



# Mucosal delivery of native and recombinant protein vaccines against *Trichostrongylus colubriformis*

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## ABSTRACT

This paper describes a series of five pilot trials to test the feasibility of inducing a protective mucosal immune response against a non-blood-feeding intestinal nematode by delivery of antigens across the mucosal epithelium. A number of antigen preparations from *Trichostrongylus colubriformis* (viable larvae, larval homogenate and recombinant 17 kDa excretory–secretory protein) were delivered to the luminal surface of the mucosal epithelium overlying jejunal or rectal lymphoid tissue in cellulose or chitosan formulations. Significant protection was induced following delivery of viable larvae, larval homogenate or recombinant protein to the epithelium overlying rectal Peyer's patches, and recombinant protein to the epithelium overlying jejunal Peyer's patches. Viable larvae were associated with a jejunal IgE/IgG1 response, while the 17 kDa antigen was associated with a jejunal IgA response. The results demonstrate that delivery of *Trichostrongylus* native and recombinant antigens across the epithelium overlying rectal lymphoid patches can result in significant protective immunity even in the absence of adjuvant. They warrant the further investigation of appropriate mucosal delivery methods and adjuvants for induction of protective mucosal responses to stages and species of gastrointestinal helminths which do not ingest serum antibodies.

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## 1. Introduction

Anthelmintics are still the method of choice for control of gastrointestinal parasitism, however, the increase in resistance of parasites, the ever decreasing profit margins for grazing enterprises and the issue of chemical residues mean that ultimately a more sustainable strategy must be found. Given the economic and social pressures to increase rather than decrease stocking rates, a promising candidate is the enhancement of host immune-mediated resistance. Vaccines are one way of achieving this enhancement and could be a major component of strategies for the prevention and control of gastrointestinal parasitism. They will permit the safe exposure of young, naïve or pregnant livestock to the parasite antigens, an exposure essential to the acquisition of immunity, thus allowing animals to develop immunity before a combination of stress, inadequate nutrition, adverse climate and availability of larvae results in a period of high risk.

Much of the emphasis in the search for vaccines has been placed on the antigen, and to a lesser extent the adjuvant. This is well and good when high serum antibody concentration is protective, but where mucosal responses are required we do not yet know how to generate these. Research has largely neglected the formulation

and delivery route necessary to achieve optimal protective mucosal immune responses and thus efficacy. Until these aspects are addressed, there is little point in identifying or producing recombinant versions of, new antigens. While effective vaccines against blood-feeding nematodes of ruminants are delivered by s.c. or i.m. routes and operate via ingestion by the worm of serum antibodies (reviewed by Knox et al., 2003; Smith and Zarlenga, 2006), consistent protective vaccine-induced immunity against the non-haematophagic nematode species has not been reported, despite many years of efforts to identify antigens and to trial them with various adjuvants (reviewed by Emery, 1996; Knox, 2000; Hein and Harrison, 2005). Some of the inconsistency in efficacy observed with these experimental vaccines is attributable to a failure to avoid factors such as genotype, liveweight, weaning, nutrition and co-infection which can predispose animals to sub-optimal development of immunity (Windon, 1996; McClure and Emery, 2007; Coop and Sykes, 2002). It is likely that sub-optimal formulation, adjuvantation and delivery also account for some of the variation in protection data. Appropriate strategies for the formulation and delivery of vaccines against larvae or browsing nematode species need to be developed before the identification of antigens can result in consistently effective vaccines (Hein and Harrison, 2005; Smith and Zarlenga, 2006).

For non-haematophagic nematodes and for the larval stages of blood-feeding species, it appears that local anamnestic immunity

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is critical to protection. Host resistance to these involves a complex range of local responses (Rothwell, 1989; McClure et al., 1992; Miller, 1996; Anthony et al., 2007), not all of which are classically immunological, and no one of which is essential or sufficient on its own. The chief site of induction of mucosal immunity is the jejunal Peyer's patch and draining mesenteric lymph node (Russell-Jones, 2000; Neutra and Kozlowski, 2006), and the generation of mucosal responses is usually better achieved by delivery of the antigen to the gut, for example by i.p. or intra-Peyer's patch routes, than by systemic routes (Husband et al., 1996; Zhou and Neutra, 2002). Surgical vaccination of the small intestine is impracticable in the field, the ruminant digestive system complicates oral delivery of vaccines by necessitating systems which protect the antigen during transit, and the marketing of vaccines requiring i.p. injection is considered by the pharmaceutical industry to be problematic. An alternative approach is to target the two remaining accessible mucosal sites which have constitutive lympho-epithelial tissue and which are included in the common mucosal immune system.

