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Immunity in pregnant ewes following a live-dead vaccination and infection with *Salmonella* Brandenburg

H. LI, R.G. MCFARLANE, O.Y. AMOAFO and X.Z. ZHANG[1]

Agriculture and Life Sciences Division, P.O. Box 84, Lincoln University, Canterbury, New Zealand

ABSTRACT

The aim of this study was to investigate the efficacy of a combined live–dead vaccination protocol to protect sheep against *Salmonella* Brandenburg infection. Thirty ewes were randomly divided into two groups, one of which was vaccinated and the other acted as a control. The vaccinated group received a live modified *Salmonella* Typhimurium vaccine (given by eye-drop) followed 4 weeks later by a sub-unit preparation derived from *S.* Brandenburg (given subcutaneously with the adjuvant Quil A). Following challenge with wild type *S.* Brandenburg (4 weeks after the booster), 43% of the ewes aborted in the vaccine group as compared with 53% of the controls. However, a higher mortality rate was found in the vaccine group (43% vs 20%). Using an enrichment/selection procedure, *S.* Brandenburg was detected in the faeces of all challenged animals until 3 weeks post challenge. By 6 weeks and 9 weeks post challenge approximately 50% and 30% of animals respectively were shedding, irrespective of treatment. Following administration of the live primary and subunit (secondary) vaccines, levels of total IgG, IgG1 and IgM antibody (measured with an indirect ELISA) were significantly increased in comparison with the control group ($P < 0.01$). The cell-mediated immune response (interferon gamma from whole blood cultures) was also enhanced following use of the live attenuated vaccine ($P < 0.01$), but was much less pronounced after boosting with the subunit vaccine and following challenge. In summary, vaccination evoked immune responses but did not give protection to experimental infection with *S.* Brandenburg.

Keywords: humoral immunity; cell-mediated immunity; vaccine; *Salmonella*.

INTRODUCTION

Salmonella Brandenburg is a facultative intracellular pathogen that causes systemic infection in pregnant ewes that may result in abortion and death. In addition, it has been considered a major contributor to the increased prevalence of human salmonellosis in the last decade (Clark *et al*., 2004).

Immunity to disease caused by intracellular bacteria requires early development of the cell-mediated immune response involving T lymphocytes and immunologically activated macrophages (Collins, 1979), and a subsequent humoral immune response (Collins, 1971). The ability of T cells to produce interferon gamma (IFNγ) is widely accepted as a strong indicator of protective immunity for intracellular pathogens in mice (Mastroeni *et al*., 1992), chickens (Farnell *et al*., 2001), sheep (Montagne *et al*., 2001), humans (Salazar-Gonzalez *et al.,* 2004), although cytokines such as IL-12 and IL-23 are also important with *Salmonella* in humans (MacLennan *et al*., 2004). However, the absence of antibodies also results in inefficient microbial clearance (Moore *et al*., 2003). Protection against *Salmonella* infection has been associated with antibody responses directed towards the cell wall of *S.* Typhimurium (Mates and Yosipovici, 1976), outer membrane protein of *S. enteritidis* (Meenakshi *et al*., 1999), porins (Singh *et al*., 1996), lipopolysaccharides (Iankov *et al*., 2002), fimbriae (De Buck *et al*., 2005) and flagellae (Mukkur *et al*., 1987). Close cooperation between T and B cells is of fundamental importance for the establishment of solid, acquired immunity to salmonellosis (Mastroeni and Menager, 2003) and effective vaccination strategies should be designed to enhance both humoral and cellular immunity.

We have previously discussed how a subunit vaccine can confer protection against mortality caused by *S.* Brandenburg in pregnant ewes, (Li *et al*., 2005, in press). In that trial, a live attenuated *S.* Typhimurium vaccine did not produce a detectable antibody response, but did stimulate cellular immunity, as measured by lymphocyte proliferation *in vitro* to *S.* Brandenburg antigen. A live-dead vaccine combination has been successfully used to immunize livestock against salmonellosis in calves (Meyer *et al*., 1977). The aim of this study was to investigate the efficacy of vaccination against *S.* Brandenburg using a live attenuated vaccine (sensitisation) followed by a subunit vaccine (booster).

