the total proteins identified in the PPDA preparations.

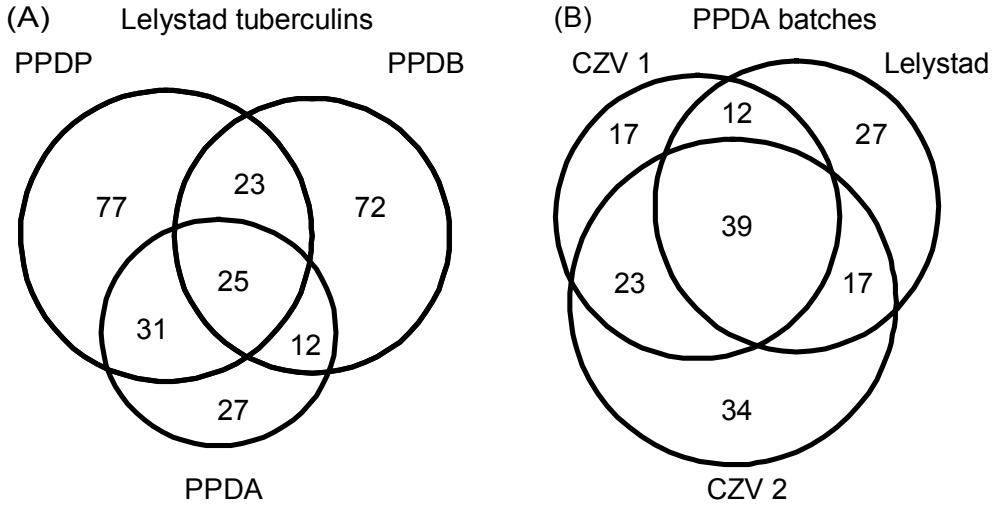

Figure 1 Panel A: venn diagram showing the overlap of the proteins identified in PPDP, PPDA and PPDB from CVI-Lelystad. Panel B: venn diagram showing the overlap of the proteins identified in three PPDA batches, from CZ Veterinaria (CZV 1 and CZV 2) and CVI-Lelystad. Numbers of proteins per section are indicated.

Nineteen proteins were identified in each of the PPDs analyzed (table 1). The average homology on protein level as established with TBLASTN analysis for this group of proteins compared to PPDP was 99% (range 99–100%) for PPDA and 87% (range 67–96%) for PPDB homologues. Of these 19 proteins 8 and 5 proteins have been described as immunogenic in bovine paratuberculosis and bovine tuberculosis, respectively.

Table 1 Proteins identified in all PPDs analyzed (*N*=19)

Protein name	Locus tag MAP	Locus tag MAA	Locus tag MB	MW (Da)	ParaTB antigen	TB antigen	Reference
35kd antigen	MAP_2855c	MAV_3635	Mb2765c	29.442	ND	ND	
Aconitate hydratase 1	MAP_1201c	MAV_3303	Mb1511c	101.097	ND	ND	
Acyl carrier protein	MAP_1997	MAV_2193	Mb2268	12.467	ND	ND	
Adenosylhomocysteinase	MAP_3362c	MAV_4211	Mb3276c	54.476	ND	ND	
Antigen 85-A	MAP_0216	MAV_0214	Mb3834c	36.078	Yes	Yes	(Rhodes et al., 2000; Rosseels et al., 2006)
Antigen 85-B	MAP_1609c	MAV_2816	Mb1918c	34.676	Yes	Yes	(Rhodes et al., 2000; Rosseels et al., 2006)
Antigen 85-C	MAP_3531c	MAV_5183	Mb0134c	38.035	Yes	Yes	(Rhodes et al., 2000; Rosseels et al., 2006)
ATP synthase F1, beta subunit	MAP_2451c	MAV_1527	Mb1342	53.080	ND	ND	
Bacterioferritin	MAP_1595	MAV_2833	Mb1907	18.462	Yes	ND	(Sugden et al., 1991)
Chaperone protein DnaK	MAP_3840	MAV_4808	Mb0358	66.510	Yes	Yes	(Koets et al., 1999; Rhodes et al., 2000)
60 kDa chaperonin 2	MAP_3936	MAV_4707	Mb0448	56.597	Yes	Yes	(Koets et al., 1999; Rhodes et al., 2000)
LprG protein	MAP_1138c	MAV_3367	Mb1446c	24.401	Yes	ND	(Dupont et al., 2005)
Malate dehydrogenase	MAP_2541c	MAV_1380	Mb1272	34.587	ND	ND	
ModD protein	MAP_1569	MAV_2859	Mb1891	37.512	Yes	ND	(Cho et al., 2007)
Ribosomal protein L7/L12	MAP_4126	MAV_4507	Mb0671	13.413	ND	ND	
Translation elongation factor Tu	MAP_4143	MAV_4489	Mb0704	43.752	ND	ND	
Trypsin	MAP_3527	MAV_5187	Mb0130	36.698	No	ND	(Cho et al., 2007)
Universal stress protein family protein	MAP_1339	MAV_3137	Mb1662	13.708	ND	ND	
Wag31 protein	MAP_1889c	MAV_2345	Mb2169c	26.988	ND	ND	

The locus tag of each protein for all three mycobacterial species analyzed is depicted. Antigenicity of each protein within bovine paratuberculosis and bovine tuberculosis as described in literature is shown. MW: molecular weight in Da, ND: not described.

Next, we selected specific proteins for MAP (table 2). Since MAP and MAA genomes are known to be highly similar, and as a consequence very few MAP-specific proteins exist, we also selected MAP proteins differentially expressed in mycobacteria. To define MAP-specific proteins we selected MAP proteins present in PPDP that were not identified in PPDA, and had no homologues in the sequenced MB genome. Only hypothetical protein MAP1718c had no homologue identified in the sequenced MAA genome and was therefore selected as MAP-specific protein. For the remaining proteins in PPDP homologues are present in the MAA genome with an average over 98% similarity on protein level, which was established with TBLASTN analysis. The hypothetical protein MAP3515c was selected as a first protein, based on differential expression in MAP and MAA, to be screened for immunogenicity. Furthermore, MAP1138c (LprG), a conserved protein present in all PPDs, was selected for evaluation of immunogenicity in paratuberculosis infection, based on immunogenicity of the *M. tuberculosis* homologue. The homology on protein level of MAP1138c as compared with the M. bovis LprG protein is 67%.

Table 2 Candidate MAP-specific proteins.

Protein name	Locus Tag MAP	Locus tag MAA	MW (Da)
Hypothetical protein	MAP_0143	MAV_0139	11.617
Hypothetical protein	MAP_0151c	MAV_0146	16.518
Hypothetical protein	MAP_0494	MAV_0588	32.672
Hypothetical protein	MAP_1718c		15.518
Hypothetical protein	MAP_1754c	MAV_2494	31.093
FdxC_1	MAP_2039	MAV_2150	12.245
Probable cysteine desulfurase	MAP_2120c	MAV_2055	69.971
Hypothetical protein	MAP_2677c	MAV_1244	14.559
Hypothetical protein	MAP_2770	MAV_3539	19.488
Hypothetical protein	MAP_2786c	MAV_3558	11.708
Hypothetical protein	MAP_3515c	MAV_5200	22.240
Hypothetical protein	MAP_3560	MAV_5153	20.058
Hypothetical protein	MAP_3659	MAV_4950	53.808
Hypothetical protein	MAP_3804	MAV_4844	30.298

Proteins identified in PPDP, which were not identified in PPDA, and do not have a homologue in the sequenced Mycobacterium bovis genome as established with Geneplot comparison (N = 14). MW: molecular weight in Da.

3.2 Immunoassays

The selected proteins (MAP1718c, MAP3515c and MAP1138c) were successfully cloned and expressed in *E. coli*. The purified proteins were used to evaluate their potential as T cell antigen in a proliferation assay. The results are summarized in figure 2 (A-D). Animals infected with paratuberculosis showed higher responses to PPDP as compared to control animals ($p<0.05$). No significant differences between groups were observed for the recombinant MAP1718c, MAP3515c and MAP1138c (LprG) proteins. The purified proteins were also evaluated for immunogenicity in a serum antibody ELISA. The results are summarized in figure 2 (E-H). No significant differences between groups were observed for the recombinant MAP1718c and Map3515c protein. However, high shedders showed significant higher responses than control animals ($p<0.01$) for the MAP1138c (LprG) protein.

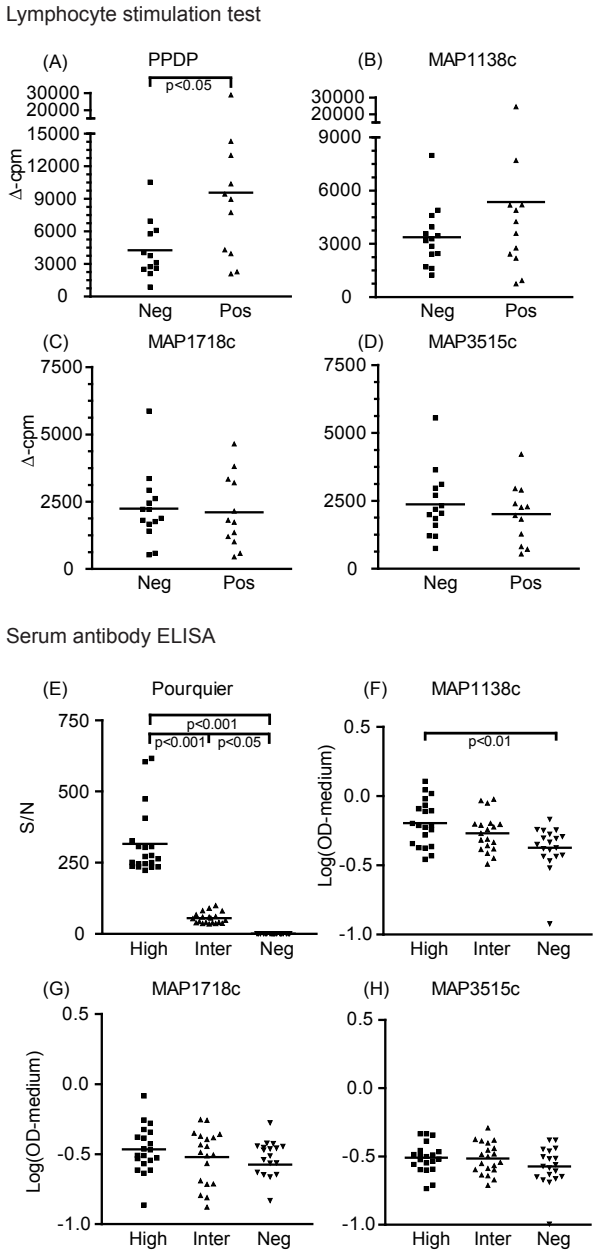

Figure 2 Panels A–D: proliferative responses of PBMC to (A) PPDP, (B) MAP1138c, (C) MAP1718c and (D) MAP3515c expressed as delta CPM for control animals (Neg, N = 14), and MAP shedding animals (Pos, N = 12). Panels E–H: serum antibody responses. Results of the commercial Pourquier absorbed ELISA are depicted in (E). Results for (F) MAP1138c, (G) MAP1718c and (H) MAP3515c expressed as the logarithmic optical density corrected for medium value for high shedding (High), intermediate shedding (Inter) infected animals and control animals (Neg) (N = 20 for each group). The horizontal line marks the group average value. Statistically significant differences ($p < 0.05$) between the different groups are indicated.

4. Discussion

This study describes a qualitative survey of the proteins present in mycobacterial PPDs. Proteomics has been used to identify proteins in mycobacterial culture filtrates and cell wall extracts (Cho et al., 2006; Xiong et al., 2005). However, we were interested in the protein composition of PPD, the most widely used antigenic preparation in mycobacterial immunodiagnostics. To improve current diagnostic assays, knowledge on the composition of PPD may enable the rational design of a synthetic tuberculin with selected immunogenic non-cross-reactive proteins or peptides.

The proteome analysis showed that the PPDs have a high range of shared proteins most likely partly responsible for the limited specificity of PPDs. In this group well-characterized and evolutionary conserved antigens are present like the 85 A, B, C complex proteins, heat shock protein 70 and Antigen D (bacterioferritin). The protein homology for these proteins is high, especially between the closely related species MAP and MAA, and likely also non-pathogenic environmental species. However, based on the range of protein homology, we speculate that specifically when MAP/MAA and MB proteins are compared, there could be peptides or protein regions within these conserved proteins that elicit pathogen-specific immune responses.

The majority of proteins identified in PPDB have been previously identified in culture filtrates or cell membrane analysis (Xiong et al., 2005). Tuberculosis-specific diagnostic antigens, like ESAT-6 and CFP-10, (Cockle et al., 2002) are present in PPDB. Andersen et al. described 30 antigens of *M. tuberculosis* recognized by T cells or effective as vaccine in animal models (Andersen and Doherty, 2005). Sixteen of these proteins were identified in PPDB in this study.

The hypothetical protein MAP1718c was identified as MAP-specific protein in PPDP, because it was absent from the sequenced genome MAA strain 104. However, in the current study a 99% identical homologue of the MAP1718c gene could also be amplified from MAA strain D4 and it was shown that a large sequence polymorphism containing MAP1718c was present in 44 of the 96 non-MAP isolates (Semret et al., 2005). Apparently strain variation within mycobacterial species may further complicate the search for diagnostic candidates.

The other potential MAP-specific proteins we have selected are based on differential expression of these proteins in the context of the tuberculin production process, in PPDP as compared to PPDA, rather than on the absence of the homologous gene in MAA. The immunoassay data showed that MAP1718c and MAP3515c are not immunodominant paratuberculosis antigens, unlike MAP1138c (LprG) that is recognized by antibodies during the course of paratuberculosis infection. LprG was recognized by high shedders to a larger extent than by intermediate shedders which may be related to the stage of the disease these animals are in. This phenomenon was also observed in other serodiagnostic studies using crude antigens (Koets et al., 2001). Recently, MAP LprG was also identified by screening of a MAP gene fusion library and found to be immunogenic in bovine paratuberculosis (Dupont et al., 2005). However, the sensitivity of the LprG ELISA appears to be much lower than the Pourquier ELISA.

Genomic approaches have led to the identification of MAP-specific ORFs by comparative sequence analysis followed by heterologous expression and immunogenicity evaluation (Bannantine et al., 2004). This has not yet resulted in novel specific antigens useful for diagnostic approaches. A disadvantage of this approach is the lack of knowledge on transcription of the identified ORFs. Proteome analysis identifies proteins which are actually transcribed under the conditions studied. Future work should also aim to identify differential expressed antigens, as this could be a key in detecting novel diagnostic antigens. If the induced proteome of MAP during infection is different from related environmental mycobacteria, resulting in a differential induction of the immune response, we propose that conserved antigens can be useful as diagnostic targets. An interesting approach in this respect is the recent study by Hughes et al., who compared the proteome of in vitro cultured MAP and MAA bacteria and selected 32 differentially expressed MAP proteins (Hughes et al., 2008). Eight proteins were also identified in the current study, of which only MAP3627 was found to be immunogenic in ovine paratuberculosis.

In conclusion, we have used proteomics as a tool to qualitatively analyze tuberculins. Three MAP proteins (MAP1718c, MAP3515c and MAP1138c) were tested in immunodiagnostic assays. The evolutionary conserved recombinant MAP1138c protein is best recognized of these three proteins by paratuberculosis infected cows in a serum antibody ELISA. These results support the line of thought that there could be evolutionary conserved proteins or parts thereof that elicit pathogen- and/or infection-specific immune responses. They warrant further study as many of these proteins are important targets for the host immune system.

Acknowledgement

The authors express their thanks to Jolanda Meijlis (CVI-Lelystad) and Eugenia Puentes (CZ Veterinaria) for the gifts of tuberculins. This work was supported by the Netherlands Proteomics Centre.

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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).