The naso-pharynx contains lympho-epithelial nodules which have been suggested as sites for the generation of responses at distant mucosal sites via the common mucosal immune system (Stanley et al., 2001). Intra-nasal administration of antigen to sheep has resulted in systemic humoral and cellular responses to inactivated influenza (Coulter et al., 2003), KLH (Premier et al., 2004) and extracts of *Toxoplasma gondii* tachyzoites (Stanley et al., 2004). Early studies utilising intra-nasal delivery of recombinant protein antigens did not demonstrate increased protection against challenge with *Trichostrongylus colubriformis* (McClure, unpublished observation), although this may change with identification of more appropriate adjuvants. The terminal rectum of ruminants also contains aggregates of lymphoid follicles (Carlens, 1928, quoted by Aleksandersen et al., 1991) with follicular associated epithelium which resembles that of the ileal Peyer's patch (Aleksandersen et al., 1991), and a lymphocyte population resembling that of the jejunal patch (Aleksandersen et al., 1990; Sedgmen et al., 2002), except that the rectal patch contains a much higher proportion of Tcr $\gamma\delta^+$  cells. This population of T lymphocytes expresses the  $\gamma\delta$  form of the surface receptor for antigen. They are a minor population in blood but constitute a higher proportion of the intestinal T cell population, and are especially prevalent within the intestinal epithelium. Their function is unclear, but they are thought to have roles in the homeostatic regulation of epithelial tissues and in tissue-associated immune regulation (Born et al., 2007). Uptake of ferritin across the rectal mucosa has been demonstrated in calves (Leibler et al., 1991). A previous study of mucosal methods for

the delivery of ovalbumin to sheep demonstrated that trans-epithelial rectal delivery of ovalbumin with Quil A primed for significant increases in Tcr $\gamma\delta^+$  cells, CD1 $^+$  cells and nerve fibres in the jejunum following intra-duodenal challenge (McClure et al., 1994).

This paper describes a series of pilot trials to test the feasibility of inducing a protective mucosal immune response against the intestinal nematode *T. colubriformis* by delivery of native or recombinant worm antigens across the rectal mucosal epithelium. As a first pass, these trials explored a range of experimental conditions in terms of dose and challenge regime and time of challenge, parameters expected to influence the rate of development of immunity.

## 2. Materials and methods

### 2.1. Animals and experimental design

Merino ewes (medium wool for Experiments 1–3 and fine wool for Experiments 4 and 5) were pasture-fed until 3 weeks before lambing, when they were brought into raised group pens, drenched with ivermectin/praziquantel (Firstmectin, Virbac, Milperra NSW), albendazole/levamisole (Rotate, Novartis, North Ryde NSW) and naphthalophos (Rametin, Bayer, Pymble NSW) at the recommended dose rates and fed a pelleted diet. For Experiments 1–3 this consisted of oat grain:lucerne (40:60 w/w) plus 1% of a mineral mixture (Siromin, Compass Feeds, Mt. Compass, South Australia) and containing at least 12% crude protein. For Experiments 4 and 5 the diet consisted of lucerne:wheat:pollard:bran (5:1:1:1.8 w/w), containing 14–17% crude protein (CP) and 9 MJ metabolisable energy (ME) kg dry matter (DM) $^{-1}$ . The helminthologically naïve lambs were offered these diets before and after weaning at a rate of 800 g d $^{-1}$ , and were weaned at 4 months of age. Five experiments were conducted. The experimental design is summarised in Table 1.

All procedures involving the use of experimental animals were approved by the CSIRO Prospect or Armidale Animal Ethics Committees.

#### 2.1.1. Experiment 1

Twenty-eight ewe and wether lambs were 6 months of age at the start of the experiment, when they were assigned to three groups so that each group contained equal numbers of males and females. Group 1,  $n = 12$ , received no vaccination or surgery. Lambs in Groups 2 and 3 were surgically vaccinated following general anaesthesia and midline laparotomy, on Days 0 and 28. Group 2

**Table 1**  
Experimental design. Jejunal and rectal routes involved delivery of vaccine to the luminal surface of the epithelium; iJPP, injection into the jejunal Peyer's patches. Group 1 in Experiments (Expt) 1–3 was not vaccinated. In Expts 4 and 5, Group 1 was vaccinated with the formulant minus antigen. In Expt 1, the antigen was 17 kDa recombinant protein, formulated in cellulose gel (Group 2) or alhydrogel (Group 3). In Expt 2, the antigen was viable exsheathed larvae formulated in cellulose gel. In Expt 3, the antigen was non-viable homogenised larvae formulated in cellulose gel. In Expt 4, the antigen was non-viable homogenised larvae formulated in chitosan gel. In Expt 5, the antigen was either non-viable homogenised larvae formulated in chitosan sponge (Group 2) or 17 kDa recombinant protein formulated in chitosan sponge (Group 3).

