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Study of the gene expression of Th1 and Th2 cytokines in the immune response of cows vaccinated with *Brucella abortus* RB51

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Summary

A reverse transcriptase real-time polymerase chain reaction was developed to estimate the expression of the gamma-interferon (IFN-γ) and interleukin-4 (IL-4) in cattle vaccinated with *Brucella abortus* RB51. Peripheral blood mononuclear cells of heifers vaccinated with *B. abortus* RB51 were stimulated *in vitro* with the same antigen and with Concanavalin A. The data obtained (presence of IFN-γ expression and absence of IL-4 expression) confirmed the cell-mediated immune response to strain RB51 antigen. Furthermore, the expression of these two cytokines was quantified and the results showed values of IFN-γ expression to be significantly higher in vaccinated than non-vaccinated animals.

Keywords

Brucella abortus, Brucellosis, Cattle, Cell-mediated immunity, Cytokines, Interferons, Reverse transcriptase polymerase chain reaction.

Introduction

Brucella abortus RB51 is a mutant, riphampicine-resistant strain that is unable to produce the N-formyl-4-amino,4-6-dideoxy mannose homopolymer (chain-O) that is normally present in the smooth lipopolysaccharide (S-LPS) wall of *Brucella* sp. (28). The efficacy and safety of the RB51 vaccine strain have been widely demonstrated (8, 9, 15, 19, 26). For the first time, the European Union approved the use of *B. abortus* strain RB51 vaccine (RB51) for the immunisation of cows at risk of infection from *B. abortus* in 2002 (12). This strain is unable to induce antibody production against the S-LPS in vaccinated animals (30, 34). Consequently, differentiation between vaccinated and unvaccinated animals is impossible with serological tests listed by European legislation (30, 32). Recent data have shown that the vaccinal strain can synthesise parts of the O-antigen (11). Therefore, animals vaccinated with the RB51 (vaccinal) strain could give positive or ambiguous results to conventional serological tests (23) based on the presence of IgG1 antibodies against S-LPS epitopes of *Brucella* sp. (3, 6, 17, 22). Nevertheless, cattle infected with *B. abortus* can reveal a serological reaction to a conventional test, even if they have been vaccinated with RB51 (9). It has been proved that *B. abortus* RB51 principally induces a cell-mediated immunity (CMI) (4), with the induction of IFN-γ but not of IL-4 (14, 37, 38, 39). Moreover, *B. abortus* is a strong CMI stimulator

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(13, 41) and macrophage activation with IFN-γ is an important control factor of *Brucella* infection (5).

Cytokines play an important role in the modulation of immunity response: depending on their type and quantity, cytokines induced a cell-mediated (Th1) or humoral (Th2) response (21, 36).

The Th1 cells mainly produced interleukin 2 (IL-2), gamma interferon (IFN-γ), tumoral necrosis factor (TNF-a) and IL-12, whilst the Th2 cells produced IL-4, IL-5, IL-6 and IL-10. The principal discriminating markers between the Th1 and Th2 immune response are considered to be IFN-γ and IL-4 (7, 20).

A reverse-transcriptase real-time polymerase chain reaction (RT-PCR) was developed to evaluate the IFN-γ and IL-4 gene expression in bovine peripheral blood mononuclear cells (PBMC) obtained from animals vaccinated with strain RB51.

Materials and methods

Animals

Fifteen steers from brucellosis-free flocks were used in the study. The animals were kept in an isolated cattle shed to prevent the introduction of *Brucella* sp. Ten steers were vaccinated subcutaneously with 2 ml of *B. abortus* RB51 (5×10^9/ml (26) and five steers with 2 ml of saline fluid (control group).

Lymphocyte isolation, cell culture and stimulation experiments

Bovine PBMCs were isolated from ethylene-diaminetetra-acetic acid (EDTA)-treated blood samples by density gradient centrifugation through Ficoll-Hypaque (density: 1 083, Sigma) (18). Cells were counted with trypan blue (TB) in a Burker chamber. The mononuclear cell fraction was washed twice in phosphate-buffered saline (PBS) and resuspended in lymphocyte growth medium RPMI (Roswell Park Memorial Institute) 1640 supplemented with 10% foetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml gentamycin, nystatin 50 IU/ml and 1% L-glutamine (18).