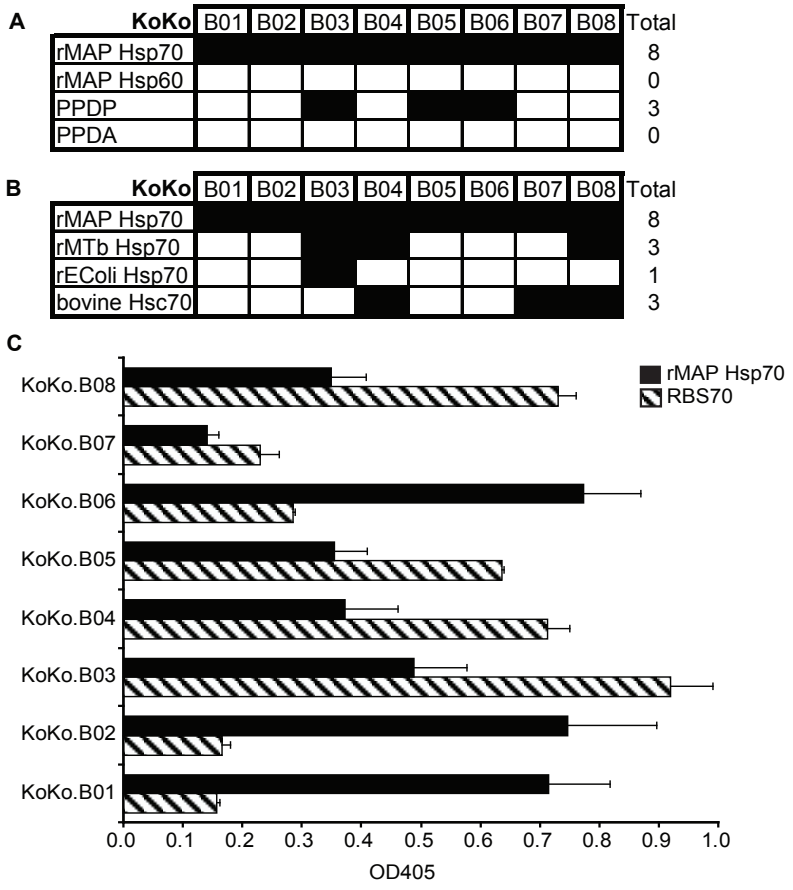

Figure 1 Characterization of rMAP Hsp70 specific hybridoma's. Panel A: reactivity of hybridoma supernatants to rMAP Hsp70 protein, a recombinant control protein (rMAP Hsp60 protein), and Johnin (PPDP) and avian tuberculin (PPDA). Panel B: reactivity of hybridoma supernatants to several Hsp70 proteins, 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).

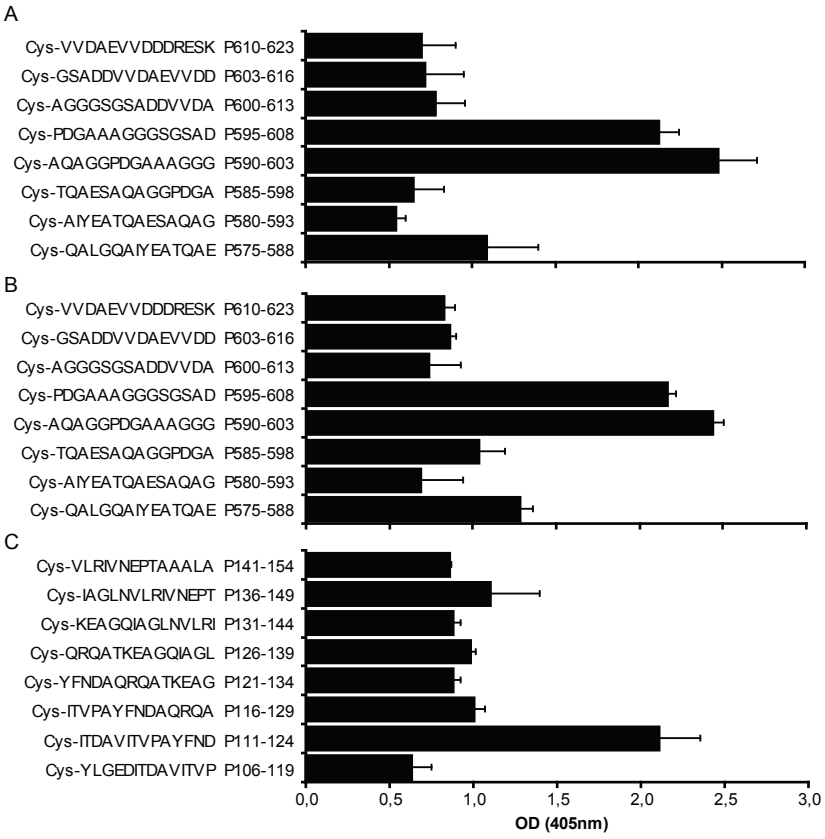

Figure 2 rMAP Hsp70 reactive monoclonal antibodies identify two linear B cell epitopes. Antibodies showing reactivity with recombinant MAP Hsp70 were tested for reactivity with synthetic MAP Hsp70 peptides. Antibody reactivity was tested on individual peptides of a positive 14-mer peptide pool, with 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).

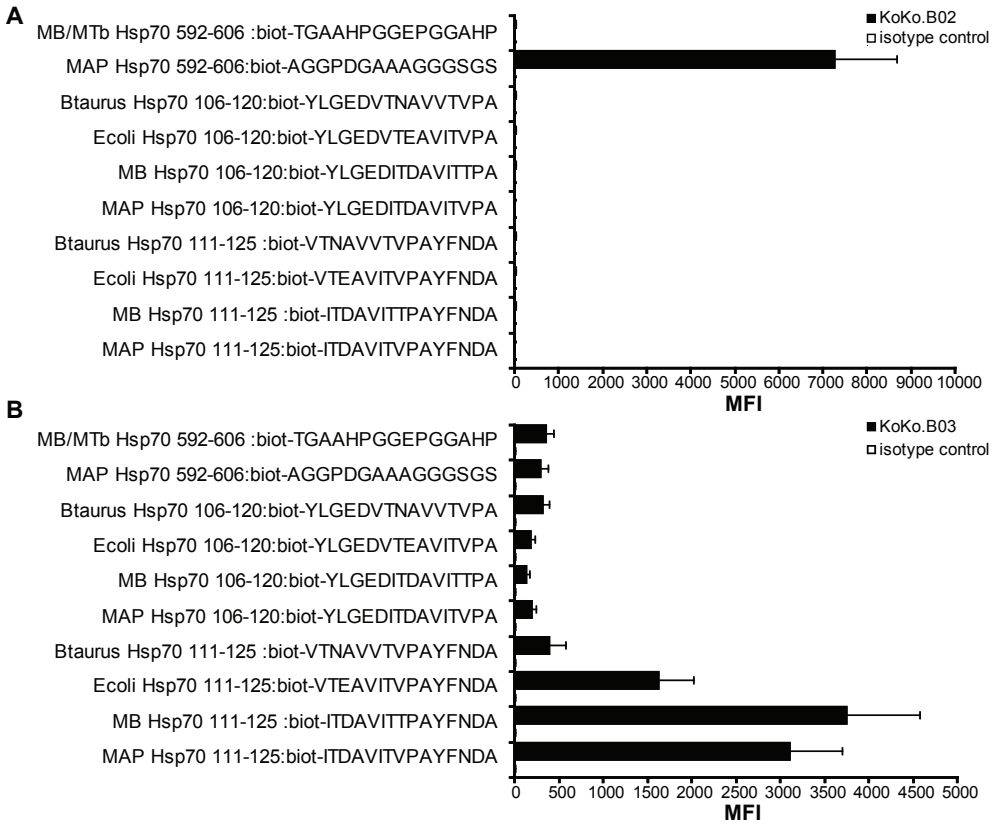

Figure 3 Specificity of KoKo.B02 and KoKo.B03 mAb's binding linear epitopes of MAP Hsp70. Specificity of (Panel A) KoKo.B02 (IgG2b) mAb and (Panel B) KoKo.B03 (IgG2a) mAb was tested on a set of 15-mer biotinylated peptides bound to avidin coated fluorescent beads (LumAv), containing relevant MAP Hsp70 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.

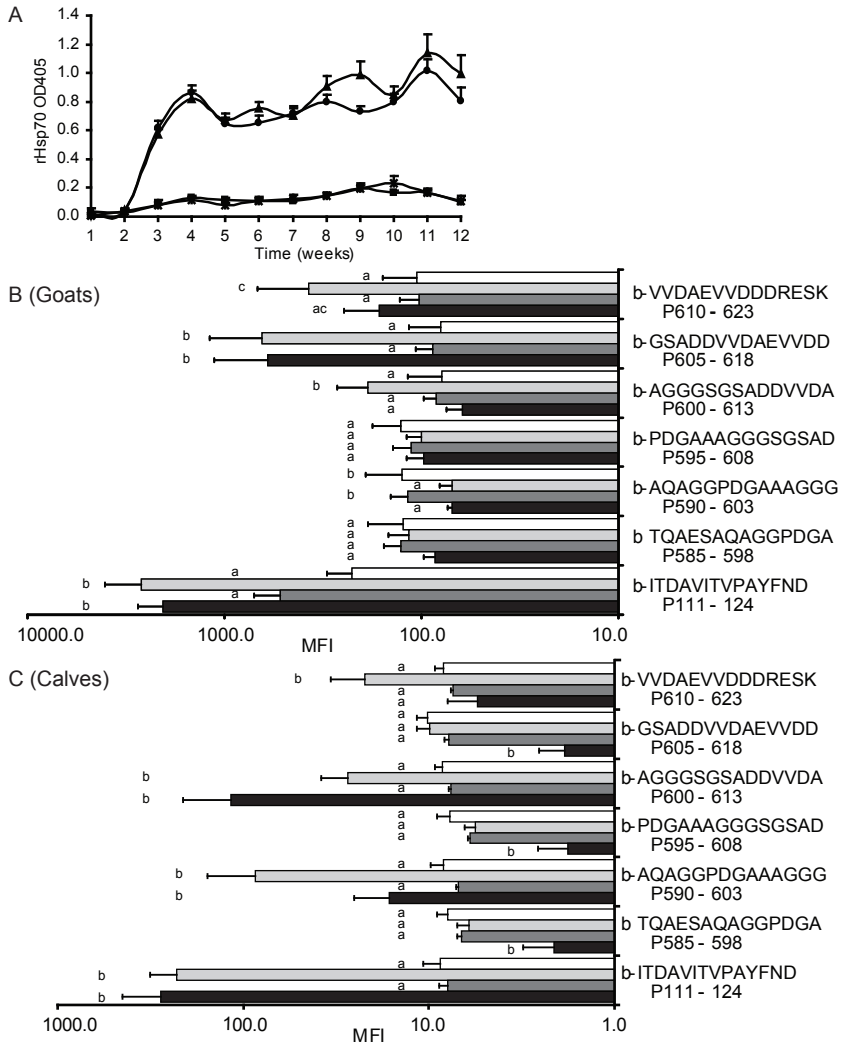

Figure 4 rMAP Hsp70 vaccinated calves and goats recognize linear B cell epitopes of MAP Hsp70. Panel A shows rMAP Hsp70-specific serum antibody responses in vaccinated goats and controls. Goats were immunized with 200g of rMAP Hsp70 in 1mL phosphate buffered saline (PBS) containing 10mg/mL DDA adjuvant (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.

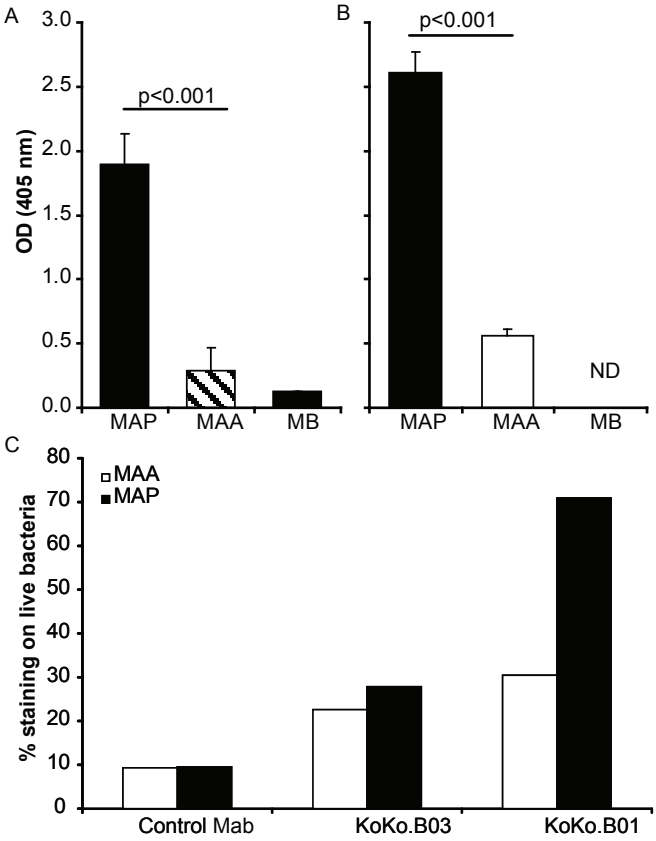

Figure 5 MAP Hsp70 linear B cell epitopes are recognized in the cell wall of MAP. Panel A, whole mycobacterial cell ELISA testing binding of antibody KoKo.Bo3 to *M. avium* spp. *paratuberculosis* (MAP), *M. avium* spp. *avium* (MAA), and *M. bovis* (MB) and panel B, testing of antibody KoKo.Bo1 (MB 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.

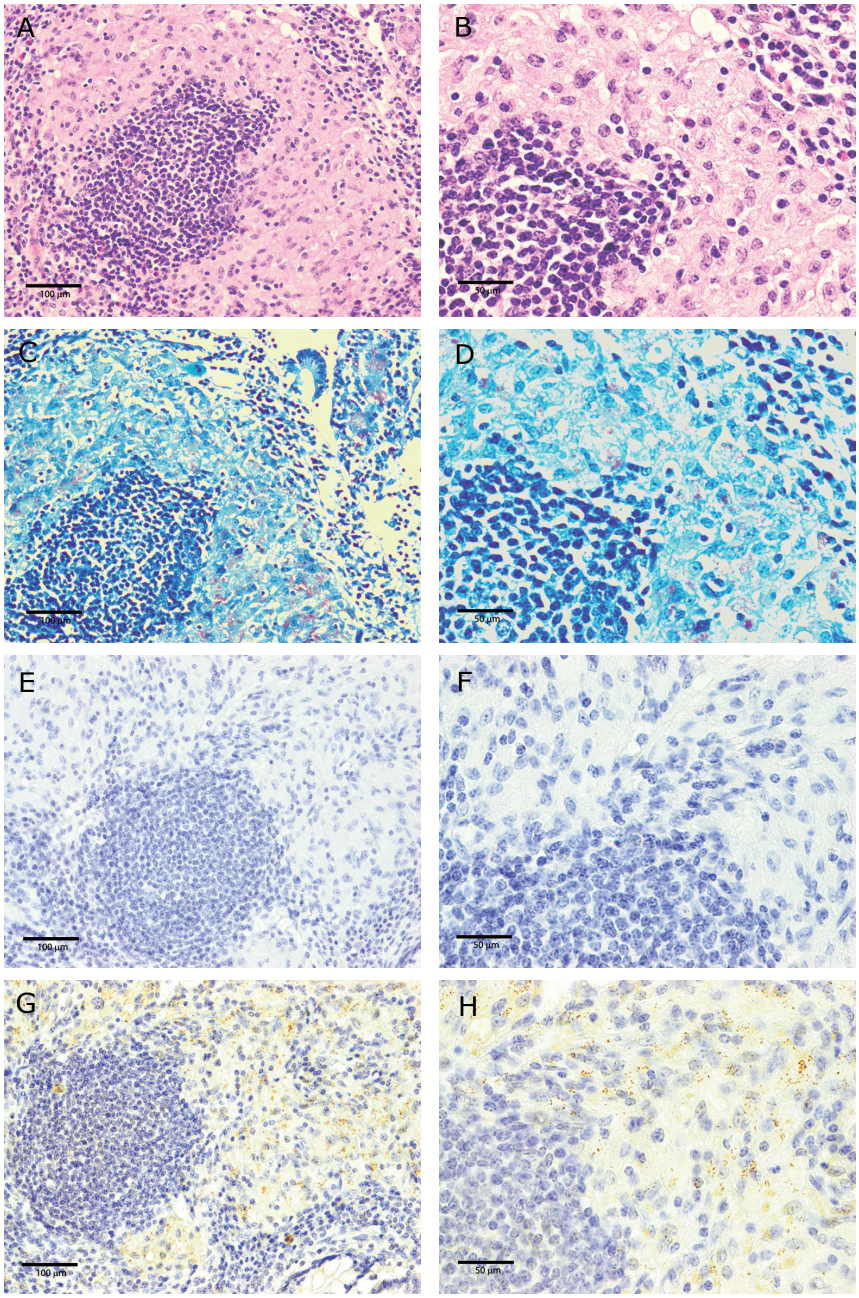

Figure 6 Staining of sequential lesional tissue sections from the ileum of a cow diagnosed with paratuberculosis by MAP Hsp70 specific monoclonal antibody KoKo.Bo2. Sequential sections of this paratuberculosis diagnosed cow, showing clinical signs, were processed using routine HE staining (panels A and 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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Chapter 5

Hsp70 vaccination-induced primary immune responses in efferent lymph of the draining lymph node

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Manuscript in preparation

Abstract

Paratuberculosis in ruminants is an infectious disease of the small intestine, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Control programs are in need of an effective vaccine, but there is no vaccine registered for use in cattle in the European Union. A candidate vaccine based on recombinant MAP heat shock protein 70 (Hsp70) has shown efficacy in an experimental challenge model. Contrary to expectation, vaccination-induced protection was associated with antibody responses, rather than with induction of specific T helper 1 cells. As peripheral responses in blood might not adequately reflect the vaccination-induced immune response, we set out to study the primary immune response after Hsp70 vaccination at the site of induction by efferent lymph vessel cannulation. From five days onwards after vaccination Hsp70-specific antibodies, predominantly of IgG1 isotype, were measured in efferent lymph. Vaccination led to increased percentages of B cells (up to 40%) in the efferent lymph, and both memory (proliferating cells: Ki67+) and antibody-secreting cells (Hsp70 B cell ELISPOT) were measured. The kinetics of the observed humoral response indicated that, although antibody production may occur during prolonged periods, the majority of memory and plasma cells are produced within two weeks. Memory CD4+ T cells (CD45RO+) were induced by vaccination, however analysis of interferon-gamma levels in lymph did not indicate a vaccine-induced Th1 response of efferent lymph cells, which did respond to Hsp70 by proliferation. These results confirmed earlier observations in peripheral blood that Hsp70/DDA vaccination primarily results in the generation of antibodies.

