Available online at www.sciencedirect.com
SCIENCE DIRECT
Vaccine 24 (2006) 2550–2559
Vaccine
www.elsevier.com/locate/vaccine

Mycobacterial 70 kD heat-shock protein is an effective subunit vaccine against bovine paratuberculosis

Ad Koets [a,b,*], Aad Hoek [b], Merel Langelaar [a,b], Marije Overdijk [b], Wiebren Santema [b], Peter Franken [c], Willem van Eden [b], Victor Rutten [b]

[a] *Department of Farm Animal Health, P.O. Box 80.165, Faculty of Veterinary Medicine, Utrecht University, 3508 TD Utrecht, The Netherlands*
[b] *Immunology Division, Department of Infectious Diseases and Immunology, P.O. Box 80.165, Faculty of Veterinary Medicine, Utrecht University, 3508 TD Utrecht, The Netherlands*
[c] *Animal Health Service Ltd., P.O. Box 9, 7400 AA Deventer, The Netherlands*

Received 9 July 2005; received in revised form 1 October 2005; accepted 9 December 2005
Available online 27 December 2005

Abstract

Paratuberculosis is a chronic granulomatous inflammation of the small intestine of cattle and other ruminants, caused by infection with *Mycobacterium avium* ssp. *paratuberculosis* (MAP). The disease can be found in ruminant herds worldwide, causing substantial economic losses at farm level due to premature culling and production losses.

In previous studies, it has been shown that immune responses to recombinant MAP Hsp70 proteins were predominantly cell mediated. As protective immunity to the intracellular mycobacterial pathogens is thought to be cell-mediated in origin, we have studied the use of a recombinant MAP Hsp70 as a subunit vaccine in cattle experimentally infected with MAP.

The results of the current study demonstrate that recombinant MAP Hsp70 can be successfully used as a subunit vaccine against bovine paratuberculosis, significantly reducing shedding of bacteria in feces during the first 2 years following experimental infection.

Keywords: *Mycobacterium paratuberculosis*; Heat-shock protein; Cattle

1. Introduction

Paratuberculosis is a chronic granulomatous inflammation of the small intestine of cattle and other ruminants, caused by infection with *Mycobacterium avium* ssp. *paratuberculosis* (MAP). The disease can be found in ruminant herds worldwide, causing substantial economic losses at farm level due to premature culling and production losses. In addition, it may represent a potential food-safety issue by transmission of MAP, the causative organism, to humans via dairy and other products [1,2].

Transmission of paratuberculosis involves the presence of the bacterium in several substrates. From infected animals, bacteria are mainly excreted in feces and when the disease progresses towards the clinical stage of infection also milk. Following excretion, the organism is able to survive in the environment for prolonged periods of time in substrates such as surface water and soil [1,3].

Eradication of paratuberculosis is hampered by a lack of reliable diagnostic tools with high specificity and sensitivity and could be aided substantially with a vaccine [4]. The currently available vaccine consists of various variations of whole bacterins with adjuvants. These vaccines have been shown to have a variable efficacy in field studies [5,6]. From these studies it has become apparent that the current vaccine prevents the occurrence of the clinical stage of the disease to a high degree, thereby limiting a substantial amount of the direct economical damage. In cattle, however, the vaccine does not prevent infection and limits the frequency of

* Corresponding author. Tel.: +31 30 2534608; fax: +31 30 2533555.
E-mail address: a.p.koets@vet.uu.nl (A. Koets).

0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.12.019

subclinically infected animals, which shed bacteria in their feces intermittently, only marginally at best. Other major drawbacks of the whole bacterin vaccines are the interference tuberculosis and paratuberculosis diagnostics and eradication due to the fact that vaccinated animals become false positive in regular tuberculosis and paratuberculosis diagnostic assays [5–9]. A third drawback is that the vaccine in its current formulation causes substantial local tissue reaction, in terms of prolonged swelling and granuloma formation at the site of injection [10].

Relative little effort has been put in studying recombinant protein antigens of MAP in general and in vaccine studies in particular. With previous work we have documented immune responses to mycobacterial heat-shock proteins (Hsp) in various stages of bovine paratuberculosis. The Hsp are a family of evolutionary conserved proteins, expressed in both prokaryotic and eukaryotic organisms. The expression of Hsp is upregulated during the cellular stress that occurs in both host and pathogen in response to infection and inflammation. We have previously shown that the Hsp are immunodominant antigens in bovine paratuberculosis [11,12], similar to other mycobacterial diseases such as tuberculosis and leprosy, eliciting strong cell-mediated and antibody responses. A number of studies on mycobacterial diseases, mainly in murine model systems, have indicated a potential use of Hsp as vaccine components. Our studies indicated that immune responses to recombinant MAP Hsp70 proteins were predominantly cell mediated contrary to recombinant MAP Hsp65, which apparently induced mainly antibody responses and minor cell-mediated responses [11,13].

As protective immunity to the intracellular mycobacterial pathogens is thought to be cell mediated in origin, we have studied the use of a recombinant MAP Hsp70 as a subunit vaccine in cattle experimentally infected with MAP. New candidate vaccines against bovine paratuberculosis should have the potential to limit transmission of infection between susceptible animals to aid in eradication strategies. In the current study we therefore studied immune parameters as well as fecal excretion in the first 2 years of experimental paratuberculosis to determine the potential of the recombinant MAP Hsp70 as a subunit vaccine in a relatively short timeframe considering the natural course bovine paratuberculosis.

2. Materials and methods

2.1. Animals and experimental design

A total of 40 female calves (aged 29 ± 9 days at the start of the experiment) were used in the current study. The calves were raised using conventional procedures and feeds and were checked daily for general health. The calves were randomly assigned to one of the four experimental groups, according to a 2×2 factorial design (Table 1). Calves in groups 1 and 2 were physically separated from calves in groups 3 and 4, and rigorous hygienic measures were taken to prevent infection of the control groups. Calves allotted to groups 1 and 2 were randomly co-mingled in group housing pens for 10 calves each, to prevent possible influence of a treatment effect. Similarly calves from groups 3 and 4 were housed mixed. Blood samples were taken every 2 weeks for the first 12 months of the experiment and monthly for the remainder. Heparinized samples were used for isolation of lymphocytes and serum samples were taken for serological analysis. Body weight was recorded on the same time points as blood samples were taken. Fecal samples were taken seven times during the experiment, at days 0, 14, 126, 280, 406, 532 and 644.

Table 1
Experimental design

Group	*n*	Infection	Immunization
G1	10	No	No
G2	10	No	Yes
G3	10	Yes	No
G4	10	Yes	Yes

2.2. Ethics

The use of animals in the experiments described in these studies was approved by the Ethical Committee of the Utrecht University and performed according to their regulations.