| Expt. | Group | <i>n</i> | Vaccine          | Route   | Frequency         | Challenge (time, type, duration) |
|-------|-------|----------|------------------|---------|-------------------|----------------------------------|
| 1     | 1     | 12       | –                |         |                   | D49, bolus, 7 weeks              |
|       | 2     | 4        | Recomb/cellulose | Jejunal | D0, 28            | D49, bolus, 7 weeks              |
|       | 3     | 12       | Recomb/AIOH      | iJPP    | D0, 28            | D49, bolus, 7 weeks              |
| 2     | 1     | 6        | –                |         |                   | D56, bolus, 6 weeks              |
|       | 2     | 6        | Viable/cellulose | Rectal  | 3/week $\times$ 6 | D56, bolus, 6 weeks              |
| 3     | 1     | 8        | –                |         |                   | D56, bolus, 6 weeks              |
|       | 2     | 9        | Homog/cellulose  | Rectal  | D0, 28            | D56, bolus, 6 weeks              |
| 4     | 1     | 9        | Chitosan         | Rectal  | D0, 28            | D35, trickle, 8 weeks            |
|       | 2     | 10       | Homog/chitosan   | Rectal  | D0, 28            | D35, trickle, 8 weeks            |
| 5     | 1     | 12       | Chitosan         | Rectal  | D0, 21, 35, 42    | D49, trickle, 7 weeks            |
|       | 2     | 12       | Homog/chitosan   | Rectal  | D0, 21, 35, 42    | D49, trickle, 7 weeks            |
|       | 3     | 6        | Recomb/chitosan  | Rectal  | D0, 21, 35, 42    | D49, trickle, 7 weeks            |

lambs,  $n = 4$ , were vaccinated by delivery to the luminal surface of the epithelium over three jejunal Peyer's patches, of 1 mg of 17 kDa recombinant protein from *T. colubriformis* (for details see Section 2.2) in 6 ml cellulose gel. Lambs in Group 3,  $n = 12$ , received 600  $\mu$ g 17 kDa in 3 mg Alhydrogel (CSL, Melbourne) by injection into three jejunal Peyer's patches. This dose for Group 3 was based on the 200  $\mu$ g used for this antigen in s.c. vaccination trials, treating each Peyer's patch as a separate site of immune induction. The dose for Group 2 was arbitrarily increased to allow for intra-luminal wastage. On Day 42, all lambs were challenged with a bolus of 30,000 *T. colubriformis* L3s). Bolus challenge was used in the initial trials in order to expose the lambs to sufficient larvae to induce immunity immediately. This would allow an estimate of the rate of response. As natural exposure in the field is trickle in nature, the challenge protocol in later trials was changed, but the first dose of the trickle was 6000 larvae, a number in excess of the minimum reported to the recognised by the immune system (Dobson et al., 1990a). Faecal worm egg counts were performed at weeks 3, 4, 5, 6 and 7 after challenge, and samples collected at post mortem 7 weeks after challenge for total worm counts. In this and the subsequent trials, the cellulose and chitosan gels allow the antigen to adhere temporarily to the mucosal surface, and the sponge dissolves in the mucus to achieve the same result.

#### 2.1.2. Experiment 2

Twelve ewe lambs were approximately 7 months of age at the start of the experiment, when they were randomly assigned to two groups. Group 1,  $n = 6$ , received no vaccination.

Group 2,  $n = 6$ , were vaccinated after rectal evacuation by delivery to the rectal mucosa, 4–8 cm proximal to the anus, of 1200 exsheathed *T. colubriformis* L3s in 6 ml cellulose gel, three times per week for 6 weeks. The vaccine was delivered by 10 ml syringe without needle to the luminal surface of the mucosal epithelium. Both gels and sponges given per rectum were largely evacuated by subsequent peristalsis, with variable timing. Lambs were monitored to ensure minimal retention times of 20 min. All lambs were challenged orally at Day 56 with a bolus of 30,000 *T. colubriformis* L3s. Faecal worm egg counts were performed at weeks 3, 4, 5 and 6 after challenge, plasma was collected at challenge and 6 weeks after challenge for measurement of antibody concentration, and samples collected at post mortem 6 weeks after challenge for total worm counts and for jejunal (1 m distal to the pylorus) antibody concentration.