1. Introduction

Paratuberculosis in ruminants is an infectious disease of the small intestine and a global problem of the livestock industry (Harris and Barletta, 2001; Nielsen and Toft, 2009), affecting animal welfare and leading to substantial economic losses (Ott et al., 1999). The disease is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Young calves seem most susceptible to acquire infection via oral uptake of the bacterium and eventually shed the bacterium via feces into the environment. A proportion of the infected animals develops an incurable protein-loosing enteropathy, with chronic diarrhea and severe emaciation (Sweeney, 1996). The exposure of humans to MAP via milk and the association of MAP with human Crohn's disease has led to implementation of control programmes by the dairy industry to limit human exposure to MAP (Behr and Kapur, 2008; Grant et al., 2002).

Paratuberculosis disease control strategies have been directed at test and cull of infected animals, limiting exposure of susceptible calves to the infected environment by hygienic measures, and vaccination of susceptible animals. Currently there is no vaccine registered for use in cattle in the European Union. Studies with whole cell bacterins have shown that infection is not prevented, but occurrence of clinical disease is reduced (Wentink et al., 1994). The disadvantage of whole cell vaccines is their interference with tuberculosis diagnosis (Kohler et al., 2001).

The development of new vaccines is based on the dogma that induction of an effective cellular response will limit infection. We previously showed that cows naturally infected with MAP mount cellular responses against heat shock protein 70 (Hsp70) (Koets et al., 2002; Koets et al., 1999). Peripheral blood mononuclear cells (PBMC), as well as lymphocytes isolated from the draining mesenteric lymph node, and intra-epithelial lymphocytes of the ileum, showed proliferation upon stimulation with Hsp70 in vitro. In contrast, Hsp70-specific antibody responses were marginal in naturally infected animals (Koets et al., 2001).

In a subsequent experiment calves were vaccinated with Hsp70, using DDA (dimethyl dodecyl ammonium bromide) as an adjuvant, and experimentally challenged with MAP simultaneously (Koets et al., 2006). Vaccination resulted in significantly reduced fecal shedding of MAP. In this setting the major immunological outcome was the induction of an Hsp70-specific antibody response. A vaccine-specific interferon-gamma (INF-gamma) response could not be detected in PBMC. Also Hsp70-specific proliferation did not differ between controls and vaccinated animals.

Follow-up studies analyzed the T and B cell response generated after Hsp70 vaccination with the goal to understand the protective immune response. T cell epitopes of Hsp70 were defined that activate MHC class II restricted CD4+ T cells in a large proportion of outbred cattle. Hsp70 vaccination induces short-lived effector T cells, which produce IFN-gamma (Hoek et al., 2010). Recently, it was shown that the Hsp70 molecule is present in the cell wall of MAP and accessible to vaccination-induced antibodies (Santema et al., 2011).

Combined these studies indicated that vaccination-induced immune responses as measured in peripheral blood did not at all resemble infection-induced immune responses to Hsp70. As peripheral responses might not adequately reflect the vaccination-induced immune response, we set out to study early-induced immune responses at the site of Hsp70 vaccination. As the primary response after vaccination is induced in the draining lymph node of the neck region, where the vaccine is applied, we used a prescapular lymph vessel cannulation strategy to measure primary immune responses (Gohin et al., 1997; Hope et al., 2006; Nichani et al., 2003; Van Rhijn et al., 2007). By cannulation of efferent lymph vessels we were able to directly monitor the generated primary immune response.

Material and methods

2.1 Animals

Three Holstein-Friesian calves (3 months of age), from farms enrolled for over 10 years in the national Veterinary Health Service annual test-and-certification program and with the highest level of paratuberculosis unsuspected status, were used in this study. Calves were housed in a conventional animal house, fed according to requirements and checked daily for general health.

The use of animals in the present study was approved by the experimental animal committee of Utrecht University and conducted according to existing regulations.

2.2 Antigens

Recombinant MAP Hsp70 was produced according to methods described in detail earlier (Koets et al., 2001). Ovalbumin (control protein) was obtained from Sigma (USA) and purified protein derivative, prepared from *Mycobacterium avium* subspecies *paratuberculosis*, from the Central Veterinary Institute (Lelystad, the Netherlands).

2.3 Experimental design and surgery

Surgical procedures were described previously (Van Rhijn et al., 2007), with the modification that the efferent lymph vessel of the prescapular lymph node was cannulated (without surgical removal of the prescapular lymph node). Efferent lymph was continuously collected in 75 cm^2 culture flasks (Corning), containing 5 ml PBS, supplemented with 50 IU/ml penicillin and 50 mg/ml streptomycin (Gibco) and 1000 IU heparin (Leo Pharmaceuticals). Once daily, each calf was injected with 5000 IU heparin subcutaneously in the neck in the draining area of the prescapular lymph node.

Animals were vaccinated subcutaneously in the neck region, when the lymph flow stabilized, with 200 µg Hsp70 in 10 mg/ml DDA (dimethyl dioctadecyl ammonium bromide), formulated as published previously (Koets et al., 2006). Lymph flow was collected up to 17 days post-vaccination.

2.4 Hsp70 protein ELISA

Hsp70-specific antibodies were measured in lymph en serum by ELISA, described in detail earlier (Santema et al., 2009). Lymph was used in a 1:2 dilution in blockingbuffer (Roche).

2.5 B cell ELISPOT

For the analysis of Hsp70-specific antibody-secreting cells we used the protocol of Lefevre et al. (Lefevre et al., 2009), with minor modifications. Wells were coated with 100 µl of 20 µg/ml Hsp70. We used alkaline phosphatase-labelled sheep anti-bovine IgG (Serotec) as secondary antibody (1:1000) and developed with NBT-BCIP. Lymph cells were added in two-fold serial dilution, starting from $5*10^5$ cells/well.

2.6 Multiplex MSD cytokine/chemokine assay

Simultaneous detection of IFN-gamma, IL-1β, IL-4, IL-10, IL-12, and MIP-1β was performed in direct ex-vivo efferent lymph supernatants using a custom bovine 7-plex cytokine/chemokine assay (MSD), according to methods published previously (Coad et al., 2010). TNF-α was not analyzed, because of problems with assay reproducibility.

2.7 Interferon-gamma ELISPOT

Interferon-gamma producing cells were detected by ELISPOT. Interferon-gamma antibodies were purchased from Mabtech (Sweden), and the assay was performed according to their instructions. A total of $4*10^5$ efferent lymph cells were added per well. Antigens were added to 96-well filter plates (Milipore): Concanavalin A (mitogen) at 2.5 µg/ml; PPDP and ovalbumin at 10 µg/ml; Hsp70 at 10, 2 and 0.4 µg/ml. Medium was used to evaluate background spots. All tests were done in triplicates. Results were expressed as delta spot forming units (SFU), the mean spot value of medium control wells was subtracted from the mean spot value of antigen stimulated wells, and expressed per $1*10^6$ efferent lymph cells.

2.8 Lymphocyte stimulation assay

Antigens were added to 96-well flat bottom culture plates (Corning): Concanavalin A (mitogen) at 2.5 µg/ml; Hsp70 and ovalbumin at 10 µg/ml. Medium was used to evaluate background proliferation. Lymph cells were added at $4*10^5$ cells per well and incubated in a 5% CO2 incubator at 37°C for 3 days. Proliferation was measured by a 7 h pulse of 1 µCi of [3H]thymidine before cells were harvested and counted for β-emissions. Stimulation indices (SI) represent the mean counts per minute of triplicate wells stimulated with antigen divided by the mean counts per minute of triplicate wells stimulated with medium. Outlier values of triplicates were omitted when the coefficient of variance exceeded 1.0.

2.9 Flow cytometry

The following primary antibodies were used for flow cytometry analysis: anti-CD4-PE (CC8, Serotec), anti-CD8-PE (CC63, Serotec), anti-N24 (γδ T cell receptor, gb21-A, IgG2b, VMRD), anti-CD3 (MM1A, IgG1, VMRD), anti-CD21-like (B cells, GB25A, IgG1, VMRD), anti-CD62L (CC32, IgG1, Serotec), anti-Ki67-Alexa647 (b56, BD), anti-CD45RO (IL-A116, IgG3, VMRD). Secondary antibodies used were: goat-anti-mouse IgG1-biotin, goat-anti-mouse IgG2-PE, goat-anti-mouse IgG3-FITC (Southern Biotech). The secondary reagent used to visualize biotin-conjugated antibodies was streptavidin-eFluor® 450 (eBioscience). Matched isotype-controls were used if appropriate.

In short, $1*10^6$ lymph cells were labelled with antibodies diluted in 100 µl FACS

buffer (PBS with 2% FCS and 0,01% azide) for 30 minutes on ice and washed twice in between with 200 µl FACS buffer. Staining of Ki67 was performed with the intracellular staining kit (BD). FACS analysis was conducted using a FACSCantoII (Becton-Dickinson) machine. FACS data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

2.10 Statistics

For statistics R statistical software (www.R-project.org) was used. Pre- and post-vaccination responses for the flow cytometry data were compared with a Mann-Whitney U test. P-values below 0.05 were considered significant.

3. Results

3.1 Lymph flow characteristics

Efferent lymph was collected from three calves. Exact calculation of cellular output in efferent lymph was not possible due to the fact that lymph flow exceeded the storage capacity of culture flask on multiple time points. Therefore cellular output was estimated based on those time points for which the maximum storage capacity was not exceeded. Animal 0020 had an average cellular output of ~$1500*10^6$ (SD $500*10^6$) cells/hour; animal 5502 ~$330*10^6$ (SD $90*10^6$) cells/hour; and animal 5524 ~$720*10^6$ (SD $80*10^6$) cells/hour. Vaccination with Hsp70 did not lead to a lymph node shutdown phenomenon, as no significant decline in cell output numbers was observed.

3.2 Antibody response in efferent lymph after Hsp70 vaccination

From five days after immunization with Hsp70/DDA onwards Hsp70-specific antibodies were measured in the efferent lymph, leaving the draining prescapular lymph node (Figure 1A). The predominant isotype was IgG1, followed by IgG2 and IgM. Limited IgA responses were found. IgG1 antibodies increased gradually with a first peak at nine days and a second peak at day 13, after which they decreased slowly in the remainder of the observation period. IgG2 followed the same trend as IgG1, albeit on a lower level. IgM levels returned to baseline around day 13. The lymph antibody isotype distribution was reflected in the peripheral serum response at day 18 after immunization (Figure 1B). At day 7 and 14 after vaccination Hsp70-specific antibody-secreting cells (IgG isotype) were measured in efferent lymph, indicating migration from the site of induction towards the periphery.

3.3 Direct ex-vivo cytokine/chemokine responses in efferent lymph before and after Hsp70 vaccination

To investigate the effect of Hsp70 vaccination on cytokine production, we measured levels of IFN-gamma, IL-1β, IL-4, IL-10, IL-12, and MIP-1β directly ex-vivo in efferent lymph supernatants from three animals using a luminescence based multiplex assay (Figure 2). For IL-4 responses were below the limit of detection (data not shown).

Two animals responded with a peak production of IFN-gamma at three and ten days post-vaccination, respectively (Figure 2A). For IL-1β considerable variation was observed between animals (Figure 2B). One animal did not respond at all,

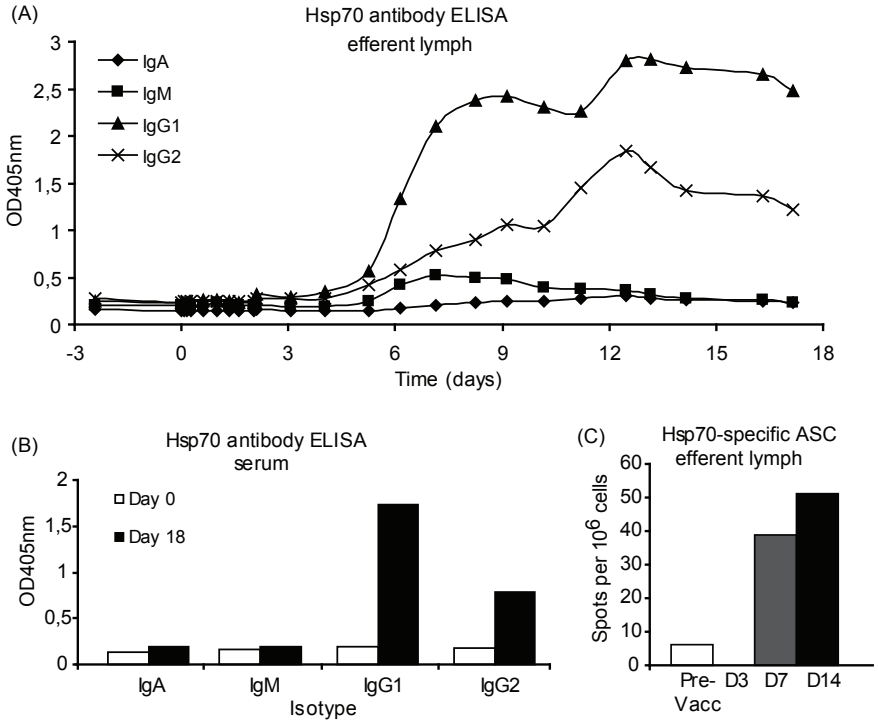

Figure 1 Hsp70-specific humoral response. Animal (0020) was vaccinated subcutaneously with Hsp70/DDA in the draining area of the prescapular lymph node at day 0. Responses shown are representative for the three animals. (A) Dynamics of Hsp70-specific antibody response in the efferent lymph of the prescapular lymph node. Results for IgA, IgM, IgG1 and IgG2 isotypes are shown. (B) Serum Hsp70-specific antibody response at D0 and D18 after immunization. (C) Dynamics of Hsp70-specific antibody-secreting cells (ASC) in efferent lymph before vaccination and at day 3, 7 and 14 after vaccination.

one animal had fluctuating responses before and after vaccination, and one animal had a peak response one day after vaccination. Vaccination did not lead to increased IL-10 responses (Figure 2C). Already before vaccination decreasing IL-10 levels were observed in all animals. IL-12 levels did not differ before and after vaccination, with the exception for a peak at day three in animal 5524 that co-incided with the peak in IFN-gamma level (Figure 2D). The chemokine MIP 1-β showed a peak response in animal 0020 at 24h after vaccination, but in the other two animals no link with vaccination was observed (Figure 2D).

To assess the presence of Hsp70-specific IFN-gamma+ effector T cells in efferent lymph, INF-gamma ELISPOT analysis was performed. Mitogen (ConA) stimulation induced between 50-200 spot forming units (SFU) per 10^6 efferent lymph cells (Figure 3A). For the control antigens, PPDP and ovalbumin, no differences in the number of spots before and after vaccination were observed. For the highest Hsp70 concentration (10 µg/ml) 18-25 spot forming units (SFU) per 10^6 efferent lymph cells were observed, but vaccination did not lead to increased number of spots (Figure 3B).

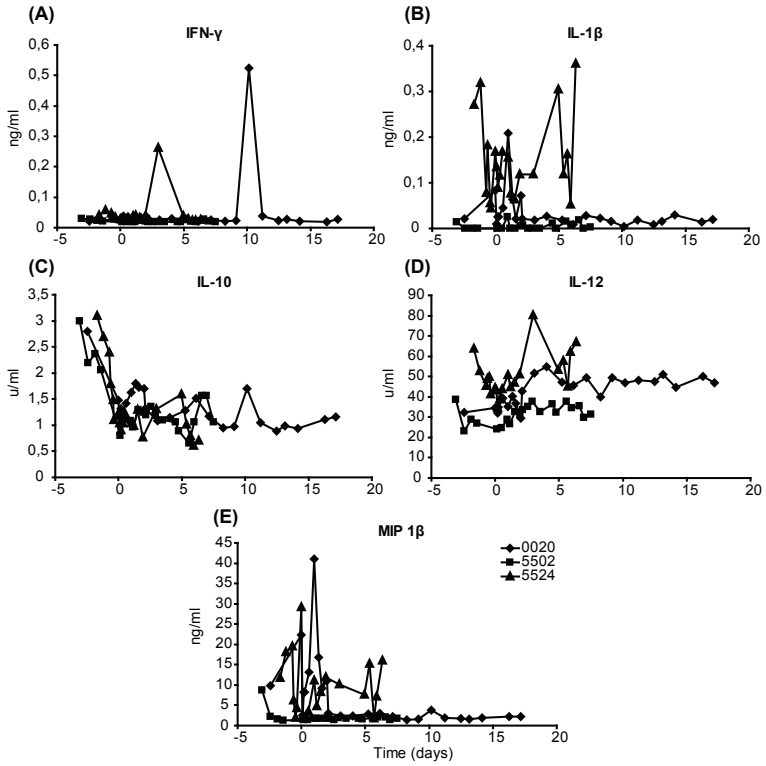

Figure 2 Direct ex-vivo analysis of (A) IFN-γ, (B) IL-1β, (C) IL-10, (D) IL-12 and (E) MIP-1β in efferent lymph supernatants of three animals vaccinated with Hsp70 at day 0.