2.3. Infection of calves

Calves assigned to groups 3 and 4 were infected orally using feces from a MAP infected cow, which was characterized as a consistent shedder by fecal culture of the mycobactin-J-dependant and IS900 PCR positive MAP. The calves received nine doses of 20 g of feces, mixed with 100 ml milk replacer per dose by gavage feeding, during the first 21 days of the experiment at regular intervals. Semi-quantitative fecal culture indicated that >100 cfu/g of feces were present in the inoculum. Hence, calves received a minimum total dose of 1.8×10^4 cfu each.

2.4. Immunization of calves

Calves 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 *M. a. paratuberculosis* Hsp70 in 1 ml phosphate buffered saline (PBS) containing 20 mg/ml dimethyl dioctadecyl ammonium bromide (DDA) adjuvant (Sigma-Aldrich, USA), subcutaneously in the dewlap. The same animals received a second immunization at day 308 of the experiment. Recombinant *M. a. paratuberculosis* Hsp70 was produced as published previously [11].

Table 2
Fecal culture score

	Time to positive culture (weeks)		
	16	12	8
Negative	0	0	0
1–10 cfu/gr	1	2	3
10–100 cfu/gr	4	5	6
>100 cfu/gr	7	8	9

2.5. Fecal culture of MAP

Diagnosis of paratuberculosis infection was performed using the routine fecal culture system, based on published methods [14], at Veterinary Health Service, Deventer, The Netherlands. Samples were checked for bacterial growth by colony count every 4 weeks, the first observation at 8 weeks post inoculation, and considered negative if after a culture period of 16 weeks no bacterial growth was observed. Bacterial growth was confirmed to be *M. avium* ssp. *paratuberculosis* based on mycobactin dependence of the culture and the confirmation of the presence of the specific IS900 insertion sequence by PCR [15]. Results of fecal culture were scored semi-quantitatively between 0 and 9 based on the combination of time to positive (TTP), respectively, 8, 12 or 16 weeks and the number of colonies counted (CFU) per gram of feces, as outlined in Table 2.

2.6. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from aseptically taken, heparinized blood samples using density gradient centrifugation and cultured as published previously [12].

2.7. Flowcytometric analysis of lymphocyte subsets

Three-colour flowcytometric analysis was performed with monoclonal antibodies against bovine lymphocyte markers that have been described earlier (see Table 3). Three-colour flowcytometry was performed as follows: approximately 200,000 cells per well, suspended in 200 μl of facs buffer (FB; PBS, 1% BSA, 0.01% Sodium-Azide), were put in 96-well plates in seven-fold, and centrifuged for 2 min at 1200 rpm in a cooled centrifuge (4 °C). Subsequently, the FB was discarded and the pellet resuspended. This wash step was repeated once more before the unlabeled antibodies (see Table 3) were added to the cells, in predetermined optimal concentration diluted in FB. In one well no primary antibody was added as negative control, in another well an optimized mixture of the unlabeled antibodies against CD4, CD8, N12, CD14 and CD21 was added to enable an estimation of the total percentage of cells that could be identified per sample. Cells were incubated for 15 min in the dark at 4 °C. Next, cells were washed twice as described above. Subsequently a phyco-erythrin (PE) conjugated secondary goat anti-mouse antibody (Southern Biotech, USA) was added in predetermined optimal concentration diluted in FB, and again cells were incubated for 15 min in the dark at 4 °C. Following two more washes the biotinylated anti-CD2 and the FITC-labeled ILA-59 were added and incubated for 15 min in the dark at 4 °C. Again cells were washed twice and streptavidin-ALEXA633 (Molecular Probes, USA) was added and incubated as described above. Finally, cells were washed twice and resuspended in 100 μl FB prior to measurement on a Facs Calibur flowcytometer (Becton-Dickinson, USA). Data of at least 10,000 events were analyzed using Cellquest software (Becton-Dickinson, USA).

2.8. Antigens

Recombinant *M. a. paratuberculosis* Hsp 65 kD and Hsp70 kD were produced according to methods described in detail earlier [11,16]. Purity of the recombinant Hsp65 and Hsp70 was checked using SDS-PAGE and preparations were tested for LPS contamination by Limulus assay (Sigma, St. Louis, USA).

Purified protein deviate was prepared from *M. a. paratuberculosis* strain 3 + 5/C culture supernatant (PPD-P) and *M. a. avium* strain D4 culture supernatant (PPD-A) according to the OIE manual [17] at the Institute for Animal Health and Science (Lelystad, The Netherlands).

M. a. paratuberculosis strain 316F and *M. a. avium* strain D4 were grown at the Institute for Animal Health and Science (Lelystad, The Netherlands). *Escherichia coli* strain DH5α was grown overnight in Luria Bertani (LB) medium at 37 °C.

Concanavalin A was used as a positive control (2.5 μg/ml) and medium alone as a negative control.

Table 3
Monoclonal antibodies (mAb) used in flow cytometric analysis

mAb	Isotype	Antigen identified	References	Modification
ILA-42	IgG2a	Bovine CD2	[38]	Biotinylated
ILA-12	IgG2a	Bovine CD4	[39]	–
CACT138A	IgG1	Bovine CD4	[40]	–
ILA-51	IgG1	Bovine CD8	[41]	–
ILA-29	IgG1	Bovine WC1 (γδ T cells)	[42]	–
CACT61A	IgM	Bovine TCR1-N12 (γδ T cells)	[43]	–
GB25A	IgG1	Bovine CD21-like (B-cell)	[44]	–
ILA-59	IgG1	Bovine immunoglobulin light chain	[44,45]	FITC labeled