MATERIALS AND METHODS

Animals

Two-year-old pregnant ewes were obtained from a commercial farm in mid-Canterbury where there was no

[1]Animal Science Division, Hebei Normal University of Science and Technology, Hebei Province, China

recorded presence of *S*. Brandenburg. The ewes did not shed *Salmonella* spp. in their faeces before the experiment began and were serologically negative (ELISA) to *S*. Brandenburg antigen. The animals were initially housed on the Lincoln University Research Farm before being moved to a quarantined farm site prior to exposure to *S*. Brandenburg. All procedures were conducted in accordance with the requirements of the Lincoln University Animal Ethics Committee and the Lincoln University Biosafety Committee.

Immunization and challenge

Thirty pregnant ewes were stratified into two groups on the basis of live weight. Ewes in Group A were primed with a live attenuated *S*. Typhimurium (Δ*cya* Δ*crp*) vaccine administrated by eye drop (1 x 10^9 cfu) and subcutaneously boosted with a subunit vaccine made up from a cell wall fraction (CWF) of *S*. Brandenburg given at 500 μg per dose, 4 weeks later. Animals in Group B served as un-vaccinated controls and were given 0.25 ml of phosphate buffered saline solution (PBS) as a placebo into each eye. All ewes were challenged with an ovine-virulent *S*. Brandenburg isolate (ESR# 3684) 4 weeks after booster (about 5 weeks prior to their predicted parturition date), at a dose of 2.1 x 10^9 cfu (0.25 ml into each eye, 6.5 ml orally). Blood and faeces were sampled weekly throughout the experiment, which was terminated 9 weeks after challenge.

Preparation of the subunit vaccine

The subunit vaccine was prepared as previously described by Li *et al.* (2005). Briefly, a semi-purified preparation containing elements of the bacterial CWF was quantified and mixed with Quil A adjuvant.

Isolation of bacteria

The presence of *Salmonella* bacteria in faeces, blood and internal organs after necropsy was carried out by an enrichment procedure utilizing peptone water, Rappapport-Vassiliadis R10 broth and brilliant green agar (Difco) as previously described (Li *et al*., 2005).

Antibody production

Humoral responses against *S*. Brandenburg antigen (CWF) were determined by indirect enzyme-linked immunosorbent assays (ELISA) with modifications (Leitner *et al*., 1990). Briefly, antibodies that bound to CWF antigen that had previously been absorbed onto an ELISA plate were detected using either mouse anti-sheep IgG (Serotec, UK), or IgG1 (culture supernatant from AgResearch Ltd NZ, originally prepared by CSIRO Australia) or IgM (Serotec, UK) monoclonal antibodies following by horse radish peroxidase-linked rabbit anti-mouse IgG (Serotec, UK) as secondary antibodies. The antibody-enzyme conjugate levels were measured using 3,3',5,5' tetra-methyl-benzidine as a substrate. The results were expressed as an optical density (OD) ratio; absorbance divided by the negative control.

Whole blood culture for measurement of interferon gamma (IFNγ)

Heparinised blood (1 ml) was dispensed into a 24-well plate and 100 μl of either antigen (CWF, 50 μg/ml) extracted from *S*. Brandenburg or PBS was added and cultured at 37^0C in 5% CO_2 for 24 hours and the plasma supernatant was collected and frozen at -20^0C. Levels of IFNγ in the supernatants were detected using a Bovine Gamma Interferon Test kit (Bovigam^{TM}, CSL, Australia) and expressed as OD ratio: (sample stimulated with CWF/negative control well) ÷ (sample unstimulated/negative control well).

Statistical analysis

Treatment effects from data composed of discrete variables (numbers of deaths and abortions) were analysed using the Mantel Haenszel chi-squared test included in Epi Info 2000 (version 1.0). Differences between treatment groups were compared using repeat-measurement analysis of variance with the Genstat Software Package (6th Edition UK).