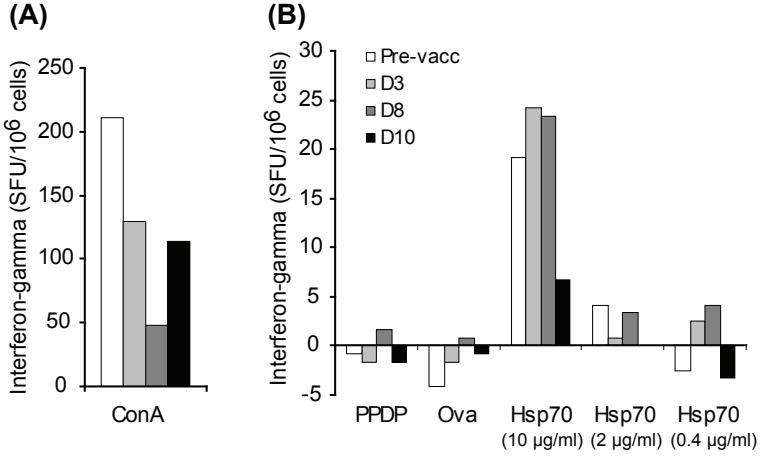

Figure 3 Interferon-gamma ELISPOT. Results are expressed as delta spot forming units (SFU) per 1*106 efferent lymph cells, mean spot value of medium control was subtracted. (A) positive control with Concanavalin A (ConA). (B) Antigen-specific INF-gamma responses for PPDP, Ovalbumin (Ova), and three Hsp70 concentrations. Efferent lymph cells from four time points of animal 0020 were analyzed, pre-vaccination, and day 3, 8, and 10 post-vaccination.

3.4 Temporal changes in lymphocyte kinetics and activation in efferent lymph after Hsp70 vaccination

To investigate changes in cellular efflux of the draining lymph node during the primary immune response after Hsp70 vaccination, flow cytometry was used to phenotype the major leukocyte populations in efferent lymph (Figure 4). The proportion of CD4+ and CD8+ T cells varied between 15-30% and 8-20%, respectively, but there were no significant differences before and after vaccination. The proportion of B cells increased significantly after vaccination with Hsp70, and constituted the major cell population in the first 14 days after vaccination. Consequently, the proportion of γδ T cells decreased significantly after vaccination from maximum 70% to around 30%.

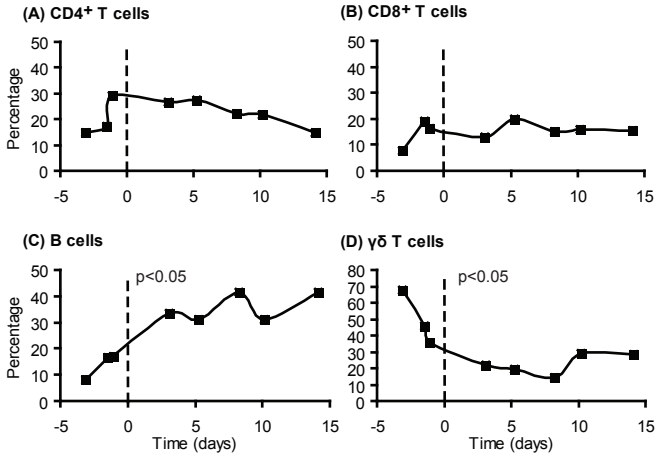

Figure 4 Dynamics of the lymphocyte distribution in efferent lymph of the prescapular lymph node. The percentage fluorescent labeled cells in the live gate are shown for (A) CD4+ T cells, (B) CD8+ T cells, (C) B cells (CD21+) and (D) γδ T cells (N24+). The vertical dashed line indicates the moment of vaccination with Hsp70 in DDA adjuvant subcutaneously in the draining area of the prescapular lymph node. Pre and post-vaccination responses were compared by a Mann-Witney U test. Significant differences were found for B cells and γδ T cells.

To evaluate whether Hsp70 vaccination induced proliferation of lymphocytes leaving the draining lymph node, flow cytometric analysis of the proliferation marker Ki67 was used. For CD4+ T cells expression of Ki67 varied between 1-2% before vaccination. From three days after vaccination onwards an increase in proliferating cells was observed, with a maximum of 7% at day 14 (Figure 5A). Proliferating CD4+ T cells resided mainly in the CD45RO+ memory population (93-99%). For CD8+ T cells there was also an increase in percentage of proliferating cells, however at a lower level with a maximum of 3% (Figure 5B). Up to 10% of proliferating B cells were observed with the peak at day five after vaccination. Following the peak at day 5, B cell proliferation waned in the remainder of the observation period to 1 % (Figure 5C). The γδ T cells showed a significant increase of proliferating cells after vaccination, although the maximum response was not higher than 1% (Figure 5D).

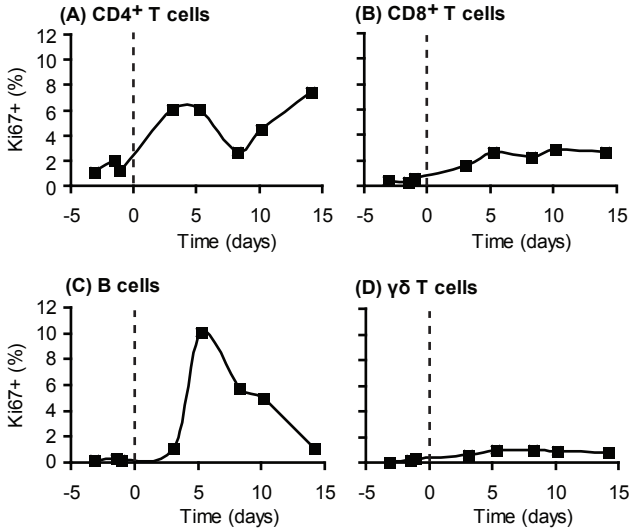

Figure 5 Temporal increase of proliferating cells after Hsp70 vaccination. Efferent lymph cells from the prescapular lymph node were analyzed by flow cytometry at three time points before and five time points after vaccination. Animal (0020) was vaccinated at day 0 with Hsp70/DDA subcutaneously in the draining area of the prescapular lymph node. Cells were gated for (A) CD4+ T cells, (B) CD8+ T cells, (C) B cells, and (D) γδ T cells, and percentage of Ki67+ cells (proliferation marker) is shown. The dashed vertical line indicates time point of vaccination. Pre and post-vaccination responses were compared by a Mann-Witney U test. Significant differences were found for all subsets.

3.5 Antigen-specific proliferation after Hsp70 vaccination in efferent lymph

To evaluate whether efferent lymph cells have the capacity to proliferate in an antigen-specific manner, a lymphocyte stimulation test was performed. Figure 6 A shows the proliferation after stimulation with mitogen Concanavalin A. There was a clear difference in stimulation index before and after vaccination. This is likely related to differences in phenotypic composition of efferent lymph. As shown in figure 5 γδ T cells, which are the main cell type present in efferent lymph before vaccination, showed limited proliferation, indicated by low Ki67 staining.

Antigens-specific proliferation against Hsp70 showed a clear increase in stimulation index after vaccination. Stimulation with ovalbumin, a control protein, did not show proliferation.

4. Discussion

Primary immune responses are generated in secondary lymphoid tissues. Subsequently, in the case of lymph nodes, immune cells will move via efferent lymphatics to various sites to execute their function. We hypothesized that analyzing the responses in efferent lymph, as compared to peripheral blood, would give a more accurate insight in the primary immune response generated after vaccination. The threshold or limit of detection in efferent lymph is anticipated to be lower than in peripheral blood, where vaccine-induced cells will be diluted in the circulating blood volume.

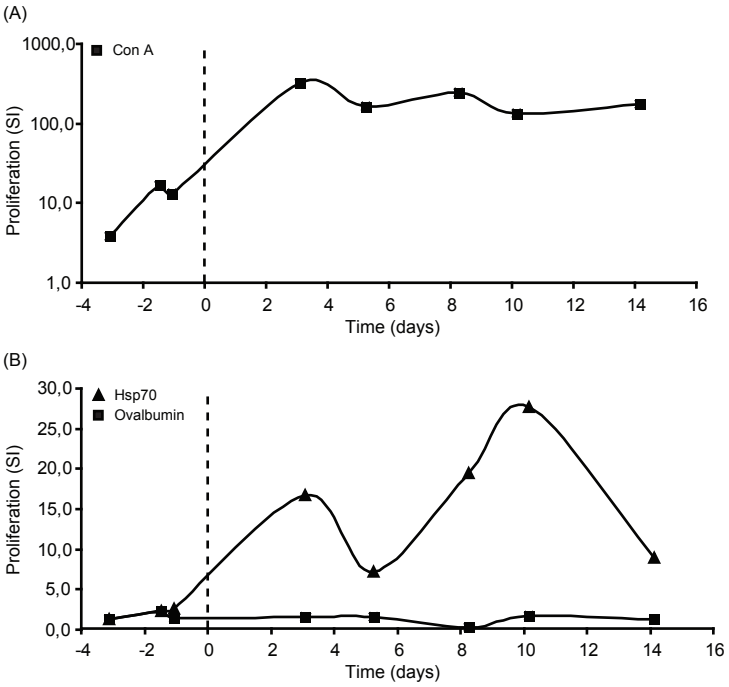

Figure 6 Hsp70 vaccination induces proliferative responses in efferent lymph of the draining prescapular lymph node. (A) Concanavalin A stimulated efferent lymph cells. (B) Hsp70 and Ovalbumin (control protein) stimulated efferent lymph cells. Results are expressed as stimulation index (SI). The vertical dashed line indicates the moment of vaccination with Hsp70 in DDA adjuvant subcutaneously in the draining area of the prescapular lymph node.

Vaccination leads to the introduction of foreign antigen at the site of application, which will be transported either as soluble molecules or by antigen presenting cells to the draining lymph node. Signals are given to attract T and B cells searching for their cognate antigen by interacting with antigen presenting cells in the draining lymph node. We showed that Hsp70 vaccination changed the cellular composition of efferent lymph. There was a significant increase of B cells, accounting for up to 40% of efferent lymph cells post-vaccination. These cells included naive B cells, evidenced by low Ki67 proliferation marker expression (Wirths and Lanzavecchia, 2005), not activated by their cognate antigen. From day three after vaccination, with a peak at day five, there was a marked increase of proliferating B cells, which can be both memory B cells and plasma blast cells (Wirths and Lanzavecchia, 2005). This correlated with the appearance of Hsp70-specific antibody-secreting cells in efferent lymph, which can be both plasma blasts and plasma cells, from seven days after vaccination until the end of the observation period (14 days). They leave the lymph node towards the periphery, most likely the bone marrow, where they exert their function of antibody production (Lanzavecchia and Sallusto, 2009; Radbruch et al., 2006). In a previous study in cattle the occurrence of plasma cells in peripheral blood was restricted to 7-8 days after primary immunization with ovalbumin, as a model antigen (Lefevre et al., 2009). The expression of Ki67 on B cells returned to baseline within 14 days after vaccination, indicating that the

primary B cell response has produced its majority of memory and plasma cells within two weeks.

In efferent lymph Hsp70-specific antibodies appeared from seven days after immunization. Hsp70-specific IgM antibodies return to baseline within 14 days, indicating class-switching within the first two weeks after vaccination. Serum antibodies were measured at day 18 after immunization, although efferent lymph from the prescapular lymph node was continuously collected and removed from the circulation. This indicates that the transport of antigen from the injection site is not exclusively limited to the prescapular lymph node, the primary draining lymph node. Accessory lymph nodes present in the neck region are likely also involved in the post-vaccination immune response. Serum Hsp70-specific antibody responses were similar as observed in previous studies (Koets et al., 2006; Santema et al., 2009).

From previous studies we found that vaccination of cattle with Hsp70/DDA induced limited Th1 type immune responses in peripheral blood (Hoek et al., 2010; Koets et al., 2006; Santema et al., 2009). In this study we showed that efferent lymph cells, leaving the lymph node that drains the area where the vaccine was applied, also produced limited Hsp70-specific IFN-gamma. Furthermore, detection of IFN-gamma directly in lymph, which can originate from the lymph node or from lymph cells, showed non-sustained IFN-gamma production. Also the lack of vaccination-induced IL-12, which can stimulate production of IFN-gamma, adds to the observations that Hsp70 vaccination does not induce Th1 driven IFN-gamma responses. We did measure increased Ki67 proliferation in CD45RO+ CD4+ T cells after vaccination, indicating that memory T cell responses are induced. Additional analysis should look into the generation of Hsp70-specific memory T cells, which can be measured by a cultured ELISPOT assay (Vordermeier et al., 2009). Collectively, this does support earlier observations that Hsp70 vaccination does not induce a robust IFN-gamma+ Th1 T cell response.

The main immunological outcome of the Hsp70/DDA subunit vaccination is a Th2 type immune response with abundant Hsp70-specific IgG1 antibody production. This warrants the question if Hsp70-specific antibodies can contribute to the observed protective effect of Hsp70 vaccination in calves. We showed that Hsp70 is present in the cell wall of MAP and accessible to Hsp70 vaccination-induced antibodies (Santema et al., 2011). After the release of MAP from the infected host cell and prior to infection of new cells, antibodies can interact with bacteria in the extracellular fluid, and possible alter their cellular fate by increasing lysosomal degradation. One of the mechanisms could be Fc receptor engagement by antibodies, which renders the host cell non-permissive for bacterial replication and targets the pathogen to lysosomes (Joller et al., 2010). Passive transfer studies with Hsp70-antibodies could elucidate their role in protection against MAP.

In conclusion, efferent lymph vessel cannulation is an elegant way of analyzing primary immune responses in cattle. If more tools become available for defining memory T and B cells in cattle, this technique could help elucidating the complex interaction between vaccine composition and subsequent quality and breadth of the generated immune response, and assess correlates of protection. The analysis

of the immune response in the primary draining lymph node following Hsp70/DDA vaccination showed that a short-term transient IFN-gamma response is generated in some animals. The main post-vaccination adaptive immune response is a long term IgG1 antibody response confirming previous observations.

Acknowledgements

The authors thank Thijs van Loon for expert support with anesthesia during the surgical procedures; Frans van Kooij for providing general assistance with the surgical procedures; and the animal caretakers of the Department of Farm Animal Health for expert pre-and post-surgical care of the calves.

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Chapter 6

Correlates of protection in cattle naturally and chronically infected with *Mycobacterium avium* subspecies *paratuberculosis*, therapeutically vaccinated with recombinant Hsp70

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Manuscript in preparation

Abstract

The control of important chronic bacterial infectious diseases with high prevalence in endemic areas, including paratuberculosis in cattle, would strongly benefit from availability of post-exposure vaccines. For mycobacterial infections, such as tuberculosis, absence of experience in natural hosts, and safety concerns due to adverse effects in mouse models hamper their development. Paratuberculosis in cattle is both an important mycobacterial disease in ruminants worldwide and a natural host model for mycobacterial infections in general. The present study showed beneficial effects of therapeutic vaccination in cattle, naturally and chronically infected with *Mycobacterium avium* subspecies *paratuberculosis* (MAP), with recombinant MAP heat shock protein 70 (Hsp70). Vaccination-induced protection was associated with antibody responses, rather than with induction of specific T helper 1 cells. Targeted therapeutic post-exposure vaccination complementary to selective use of antibiotics could be an effective approach for control of paratuberculosis and other mycobacterial infections. Based on high Hsp70 amino acid sequence conservation between mycobacterial species, and immunopathogenic parallels, this post-exposure immunization approach may also prove of interest for tuberculosis control in cattle and humans.

Text

Pre-exposure vaccination is the key preventive measure against many infectious diseases. For high-endemic areas, natural disaster areas, emerging infections, and diseases where vaccine efficacy is waning, there is however a need for reliable post-exposure vaccines (1-3). Post-exposure immunoprophylaxis is an accepted approach in a limited number of (mostly viral) infections, exemplified by rabies in humans (4). For bacterial pathogens there is little experience with post-exposure vaccination. Despite increasing occurrence of multidrug resistant strains, antibiotics are still the mainstay post-exposure treatment for bacterial infections such as tuberculosis, brucellosis, anthrax, plague and Q-fever.