Freshly isolated PBMCs were dispensed in a 48-well plate (2×10^6 cell/well) and incubated at 37°C and 5% CO_2 (10) with Concanavalin A (ConA) (10 µg/ml) or *B. abortus* RB51 (100 particles for each cell) or only with RPMI as the negative control. After 6, 9, 12 and 24 h post stimulation, the cells were prepared for RNA extraction.

RNA extraction

Total RNA (tRNA) was extracted from lysed cells using the RNeasy mini kit (Qiagen), according to the instructions of the manufacturer. The extracted tRNA was treated with RNase-Free DNase Set 50 to remove contaminating genomic DNA (gDNA), and eluted in 50 µl of RNase-free water.

Primers and TaqMan probes

The gene sequence for bovine IL-4 was obtained from GenBank (accession number AH003241) and the primers and TaqMan probe for bovine IL-4 were designed using Primer Express software ver. 1 (Applied Biosystems). The sense and antisense primers and the TaqMan probe were placed in two consecutive exons of the respective gene to ensure discrimination between cDNA and gDNA. Primers and TaqMan probes for bovine glyceraldehyde-phosphate-dehydrogenase (GAPDH) and bovine IFN-γ are identical to those used by Leutenegger *et al.* (18). Each probe was labelled at the 5´-end with the reporter dye 6FAM (6-carboxy-fluorescein) and at the 3´-end with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine) and was phosphate blocked at the 3´-end to prevent extension by the AmpliTaq Gold polymerase enzyme. All primers and probes were synthesised (Applied Biosystems) (Table I).

Real-time TaqMan RT-PCR one-step for quantification of cytokine

Real-time TaqMan RT-PCR for bovine GAPDH and the cytokines were run in separate wells of 96-well optical plates. The RT-PCRs were performed

Table I
Oligonucleotide sequence used

Name	Primer	Sequence (5'-3')	Probe	Probe sequence (5'-3')
GAPDH	GAPDH.463f GAPDH.582r	GGCGTGAACCACGAGAAGTATAA CCCTCCACGATGCCAAAGT	GAPDH.489p	ATACCCTCAAGATTG TCAGCAATGCCTCCT
IFN-γ	IFN.287f IFN.12.737r	TGGATATCATCAAGCAAGACATGTT GGTCTCAGTTGCAGGTTCTTGG	IFN.378p	GGAATTTGAATCAGC CAGATCATCCACC
IL-4	IL-4f IL-4r	CCATGGACACAAGTGTGATATTACC TACAGCAGCTCCATGCATG	IL-4p	CGCTGAACATCCTCAC AACGAGAAAG

in a single buffer system and reactions contained 1 µl RNA (approximately 10-50 ng of tRNA), 400 nM of each primer, 80 nM of the TaqMan probe, 12.5 µl of the 'one-step' RT-PCR Mastermix and 0.625 µl of Multiscribe in a final volume of 25 µl.

The RT-PCR amplifications were performed using the ABI Prism 7700 in the following thermal cycling conditions: 30 min at 48°C, 10 min at 95°C and 50 cycles of 15 s at 95°C and 1 min at 60°C.

Relative quantitative method of cytokines

The gene expression of IFN-γ and IL-4 was measured by relative quantitation; this method compares the threshold cycle (Ct) of the sample to the Ct generated by a reference sample termed a 'calibrator' (non-stimulated PBMC incubated for the same time period as for stimulated PBMC). Cytokine gene expression was standardised to GAPDH expression by ΔCt values ('Ct for cytokine' – 'Ct for GAPDH' for the same sample). The ΔΔCt was calculated as the difference between ΔCt values for stimulated PBMCs and non-stimulated PBMCs. Finally, the relative difference in cytokine expression between stimulated PBMC with ConA or with *B. abortus* RB51 and the respective non-stimulated PBMC was determined using the equation: $2^{-\Delta\Delta Ct}$. This formula is valid only if the amplification efficiencies of IFN-γ and IL-4 are approximately equal to the amplification of housekeeping gene (GAPDH) and if the levels of gene expression of the housekeeping gene are not affected by the stimulus used. To confirm this, six dilutions of tRNA preparations from stimulated PBMC with ConA in triplicate were amplified to obtain standard curves. The absolute value of the