#### 2.1.3. Experiment 3

Seventeen ewe lambs were 6 months of age at the start of the experiment, when they were randomly assigned to two groups. Group 1,  $n = 8$ , received no vaccination. Group 2,  $n = 9$ , were vaccinated by delivery to the rectal mucosa as above of homogenate (non-viable) of 40,000 *T. colubriformis* L3s in 6 ml cellulose gel applied as single doses on Day 0 and Day 28. All lambs were challenged orally at Day 56 with a bolus of 30,000 viable *T. colubriformis* L3s. Faecal worm egg counts were performed at weeks 3, 4, 5 and 6 after challenge, and samples collected at post mortem 6 weeks after challenge for total worm counts.

#### 2.1.4. Experiment 4

Nineteen ewe lambs were 9 months of age at the start of the experiment, for logistic reasons 2–3 months older than in other trials. They were randomly assigned to two groups. Group 1,  $n = 9$ , received chitosan gel without antigen. Group 2,  $n = 10$ , were vaccinated by delivery to the rectal mucosa as above of homogenate of 50,000 *T. colubriformis* L3s in 6 ml chitosan gel applied as single doses on Day 0 and Day 28. Chitosan was chosen as it has been reported to have mucosal adhesive properties, and may deliver a consequent increase in antigen retention time at the mucosa

(Senel and McClure, 2004). From Day 35 (i.e. 2–3 weeks earlier than in the previous trials) all lambs were challenged with 30,000 viable *T. colubriformis* L3s given as a trickle (6000, then 2000 three times/week for 4 weeks). Faecal worm egg counts were performed at weeks 3, 4, 5, 6, 7 and 8 after challenge, and samples collected at post mortem 8 weeks after challenge for total worm counts and for measurement of serum and jejunal (1 m distal to the pylorus) antibody titres.

#### 2.1.5. Experiment 5

Thirty ewe and wether lambs were 6 months of age at the start of the experiment, when they were assigned to four groups, each containing equal numbers of each sex. Group 1,  $n = 12$ , received chitosan sponge without antigen. Groups 2 and 3 were vaccinated on Days 0, 21, 35 and 42. Group 2 lambs,  $n = 12$ , were vaccinated by intra-rectal delivery as above of homogenate of 30,000 *T. colubriformis* L3s in chitosan sponge. The sponge was cut into four pieces for application with gentle pressure to the mucosal surface of the rectum. The sponge was tried in an attempt to increase the retention time of the vaccine in the rectum. Group 3,  $n = 6$ , received 500  $\mu$ g 17 kDa recombinant protein in chitosan sponge, delivered intra-rectally as for Group 3. All lambs were challenged from Day 49 with 30,000 viable *T. colubriformis* L3s given as five doses of 6000 over 10 days. Faecal worm egg counts were performed at weeks 3, 4, 5, 6 and 7 after challenge, and samples collected at post mortem 7 weeks after challenge for total worm counts, and measurement of antibody concentrations in jejunum (1 m distal to the pylorus) and internal iliac lymph node. Blood samples were collected from the jugular vein at days 0, 49, 70 and 98 for measurement of serum antibody concentration.

### 2.2. Parasitology

The larvae used throughout were McMaster levamisole-susceptible *T. colubriformis*. Larvae were exsheathed as described by McClure et al. (1992). The homogenate was prepared by grinding *T. colubriformis* L3s in PBS containing 5 mg g tissue<sup>-1</sup> of protease inhibitor cocktail (P2714, Sigma, St. Louis, USA) in a glass homogeniser until no intact larvae were detected by microscopic inspection. The 17 kDa recombinant protein from *T. colubriformis* was cloned and expressed in *Escherichia coli* (Dopheide, T.A.A., Frenkel, M.J., Grant, W.N., Savin, K.W., Wagland, B.M., 1990. Patent Corporation Treaty Application WO-90/03433). This protein is a larval excretory/secretory molecule with sequence similarity to globins. Faecal worm egg counts were performed using the modified McMaster technique (Whitlock, 1948) and total worm counts as described by Gregg et al. (1976).

#### 2.3. Preparation of formulations

The cellulose gel consisted of 3% hydroxy-propylmethyl-cellulose (Sigma St. Louis MO, USA) and 20% propylene glycol in distilled water, with the larvae, homogenate or recombinant protein added to the water immediately before mixing. The chitosan gel consisted of 2% chitosan (medium molecular weight, Aldrich St. Louis MO, USA) in 1.5% lactic acid solution, to which the homogenate was added approximately 30 min before application to the sheep. For preparation of the sponge, the homogenate or recombinant protein was added to the gel before the gel was freeze-dried for 48 h at  $-20^{\circ}\text{C}$  under vacuum to form a sponge.