RESULTS

Clinical observations

The challenge infection induced a disease typical of *S*. Brandenburg. Eight of the total 28 (1 died) (29%) ewes developed mild diarrhoea for approximately 1 week after challenge and all animals were shedding challenge organisms in their faeces at that time. However, by the second week, nine of the surviving 27 (33%) animals were not shedding with no difference between treatment groups. No *Salmonella* bacteria were isolated from blood one week after challenge. Death and abortion occurred during a 4-week period following challenge (Table 1). The overall mortality rate of the vaccinated group was 43% as compared with 20% in the control group. Abortion in the control group started during the second week when 75% of abortions occurred, whereas in the vaccine group it started during the 3rd week when all abortions occurred. Number of ewes that died or aborted in the vaccine and control groups were 79% and 73% of the total, respectively. *S*. Brandenburg was isolated from 4/6 vaccinated and 2/3 control ewes that died and 33/34 of the aborted lambs.

Analysis of plasma antibody by ELISA

Conjunctival vaccination with the live attenuated mutants at a dose of 1 x 10^9 CFU induced a significant antibody response to CWF, compared with the control group (P < 0.01) (Figure 1). Both IgG1 and IgM titres peaked three weeks after sensitisation with the live vaccine, whereas, total IgG peaked at two weeks.

Boosting with the subunit vaccine induced a quick and significant increase in all antibodies ($P < 0.01$). By the time of challenge at week 8, the vaccinated group showed levels of antibody IgG, IgG1 and IgM, that were significantly higher than the controls ($P < 0.01$). Challenge with virulent *S*. Brandenburg evoked a stronger antibody response in the control than in vaccinated animals because of the lower initial starting point, although resultant IgG and IgG1 antibody levels and pattens in both groups were similar. Levels of IgM were continuously higher in the control than the vaccine group following challenge infection ($P < 0.01$).

TABLE 1: Death and abortion of ewes following challenge with *S*. Brandenburg.

Weeks post challenge	Death/total		Abortion/survivors	
	Vaccine	Control	Vaccine	Control
1	0	1	0	0
2	1	0	0	6
3	4	2	5	3
4	1	0	0	0
Total	**6/14**	**3/15**	**5/8**	**9/12**

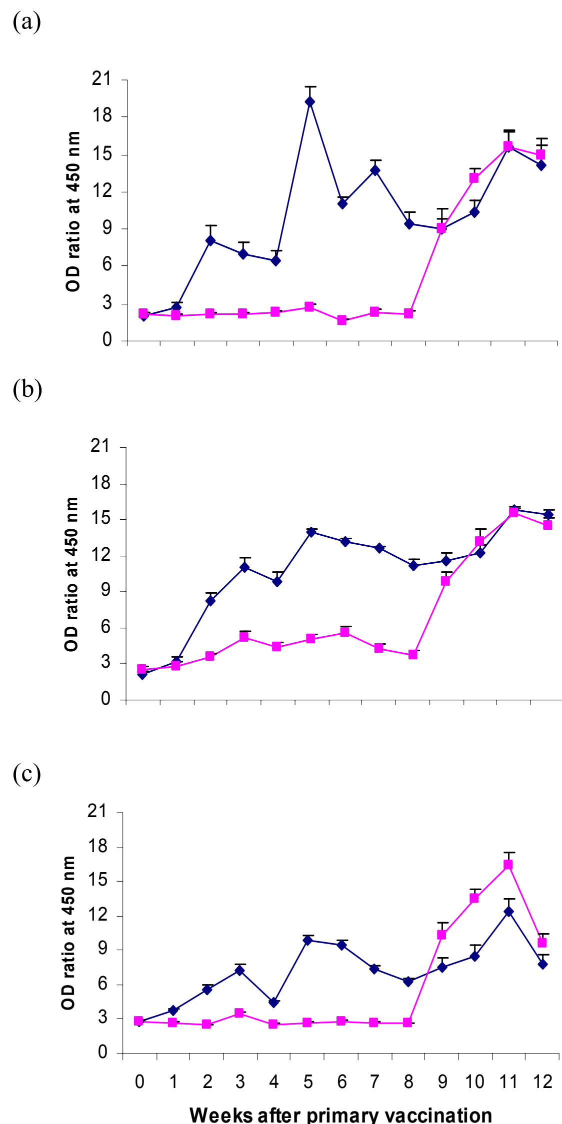
Interferon gamma (IFNγ) in whole blood culture