2.9. Elispot assay for bovine IFN-γ secreting cells

The sterile 96-wells PVDF filter plates (Millipore, USA) were coated overnight at 4 °C with 100 μl/well anti-bovine IFN-γ capture monoclonal antibody 5D10 (1 μg/ml, Biosource, USA) in filter sterilized bicarbonate coating buffer (pH 9.6). Subsequently, the plate was blocked with 200 μl RPMI1640 supplemented with 10% fetal calf serum (FCS) for 2 h at 37 °C. After 2 h the blocking medium was replaced with culture medium (CM) (RPMI1640 supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine) with or without antigen according to the description below. Subsequently, we added 2×10^5 cells per well in 100 μl CM to the Elispot plate and cultured the plate for 20–24 h in humidified incubator at 37 °C and 5% CO_2. Following incubation, spots were developed as follows. The wells were washed twice with H_2O_d and then trice with PBS-0.05% Tween 20 (PBS-T). Subsequently, 100 μl/well rabbit anti-bovine IFN-γ (1:200 in PBS-0.05% Tween 20-2% BSA (PBS-TB)), a generous gift from Martin Vordermeier, VLA Weybridge, UK, was added and incubated for 30 min at room temperature. Then plates were washed three times with PBS-T, and wash fluid was removed thoroughly. Next, 100 μl/well of monoclonal anti-rabbit IgG-alkaline phosphatase conjugate (Sigma, USA, diluted 1:2000 in PBS-TB) was added and incubated for 30 min at room temperature. Subsequently, plates were washed six times with PBS-T. Then 100 μl/well of BCIP/NBT (Sigma, USA) dissolved in 10 ml H_2O_d was added. Once the spots developed, substrate was removed and plates were washed with copious amounts of tap water. Plates were air dried and stored in the dark before counting the spots. Spots were counted, and total spot area was calculated using an automated Elispot reader according to instructions provided by the manufacturer (A.EL.VIS GmbH, Hanover, Germany). Spot-counting results were expressed as delta-spot forming cells (dSFC), calculated by subtracting the number of spots in medium control wells from the number of spots in antigen-stimulated wells, unless stated otherwise. Medium alone was used as a negative control; concanavalin A (2.5 μg/ml) was used as positive control. PPD-P, PPD-A, Hsp70 and Hsp65 were used at a predetermined optimal concentration of 10 μg/ml. *M. a. paratuberculosis* strain 316F, *M. a. avium* strain D4, and *E. coli* DH5α were used at MOI 1:1 with the PBMC. All tests were performed in triplicate. To perform a relative estimation of the amount of IFN-γ produced, the percentage of area that the spots covered per well was calculated and expressed as percentage of the total area of the well. Subsequently the antigen specific spot area was calculated by subtraction of medium control values from values in antigen-stimulated wells.

2.10. Serology of calves

Serological responses to recombinant *M. a. paratuberculosis* Hsp70 protein were measured using a previously described ELISA technique, with modifications [11]. All sera were diluted 10 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. The modifications consisted of the use of a biotinylated anti-bovine IgG secondary antibody (Sigma-Aldrich, USA) and an avidin-peroxidase conjugate (Sigma-Aldrich, USA), both according to instructions provided by the manufacturer, before adding substrate. Results are expressed as S/P (sample to positive) ratio.

For a general serological screening of the samples, a commercially available absorbed ELISA assay for the serodiagnosis of paratuberculosis was used according to instructions provided by the manufacturer (Institute Pourquier, Montpellier, France). The antigens used in this ELISA are derived from a protoplasmic extract of MAP. Absorbence values were expressed as S/N ratio (sample to negative), and values higher than S/N ratio 59 were considered positive [18].

2.11. Statistical analysis

The data from the fecal culture test were analyzed using the R program version 2.0.1 [19] for statistical analysis using a logistic regression for non-normal repeated measurements models [20]. Cow was taken as the random effects term. Fecal culture, scored semi-quantitatively (Table 2), was used as the outcome in the model. Based on the lowest AIC for best fit, the final model contained the continuous variable time (month 1 and month 2), the factor treatment (treated and untreated) as well as the interaction month × treatment as explanatory variables. A linear mixed effects model has been performed to analyze Elispot data as the response variable and group, time and the interaction between group and time as explanatory variables. For the Elispot medium control data the logarithm was taken to meet the normality assumption. To model the correlation between consecutive time points an autoregressive order 1 (AR1) correlation structure was introduced. The variance was not constant in time; for this a variance structure was used, which allows a different variance at each time point. Cow was added to the model as the random effects term to take into account the dependence of the data. For the analysis of the data the program R Version 2.1.1 was used with the nlme library using maximum likelihood estimation of the effects. The likelihood ratio test was used for best model fit using a significance level of 0.05. Multiple comparisons were made, following a Bonferroni correction, between groups per time point. The relationship between the number of spots and the area covered by the spots was calculated using the least squares fit for a line represented by $y = mx + b$ in which m is the slope and b is the intercept. R squared was used to judge goodness of fit. A linear mixed effects model was performed to analyze flow cytometry data as the response variable and group, time and the interaction between group and time (age) as explanatory variables. To additionally analyze the age-related differences in subset frequencies, the flow cytometric data was grouped in five age intervals, namely animals aged from 20 to 43 days, 44 to 120 days, 121 to 240 days, 241 to

360 days and 360 to 644 days. Analysis of variance was performed on the flow cytometric data using the Kruskal–Wallis test, followed by Dunn's multiple-comparisons test to test for differences between the age intervals.

3. Results

3.1. Observations on general health status

The growth of the animals was monitored throughout the experiment and the results indicated that there were no differences in growth between the different treatment groups (data not shown), Around day 56, an outbreak of ringworm skin disease, caused by *Trichophyton verrucosum,* affected all animals in groups 3 and 4; this infection resolved spontaneously. Around day 70, 16 out of 20 animals in groups 1 and 2 showed signs of a respiratory infection; while 10 cases resolved spontaneous, 6 were successfully treated with antibiotics and anti-inflammatory drugs. During the experiment three animals had to be culled for reasons unrelated to paratuberculosis. These animals belonged to three different treatment groups. Two animals were culled based on severe respiratory infections that were non-responsive to treatment; one animal had to be culled due to paresis posterior following a fall.

3.2. Side effects of vaccination

The effect of primary vaccination with Hsp70 with DDA adjuvant in the dewlap was a palpable swelling with a maximum diameter between 2 and 5 cm at a week post immunization in animals of both the vaccinated groups G2 and G4. The swelling was in the majority of cases not painful and resolved to a small, apparently inert, nodule of approximately 1 cm diameter in the course of 3 weeks. The effect of the second immunization at 308 days was similar in animals of group G2 (vaccination, no infection); however, less swelling (maximum diameter 3 cm, and four cases with no palpable swelling, one week post immunization) was observed in animals of group G4 (vaccination and infection).

3.3. Fecal culture results

The results from the fecal culture tests are summarized in Fig. 1. The animals on the control groups 1 and 2 remained culture negative throughout the experiment. From the calves in group 3, infection with MAP only, in total 8 out of 10 animals were tested positive for MAP at least once of seven time points tested, and 2 animals tested positive five out of seven time points. In group 4 calves, which were both infected and immunized, 4 out of 10 animals tested positive once at day 126 of the experiment. Only one animal tested positive for MAP on two subsequent occasions. Based on the logistic regression model, the vaccinated animals in G4 shed bacteria in their feces significantly less frequently compared to unvaccinated calves in G3 ($p < 0.01$) (Fig. 2).