Transmission of infection through early exposure to pathogens from environmental sources and/or subclinical carriers is an important obstacle for classical preventive vaccination, notably in case of mycobacterial infections (5-7). This applies to important human pathogens (*Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Mycobacterium avium*) as well as pathogens of food producing animals and wildlife (*Mycobacterium bovis*, *Mycobacterium avium* subspecies *paratuberculosis*). Therefore control of especially this group of pathogens has a particular need for vaccines that can be administered to already exposed individuals, to reduce clinical disease and possibly population transmission. For bovine paratuberculosis a bacterin vaccine has been used that slows progression to the clinical stage of the disease, but has no effect on infection and shedding of bacteria into the environment (8). In addition, the use of this vaccine interferes with bovine tuberculosis control and its usefulness is therefore limited (9). The current tuberculosis vaccine BCG (bacillus Calmette-Guérin) has limited efficacy against pulmonary tuberculosis infection (10, 11). Furthermore, there are safety concerns regarding vaccination of exposed individuals with BCG, because vaccination of tuberculosis infected mice was shown to cause IL-17-dependent pathology (12).

Mycobacterial infections of cattle provide good models for human tuberculosis (13-15). Cattle are within the natural host range of several pathogenic mycobacteria (*M. bovis*, *M. tuberculosis* and *M. avium* spp. *paratuberculosis* (MAP)). Similarities between bovine and human mycobacterial infections relate to infectious dose, pathology, prolonged latency periods, clinical wasting disease and immune response (13-15). Cattle studies with tuberculosis require experimental infection to limit variation in infection status between animals, because the moment of exposure in natural infected animals can not reliably be assessed. Furthermore, they require BSL-3 facilities, which limit the number of animals per study. For those reasons, a related pathogenic mycobacterial infection of cattle, called bovine paratuberculosis, which causes chronic granulomatous enteritis and transmits via shedding of bacteria in feces (16) is a more appropriate animal model. As this disease is endemic and highly prevalent in cattle populations worldwide, naturally infected individuals are readily available for post-exposure vaccine trials. Furthermore, animals younger than 6 months are most susceptible to infection; clinical signs do not develop for 4 to 5 years post infection. As a result, naturally chronically infected individuals have had a much more synchronic exposure compared to bovine tuberculosis exposed individuals. It is essential that new tuberculosis vaccines are evaluated in natural hosts. There has recently been

evaluation of pre-exposure vaccines in experimental challenge models in natural hosts (17-19). However, we evaluate a post-exposure vaccine in a natural host with natural infection to mimic field conditions as closely as possible.

We studied the efficacy of an Hsp70 subunit vaccine candidate (20) in cattle chronically infected with MAP. Two vaccine efficacy parameters were assessed, namely reduction of fecal MAP shedding and survival. The cattle selected for this study were all naturally infected in the field and had documented shedding of MAP via feces. The study design included two vaccination groups that differed in the primary vaccination scheme (G2: day 28; G3: day 0 and 28), which both received additional vaccinations at day 112 and 196, and a control group of 15 animals each. In view of antigen-specific immune suppression known to occur in the course of chronic mycobacterial infections (21, 22), particular care was given to control for the effect of known confounding factors, like age and mycobacterium-specific T and B cell responses, in stratification of animals into homogeneous groups (Table 1). Experimental groups created did not show significant differences in age, MAP-specific antibody ELISA response, and mycobacterial antigen-specific T cell proliferation.

Fecal shedding was used as a proxy for disease activity in the small intestine, the primary locus of infection. The level of fecal shedding of MAP was used as a first measure of vaccine efficacy. Before the start of the vaccination (6 or 7 time points per animal) no significant differences in the percentage of negative MAP fecal cultures were observed between the vaccinated groups and the control group (Table 2). After the start of vaccination animals were followed for a maximum of 15 time points with three weeks intervals (45 weeks in total). The proportion of negative fecal cultures was higher in both Hsp70/DDA vaccinated groups as compared to controls, and for G2 (3x vaccinated) this effect was statistically significant ($p=0.02$). This indicates that Hsp70 vaccination likely has direct effects on the chronic intestinal inflammation and subsequent shedding of mycobacteria in feces. However, there is no direct explanation for the differences observed between the two vaccinated groups.

In addition, the contribution of vaccination to survival was assessed. In total five (33.3%) control animals and two (13.3%) vaccinated animals in each group reached predetermined humane endpoints due to end stage clinical signs of paratuberculosis. Survival analysis did not indicate these differences to be statistically significant between groups, which can partly be explained by the limited number of animals and events (Fig 2A).

Hsp70 vaccination in chronically infected cattle induced antibody titers. In contrast to previous experiments with uninfected animals (20, 23), considerable variation in the magnitude of antibody titers was observed (Fig 1A). Control animals had limited Hsp70-specific antibodies, which fits previous observations in naturally infected animals (24). Vaccination did not induce or boost Hsp70-specific interferon-gamma (IFN-gamma) responses in peripheral blood, although circulating IFN-gamma positive T cells were present in a subset of animals, irrespective of vaccination status (Fig. 1B).

Table 1 Stratification parameters. Animals were assigned to three experimental groups (N=15). Mean values (+SD) are shown for age, MAP-specific antibody ELISA (Pourquier) and mycobacterial-specific lympho-proliferative responses. No significant differences between the groups were found as analyzed by ANOVA for all parameters. S/P: sample to positive ratio; SI: stimulation index; PPDA (tuberculin from Mycobacterium avium spp. avium); PPDB (tuberculin from M. bovis).

	G1: Non-vaccinated	G2: Vaccinated (3x)	G3: Vaccinated (4x)
Age (months)	62.60 (15.16)	61.47 (17.37)	55.13 (17.54)
Pourquier ELISA (S/P)	239.00 (32.92)	229.07 (61.55)	207.13 (99.23)
PPDA (SI)	8.67 (7.49)	6.23 (3.42)	7.86 (10.89)
PPDB (SI)	4.53 (4.49)	3.03 (1.50)	5.68 (9.60)

Table 2 MAP fecal culture results. The number of negative fecal cultures (FCneg) for MAP is shown in the periods before and after the start of vaccination for the control group and two vaccination groups. Animals were vaccinated with recombinant MAP Hsp70 in DDA adjuvant. Groups were compared with a logistic regression model. The p-values indicate the comparison of the vaccinated groups with the control group after the start of vaccination.

	Before vaccination			After vaccination			
Group	FC^{neg}	N	Percentage	FC^{neg}	N	Percentage	P-value
G1: Control	3	100	3,00%	14	171	8,19%	
G2: Vaccinated (3x)	4	101	3,96%	33	189	17,46%	0.02
G3: Vaccinated (4x)	4	88	4,55%	30	220	13,64%	0.14

Some vaccinated animals were not able to produce high Hsp70-specific antibody titers. To pinpoint the background of this non-responsiveness to vaccination, immune responses were characterized to find evidence for a potential role of disease-induced immune suppression. There were no differences between low-antibody responders and high-antibody responders in their ability to produce antibodies against a viral vaccine, administered at the start of the experiment (Fig. 1C) (see M&M), and both groups did not significantly differ from the control group and the animals that reached end stage clinical paratuberculosis in the course of the observation period. Furthermore, there were no differences between antibody responder types in their lymphocyte proliferation responsiveness towards a complex mycobacterial antigen (PPDA) (Fig. 1D). This indicated that these low-antibody responders were not likely to have a general immune suppression, and also were still capable to mount specific anti-mycobacterial responses, but their ability to mount a robust Hsp70-specific antibody response was limited in an antigen-specific manner.

Given the observation that some animals were less able to mount Hsp70-specific antibodies, the major immune parameter associated with protection in our previous study, one could argue that in these low responders less vaccine efficacy is expected, because antibodies might be (the major) part of the protective response. To test this hypothesis, the vaccinated animals were divided into low and high-antibody responders based on the median value of the Hsp70-specific antibody level (Fig. 1A). Subsequently, the effect of responder types on vaccine efficacy parameters was compared with the control animals, under the assumption that there is no strong correlation between the inability to produce Hsp70-

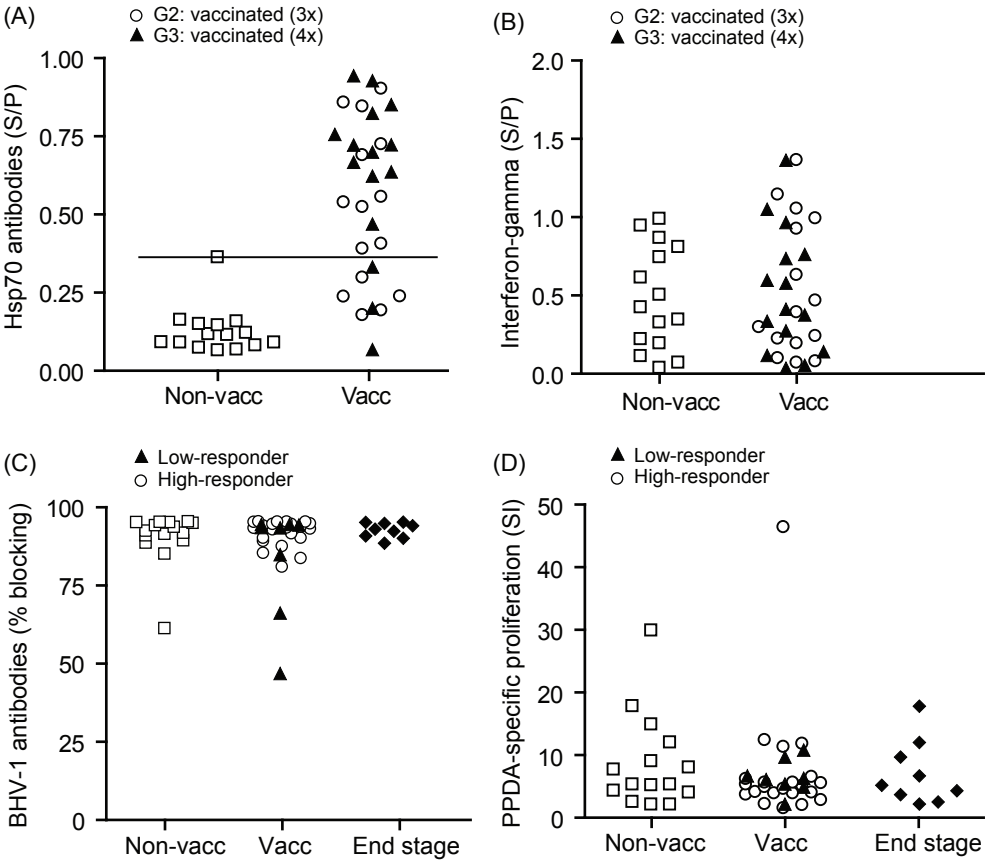

Figure 1 Hsp70 vaccination induces a variable antibody response in cattle with natural chronic paratuberculosis infection, but no Hsp70-specific IFN-gamma. (A) Hsp70-specific serum antibody response at day 49 after the primary Hsp70/DDA vaccination round. Results are expressed as sample to positive ratio (S/P) for vaccinated animals (open circles for G2 (3x vaccinated; day 28, 112 and 196) and closed triangles for the G3 (4x vaccinated; day 0, 28, 112 and 196), N=15 per group) and non-vaccinated animals (open squares, N=15). Animals were classified into low and high-antibody responders based on the median value (horizontal line) of all animals. (B) IFN-gamma response expressed as S/P ratio for Hsp70-stimulated peripheral blood mononuclear cells at day 49, three weeks after the primary Hsp70 vaccination round for vaccinated and non-vaccinated animals. (C) Antibody responses against bovine herpes virus 1 (BHV-1) glycoprotein B. Results are expressed as percentage blocking of monoclonal antibody binding. All animals were vaccinated against BHV-1 at the start of the experiment. Results are shown for control animals (open squares), vaccinated: low (closed triangle) and high (open circle) antibody responders, and animals that reached end stage clinical paratuberculosis in the course of the observation period (closed diamonds). No significant differences between the groups were measured with a non-parametric Kruskal-Wallis test. (D) PPDA (complex mycobacterial antigen) specific lymphocyte proliferation responses expressed as stimulation index (SI), measured before the start of the vaccination. Results are shown for control animals (open squares), vaccinated: low (closed triangle) and high (open circle) antibody responders, and animals that reached end stage clinical paratuberculosis in the course of the observation period (closed diamonds). No significant differences between the groups were measured with a non-parametric Kruskal-Wallis test.

specific antibodies and disease-induced immune suppression with subsequent disease progression. There is no direct way of assessing this correlation, because non-vaccinated paratuberculosis infected animals have limited Hsp70-specific antibodies. Arguments in favor for our assumption include above mentioned observations, which showed no measurable differences in immune responses between high and low antibody responders for complex mycobacterial and viral antigens at the start of the experiment, close to the moment of defining antibody responder types. High Hsp70-specific antibody levels significantly ($p=0.01$) correlated with reduced fecal shedding as compared to controls. In contrast, fecal shedding in low-antibody responders did not differ from controls. Furthermore, high-antibody responders had a significantly ($p=0.047$, likelihood ratio test) higher survival rate (Cox regression) as compared to controls (Fig 2B). Therefore, the magnitude of the antibody response generated by Hsp70 vaccination was significantly associated with reduced fecal shedding and higher survival, which could indicate that the generation of Hsp70-specific antibodies after vaccination is needed for efficacy.

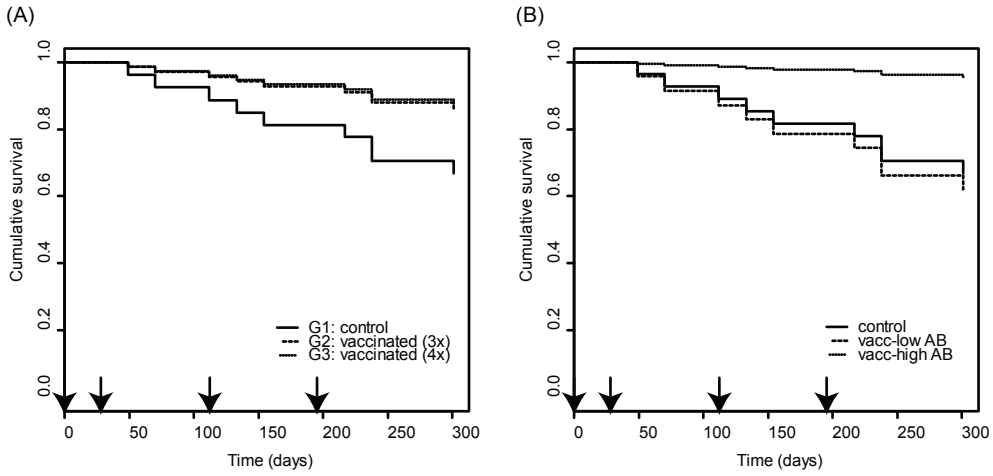

Figure 2 Survival analysis. Animals that reached pre-determined humane end-points due to clinical paratuberculosis were culled. The two vaccination groups (G2 and G3) differed in the number of Hsp70/DDA vaccinations. G2 received a vaccine at day 28, 112 and 196, and G3 at day 0, 28, 112 and 196 (indicated with arrows). (A) The survival rate of the two vaccination groups was not significantly different as compared with the control group. (B) Vaccinated animals were classified into low and high antibody-responders based on the median value of an Hsp70 ELISA (Figure 1A) and their survival was compared with the control group. High antibody-responders had a significantly ($p=0.047$) higher survival rate as compared to non-vaccinated controls. Low-antibody responders did not significantly differ from controls. Survival of groups was compared with a Cox-proportional hazards model.

The composition of the Hsp70 vaccine together with the route of application does not lead to induction of a Th1 type response. Repeated vaccination with Hsp70 did not induce IFN-gamma positive cells in peripheral blood of our MAP-infected animals (Fig 3A). This is similar to what was previously found in uninfected animals (23). The immune response to the bacterium, indicated by PPDA-specific T cell responses, during the course of infection in this experiment was variable between individual animals, but not different between the groups of low or high Hsp70 antibody responders. Therefore, it is expected that these groups are comparable with respect to immune competence and progression of the disease (Fig 3B).