The amount of IFNγ in the supernatant of the cultures from the vaccinated group was significantly higher than for controls ($P < 0.01$) following sensitisation with the live vaccine (Figure 2). Boosting with the subunit vaccine did not increase the level of IFNγ production *in vitro*. Following challenge, higher levels of IFNγ were detected in the cultures from control, as compared with vaccinated, animals ($P < 0.01$). The protected animals (did not die or abort) in the control group had higher levels of IFNγ ($P < 0.01$) than non-protected animals, across 3 time-points, but protection was not associated with IFNγ in the vaccinates.

DISCUSSION

The purpose of this study was to develop a combined vaccination schedule for protection against *S*. Brandenburg where a live attenuated *S*. Typhimurium vaccine was given to ewes followed by a subunit vaccine derived from *S*. Brandenburg. This combination induced an immune response but was not protective against the experimental infection with *S*. Brandenburg.

FIGURE 1: Kinetics of plasma IgG (a), IgG1 (b) and IgM (c) production, reactive to CWF, in vaccinated (♦) and unvaccinated ewes (■). Vaccinates were primed with live vaccine at week 0, boosted with subunit vaccine at week 4 and all ewes were challenged at week 8.

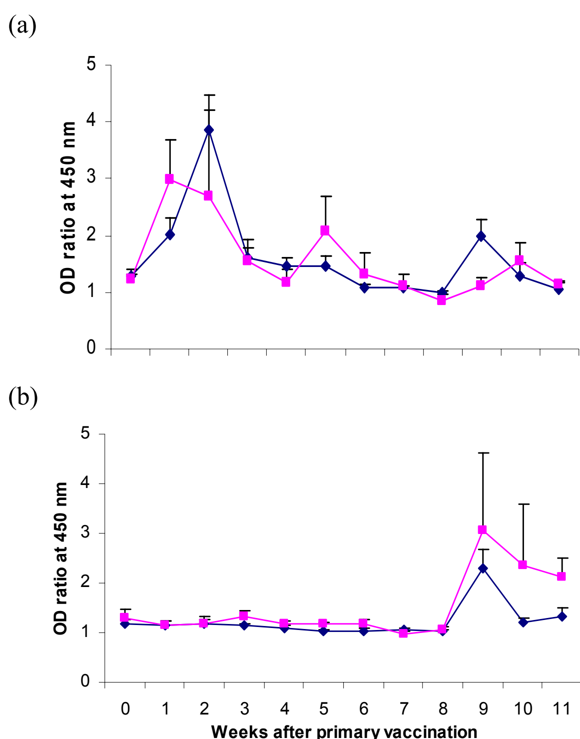


In the present study, the dose of the live vaccine for sensitization was 10^9 cfu per animal, instead of 5×10^8 cfu which did not induce a detectable antibody response in a previous trial (Li *et al*., 2005). At this increased dose, there was detectable IgG, IgG1 and IgM suggesting that a minimum dose (threshold) is required for activation of the immune system in ewes using this attenuated *S*. Typhimurium mutant (Δ*cya* Δ*crp*). Active immunity has been also produced by the same attenuated vaccine in mice and chickens (Curtiss & Kelly, 1987), but to our

knowledge, this is the first report on the immunogenicity of this mutant in ruminants.

It is not surprising that a significant antibody response developed after boosting with the *S.* Brandenburg subunit vaccine in ewes that were sensitized with the live *S.* Typhimurium vaccine. The subunit preparation given as a double vaccine strongly induced a humoral immunity (unpublished data).

FIGURE 2: Kinetics of IFNγ release from whole blood cultures incubated with CWF from vaccinated (a) and control (b) groups, and shown as protected (did not die or abort) (■) and unprotected (♦) ewes. Vaccinates were primed with live vaccine at week 0, boosted with subunit vaccine at week 4 and all ewes were challenged at week 8.