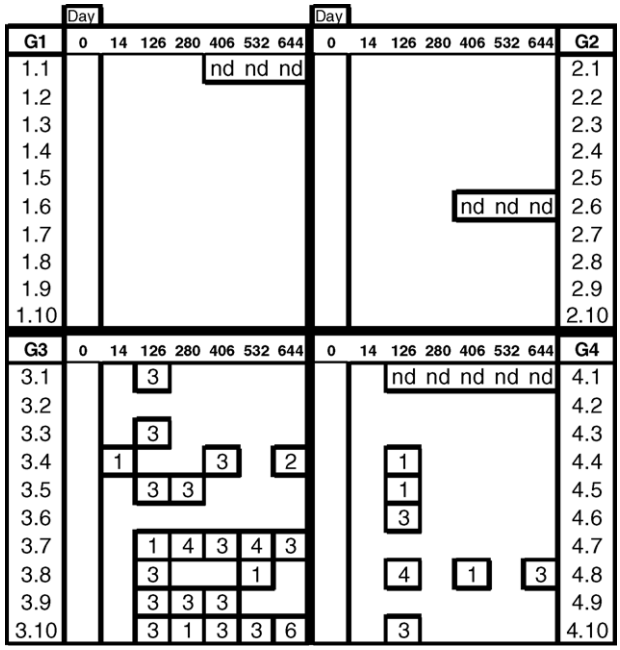

Fig. 1. In the four panels all the results from the fecal culture tests are shown per treatment group. G1 represents the animals that were neither immunized nor infected, G2 animals only received the immunization, G3 animals only received the infection and G4 contains the animals that were both immunized and infected. Squares with scores >0 indicate a positive fecal culture for MAP for a given animal and a given time point scored according to Table 2; (nd) indicates that time points of respective animals were not tested because they were culled from the experiment.

3.4. Phenotyping of PBMC

With the panel of monoclonal antibodies we defined seven subtypes of lymphocytes in the PBMC as is outlined in Table 4. The use of the mixed set of antibodies as defined in Section 2 also led to the identification of a subset of lymphocytes that stained positive for CD2; however, did not stain with any of the other markers. Based on staining similarities we designated this population as NK-like cells. Data analysis indicated that there were no significant differences between the four treatment groups during the study (data not shown). On the other hand, age was a major factor regarding observed frequency differences in certain lymphocyte subsets. For analysis of the age effect, the data was subsequently grouped for all animals in different age groups (20–43, 44–120, 121–240, 241–360, 360–644 days of age), as shown in Table 4. The $CD4^+$ cells were present at relatively stable frequencies, 20.1–21.4%, when comparing the age intervals with the exception of the interval between 44 and 120 days when a significant elevation to 26.2% was observed ($p < 0.001$ for all comparisons). In the $CD8^+$ T cells, there were more frequency fluctuations during the first 2 years of life, while an average frequency of 8.6% was measured overall, most notably the period 20–43 days and 241–360 days

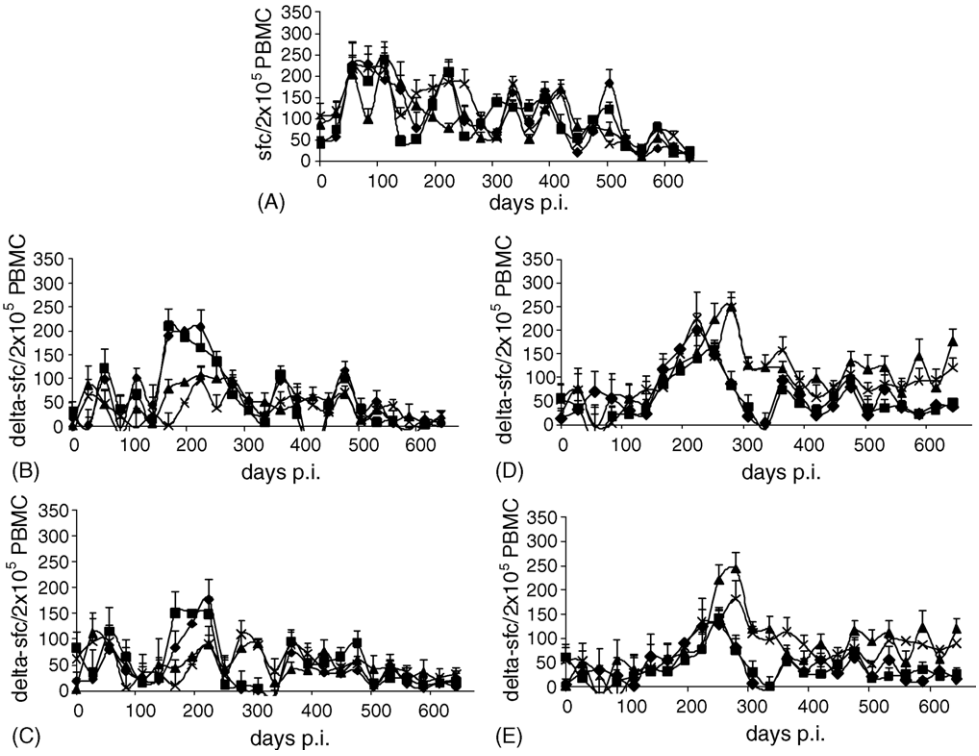

Fig. 2. The production of IFN-γ in the IFN-γ Elispot assay is expressed as the number of spot forming cells (sfc)/2 × 10^5 PBMC for the medium control (panel A) and as the delta-sfc, that is the number of spots in the antigen-stimulated wells minus the number of spots in medium control wells, for antigens *E. coli* (panel B), MAP Hsp70 (panel C), PPDP (panel D) and MAP (panel E). The four treatment groups are indicated by (♦) for G1 (no infection, no immunization); (■) for G2 (no infection, Hsp70 immunization); (▲) for G3 (infection with MAP, no immunization); and (×) for G4 (infection with MAP and Hsp70 immunization).

showed a similar and significantly lower frequency of 8.0% and 7.2%, respectively. The $N12^+CD2^+WC1^-$ subset of γδ T cells was low (2.5%) in youngest animals then rose in frequency in age intervals 44–120 and 121–240, up to 6%, and subsequently declined again to 3.3% in age interval 360–644. The $N12^+CD2^-WC1^+$ subset of γδ T cells was highest in the lowest age interval (26.0%), fluctuated between 16.2 and 20.6% in the intermediate intervals to decline to 15.1% in the highest age interval. The B-cell frequency was lowest in the 20–43 day (10.2%, $p < 0.001$ for all comparisons) and 44–120 day (18.9%, $p < 0.001$ for all comparisons) interval to stabilize between 32.3 and 35.8% in the remaining three age intervals. In addition, we observed a high frequency of $CD14^+$ monocytes in young calves (29.8%, $p < 0.001$ for all comparisons), which subsequently declined to an average frequency of 8.3% in the highest age group.

3.5. IFN-γ Elispot

In the young calves there was a high number of cells, which spontaneously produced IFN-γ following density gradient isolation and overnight culture as is shown in Fig. 3A (medium control values). This background was most prominent in the first 3 months of life; however, to a certain extend it remained present during the first 2 years of life.