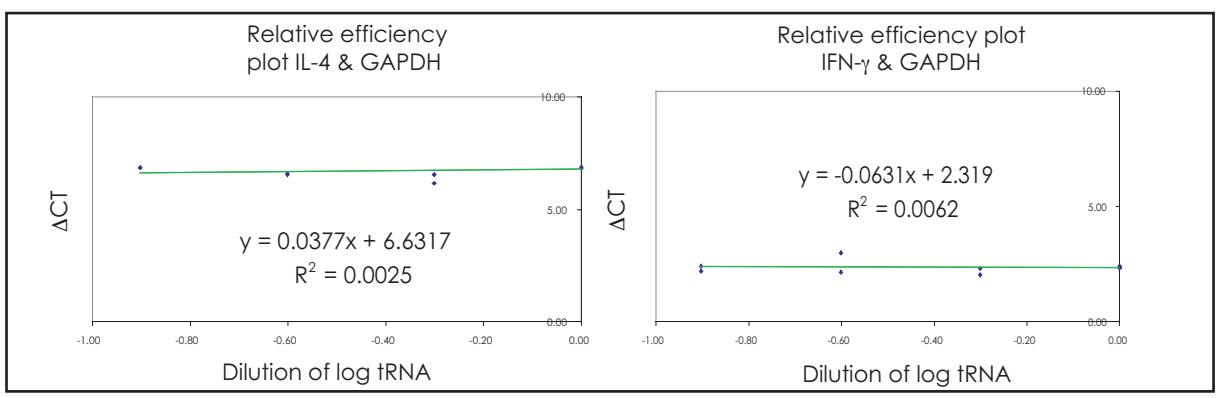

Figure 1
Plot of log input amount versus ΔCt calculated for glyceraldehyde-phosphate-dehydrogenase (GAPDH) and each gene involved

Table II
Mean glyceraldehyde-phosphate-dehydrogenase Ct values measured at various times from stimulated or non-stimulated bovine PBMC

Peripheral blood mononuclear cells	6 h	9 h	12 h	24 h
Non-stimulated	23.66±0.66	23.71±0.67	26.33±2.60	26.32±3.51
Stimulated with ConA	23.52±0.56	23.18±0.65	24.37±2.31	23.06±4.55
Stimulated with *Brucella abortus* RB51	24.40±.50	24.23±0.30	23.61±1.80	24.11±2.20

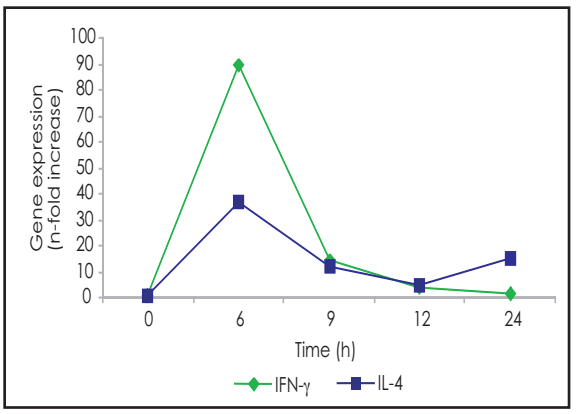

Figure 2
Kinetics of IFN-γ and IL-4 gene expression by ConA-stimulated peripheral blood mononuclear cells (PBMC).
RNA was extracted from ConA-stimulated and non-stimulated PBMC at 6, 9, 12 and 24 h. Results are expressed as n-fold increases in cytokine expression by ConA-stimulated cells compared to media treated (non-stimulated) cells

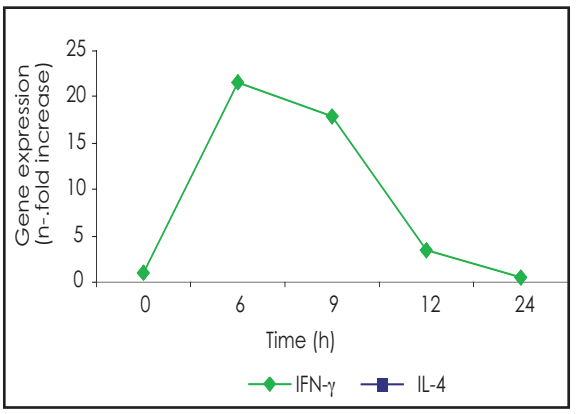

Figure 3
Kinetics of IFN-γ gene expression by *Brucella abortus* RB51 stimulated peripheral blood mononuclear cells (PBMC).
RNA was extracted from RB51-stimulated and non-stimulated PBMC at 6, 9, 12 and 24 h. Results are expressed as n-fold increases in cytokine expression by *B. abortus* RB51-stimulated cells compared to media treated (non-stimulated) cells

slope of the log of the tRNA dilutions plotted against the ΔCt, calculated for GAPDH and each gene of interest, was less than 0.1 (Fig. 1).
Moreover, in different experimental conditions there was no difference between the mean GAPDH Ct values generated by non-stimulated and ConA-stimulated samples (Table II).