In addition, *S.* Typhimurium and *S.* Brandenburg are closely related (both in group B) and cross-react immunologically as shown by Western blot (unpublished data). In addition, we observed similar results for the ELISA when the primary antigen (*S.* Typhimurium) was used in the assay (unpublished data). Different serotypes of *Salmonella* may carry common antigenic determinants in their outer membrane resulting in antigenic cross-reactivity with each other that could provide protection. Indeed, the live vaccine used in this trial has been used successfully to protect against *S.* enteritidis (Group D) infection in poultry (Hassan & Curtiss 1997). Interestingly, IFNγ release was less than the primary response after boosting with the subunit vaccine and at the time of challenge. These results are in contrast to previous studies where *Salmonella* subunit vaccines activated cellular immunity as indicated by lymphocyte proliferation, delayed type hypersensitivity and IFNγ release (Matsui & Arai, 1992). The reason for this disparity may be due to the fact that T-cell epitopes were not present in the subunit vaccine and where ewes had been primed with the live vaccine, no re-stimulation of T cells occurred. However, a reduced release of IFNγ occurred in vaccinated animals after challenge when T cell epitopes should have been plentiful, so perhaps another mechanism is present.

Previously, we have discussed a challenge model for infection with *S.* Brandenburg in pregnant ewes (Li *et al.*, 2005) in which 2 x 10^{10} cfu per animal was used for challenge dose. This dose caused a high abortion rate in ewes, so in the present trial, 2.1 x 10^{9} cfu was given in an attempt to better mimic a typical field exposure. The vaccination schedule was not effective for protection against this experimental challenge, as 6 of 14 ewes died from *S.* Brandenburg exposure which did not differ significantly from the controls where 3 of 15 ewes died ($P = 0.19$). In a previous trial, the subunit vaccine given twice stimulated a strong humoral response and also greatly reduced the mortality caused by challenge with the same strain used in this trial (Li *et al.*, 2005). The trial shows the importance of boosting the subunit preparation with an identical product (CWF). The lack of protection of vaccinates observed in the current trial may indicate immune suppression, as shown by the low level of IFNγ production after challenge. Matsui and Arai (1993) demonstrated that cell mediated immunity can be suppressed when mice were immunized with either viable cells of, or a sonicate from *S.* Typhimurium. This was due to suppression of protein kinase C (PKC) activity and down-regulation of tyrosine phosphorylation. IFNγ production after challenge was associated with protection, but only in previously naïve (to *Salmonella*) animals. Muotiala and Makela (1993) also demonstrated that in naïve mice infected with *S.* Typhimurium, a high level of IFNγ release from the spleen cells was observed in the presence of high titres of bacteria, as compared with immune animals that had low IFNγ levels.

Abortion is a typical sequel to *S.* Brandenburg infection in the field. Our results showed that a ten-fold reduction of the challenge dose from a previous trial did not change the high abortion rate in both treated and untreated groups. Although a lower abortion rate was found in the vaccine group, it was accompanied with a higher mortality; the total loss (abortion plus death) in both groups was the same. It is well known that the pathogenicity of bacterial infections can be increased when stress factors exist. In this trial all animals were fasted for 36 hours prior to challenge and showed a greatly increased level of plasma β-hydroxybutyrate (data not shown). During and subsequent to the challenge period, Canterbury experienced extremely cold and wet

climatic conditions, and this probably contributed to the high mortality of the ewes. As the trial was conducted on a farm site where there was substantial risk to personnel due to inadvertent zoonotic transmission, in-depth necropsies were not conducted to establish the definitive cause of death (e.g. exposure) in these ewes, where liver biopsies were negative to *S.* Brandenburg (33% of samples from both groups). Nevertheless, all dead ewes were shedding *Salmonella* in their faeces at the time of death and *S.* Brandenburg was isolated from the dead fetuses inside all of the ewes that died.

Our conclusion is that the dual immunization procedure, of a live followed by the subunit vaccine, gives no protection against mortality, abortion and bacterial shedding caused by *S.* Brandenburg in pregnant ewes, despite stimulation of certain immune responses.

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