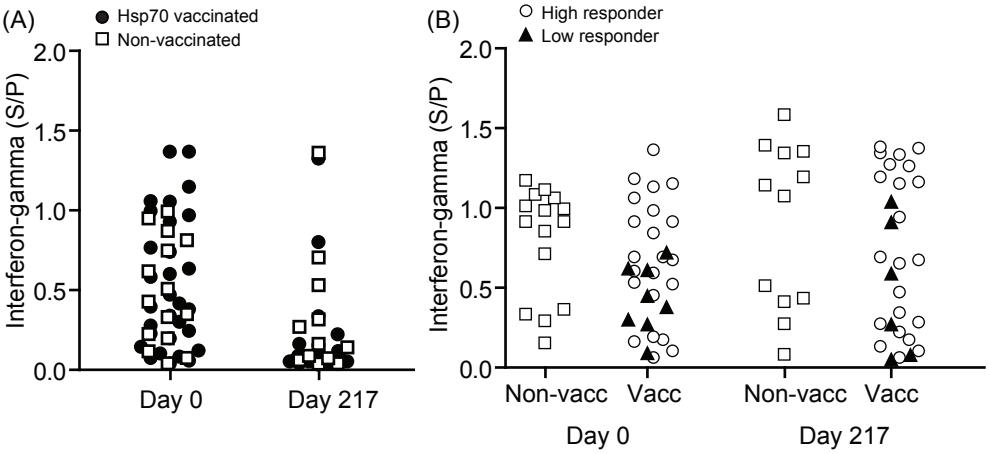

Figure 3 Hsp70 vaccination induces no IFN-gamma responses. (A) Hsp70 vaccination does not induce IFN-gamma responses after repeated applications. Results are shown before vaccination (Day 0) and three weeks after the secondary booster vaccination (Day 217) for non-vaccinated (open squares) and Hsp70 vaccinated (closed circles) animals. (B) PPDA-specific INF-gamma responses expressed for non-vaccinated (open squares), and vaccinated low (closed triangles) or high (open circles) Hsp70 antibody responders did not change in the course of infection between groups. Groups were compared with a non-parametric Kruskal-wallis test. All results are expressed as sample to positive ratio (S/P).

The demonstration of an association between antibodies and vaccine efficacy warrants further study into the exact mechanism of the protective immune response. From our study, vaccination-induced antibodies appear to confer the therapeutic modality of the vaccine. This is in contrast to the current dogma that cell-mediated immunity is the most important defense against intracellular infections. We have previously shown that Hsp70 is present in the cell wall of MAP and accessible for Hsp70-specific antibodies, induced by vaccination (25). MAP infects macrophages and is able to arrest phagosomal maturation to enable intracellular survival similar to Mycobacterium tuberculosis. However, following a stage of replication, bacteria need to escape from their infected host cell to infect new cells. In this process bacteria are present in the extracellular environment and become a target for humoral responses. One defense mechanism could be that Fc receptor engagement of antibodies can protect by altering intracellular trafficking of mycobacteria into lysosomal compartments (26). Moreover, the repeated post-exposure vaccination strategy with Hsp70 led to antigen-specific skewing away

from IFN-gamma driven Th1 type response (Fig 3A). These observations are further supported by the fact that Hsp70 vaccination does not induce IFN-gamma responses in peripheral blood, but instead induces production of IgG1 isotype antibodies, a marker for a Th2 type response (27).

In light of the growing incidence of multidrug resistant mycobacteria it is anticipated that new post-exposure vaccines can be beneficial in a targeted approach complementary to anti-mycobacterial drugs (28). Apart from the beneficial effects for the vaccinated individual, population effects of vaccination need to be considered, most importantly reduction of transmission. We show that vaccination reduced fecal shedding, the natural way of transmission, of mycobacteria in individuals with chronic infection, and improved survival in a subset of vaccinated animals. For bovine and human tuberculosis transmission of infection is related to the presence of individuals with active pulmonary disease. Post-exposure vaccination should ideally limit transmission from active tuberculosis diseased individuals. It is important that the effect on population transmission of post-exposure vaccines, but in fact also of prophylactic vaccines, is evaluated in transmission studies.

Overall, our findings suggest that vaccination of individuals chronically infected by MAP has a therapeutic effect improving longevity of infected individuals. This is the first time that post-exposure (subunit) vaccine efficacy against a mycobacterial infection is shown in a natural host after natural infection. Recently, Aagaard et al. have shown post-exposure efficacy of a subunit vaccine in an experimental challenge model of tuberculosis in mice (29). We did not observe adverse effects associated with repeated post-exposure vaccination at the site of application or systemically in this and previous experiments. The high conservation of Hsp70 between mycobacterial species (94% amino acid sequence identity between MAP and *M. bovis*/*M. tuberculosis*), and similarity in pathogenesis, entails the potential that comparable vaccine-induced protection can be translated to other mycobacterial infections, notably tuberculosis infections of humans. The candidate Hsp70 vaccine has shown dual-action (i.e., has pre- and post-exposure efficacy) in uninfected (20) as well as infected individuals, which for tuberculosis control is the most desired vaccination strategy (28).

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Acknowledgements

We thank Jeroen Hoeboer for technical assistance; Jan van den Broek for statistical advice.

Material and methods

Animals

Animals originated from Dutch dairy holdings and were selected by Animal Health Services (GD, Deventer, the Netherlands) via their regular paratuberculosis testing program. The inclusion criterion for natural chronic paratuberculosis infection was that animals were shedding the bacterium in the feces. In total 59 animals were purchased and evaluated for general health. A total of 14 animals were excluded due to pre-existing lameness, udder infection or poor body condition. The remaining forty-five female cattle (average age at time of inclusion 5.0 years (range 2.6 – 7.8 years)) were used in this study. After arrival at the experimental farm cows were vaccinated against bovine herpes virus type 1 and ringworm (intranasal Bovilis IBR live marker and Bovilis Ringvac, Intervet/Schering-Plough Animal Health, The Netherlands).

The cows were housed together in a conventional animal house, fed according to requirements and checked daily for general health. Local side effects after vaccination were recorded. The decision to cull an animal due to end stage paratuberculosis was blinded and executed by an independent veterinarian. Clinical features of end stage paratuberculosis were cachexia, submandibular oedema, and chronic diarrhea.

The use of animals in the present study was approved by the experimental animal committee of Intervet/Schering-Plough Animal Health and conducted according to existing regulations.

Hsp70 Antigen

Recombinant MAP Hsp70 was produced according to methods described in detail earlier (1). Purity of the recombinant Hsp70 was checked using SDS-PAGE and the endotoxin concentration was below the detection limit of the assay (LAL assay, Pierce).

Experimental design

The animals were assigned to one of three experimental groups of fifteen animals each. Assignment of animals to groups was stratified based on age, and anti-mycobacterial T and B cell responses to PPDA and PPDB, using previously described lymphocyte proliferation assays (4) and antibody ELISA (1). Control cattle were sham-immunized (G1) at D28. Recombinant MAP Hsp70 protein vaccine was formulated as published previously (2). Immunization consisted of subcutaneous administration of Hsp70/DDA, 200 µg 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 neck. Group 2 animals were vaccinated at D28 (G2). Group 3 animals were vaccinated at D0, followed by a booster vaccination at D28 (G3). G2 and G3 received an additional booster vaccination with Hsp70/DDA at D112 and D196.

Heparinized blood was collected aseptically from the tail vein of all animals every three weeks throughout the experiment, starting at –D84 prior to vaccination. Serum samples were taken at the same time points. Fecal samples were collected from the rectum of all animals every three weeks and processed for fecal culture.

The post-vaccination observation period included maximum 15 time points (42 weeks).

Diagnosis of paratuberculosis infection

Diagnosis of paratuberculosis infection was performed by fecal culture in the automated TREK-DS paraJEM ESP culture system. Samples were processed according to manufacturers' protocol and incubated for 42 days. After the incubation time all the samples were processed for Ziehl-Neelsen staining of culture broth and bacterial growth was confirmed to be MAP based on PCR for the specific IS900 insertion sequence (5).

Hsp70 specific serology

Serological responses to recombinant MAP Hsp70 protein were measured using an ELISA technique as described previously (3).

IBR gB ELISA

Glycoprotein-B-specific antibodies to bovine herpesvirus-1 were detected in bovine serum with the HerdCheck IBR gB ELISA test kit (Idexx, USA).

Antigen-specific INF-gamma ELISA

Antigen specific INF-gamma responses were measured using the whole blood culture Bovigam® assay (Prionics, Switzerland) according to instructions provided by the manufacturer. In brief, 1.5 ml heparinised whole blood was incubated with bovine and avian tuberculin antigens (PPDA, PPDB) in a 24 well tissue culture plate for 24 hours in a humidified incubator at 37°C. Nil antigen (PBS) was used to determine spontaneous release of INF-gamma in the blood culture. Subsequently the supernatant plasma was collected and stored at -20°C until analysis. In addition, recombinant MAP Hsp70 was used as antigen at a final concentration of 20 µg/ml. The production of bovine INF-gamma was measured using a monoclonal antibody-based sandwich enzyme immunoassay. Results were expressed as S/P ratio calculated as OD450(antigen stimulated plasma)/ OD450(positive control plasma).

Statistical analysis

Fecal shedding was analyzed with generalized linear models, available in R statistical software (2.10.1, www.R-project.org). The dependent variable was the proportion negative fecal culture samples relative to the total number of samples per animal in the post-vaccination observation period (maximum 15 time points). Variability in shedding pattern was corrected for by inclusion of a fixed factor that described whether animals had a negative fecal culture in the pre-vaccination observation period (six or seven time points). The first model evaluated the question if the original experimental groups influenced fecal shedding, which included group as fixed factor. The second model evaluated the question if low or high Hsp70 antibody responders in the vaccinated animals influenced fecal shedding levels as compared to controls. Models were selected based on lowest AIC.

The stratification parameters were compared with ANOVA. BHV-1 antibody responses, IFN-gamma and lympho proliferative responses were analyzed with a non-parametric Kruskal-Wallis test.

Survival was analyzed by a Cox proportional hazard model, available in the survival package of R statistical software (2.10.1). An event was defined as the moment an animal reached predetermined humane end points, which was observed by an independent, blinded veterinarian. Explanatory factors included the original experimental groups and in a subsequent model the Hsp70 antibody responder types. Age, as potential confounding factor, did not significantly impact the outcome. The best model fit was selected based on lowest AIC.

The level of significance was set at $p<0.05$.

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Chapter 7

Summarizing discussion

Summarizing discussion

In this thesis data are presented that support the development of Hsp70/DDA as a subunit vaccine for paratuberculosis. To increase our knowledge on the Hsp70/DDA vaccination-induced immune response three main research topics were addressed. First, potential interference of vaccination with diagnosis of tuberculosis (TB) and paratuberculosis was evaluated. Second, the efficacy of Hsp70/DDA as post-exposure vaccine was assessed in chronic naturally infected paratuberculosis cows. Lastly, immune responses induced by Hsp70/DDA were analyzed to search for correlates of protection.

1. Hsp70 vaccination and interference with diagnosis of bovine TB and paratuberculosis

Regulatory authorities demand proven efficacy and safety of candidate vaccines prior to licensing. As an additional requirement new paratuberculosis vaccines should not interfere with diagnostic assays for bovine tuberculosis. In other words, paratuberculosis vaccination should not compromise interpretation of diagnostic tests for bovine tuberculosis, by influencing either test sensitivity or specificity. Lower sensitivity could arise if for example a comparative skin test is used, comparing responses against PPDB (crude antigen from *Mycobacterium bovis*) and PPDA (crude antigen from *Mycobacterium avium* subspecies *avium*). If PPDA responses are increased by paratuberculosis vaccination, the difference will be smaller and fewer TB infected animals will be identified. This is important in countries with endemic bovine tuberculosis, for example the United Kingdom. Lower specificity could relate to the presence of cross-reactive antigens in PPDA and PPDB, shared with proteins in MAP. Immunization with MAP antigens could prime responses against cross-reactive antigens. Recall of responses in skin testing by injection of tuberculins will then lead to skin induration and false positive read outs. This may compromise TB testing in tuberculosis-free countries.

In chapter 2 we evaluated whether our candidate paratuberculosis vaccine interferes with current immunodiagnostic tests for bovine tuberculosis and paratuberculosis. Vaccination with recombinant MAP Hsp70 in DDA adjuvant did not result in priming of immune responses influencing skin test outcome in TB-negative animals. In contrast, vaccination with an inactivated MAP bacterin did lead to pronounced skin swelling after injection of tuberculins and can compromise tuberculosis diagnosis. The specificity of the skin test is not altered in Hsp70 vaccinated animals. Therefore we conclude that immunizing tuberculosis-free cattle with our candidate paratuberculosis vaccine does not compromise diagnostic assays for bovine tuberculosis. Additional studies will have to evaluate the effect of this vaccine on the sensitivity of diagnostic assays for bovine tuberculosis in tuberculosis-exposed/infected animals. However, based on the limited induction of skin swelling in Hsp70 vaccinated animals, it is anticipated that the effect on sensitivity is limited.

Diagnosis of paratuberculosis, based on measurement of antibodies directed to components of crude MAP extracts, present in current commercially available diagnostic kits (Pourquier, ID-Vet), is compromised when animals are vaccinated

with Hsp70 or MAP bacterin. However, these kits can be adapted by inclusion of Hsp70 in the pre-absorption buffer to remove Hsp70-specific antibodies. In this way Hsp70 vaccinated animals stay negative in the paratuberculosis ELISA, allowing vaccinated animals to be differentiated from infected animals. This approach does not work for MAP bacterin vaccinated animals as they remain false positive in the adapted Hsp70-pre-absorbed antibody test.

Both from the point of view of vaccine development, and from the perspective of improving diagnostic assays, insight in the protein composition of tuberculins is crucial. In chapter 3 individual proteins of mycobacterial tuberculins, identified by proteomics techniques, were described. Diagnostic assays need specific MAP antigens to improve their performance. However, the identification of specific antigens is challenging. Although MAP-specific proteins were identified in PPDP, when evaluated in immunoassays, they showed limited antigenicity. One of the explanations could be that these specific proteins, compared to proteins that are conserved between mycobacterial species, have lower expression levels. In follow-up studies the relative abundance of proteins in multiple batches of tuberculins was determined. We hypothesized that more abundant proteins are more likely to interact with the immune system. For PPDB the most abundant proteins are ESAT-6 (early secretory antigenic target 6 kDa) and CFP-10 (culture filtrate protein 10 kDa). These are the most promising candidates for improvement of tuberculosis cellular diagnostic assays. Furthermore, these proteins are not present in the tuberculosis vaccine BCG (bacillus Calmette-Guérin), and can be used to discriminate infected from BCG vaccinated individuals. The most abundant proteins in PPDP (MAP) are Hsp70 and Hsp65. However, these proteins are highly conserved between mycobacterial species, and therefore not suitable as specific antigen for diagnostic assays.

Alternatives for the skin test are searched for, also in the light of the development of new tuberculosis vaccines. The use of these vaccines, for example modified BCG, asks for diagnostic tests that can distinguish between infected and vaccinated animals. Research is focused at understanding which antigens are good candidates for diagnostic assays, and ideally there are other antigens suitable to include in novel vaccines to induce protection. Recent developments have been directed at the generation of a new skin test reagent that identifies *Mycobacterium bovis* infected animals, but not BCG vaccinated animals (Whelan et al., 2010a). Other approaches look at the development of a multiplex antibody-based test for bovine tuberculosis, most suitable to be implemented in control programs (Whelan et al., 2010b). A comprehensive study profiling antibody responses in human tuberculosis showed that there is a small number of proteins (0,5% of the total proteome) that can identify humans with active disease (Kunnath-Velayudhan et al., 2010). Bringing these profiling studies to the field of paratuberculosis could accelerate the development of more accurate diagnostic tests. A very promising initiative to mention in this respect is the production of recombinant MAP proteins by Bannantine et al. So far, they have produced over 700 MAP proteins in *E. coli* (Bannantine et al., 2010), which will be screened for immunogenicity in cellular and antibody assays. One of the drawbacks of recombinant protein production in *E. coli* is the absence of some post-translational enzyme modifications, like

glycosylation. It has been shown for lipoproteins that particular enzymatic modifications are necessary to stimulate T cell responses (Sieling et al., 2008). Alternative recombinant protein production systems, which use *Mycobacterium smegmatis* as host, may result in proteins with the proper enzymatic modifications.

In summary, it was shown that Hsp70/DDA vaccination does not interfere with diagnosis of bovine tuberculosis, an important prerequisite for licensing of new paratuberculosis vaccines. Although Hsp70 is a T cell antigen in naturally paratuberculosis infected animals, and abundantly present in PPDs, the vaccination-induced immune response did not prime T cells which play a role in the tuberculin skin test. This discrepancy between infection-induced and vaccination-induced immune responsiveness stresses the necessity to perform these vaccination experiments in the target animal.

2. Hsp70 vaccination: role for a post-exposure vaccine

It has long been recognized that paratuberculosis control is in need of an effective vaccine. Efficacy of paratuberculosis vaccines can be evaluated in different ways, depending on the goal to be achieved. These goals can be defined for the individual animal and the animal population.