Table 4
Age related differences in lymphocyte subset frequencies in blood mononuclear cells

Lymphocyte subset	Age(days)									
	20–43		44–120		121–240		241–360		360–644	
$CD4^+$	20.8^a	(19.5–22.0)	26.2^b	(24.7–27.7)	21.4^a	(20.4–22.4)	20.1^a	(19.0–21.1)	21.1^a	(20.2–22.2)
$CD8^+$	8.0^a	(7.2–8.8)	9.7^{ab}	(8.5–10.8)	9.9^b	(9.0–10.8)	7.2^a	(6.4–8.1)	9.0^{bc}	(8.2–9.8)
$N12^+CD2^+WC1^-$	2.5^a	(2.2–2.8)	5.7^{bc}	(4.8–6.5)	6.0^b	(5.3–6.6)	4.9^{bc}	(4.4–5.5)	3.3^d	(3.0–3.6)
$N12^+CD2^-WC1^+$	26.0^a	(23.9–28.2)	16.2^b	(15.0–17.5)	17.5^{bcd}	(16.2–18.8)	20.6^d	(18.8–22.4)	15.1^{bc}	(14.1–16.1)
B cell	10.2^a	(9.1–11.4)	18.9^b	(17.4–20.4)	35.8^c	(34.4–37.3)	33.1^c	(31.1–34.9)	32.3^c	(30.7–33.9)
$CD14^+$	29.8^a	(27.4–32.2)	15.6^b	(14.1–17.0)	12.1^{bd}	(10.9–13.3)	9.7^{cd}	(8.7–10.6)	8.3^c	(7.2–9.4)
$CD2^+$NK like	11.2^a	(10.0–12.5)	8.9^{ab}	(8.0–9.8)	10.2^a	(9.3–11.1)	8.2^{bc}	(7.1–9.2)	6.7^c	(5.7–7.7)

The average frequencies (95% confidence interval) of the seven different lymphocyte subsets detected in the blood mononuclear lymphocytes are shown for the five different age (days) groups. Lymphocyte frequencies between age groups that do not share superscripts (a,b,c,d) are significantly different ($p < 0.05$). As no differences between treatment groups were detected, the results of the animals from the different treatment groups have been combined.

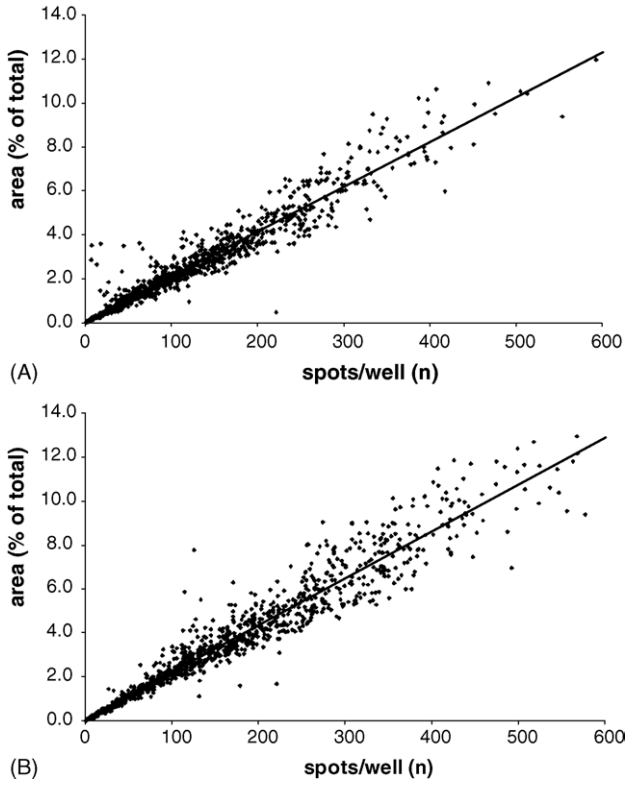

Fig. 3. The correlation between the number of spots per well and the percentage of area covered by the spots per well is shown in panel A for the medium controls and in panel B for well-stimulated with recombinant MAP Hsp70 protein. For the medium control wells the relation between total spot number and percentage area covered was characterized by $y = 0.0203x + 0.0754$ ($R^2 = 0.93$), and for the Hsp70 stimulated wells this was $y = 0.0213x + 0.0937$ ($R^2 = 0.93$).

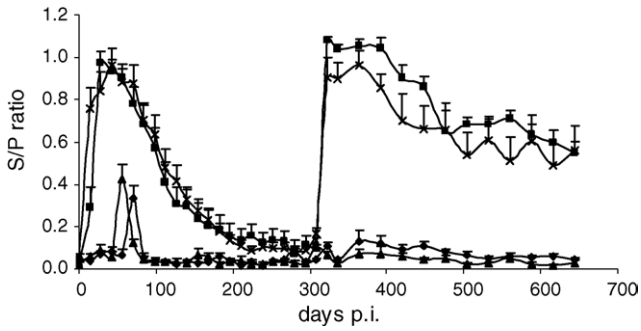

Fig. 4. Total IgG responses to Hsp70 protein measured by ELISA and expressed as the S/P ratio + S.E.M. Antibody responses of immunized animals (G2, ■, non infected; and G4, ×, infected) and non-immunized (G1, ♦, non infected; and G3, ▲, infected) are shown during the course of the experiment. Animals of G2 and G4 were immunized at day 0 of the experiment. A second immunization was performed at day 308 of the experiment.

In general, most differences in IFN-γ production were observed in the period between 224 and 336 days post infection for both PPDP (Fig. 3D) and MAP (Fig. 3E) antigen. Statistically significant differences in IFN-γ responses to PPDP between the infected group G3 and control groups G1 and G2 were observed at 308 days post infection. For the animals in G4, significant differences were observed at the three consecutive time points between 280 and 336 days post infection. Significant differences in IFN-γ responses to MAP between the infected group G3 and control groups G1 and G2 were observed at the two time points between 308 and 336 days post infection. For the animals in G4 significant differences were observed at the four time points between 252 and 336 days post infection. To a lesser extend a similar distinction between exposed/infected and uninfected controls could be made during the final part of the second year. Both at 588 and 644 days post infection animals in G3 had statistically significant elevated IFN-γ production in response to PPDP and MAP antigens. For animals in G4 these differences were not statistically significant. With respect to IFN-γ response to Hsp70 only at days 280 and 308 post infection a statistical significant difference between G4 and control groups G1 and G2 was observed. No other statistically significant differences between groups were identified for stimulation with Hsp70.