Results

The PBMC stimulated *in vitro* with Con A, showed the maximal gene expression for IFN-γ and IL-4 after 6 h post stimulation and the mean levels were 90 and 37 times greater than the expression of non-stimulated cells, respectively (Fig. 2). The PMBCs stimulated with *B. abortus* RB51 showed a similar IFN-γ gene expression after 6 h and its level was 22 times greater than in the non-stimulated cells (Fig. 3), whereas there was no demonstration of IL-4 gene expression.
Moreover, in PBMCs from the blood of animals vaccinated with *B. abortus* RB51 stimulated *in vitro* with the same antigen, differences were observed in the IFN-γ gene expression between the vaccinated and non-vaccinated animals. The gene expression in the vaccinated animals was 22 and 15 times

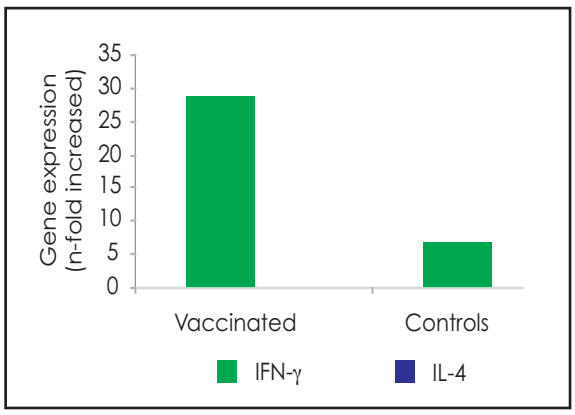

Figure 4
IFN-γ and IL-4 gene expression by peripheral blood mononuclear cells-stimulated with *Brucella abortus* RB51 at 6 h.
The IFN-γ gene expression was significantly higher in animals vaccinated with *B. abortus* RB51 when compared to the non-vaccinated group ($p<0.05$)

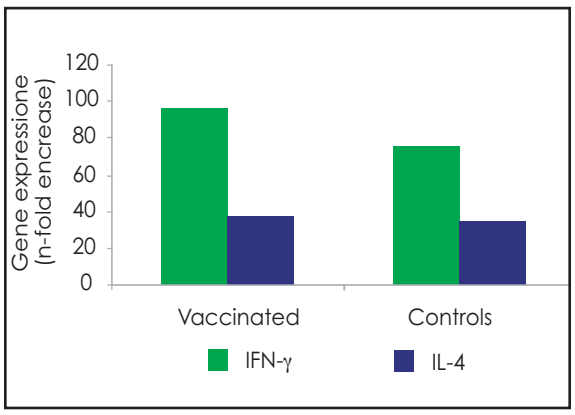

Figure 6
IFN-γ and IL-4 gene expression by peripheral blood mononuclear cells-stimulated with ConA at 6 h.
IFN-γ and IL-4 gene expression was not significantly different in animals vaccinated with *Brucella abortus* RB51 when compared to the non-vaccinated group

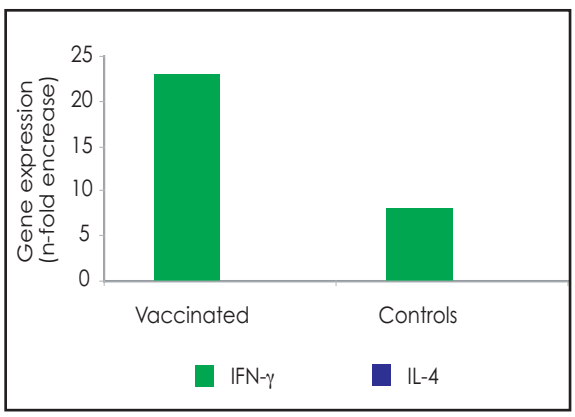

Figure 5
IFN-γ and IL-4 gene expression by peripheral blood mononuclear cells-stimulated with *Brucella abortus* RB51 at 9 h.
The IFN-γ gene expression was significantly higher in animals vaccinated with *B. abortus* RB51 when compared to the non-vaccinated group ($p<0.05$)