For individual animals the best option would be a vaccine that induces sterile immunity, in other words animals clear infection or don't get infected at all. However, this is not a realistic scenario based on past experiences with vaccine development for chronic infectious diseases (Kaufmann, 2007). A more feasible scenario would be a vaccine that controls infection in the individual animal and prevents progression to the clinical stage of paratuberculosis. The whole-cell vaccines available offer this kind of protection, but they do not prevent infection and do not lead to sterile immunity (Kalis et al., 2001; Sweeney et al., 2009; Uzonna et al., 2003; Wentink et al., 1994). Furthermore, they have limitations as discussed in the introductory chapter. Therefore, new candidate vaccines should be tested for efficacy.

As there is a long latency period before infected animals progress to the clinical phase, studies to evaluate reduction in clinical disease have a long duration. An alternative approach would be to use tissue colonization or fecal shedding as markers for disease development. However, whether short-term experimental infection models could predict efficacy in clinical disease prevention is a question that remains to be answered. Experimental challenge of calves with MAP leads to a reproducible infection (Hines et al., 2007; Koets et al., 2006; Stabel et al., 2009; Stewart et al., 2007; van Roermund et al., 2007). The first peak of fecal shedding of MAP starts at 3 months after challenge in a high proportion of animals. After 5 months the frequency of positive fecal culture samples declines and for the remainder of the follow-up period animals intermittently shed bacteria in the feces. There is a high correlation between fecal shedding and post-mortem confirmed MAP infection (unpublished observations). Previously it was shown that Hsp70 vaccination reduced fecal shedding in calves experimentally infected with MAP during two years of observation (Koets et al., 2006). In this experiment animals were vaccinated concurrent with experimental challenge to mimic field conditions.

Since paratuberculosis is an endemic disease on infected farms and MAP is able to survive in the environment for long periods of time, susceptible animals are born in a contaminated environment. Exposure with MAP can take place early after birth, before a classical pre-exposure vaccine can induce protective immunity against paratuberculosis. Hence, a more realistic scenario is the use of a vaccine in already exposed individuals, immunologically primed against MAP, so-called post-exposure vaccination.

To mimic field conditions as closely as possible we evaluated in chapter 5 whether Hsp70 provides efficacy as a post-exposure vaccine in chronic naturally infected adult cows. Vaccine efficacy showed to be correlated with vaccination-induced Hsp70 antibodies. Hsp70 vaccination reduced fecal shedding and improved survival in Hsp70-specific high-antibody responders, while no vaccination-specific Th1 responses were induced. These results are not only relevant for paratuberculosis control, but may also have a broader application to the field of tuberculosis. There are considerable similarities in disease pathogenesis, and Hsp70 is highly conserved between mycobacterial species. A post-exposure vaccine for human and bovine tuberculosis can have considerable impact on prevention of disease exacerbation, and can be given in conjunction with complementary antibiotics (Young and Dye, 2006). Recently, it was shown that a subunit vaccine composed of a hybrid protein antigen protected against TB when applied as post-exposure vaccine in an experimental mouse model (Aagaard et al., 2011). Interestingly, the main immunological immune response was a potent cellular response, which is the opposite of what can be observed with Hsp70/DDA. This opens up the possibility that different arms of the immune system can be exploited to induce a protective immune response against mycobacterial infections.

Next to vaccine efficacy in the individual, the population would benefit from a vaccine that limits transmission of infection. The basic reproduction ratio (R0), defined as the total number of secondary cases caused by a single infected individual during its entire infectious period in a susceptible population, is frequently used to quantify the transmission of infection (Diekmann and Heesterbeek, 2000). If the reproduction ratio is above one, transmission of infection can sustain in the population. A vaccine should ideally lower the reproduction ratio below 1, to prevent major outbreaks and enable eradication of infection. Limited information is available about the reproduction ratio of paratuberculosis infection in cattle. Van Roermund et al. calculated reproduction ratios for cow-calf and calf-calf transmission after experimental infection of adult cows (van Roermund et al., 2007), 2.7 and 0.9 respectively. In future experiments, efficacy of the candidate Hsp70 vaccine, as well as other candidate vaccines, on transmission of infection between vaccinated animals should be assessed. This information is particularly useful for implementation of a vaccination strategy in regional or national control strategies.

In summary, we now have shown efficacy of Hsp70/DDA vaccination in experimentally challenged calves (Koets et al., 2006), and also in chronic, naturally paratuberculosis infected animals. The next step would be to evaluate this candidate vaccine in a field study.

3. Hsp70 vaccination-induced immune responses and correlates of protection

For vaccine development it is important to define correlates of protection, which can be defined as a specific immune response to a vaccine that is closely related to protection against infection or disease (Plotkin, 2008). When a correlate of protection is known more effective vaccine candidates can be screened by focusing on the induction of the highest level of the correlate of protection. Most vaccines, mostly against viral pathogens, are effective based on the induction of specific antibodies. The definition of correlates of protection for cellular responses, either effector memory or central memory responses, are still under development. For paratuberculosis, as well as other mycobacterial diseases, no correlates of protection have been defined to date. As we showed protective effects of Hsp70/DDA vaccination, it would be interesting to look for Hsp70 vaccination-induced immune response that could indicate protective responses.

Earlier work has described the Hsp70 vaccination-induced T cell response (Hoek et al., 2010), which can be summarized as a short term Th1 response, that can not be boosted to prolonged activity by subsequent vaccinations. In chapter 3 the B cell response after Hsp70/DDA vaccination was studied. Hsp70 is present in the cell wall of MAP and accessible to vaccination-induced antibodies. In chapter 4 the primary immune response generated after Hsp70 vaccination in the draining lymph node was studied by cannulation of efferent lymph vessels. It was shown that the B cell response is the dominant immune response, with the generation of memory B cells and antibody-secreting cells. Taken together, this supports indications that antibodies may contribute to the observed protection seen after Hsp70 vaccination by interacting with bacteria during the course of infection.

The role for antibodies in intracellular mycobacterial infections has recently gained more attention (Glatman-Freedman, 2006; Maglione and Chan, 2009). In spite of the fact that most research focuses on cellular immunity against intracellular infections, it has been shown that antibodies can confer protection against mycobacterial infection (Balu et al., 2011; Pethe et al., 2001; Teitelbaum et al., 1998). Multiples modes of action for antibodies have been described, such as neutralization, complement activation, and more recently Fc receptor engagement (Joller et al., 2010). We hypothesize that, especially after the release of MAP from the infected host cell and prior to infection of new cells, antibodies can interact with bacteria in the extracellular fluid. Future passive transfer studies could elucidate a specific role for Hsp70-specific antibodies in protection against paratuberculosis.

Our finding that antibodies are important in the battle against chronic intracellular mycobacterial infection challenges the current dogma that cellular responses are most effective. Candidate vaccine antigens are frequently selected based on their induction of IFN-gamma responses in the course of mycobacterial infection. As these immune responses are the result of complex interactions between bacterium and host, part of this response can be driven by the bacterium to subverse the immune system generating the most effective immune response. As during natural paratuberculosis infection marginal Hsp70-specific antibodies are measured, the

vaccination-induced Hsp70-specific antibodies and their interaction with MAP may tip the balance towards a more effective immune response.

Concluding remarks

The goal of this thesis was to evaluate recombinant MAP Hsp70/DDA as a candidate paratuberculosis subunit vaccine in cattle. It was shown that vaccination with Hsp70 does not interfere with diagnosis of bovine tuberculosis, although Hsp70 is an abundant protein identified in tuberculin. Antibody-based assays for paratuberculosis can be adapted by inclusion of Hsp70 in the pre-absorption step to remove cross-reactive proteins and thereby differentiating infected animals from vaccinated animals. Analysis of the Hsp70 vaccination-induced immune response revealed that the B cell response was dominant above Th1 reactivity. Vaccination-induced antibodies can bind Hsp70 in the cell wall of MAP, and provide a possible explanation for the observed protection in calves.

Lastly, vaccination of chronic naturally MAP infected cows resulted in reduced fecal shedding and prolonged survival, which correlated with vaccination-induced Hsp70 antibodies. Combined with previous vaccination studies in calves, the candidate Hsp70 vaccine has shown dual action (both pre- and post exposure efficacy), which for paratuberculosis control, and the same holds true for TB, is the most desired vaccine strategy.

Overall, these results show beneficial effects of Hsp70 as a candidate paratuberculosis subunit vaccine, which warrants further research into the clinical development of this candidate vaccine.

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Nederlandse samenvatting

Introductie

Infectieziekten zorgen voor voortdurende bedreigingen voor de volks- en diergezondheid. Na de ontdekking van micro-organismen als verwekkers voor vele ziekten, aan het eind van de 19de eeuw door Robert Koch en anderen, is de kennis over het verloop, de behandeling en preventie van infectieziekten sterk toegenomen. Desondanks bevinden infectieziekten zich bovenaan de lijst van belangrijke ziekten in dierlijke productie systemen. Ze zorgen niet alleen voor een lagere economische opbrengst, maar ze zijn ook van invloed op dierwelzijn en in een aantal gevallen zijn ze in staat om ziekte bij de mens te veroorzaken (zoönose). Sommige ziekten kunnen voorkomen worden door vaccinatie (bijvoorbeeld Mond en Klauwzeer en Bovine Virus Diarree), maar voor andere, in het bijzonder voor chronische infecties, is het ontwikkelen van effectieve vaccins ingewikkeld gebleken.

Het in dit proefschrift beschreven onderzoek concentreert zich op de bestrijding van paratuberculose bij het rund door vaccinatie. Paratuberculose is een wereldwijd veelvoorkomende chronische infectieziekte bij herkauwers, die mogelijk een rol speelt in de ziekte van Crohn bij de mens. Dit heeft geleid tot maatregelen om de blootstelling van de mens via zuivelproducten aan deze bacterie te beperken. Voor een effectieve bestrijding van paratuberculose is een vaccin voor gebruik in runderen gewenst, maar op dit moment niet beschikbaar. In dit proefschrift wordt een kandidaat vaccin voor paratuberculose geëvalueerd.

Paratuberculose – de ziekte

Paratuberculose is een chronische darmziekte van herkauwers, die wordt veroorzaakt door de bacterie *Mycobacterium avium* subspecies *paratuberculosis* (kortweg MAP). MAP behoort tot de familie van mycobacteriën, waaronder ook de verwekker van rundertuberculose, *Mycobacterium bovis*, valt. Een belangrijk kenmerk van MAP is dat deze bacterie in staat is om lang (> 1 jaar) te overleven in de omgeving en van daaruit dieren kan besmetten.

Jonge dieren zijn het meest gevoelig voor het oplopen van een infectie met MAP vanuit een besmette omgeving. MAP infecteert darmweefsel. Besmette dieren komen vervolgens in een subklinische fase van de infectie, gekenmerkt door het afwezig zijn van uiterlijke verschijnselen van ziekte, die een aantal jaren kan duren. In deze periode vindt er wel uitscheiding van de MAP bacteriën plaats via de mest naar de omgeving en kunnen andere dieren besmet raken. Sommige besmette dieren ontwikkelen klinische paratuberculose, meestal op een leeftijd tussen 3 en 5 jaar. Deze fase wordt gekenmerkt door wisselende/chronische diarree, sterke vermagering ondanks goede eetlust en oedeem (vochtophoping) tussen de kaaktakken. Op darmniveau wordt een uitgebreide chronische ontsteking waargenomen, waardoor de opname van voedingsstoffen is verstoord en er lekkage van eiwitten vanuit het lichaam naar de mest plaatsvindt. Er is geen therapie beschikbaar en deze dieren sterven uiteindelijk door uithongering.

Paratuberculose - vaccinatie

Bescherming tegen ziekteverwekkers, waaronder MAP, kan worden opgewekt door toediening van een vaccin. De ontwikkeling van een vaccin voor paratuberculose begon rond het begin van de vorige eeuw, in eerste instantie op basis van de hele bacterie, afgedood of verzwakt levend. Het meeste onderzoek werd gedaan aan een zogenaamd bacterin vaccin (afgedode MAP bacteriën). Vaccinatie met MAP bacterin kan het aantal dieren dat de klinische ziekte ontwikkeld verminderen, maar is niet in staat om de besmettingsgraad terug te dringen. Toediening van een bacterin geeft sterke weefselschade op de plaats van injectie, die bij een prikincident ook de toediener schade kan berokkenen. Daarnaast heeft een MAP bacterin vaccin als belangrijk nadeel dat het interferentie geeft met de diagnostiek van rundertuberculose. Deze bevindingen hebben ertoe geleid dat er op dit moment geen paratuberculose vaccin geregistreerd is voor gebruik in runderen in de Europese Unie.

Een van de onderzoekslijnen gericht op de ontwikkeling van nieuwe paratuberculose vaccins concentreert zich op het gebruik van subunits, onderdelen van de paratuberculose bacterie, meestal eiwitten, die versterkt worden met hulpstoffen (adjuvantia) om een effectieve afweerreactie op te wekken. Eerdere studies hebben laten zien dat het eiwit Hsp70 ('heat shock protein 70 kDa') samen met de hulpstof DDA ('dimethyl dioctadecyl ammoniumbromide') een interessant kandidaat vaccin is, vooral vanwege het feit dat gevaccineerde kalveren minder bacteriën uitscheidden via de mest na een experimentele besmetting vergeleken met controle dieren.

In dit proefschrift

In dit proefschrift worden studies gepresenteerd die de ontwikkeling van een kandidaat subunit vaccin voor paratuberculose ondersteunen. Ter vergroting van de kennis over het kandidaat vaccin 'Hsp70/DDA' werd onderzoek gedaan aan een drietal hoofdvraagstellingen. De eerste betrof de mogelijke interferentie van vaccinatie met de diagnostiek van rundertuberculose en paratuberculose. De tweede betrof de werkzaamheid van het kandidaat vaccin in chronisch besmette paratuberculose koeien. De laatste was gericht op opheldering van de wijze waarop het afweersysteem reageert op het vaccin.

1. Hsp70 vaccinatie en interferentie met de diagnostiek van rundertuberculose en paratuberculose

Voor registratieautoriteiten is het belangrijk dat kandidaat vaccins bewezen effectiviteit en veiligheid hebben, voordat tot registratie kan worden overgegaan. Voor een nieuw paratuberculose vaccin komt daar nog bij dat het niet mag interfereren met de diagnostiek van rundertuberculose. Met andere woorden, paratuberculose vaccinatie mag de interpretatie van diagnostische testen voor rundertuberculose niet in gevaar brengen, door het beïnvloeden van testgevoeligheid en specificiteit. Op dit moment wordt de huidtest als standaard test gebruikt voor het aantonen van rundertuberculose. Bij deze test wordt een eiwit extract van de rundertuberculose bacterie (PPDB; tuberculine) in de huid van de koe gespoten. Na drie dagen wordt

gemeten of er zwelling van de huid heeft plaatsgevonden, wat aangeeft dat het dier besmet kan zijn met rundertuberculose. Een lagere gevoeligheid van de huidtest kan optreden wanneer een vergelijkende huidtest wordt gebruikt, waarbij naar het verschil in huidreactie tengevolge van PPDB injectie en die tengevolge van PPDA (een eiwit extract van de vogeltuberculose bacterie; voor het meten van de achtergrondreactie tegen omgevingsmycobacteriën) injectie wordt gekeken. Toename van de PPDA-reactie door het toepassen van het paratuberculose vaccin (de vogeltuberculose bacterie en de paratuberculose bacterie zijn sterk verwant), leidt tot een kleiner verschil tussen PPDB en PPDA reacties, waardoor minder met rundertuberculose besmette dieren aangetoond zullen worden. Dit is voornamelijk van belang in landen waar rundertuberculose voorkomt, zoals in het Verenigd Koninkrijk. Een lagere specificiteit kan komen door de aanwezigheid van kruisreagerende antigenen (stoffen die een afweerreactie kunnen opwekken) in PPDA en PPDB, die gedeeld worden met antigenen in de paratuberculose bacterie. Vaccinatie met van paratuberculose afkomstige antigenen kan een afweerreactie tegen deze kruisreagerende antigenen opwekken. Als vervolgens een huidtest wordt uitgevoerd, kan dit leiden tot vals-positieve uitslagen. Dit is vooral van belang in landen die vrij zijn van rundertuberculose.

In hoofdstuk 2 is onderzocht of het kandidaat paratuberculose vaccin Hsp70/DDA interfereert met de huidige testen voor het aantonen van rundertuberculose en paratuberculose. In een dierproef werden rundertuberculose negatieve koeien gevaccineerd met het kandidaat vaccin, waarna de vergelijkende huidtest werd uitgevoerd. De toename in huiddikte is vergeleken met die in een groep controle dieren en een groep dieren gevaccineerd met een MAP bacterin vaccin. De conclusie van dit experiment is dat de specificiteit van de huidtest niet is veranderd in Hsp70/DDA gevaccineerde dieren. De vraag of de gevoeligheid van de huidtest veranderd is in Hsp70/DDA gevaccineerde dieren kan met dit experiment niet volledig beantwoord worden, maar gebaseerd op de verkregen resultaten is de inschatting dat Hsp70/DDA vaccinatie de gevoeligheid niet sterk beïnvloed.