Apart from the number of IFN-γ producing cells in response to various stimulations and controls, we also calculated the total area of the well covered by the spots as an estimation of the total amount of IFN-γ produced. The results indicated that there was a significant linear correlation between the number of spots per well and the total area the spots covered in medium control wells (Fig. 4A) and Hsp70-stimulated wells (Fig. 4B). In case of the medium control wells the relation was characterized by $y = 0.0203x + 0.0754$, $R^2 = 0.93$, and for the Hsp70 stimulated wells this was $y = 0.0213x + 0.0937$, $R^2 = 0.93$. For the other antigens tested, similar linear correlations were observed (not shown).

3.6. Serological responses

The animals in group 2 and 4, which were vaccinated with Hsp70 at day 0 and day 308 showed clear and prolonged antibody responses to the immunogen as is shown in Fig. 4. The second immunization showed a more prolonged antibody response compared to the primary immunization. Where levels were close to baseline an year after primary immunization, they remained high until the end of year 2. No antibody responses were observed in the uninfected control group G1 and the infected non-immunized group G3 except for minor peaks at day 56 and day 70 of the experiment.

The results from the commercially available absorbed ELISA are depicted in Fig. 5. Serum antibody responses were determined in samples from the same time points at which fecal samples were tested. The first serological responses can be seen at 406 days post infection, 98 days past the second immunization with Hsp70 in G2 and G4. Apart from responses in the MAP infected groups G3 (4 of 10) and G4 (1 of 9), responses were also detected in four of nine G2 animals, while none of the G1 animals showed responses. At day 532 one animal in G3 and three animals in G4 were positive, while animals in G1 and G2 were all negative. At day 644

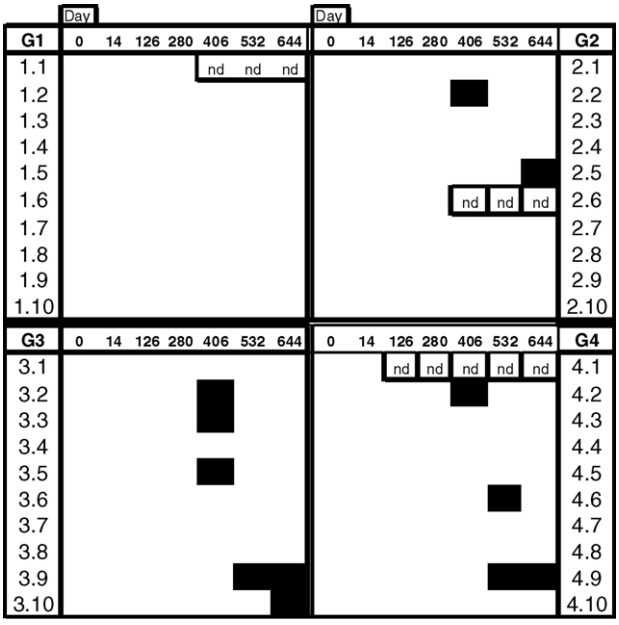

Fig. 5. In the four panels all the results from the absorbed ELISA are shown per treatment group. G1 represents the animals that were neither immunized nor infected, G2 animals only received the immunization, G3 animals only received the infection and G4 contains the animals that were both immunized and infected. Black squares (■) indicate a positive ELISA S/N ratio for a given animal and a given time point; (nd) indicates time points of respective animals were not tested because they were culled from the experiment.

one animal in G2, two animals in G3 and one animal in G4 tested positive.

4. Discussion

To our knowledge this is the first report describing a heat-shock protein-based subunit vaccine approach to bovine paratuberculosis, which leads to significantly decreased shedding of bacteria into the feces. Hsp have been shown to be dominant antigens for the immune system in many infectious diseases (reviewed in [21]). The Hsp70 of *M. tuberculosis* and *M. bovis* has been used previously in DNA vaccination studies, both in calves and in murine model systems, with variable success as therapeutic [22] or a preventive [23] vaccine against tuberculosis. Our previous studies provided evidence that the mycobacterial Hsp70 protein was a dominant T cell antigen in bovine paratuberculosis [12,13]. In addition, we also showed that in naturally infected animals the antibody responses to Hsp70 were generally low and did not allow for discrimination between infected and uninfected animals. This was in contrast with the immune responses to the Hsp60 antigen, which induced little T cell activation and high levels of antibody in MAP-infected animals shedding the bacteria in the feces [11]. As protective immunity against mycobacterial infection is considered to be cell mediated in origin (reviewed in [24,25]), we decided to use the recombinant Hsp70 protein in a subunit vaccine using DDA as a Th1 skewing adjuvant [26]. Based on the current insights in transmission of bovine paratuberculosis, characterized by the fact that the most susceptible individuals, neonates, are born in an infectious environment [1], we did not use a classical prime-boost vaccination before experimental infection as in our view this would not represent the natural sequel of events. Therefore, vaccination and challenge were synchronized, and in addition, a single vaccination was used to enable monitoring of a potential differential development of immune responses due to the concomitant infection in comparison to vaccination in uninfected control animals.

The results of the current study demonstrate that recombinant MAP Hsp70 can be successfully used as a subunit vaccine against bovine paratuberculosis in terms of significantly reducing shedding of bacteria in feces during the first 2 years following experimental infection. Our study is too short to evaluate the protective effect of the vaccination in terms of preventing the progression to the clinical stage of the disease, which is a hallmark of the whole bacterin vaccine [27]. However, the fact that the subunit vaccine significantly reduces shedding, which is something the whole bacterin fails to do [8,28], is a promising aspect of the Hsp70 immunization indicating a higher level of control on bacterial growth. Although we selected the Hsp70 protein based on its cell-mediated immunity stimulating properties in our own and other studies, the lack of clearly different IFN-γ production in the vaccinated animals was unexpected. Antigen-specific IFN-γ production was present from day 280 onwards in the study; however, while significant differences could be measured, they were related to challenge, not to treatment. The effect was similar when comparing PPD-P and whole MAP bacteria as antigen. At time points were significant differences in Hsp70 induced IFN-γ could be measured the difference was also related to challenge rather than treatment. These results indicated that the Elispot assay could detect MAP-infected individuals in different stages post challenge based on IFN-γ. There was IFN-γ production in response to Hsp70 protein during the 2 years of the study only to reach baseline at the end of the second year. Possibly, as Hsp70 is an evolutionary conserved antigen with high homology with other bacterial and even eukaryotic Hsp70, cross-reactive T-cell responses obscured vaccine-specific T-cell responses. In addition, it has also been shown that mycobacterial Hsp70 specifically binds to innate receptors present on macrophages [29] and dendritic cells [30], leading to production of pro-inflammatory signals, which may in turn lead to IFN-γ production by T and NK cells. The observation that all animals in this study have high frequencies of monocytes, gradually decreasing with age, adds to this hypothesis.