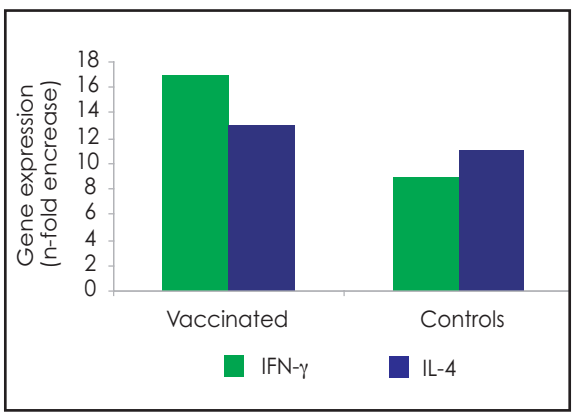

Figure 7
IFN-γ and IL-4 gene expression by peripheral blood mononuclear cells-stimulated with ConA at 9 h.
The IFN-γ and IL-4 gene expression was not significantly different in animals vaccinated with *Brucella abortus* RB51 when compared to the non-vaccinated group

greater after 6 and 9 h, respectively (Fig. 4) than in the non-vaccinated animals (Fig. 5).

The Mann-Whitney test showed a statistically significant difference ($p<0.05$) between the vaccinated and non-vaccinated animals at 6 and 9 h post stimulation, while no statistically significant differences were observed 12 and 24 h post stimulation.

However, the results of PBMCs stimulated for 6 h with ConA showed higher values of IFN-γ and IL-4 gene expression than those stimulated with RB51 antigen (Fig. 6), but the differences between the vaccinated and non-vaccinated animals were not statistically significant (Fig. 7).

Discussion

Vaccination with *B. abortus* RB51 confers protection against pathogenic challenge infection in both mice (16, 31, 33, 35) and cattle (19, 25, 26, 32). Protection induced by vaccination with *B. abortus* RB51 is based on cell-mediated immunity while antibody plays a minor role in protection (16, 31, 35). Our data have shown IFN-γ expression and no expression of IL-4 in the PBMCs stimulated *in vitro* with *B. abortus* RB51, in both vaccinated and unvaccinated cattle. These results confirm that antigen RB51 mainly induces the production of Th1 type cytokine and are in agreement with the conclusions of other authors (2, 4, 29, 40). Moreover, the comparison of data between the animals vaccinated with *B. abortus* RB51 and the control group (unvaccinated and brucellosis-free animals), showed IFN-γ expression values that were significantly higher in the vaccinated animals.

The results of a previous study (27) show that splenocytes stimulated *in vitro* with *B. abortus* RB51 or *B. abortus* 2308 produce INF-γ in mice immunised with *B. abortus* RB51, but they do not produce IL-4. Instead, after infection of the animals with *B. abortus* 2308, the stimulated splenocytes synthesised IL-4 also, in both vaccinated and unvaccinated mice. Moreover, IFN-γ production was revealed significantly early in spleen cells from vaccinated mice.

After an analysis of our data and those obtained from Pasquali *et al.* (27), it is reasonable to assume that IL-4 production, in splenocytes or PBMCs *in vitro* stimulated with *B. abortus* RB51, are closely correlated to the infection of the animals with virulent strain *B. abortus* 2308; while it could be possible that in single vaccinated animals there is no production of IL-4.

At present, the only tests able to identify cattle vaccinated with *B. abortus* strain RB51 are the dot-blot assay (24) and the complement fixation test with RB51 antigen (CF-RB51) (1). The dot-blot assay is capable of determining antibody titres during the first 8 weeks post vaccination; it is highly specific for bovines vaccinated with 10^{10} colony-forming units (cfu) of *B. abortus* RB51, but it does enable identification of animals vaccinated with a lower dose (10^{9} cfu of RB51). Instead, the CF-RB51 is able to identify, with high specificity, bovines vaccinated with both doses, until 18 weeks post vaccination.

Since we have demonstrated a difference of IFN-γ expression between vaccinated and unvaccinated bovines until six months after immunisation, the method used here could also be used as a diagnostic tool to discriminate between healthy cattle and bovines vaccinated with *B. abortus* RB51. However, the presence of IL-4 expression would indicate the probable interaction of the animals with virulent strain *B. abortus* 2308, independently of whether or not they have been vaccinated with *B. abortus* RB51.

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