#### 2.4. Antibody assays

Concentrations of antibody to worm antigens in serum and jejunal homogenate were measured by ELISA as previously described (Bendixsen et al., 2004). Tissue collected at post mortem consisted

of a jejunal segment, unwashed but with the gross contents removed, and a transverse segment of internal iliac lymph node containing cortex and medulla. These were homogenised at 4 °C in the presence of protease inhibitor cocktail (Sigma, St. Louis, USA) and the soluble fraction frozen at –20 °C until assayed. The antigen used was the soluble fraction of homogenised *T. colubriformis* L3s (McClure et al., 1998). Isotype-specific monoclonal antibodies used were MCM1 and MCM4 (IgG1 and IgG2, Beh, 1987), McM9 and McM10 (IgM and IgA, Beh, 1988) and 2F1 (IgE, Bendixsen et al., 2004).

### 2.5. Statistical analysis

Parasitological and antibody data were analysed by *t*-test except for worm counts in Experiment 5 and egg counts in Experiments 2 and 3, which proved difficult to normalise and for which the differences between the median values were tested using the Mann–Whitney rank sum test. In the case of Experiment 1, worm egg counts were transformed log<sub>x</sub>. Analyses were performed using Systat 9 (SPSS, Chicago, USA). Differences were considered significant where  $P < 0.05$ .

## 3. Results

### 3.1. Experiment 1

Faecal worm egg counts and total worm counts are shown in Fig. 1. The cumulative egg counts of lambs in vaccinated groups 2 and 3 were lower ( $P = 0.040$  and  $0.013$ , respectively) than those of the Group 1 controls. Worm counts were also significantly lower in lambs of Groups 2 and 3 ( $P = 0.009$  and  $P = 0.001$ , respectively) than in those of Group 1. While it is unlikely that surgery itself improved immunity, this would need to be controlled in any further work.

### 3.2. Experiment 2

Faecal worm egg counts and total worm counts are shown in Fig. 2. Cumulative egg counts were lower in lambs of Group 2

( $P = 0.002$ ), and there was a trend ( $P = 0.06$ ) for lower worm counts in this group. Jejunal antibody titres were not significantly different between treatment groups (data not shown). Serum antibody concentrations are shown in Table 2. Vaccinated lambs had higher serum IgE, G1 and G2 antibody titres in plasma at challenge than did control lambs ( $P = 0.026$ ,  $0.015$  and  $0.002$ , respectively), and higher IgE and IgG2 titres 6 weeks after challenge ( $P = 0.041$  and  $0.026$ ). Titres at these timepoints were not correlated with protection, and the only correlation between isotypes was between G1 and G2 titres (correlation coefficient =  $0.64$ ,  $P = 0.02$ ). This trial did not investigate the possibility that some worms migrated back to the jejunum after delivery to the rectum, so the assumption that the observed immune response was induced in the rectal lymphoid tissue has not been tested.

### 3.3. Experiment 3

Faecal worm egg counts and total worm counts are shown in Fig. 3. Cumulative faecal egg count was lower in the vaccinated Group 2 lambs ( $P = 0.015$ ), but the trend to decreased worm count was not significant.

### 3.4. Experiment 4

Faecal worm egg counts at 8 weeks after challenge for control and vaccinated groups were  $2767 \pm 2787$  and  $1970 \pm 2933$  eggs per gram of faeces (EPG), respectively, and total worm counts  $18,000 \pm 6500$  and  $15,000 \pm 5000$ . There were no significant differences between groups in cumulative egg count, egg count at 8 weeks after challenge or worm count, that is, no indication of protection. Nor was there any indication of immunity in the vaccinated group, with no increase in serum or jejunal antibody of any isotype (data not shown).

### 3.5. Experiment 5

Faecal worm egg counts and total worm counts are shown in Fig. 4. Lambs in Groups 2 (rectal homogenate) and 3 (rectal recombinant protein) had lower worm counts than did those in control