The whole blood IFN-γ assay performs better in populations of young cattle as compared to serum antibody ELISA [31]. However, the fact that young ruminants have high non-specific production of IFN-γ puts constrains on the use of IFN-γ-based diagnostics of bovine paratuberculosis in animals less than 2 years of age and especially less than 1 year of age [32]. A role for the abundantly present γδ T cells in young ruminants is suspected; however, the biology behind

these observations remains unclear as is the function of these cells [13,33,34]. Recently, NK cells have been implicated in the production of IFN-γ, especially in young calves, and in response to certain mycobacterial antigens [35]. The NKp46 antibody [36] was not available during our study for confirmation and our flow cytometry data indicate we overestimated the number of NK cells in the population we called NK-like, although we observe similar frequency kinetics [37]. The proportions of γδ T cells, CD4+ and CD8+ T cells were comparable to those reported previously for various age groups [37].

The fact that little antibody can be detected during the first 2 years of bovine paratuberculosis infection is a common characteristic of the natural infection, hampering the use of serum antibody ELISA diagnostic assays at these early stages of infection [31]. In the current study, when using a commercially available serum antibody ELISA for paratuberculosis, we found the first animals in both the treated and untreated infected group in the second year for the first time. In addition, we had two responders in treated but uninfected control group, which may indicate that some Hsp70 may be present in the antigen used in the commercial ELISA leading to a positive response. The data also supports previous observations that few antibodies to Hsp70 are formed during the infection with MAP [11]. The extensive and prolonged production of serum antibodies to Hsp70 was prominent and restricted to the treatment groups. No significant difference between challenged and uninfected animals was observed considering the total anti-Hsp70 IgG produced. Also, the high levels of anti-Hsp70 antibodies observed in this study subsequent to vaccination at least suggested that antibody responses to this protein in the context of paratuberculosis infection are not detrimental per se, as they were correlated with decreased shedding of bacteria. The priming of animals with Hsp70 using DDA adjuvant induced an immune response, which is clearly different from encountering the protein during infection. This difference does enable a differentiation between vaccinated and infected animals, which may be clearly an advantage in the application of the vaccine. As the Hsp70 is an evolutionary conserved antigen, it remains to be investigated whether Hsp70 vaccinated cows will become false positive in diagnostic assays for bovine tuberculosis, such as the intra-dermal skin test.

In conclusion we have demonstrated that vaccination of cattle with a Hsp70/DDA vaccine significantly reduces shedding of MAP in the feces, which in turn may reduce transmission of infection, has little direct and long term side effects and enables differentiation between vaccinated and infected animals and as such may contribute to the paratuberculosis eradication strategies.

Acknowledgements

The authors express their thanks to Hans Vernooij for expert help with the statistical analysis. This study was supported by a grant from the Technology Foundation (STW) of the Dutch Research Council (NWO), grant number STW-UDG5589.

References

[1] Sweeney RW. Transmission of paratuberculosis. Vet Clin North Am Food Anim Pract 1996;12(2):305–12.
[2] Whitlock RH, Buergelt C. Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet Clin North Am Food Anim Pract 1996;12(2):345–56.
[3] Whittington RJ, Marshall DJ, Nicholls PJ, Marsh IB, Reddacliff LA. Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. Appl Environ Microbiol 2004;70(5):2989–3004.
[4] Manning EJ, Collins MT. *Mycobacterium avium* subsp. *paratuberculosis*: pathogen, pathogenesis and diagnosis. Rev Sci Tech 2001;20(1):133–50.
[5] Kohler H, Gyra H, Zimmer K, Drager KG, Burkert B, Lemser B, et al. Immune reactions in cattle after immunization with a *Mycobacterium paratuberculosis* vaccine and implications for the diagnosis of *M. paratuberculosis* and *M. bovis* infections. J Vet Med B Infect Dis Vet Public Health 2001;48(3):185–95.
[6] Muskens J, van Zijderveld F, Eger A, Bakker D. Evaluation of the long-term immune response in cattle after vaccination against paratuberculosis in two Dutch dairy herds. Vet Microbiol 2002;86(3):269–78.
[7] Kalis CH, Hesselink JW, Barkema HW, Collins MT. Use of long-term vaccination with a killed vaccine to prevent fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in dairy herds. Am J Vet Res 2001;62(2):270–4.
[8] Koets AP, Adugna G, Janss LL, van Weering HJ, Kalis CH, Wentink GH, et al. Genetic variation of susceptibility to *Mycobacterium avium* subsp. *paratuberculosis* infection in dairy cattle. J Dairy Sci 2000;83(11):2702–8.
[9] van Eden W, van der Zee R, Paul A, Prakken B, Wendling U, Anderton S, et al. Do heat shock proteins control the balance of T-cell regulation in inflammatory diseases? Immunol Today 1998;19(7):303–7.
[10] Patterson CJ, LaVenture M, Hurley SS, Davis JP. Accidental self-inoculation with *Mycobacterium paratuberculosis* bacterin (Johne's bacterin) by veterinarians in Wisconsin. J Am Vet Med Assoc 1988;192(9):1197–9.
[11] Koets AP, Rutten VP, de Boer M, Bakker D, Valentin-Weigand P, van Eden W. Differential changes in heat shock protein-, lipoarabinomannan-, and purified protein derivative-specific immunoglobulin G1 and G2 isotype responses during bovine *Mycobacterium avium* subsp. *paratuberculosis* infection. Infect Immun 2001;69(3):1492–8.
[12] Koets AP, Rutten VP, Hoek A, Bakker D, van Zijderveld F, Muller KE, et al. Heat-shock protein-specific T-cell responses in various stages of bovine paratuberculosis. Vet Immunol Immunopathol 1999;70(1–2):105–15.
[13] Koets A, Rutten V, Hoek A, van Mil F, Muller K, Bakker D, et al. Progressive bovine paratuberculosis is associated with local loss of CD4(+) T cells, increased frequency of gamma delta T cells, and related changes in T-cell function. Infect Immun 2002;70(7):3856–64.
[14] Jorgensen JB. An improved medium for culture of *Mycobacterium paratuberculosis* from bovine faeces. Acta Vet Scand 1982;23(3):325–35.
[15] Vary PH, Andersen PR, Green E, Hermon-Taylor J, McFadden JJ. Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. J Clin Microbiol 1990;28(5):933–7.