Voor de diagnostiek van paratuberculose wordt voornamelijk gebruik gemaakt van het opsporen van antilichamen gericht tegen de paratuberculose bacterie. Koeien gevaccineerd met het kandidaat Hsp70 vaccin reageren echter ook positief in de huidige antilichaam test. Met een kleine aanpassing van deze test kan ervoor gezorgd worden dat deze dieren als negatief aangeduid worden, waardoor gevaccineerde dieren van besmette dieren onderscheiden kunnen worden. Dit in tegenstelling tot dieren die gevaccineerd worden met de MAP bacterin, deze blijven ook na de aanpassing van de test positief in de antilichaam test.

Vanuit het perspectief van de vaccinontwikkeling alsmede vanuit het perspectief van verbetering van diagnostische testen is inzicht in de samenstelling van tuberculines, comlexe eiwitmengsels, wenselijk. In hoofdstuk 3 zijn de individuele eiwitten van mycobacteriele tuberculines onderzocht door middel van 'proteomics' technieken. De analyse van tuberculines laat zien dat ze >100 verschillende eiwitten bevatten. Van een aantal van die eiwitten is bekend dat ze een rol spelen in de interactie met het afweersysteem, anderen hebben geen directe interactie en van velen is de functie onbekend. Voor diagnostische testen zijn specifieke paratuberculose antigenen nodig ten einde hun prestaties te verbeteren. Hoewel

specifieke eiwitten zijn aangetoond in PPDP, de eiwitten die tot op heden zijn getest waren niet succesvol als antigeen. Vervolgstudies zijn nodig om interessante antigenen te isoleren en identificeren ter verbetering van de paratuberculose diagnostiek. Vanuit het oogpunt van kruisreactie is ook gekeken naar de eiwitten die in alle geteste tuberculines voorkomen. Deze eiwitten kunnen ook interessant zijn als vaccinkandidaat, onder voorwaarde dat ze een afweerreactie kunnen opwekken, waarbij geen interferentie ontstaat met diagnostische testen. Een van de meest voorkomende eiwitten in alle geteste tuberculines is Hsp70. Maar ondanks de aanwezigheid van Hsp70 in de tuberculines leidt vaccinatie met Hsp70/DDA niet tot interferentie met de huidtest.

2. Hsp70 vaccinatie: rol voor een 'post-exposure' vaccin

Sinds lange tijd wordt erkend dat voor de bestrijding van paratuberculose een effectief vaccin nodig is. Effectiviteit van vaccins kan op verschillende manieren onderzocht worden, afhankelijk van het doel dat nagestreefd wordt. Deze doelen kunnen zowel voor het individuele dier als de dierpopulatie vastgesteld worden.

Voor het individuele dier is de beste optie een vaccin dat steriele immuniteit geeft. Met andere woorden gevaccineerde dieren kunnen besmetting voorkomen of de infectie aanpakken en de bacterie volledig opruimen. Gebaseerd op ervaringen tot nu toe is dit nog geen realistisch scenario gebleken voor vaccins tegen chronische mycobacteriële infecties. Een beter haalbaar doel zou een vaccin zijn dat ervoor zorgt dat de infectie in het individuele dier onder controle blijft en dat voorkomt dat de klinische fase van paratuberculose zich ontwikkeld. De bacterin vaccins geven dit niveau van bescherming, maar ze voorkomen niet de infectie en zorgen niet voor steriele immuniteit. Daarnaast hebben de MAP bacterins eerder genoemde bezwaren, waardoor ze op dit moment niet geregistreerd zijn voor gebruik in runderen.

Dierstudies om een afname van klinische paratuberculose door vaccinatie te evalueren hebben een lange tijdsduur, omdat er een lange periode zit tussen het moment van infectie en het ontwikkelen van klinische symptomen (3-5 jaar). Om dit soort studies te bekorten zou als alternatieve benadering gebruik gemaakt kunnen worden van markers voor ziekteontwikkeling, zoals uitscheiding van de bacterie in de mest of infectie op darmniveau. De vraag blijft natuurlijk of deze kortdurende experimentele infectiestudies effectiviteit voor afname in klinisch ziekte kunnen voorspellen.

In een eerdere studie is aangetoond dat Hsp70/DDA vaccinatie zorgt voor minder uitscheiding van MAP in de mest van kalveren, experimenteel besmet met MAP, gedurende twee jaren van observatie. In deze studie werden dieren tegelijk gevaccineerd en besmet om de condities in het veld na te bootsen. Omdat paratuberculose een ziekte is die endemisch (een ziekte die blijft voorkomen in een bepaald gebied) voorkomt op besmette bedrijven en MAP lang kan overleven in de omgeving, worden gevoelige kalveren geboren in een besmette omgeving. Blootstelling aan MAP kan meteen al rond de geboorte plaatsvinden, voordat een klassiek vaccin het afweersysteem kan aansturen. Het is daarom realistischer om er van uit te gaan dat vaccins ingezet worden in al blootgestelde dieren, waarvan

het afweersysteem al met de bacterie in aanraking is geweest, zogenaamde 'post-exposure' vaccinatie.

In hoofdstuk 5 wordt een experiment beschreven, dat werd uitgevoerd ter beantwoording van de vraag of vaccinatie van chronisch paratuberculose besmette koeien effect heeft. Om zo dichtbij condities in het veld te blijven zijn volwassen dieren gebruikt, die op natuurlijke wijze besmet zijn. Vaccineffectiviteit op deze wijze gemeten was gecorreleerd aan door vaccinatie opgewekte Hsp70 specifieke antilichamen. Hsp70/DDA vaccinatie zorgde voor minder uitscheiding van de paratuberculose bacterie via de mest en verlengde de overlevingsduur van dieren met een hoge Hsp70-specifieke antilichaamreactie. Deze resultaten zijn niet alleen relevant voor de bestrijding van paratuberculose, maar kunnen ook een bredere toepassing hebben richting het tuberculose onderzoeksveld. Er zijn overeenkomsten in ziekteontwikkeling en het Hsp70 eiwit is sterk geconserveerd binnen de mycobacteriën. Een 'post-exposure' vaccin voor humane en rundertuberculose kan een sterke impact hebben op de preventie van progressie naar klinische ziekte en kan gegeven worden samen met antibiotica.

Naast vaccineffectiviteit voor het individu, heeft de dierpopulatie baat bij een vaccin dat de overdracht van besmetting beperkt. In toekomstige studies is het noodzakelijk om de effectiviteit van het kandidaat Hsp70/DDA vaccin, als ook van andere kandidaat vaccins, op de overdracht van besmetting te meten. Deze informatie is voornamelijk belangrijk voor het implementeren van een vaccinatiestrategie in regionale of nationale bestrijdingsprogramma's.

Samengevat heeft Hsp70/DDA vaccinatie werkzaamheid laten zien in experimenteel besmette kalveren (eerdere studie) en in chronische, via natuurlijke weg besmette paratuberculose koeien (dit proefschrift). De volgende stap om nader onderzoek te doen aan vaccin effectiviteit zou een veldstudie kunnen zijn.

3. Afweerreacties opgewekt door Hsp70 vaccinatie en correlatie met bescherming

Voor vaccinontwikkeling is het belangrijk om 'correlates of protection' te definiëren. Dit kan gedefinieerd worden als een specifieke afweerreactie tegen een vaccin dat sterk gerelateerd is aan bescherming tegen infectie of ziekte. Als een 'correlate of protection' bekend is, kunnen effectievere vaccin kandidaten gescreend worden door te sturen op optimale/maximale activatie van de 'correlate of protection'. De meeste vaccins, vooral gericht tegen virussen, zijn effectief gebaseerd op de aanmaak van specifieke antilichamen. Voor paratuberculose, als ook voor andere mycobacteriële ziekten, zijn er tot op heden geen 'correlates of protection' gedefinieerd. Omdat Hsp70/DDA vaccinatie bescherming laat zien, is het interessant om te zoeken naar die afweerreactie die correleert met bescherming.

In hoofdstuk 4 is de specificiteit van de B cel (verantwoordelijk voor de aanmaak van antilichamen) reactie na Hsp70/DDA vaccinatie bestudeerd. Hsp70 is aanwezig in de celwand van de paratuberculose bacterie en bereikbaar voor door vaccinatie opgewekte antilichamen. In hoofdstuk 5 is de afweerreactie bestudeerd op het niveau van de lymfeknoop door het cannuleren van lymfevaten. De conclusie uit deze studies is dat de B cel reactie de dominante reactie is, die gemeten

wordt na Hsp70/DDA vaccinatie en er slechts minimale activatie is van een T cel gemedieerde afweerreactie. Samengenomen geven deze resultaten aan dat antilichamen mogelijk bijdragen aan de waargenomen bescherming na vaccinatie door een interactie aan te gaan met de paratuberculose bacterie gedurende het verloop van de besmetting. Toekomstige studies gericht op passieve overdracht van antilichamen zouden deze hypothese kunnen verhelderen.

Samenvatting

Het doel van dit proefschrift was om een kandidaat vaccin voor paratuberculose (Hsp70/DDA) te evalueren voor gebruik in runderen. Vaccinatie met Hsp70/DDA leidt niet tot interferentie met de diagnostiek van rundertuberculose, hoewel Hsp70 een in tuberculine veelvoorkomend eiwit is. Op antilichaam detectie-gebaseerde diagnostische testen voor paratuberculose kunnen aangepast worden door het eiwit Hsp70 toe te voegen aan de pre-absorptie stap, die nu al deel uit maakt van de test, om kruisreagerende antilichamen te neutraliseren. Hierdoor kunnen gevaccineerde dieren van besmette dieren onderscheiden worden. De analyse van de afweerreactie opgewekt door Hsp70 vaccinatie laat zien dat de antilichaam reactie dominant was ten opzichte van een T cel gemedieerde afweerreactie. Door vaccinatie opgewekte antilichamen zijn in staat om het Hsp70 eiwit te binden in de celwand van de paratuberculose bacterie. Dit kan een mogelijke verklaring zijn voor de bescherming die werd gezien na vaccinatie.

Vaccinatie van chronisch paratuberculose besmette volwassen koeien resulteerde in verminderde bacteriële uitscheiding via de feces en in langere overleving. Deze bevindingen correleerden met de aanwezigheid van Hsp70-specifieke antilichamen. Combinatie van deze bevindingen met die uit een eerdere vaccinatiestudie in kalveren laat voor het kandidaat Hsp70/DDA vaccin zowel pre- als 'post-exposure' effectiviteit zien. Dit is voor de bestrijding van paratuberculose, als ook voor tuberculose, de meest gewenste vaccin strategie.

In conclusie kan gesteld worden dat in dit proefschrift gunstige effecten voor Hsp70/DDA als een kandidaat paratuberculose vaccin worden beschreven, die nader onderzoek naar verdere ontwikkeling van dit kandidaat vaccin ondersteunen.

Dankwoord

Vier jaar. Dat is niet zo lang, als je aan paratuberculose werkt. De tijd is voorbij gevlogen. Het was voor mij een groot avontuur en unieke ervaring om mee te werken aan het onderzoek naar een kandidaat vaccin voor paratuberculose. Voor de hoofdstukken beschreven in dit proefschrift hebben velen een bijdrage geleverd. Ik wil graag een aantal van hen specifiek noemen.

Ad Koets, met jouw enthousiaste verhaal begon mijn avontuur. Het bracht ons tussen de koeien; in een karaokebar in Japan; priegelen met het cannuleren van lymfevaten; wachtend op de uitslag van de huidtest; overtuigd dat we deze monsters moesten bewaren voor later; dag en nacht FACSen; fluorescerende cellen terugspuiten en ook weer terugvinden; op de zoutvlakte van Chili; waar zijn nu die cellulaire responsen; in overpeinsing voor de volgende stap; in de geruststelling dat onze leeftijd gecontroleerd is een biertje drinken in Minneapolis; op zoek naar het volgende avontuur. Thanks! *Victor Rutten*, ik waardeer jouw onvermoeibare inzet om altijd voor het maximale te gaan; het nog eens vanuit een ander perspectief bekijken; en natuurlijk een gewoon praatje over de dagelijkse dingen. *Hans Heesterbeek*, bedankt voor de prettige begeleiding en de mogelijkheid om de Vet-Epi master te doen. *Willem van Eden*, bedankt voor het vertrouwen in de goede afloop. Hsp70 blijft fascineren.

Voor de experimenten die in samenwerking met Intervet zijn gedaan: dat was lekker werken! Het waren grote experimenten, waarbij ik heb kunnen meedraaien in een goed geoliede machine. Ik heb er veel van opgestoken. *Selma Hensen*, bedankt voor de prettige samenwerking en de mogelijkheid die jij mij gaf om een goede indruk te krijgen van de verschillende werkzaamheden bij Intervet. *Jacqueline Poot*, altijd mooie verhalen, maar ook heerlijke no-nonsense mentaliteit. *Ruud Segers*, het staartje kwam bij jou, maar was daarom niet minder interessant. *Daniëlle*, wat een klus: respect!; maar ook de gezelligheid, top! Niet te vergeten: *Paul Vermeij, Katja, Thea, Theo, Leonie, Sandra, Samantha, Pieter en Ed, de ASD: Lea, Eric*, *Wim* en vele anderen. *Familie Driessen*, altijd mooi weer in het land van Cuijk.

Ook aan de Faculteit Diergeneeskunde is het goed toeven. *Jeroen Hoeboer*, een Groninger en een Fries, dat praat makkelijk: 'Nait soezen'. *Susanne Eisenberg*, dan straalt de zon (in Brabant). *Kimm van Hulzen*, nog maar een paar geiten. *Marina Bouman*, alweer de slappe lach. *Manouk Vrieling*, even facsen! *Ildiko van Rhijn, Kim Anh Nguyen*, *Mirjam Nielen, Henri Heuven, Don Klinkenberg, Maite Severins, Sanne Slot*. Een dierenarts op een lab, dat vraagt om hulptroepen: *Marije Overdijk, Peter van Kooten, Aad Hoek*, bedankt voor het bijbrengen van de fijne kneepjes op het lab. Gelukkig ook altijd tijd voor een praatje. Voor de cannulatie experimenten uitgevoerd bij Landbouwhuisdieren wil ik graag bedanken: *Hans Lutz, Freek Weites, Frans van Kooij, Tijs van Loon* en de hulp van alle dierverzorgers en de ondersteunende dienst. Daarnaast zijn er ook de collega's van de divisie Immunologie, het departement Landbouwhuisdieren en de afdeling pathologie die ik wil bedanken voor het creëren van een inspirerende werkomgeving.

Dan zijn er de externe contacten/samenwerkingen, die het onderzoek weer extra impulsen geven. Voor de 'proteomics' analyses wil ik graag *Jeroen Krijgsveld, Judith Barends, Sharon Gauci, Soenita Goerdayal en Maarten Altelaar* bedanken. *Martin Vordermeier*, thanks for the opportunity to visit the VLA to use your multiplex assay, and your help with sharing protocols for the memory T cell assays. *John Bannantine*, thanks for sharing your recombinant proteins. *Geart Benedictus en Maarten Weber*, voor de handige inzichten uit het veld.

Niet onbelangrijk, familie en vrienden. *Heit en mem*, jullie gaven mij de ruimte en het vertrouwen om mij te ontwikkelen: jim binne hiel bysûnder! *Famylje Gaastra*, op 'de pleats' begon de interesse voor de koeien, het was een geweldige tijd. Mijn broers *Willem, Jehannes en Siemen*, de Daltons. *Bas, Jim en Rommert*, voor de nodige ontspanning, al geeft rood/wit heel wat stress momenten. *Astrid en Jeroen*, altijd klaar staan, niks is te gek: super! *Famylje Jorritsma*, jim binne er altyd foar ús. *Feije en Jildau*, jim bringe in soad wille. *Dieuwke*, we hawwe de ôfrûne jieren in hiel soad moaie mominten meimakke, dat hâld net op! Neffens pake Willem: 'myn stikje goud'.

Wiebren

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Curriculum Vitae

Wiebren Jehannes Santema werd geboren op 26 juli 1982 te Kûbaard. In 2000 rondde hij de middelbare school af op het Christelijk Gymnasium Beyers Naudé te Leeuwarden. In datzelfde jaar begon hij met de studie Diergeneeskunde aan de Universiteit Utrecht. In 2004 deed hij een jaar onderzoek op de afdeling Immunologie van de Faculteit Diergeneeskunde in het kader van het Excellent Tracé. In 2007 sloot hij de studie diergeneeskunde 'met genoegen' af, met als differentiatie het monotraject herkauwers. Aansluitend begon hij als promovendus bij de departementen Landbouwhuisdieren en Infectieziekten en Immunologie. In 2009 won hij de Merkal award voor talentvolle onderzoeker op het internationale paratuberculose congres in Minneapolis. In 2011 heeft hij 'cum laude' de post-graduate master 'Veterinary Epidemiology and Economics' afgerond. Vanaf mei 2011 werkt hij als dierenarts-onderzoeker bij de afdeling Immunologie aan de Faculteit Diergeneeskunde in Utrecht.