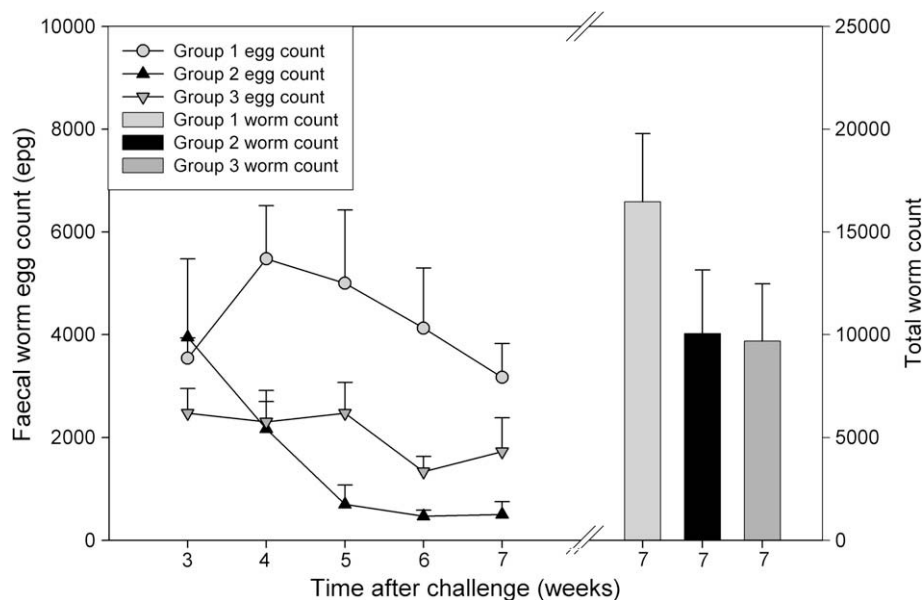


**Fig. 1.** Experiment 1 worm egg counts during challenge infection and total worm count 7 weeks after challenge infection (group means  $\pm$  SEM). Group 1, control; Group 2, 17 kDa antigen in cellulose gel applied to the luminal surface of the epithelium over three jejunal Peyer's patches; Group 3, 17 kDa antigen in alhydrogel injected into three jejunal Peyer's patches. epg, eggs per gram of faeces.

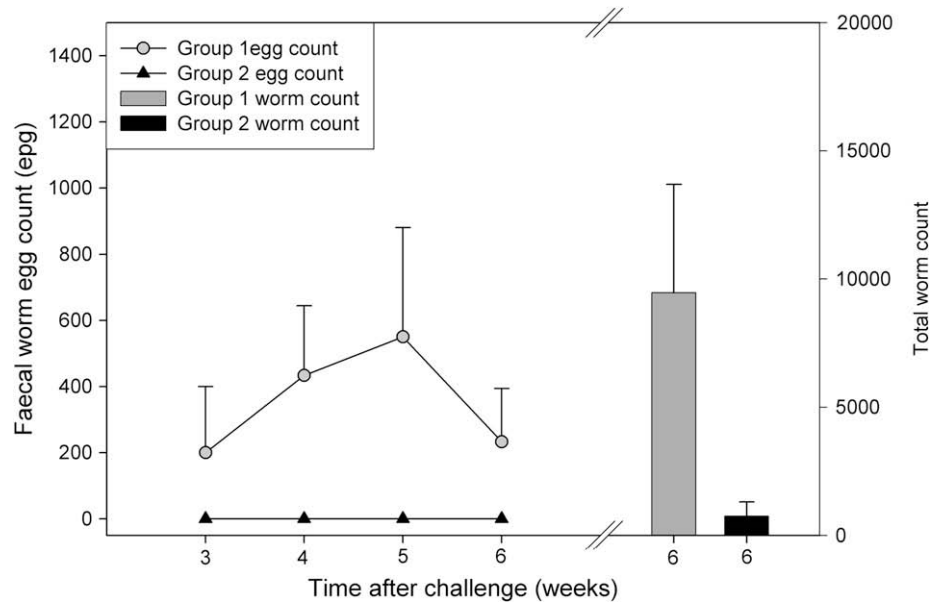


**Fig. 2.** Experiment 2 worm egg counts during challenge infection and total worm count 6 weeks after challenge infection (group means  $\pm$  SEM). Group 1, control; Group 2, viable larvae in cellulose gel delivered to the luminal surface of the rectal epithelium. epg, eggs per gram of faeces.

**Table 2**

Concentration of antibody to soluble protein from *Trichostrongylus colubriformis* L3s in sera and homogenised tissues, as measured by ELISA (group mean titre  $\pm$  SD).