[16] Colston A, McConnell I, Bujdoso R. Cloning and expression in *Escherichia coli* of DNA encoding a 60 kDa stress protein of *Mycobacterium paratuberculosis*, the causative agent of Johne's disease. Microbiology 1994;140:3329–36.
[17] Gilmour NJL, Wood GW. Paratuberculosis (Johne's disease). In: OIE manual of standards for diagnostic tests and vaccines. Paris: Office International des Epizooties; 1996. p. 218-228.
[18] Couquet C, Pourquier P. In: Manning EJB, Collins MT, editors. Evaluation of the sensitivity, specificity and detectability of a serological ELISA test for the diagnosis of paratuberculosis. Melbourne, Au: Sixth International Colloquium on Paratuberculosis; 1999. p. 494–500.
[19] R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, 2004.
[20] Lindsey, J. Non-normal repeated measurement models. R package version 1.0. 2004.
[21] Zugel U, Kaufmann SH. Role of heat shock proteins in protection from and pathogenesis of infectious diseases. Clin Microbiol Rev 1999;12(1):19–39.
[22] Lowrie DB, Tascon RE, Bonato VL, Lima VM, Faccioli LH, Stavropoulos E, et al. Therapy of tuberculosis in mice by DNA vaccination. Nature 1999;400(6741):269–71.
[23] Skinner MA, Wedlock DN, de Lisle GW, Cooke MM, Tascon RE, Ferraz JC, et al. The order of prime-boost vaccination of neonatal calves with *Mycobacterium bovis* BCG and a DNA vaccine encoding mycobacterial proteins Hsp65, Hsp70, and Apa is not critical for enhancing protection against bovine tuberculosis. Infect Immun 2005;73(7):4441–4.
[24] Stabel JR. Transitions in immune responses to *Mycobacterium paratuberculosis*. Vet Microbiol 2000;77(3–4):465–73.
[25] Raupach B, Kaufmann SH. Immune responses to intracellular bacteria. Curr Opin Immunol 2001;13(4):417–28.
[26] Lindblad EB, Elhay MJ, Silva R, Appelberg R, Andersen P. Adjuvant modulation of immune responses to tuberculosis subunit vaccines. Infect Immun 1997;65(2):623–9.
[27] van Schaik G, Kalis CH, Benedictus G, Dijkhuizen AA, Huirne RB. Cost-benefit analysis of vaccination against paratuberculosis in dairy cattle. Vet Rec 1996;139(25):624–7.
[28] Wentink GH, Bongers JH, Zeeuwen AA, Jaartsveld FH. Incidence of paratuberculosis after vaccination against *M. paratuberculosis* in two infected dairy herds. Zentralbl Vet [B] 1994;41(7–8):517–22.
[29] Bulut Y, Michelsen KS, Hayrapetian L, Naiki Y, Spallek R, Singh M, et al. *Mycobacterium tuberculosis* heat shock proteins use diverse toll-like receptor pathways to activate pro-inflammatory signals. J Biol Chem 2005;280(22):20961–7.
[30] Langelaar MFM, Hope JC, Rutten VPMG, Noordhuizen JPTM, van Eden W, Koets AP. *Mycobacterium avium* ssp. *paratuberculosis* recombinant heat shock protein 70 interaction with different bovine antigen-presenting cells. Scand J Immunol 2005;61(3):242–50.
[31] Huda A, Jungersen G, Lind P. Longitudinal study of interferon-gamma, serum antibody and milk antibody responses in cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*. Vet Microbiol 2004;104(1–2):43–53.
[32] Billman-Jacobe H, Carrigan M, Cockram F, Corner LA, Gill IJ, Hill JF, et al. A comparison of the interferon gamma assay with the absorbed ELISA for the diagnosis of Johne's disease in cattle. Aust Vet J 1992;69(2):25–8.
[33] Rhodes SG, Hewinson RG, Vordermeier HM. Antigen recognition and immunomodulation by gamma delta T cells in bovine tuberculosis. J Immunol 2001;166(9):5604–10.
[34] Kennedy HE, Welsh MD, Bryson DG, Cassidy JP, Forster FI, Howard CJ, et al. Modulation of immune responses to *Mycobacterium bovis* in cattle depleted of WC1(+) gamma delta T cells. Infect Immun 2002;70(3):1488–500.
[35] Olsen I, Boysen P, Kulberg S, Hope JC, Jungersen G, Storset AK. Bovine NK cells can produce gamma interferon in response to the secreted mycobacterial proteins ESAT-6 and MPP14 but not in response to MPB70. Infect Immun 2005;73(9):5628–35.
[36] Storset AK, Kulberg S, Berg I, Boysen P, Hope JC, Dissen E. NKp46 defines a subset of bovine leukocytes with natural killer cell characteristics. Eur J Immunol 2004;34(3):669–76.
[37] Kulberg S, Boysen P, Storset AK. Reference values for relative numbers of natural killer cells in cattle blood. Dev Comp Immunol 2004;28(9):941–8.
[38] Davis WC, Ellis JA, MacHugh ND, Baldwin CL. Bovine pan T-cell monoclonal antibodies reactive with a molecule similar to CD2. Immunology 1988;63(1):165–7.
[39] Baldwin CL, Teale AJ, Naessens JG, Goddeeris BM, MacHugh ND, Morrison WI. Characterization of a subset of bovine T lymphocytes that express BoT4 by monoclonal antibodies and function: similarity to lymphocytes defined by human T4 and murine L3T4. J Immunol 1986;136(12):4385–91.
[40] Howard CJ, Morrison WI, Bensaid A, Davis W, Eskra L, Gerdes J, et al. Summary of workshop findings for leukocyte antigens of cattle. Vet Immunol Immunopathol 1991;27(1–3):21–7.
[41] Ellis JA, Baldwin CL, MacHugh ND, Bensaid A, Teale AJ, Goddeeris BM, et al. Characterization by a monoclonal antibody and functional analysis of a subset of bovine T lymphocytes that express BoT8, a molecule analogous to human CD8. Immunology 1986;58(3):351–8.
[42] Clevers H, MacHugh ND, Bensaid A, Dunlap S, Baldwin CL, Kaushal A, et al. Identification of a bovine surface antigen uniquely expressed on CD4- CD8- T cell receptor gamma/delta+ T lymphocytes. Eur J Immunol 1990;20(4):809–17.
[43] Davis WC, Brown WC, Hamilton MJ, Wyatt CR, Orden JA, Khalid AM, et al. Analysis of monoclonal antibodies specific for the gamma delta TcR. Vet Immunol Immunopathol 1996;52(4):275–83.
[44] Brodersen R, Bijlsma F, Gori K, Jensen KT, Chen W, Dominguez J, et al. Analysis of the immunological cross reactivities of 213 well characterized monoclonal antibodies with specificities against various leucocyte surface antigens of human and 11 animal species. Vet Immunol Immunopathol 1998;64(1):1–13.
[45] Williams DJ, Newson J, Naessens J. Quantitation of bovine immunoglobulin isotypes and allotypes using monoclonal antibodies. Vet Immunol Immunopathol 1990;24(3):267–83.