| Isotype | Experiment 2          |                |                       |                 | Experiment 5            |               |               |                      |                   |                   |
|---------|-----------------------|----------------|-----------------------|-----------------|-------------------------|---------------|---------------|----------------------|-------------------|-------------------|
|         | Serum titre at Day 56 |                | Serum titre at Day 98 |                 | Jejunal titre at Day 98 |               |               | IILN titre at Day 98 |                   |                   |
|         | Group 1               | Group 2        | Group 1               | Group 2         | Group 1                 | Group 2       | Group 3       | Group 1              | Group 2           | Group 3           |
| IgA     | 0 $\pm$ 0             | 10 $\pm$ 22    | 13 $\pm$ 21           | 14 $\pm$ 33     | 1.5 $\pm$ 2.4           | 19 $\pm$ 25   | 12 $\pm$ 10   | 0.13 $\pm$ 0.30      | 0.59 $\pm$ 0.69   | 0.51 $\pm$ 0.78   |
| IgE     | 1.2 $\pm$ 2.9         | 788 $\pm$ 1822 | 1.0 $\pm$ 1.5         | 4169 $\pm$ 1011 | 1.6 $\pm$ 2.1           | 50 $\pm$ 163  | 1.5 $\pm$ 1.9 | 1.09 $\pm$ 1.48      | 11.93 $\pm$ 33.93 | 3.08 $\pm$ 3.68   |
| IgG1    | 0.8 $\pm$ 2.0         | 125 $\pm$ 115  | 800 $\pm$ 936         | 1035 $\pm$ 742  | 3.2 $\pm$ 6.5           | 25 $\pm$ 82   | 1.0 $\pm$ 1.5 | 11.74 $\pm$ 20.12    | 88.39 $\pm$ 271.6 | 17.03 $\pm$ 13.75 |
| IgG2    | 15 $\pm$ 24           | 89 $\pm$ 36    | 6.0 $\pm$ 9.3         | 168 $\pm$ 306   | 0.2 $\pm$ 0.5           | 0.8 $\pm$ 1.0 | 0.2 $\pm$ 0.3 | 6.11 $\pm$ 14.77     | 4.18 $\pm$ 5.56   | 2.45 $\pm$ 1.99   |
| IgM     | 480 $\pm$ 205         | 524 $\pm$ 299  | 1231 $\pm$ 897        | 594 $\pm$ 128   | 22 $\pm$ 33             | 37 $\pm$ 63   | 45 $\pm$ 67   | 4.07 $\pm$ 2.83      | 4.31 $\pm$ 2.69   | 5.39 $\pm$ 4.30   |

IILN, internal iliac lymph node.

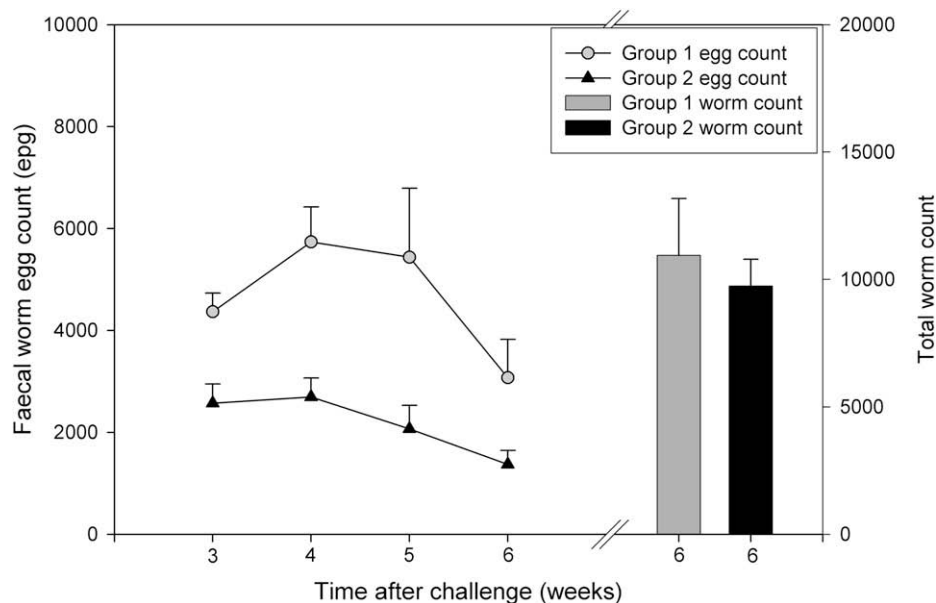


**Fig. 3.** Experiment 3 worm egg counts during challenge infection and total worm count 6 weeks after challenge infection (group means  $\pm$  SEM). Group 1, control; Group 2, non-viable homogenate in cellulose gel delivered to the luminal surface of the rectal epithelium. epg, eggs per gram of faeces.

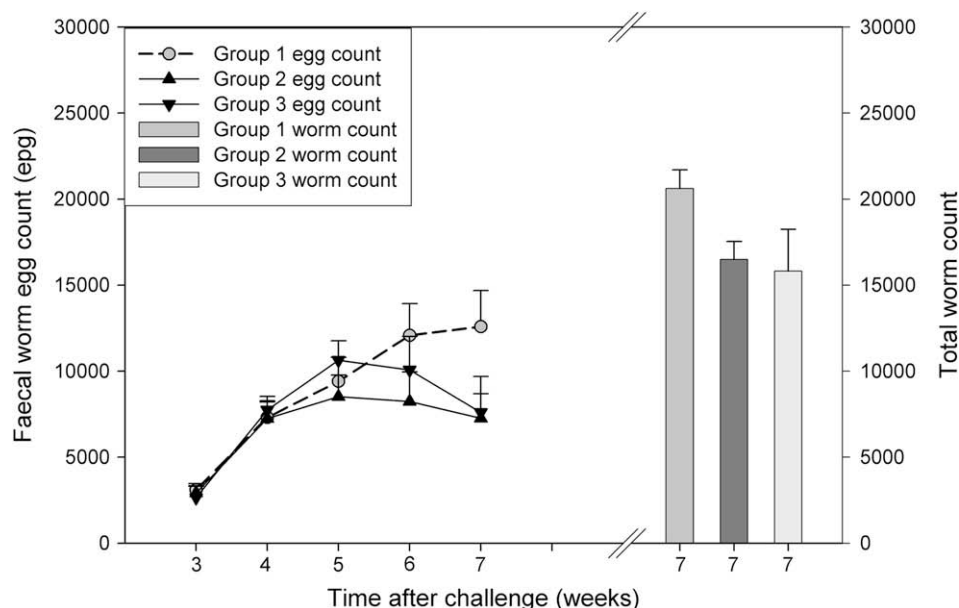


**Fig. 4.** Experiment 5 worm egg counts during challenge infection and total worm count 7 weeks after challenge infection (group means  $\pm$  SEM). Group 1, control; Group 2, non-viable homogenate in chitosan sponge delivered to the luminal surface of the rectal epithelium; Group 3, 17 kDa recombinant protein in chitosan sponge delivered to the luminal surface of the rectal epithelium. epg, eggs per gram of faeces.

Group 1 ( $P=0.012$  and  $P=0.048$ , respectively). Cumulative egg counts did not differ between groups, although there was a trend for the last egg count at 7 weeks after challenge to be lower in both vaccinated groups compared to the unvaccinated group, indicating onset of protection. There was no effect of vaccination on serum antibody titres (data not shown). Tissue antibody titres are shown in Table 2. Group 2 lambs had higher IgA titres in the jejunum ( $P=0.046$ ) and internal iliac lymph node (IILN) ( $P=0.049$ ) than did control lambs, and trends for higher IgG1 and IgE titres in both tissues. Antibody IgG1 and IgE titres were correlated in both jejunum (correlation coefficient = 0.996,  $P<0.001$ ) and IILN (coefficient = 0.989,  $P<0.001$ ). Worm count and last egg count correlated with IgA concentration in IILN ( $P=0.049$ , coefficient = -0.303), and there was a trend ( $P=0.08$ ) for the last egg count to also correlate with jejunal IgA titre.

#### 4. Discussion

These trials have demonstrated that delivery of *Trichostrongylus* native and recombinant antigens across the epithelium overlying rectal lymphoid patches can result in significant protection of a level commensurate with that achieved by jejunal vaccination, and in mucosal (IgA and IgE) immune responses. This induction of mucosal antibody responses is consistent with the results achieved with similar delivery of ovalbumin and Heliotrine toxin (Hauptli, 2006). The effect was obtained with both native (soluble plus particulate) and recombinant (soluble) antigens, with two- and four-dose vaccination regimes, and with bolus and trickle challenge regimes. The different experimental conditions may have influenced the degree of protection achieved because of differing primary or challenge exposure to worm antigen, and a definitive trial would be needed to determine the relative efficacy of these vaccine options.

The antibody data show large SD, as some vaccinated animals had high titres while others were unresponsive. This may be due to variable antigen retention times and thus variable exposure. Although IgG1 and IgE responses were strongly correlated, the

IgA response appeared to occur independently of other isotypes. The animals with the highest IgA response also had the highest degree of protection. However, such correlations, and the titres themselves, are likely to be heavily influenced by the time of sampling, and should be interpreted in terms of causality with caution.

The rectal route has been tried in ruminants with native antigen form *Haemonchus contortus* larvae deposited with cholera toxin on the surface of the rectal mucosa (Jacobs et al., 1999). In that case, significant protection compared with the adjuvant control in terms of cumulative faecal worm egg count and worm count was not obtained, although it appears that egg count at 9 weeks after challenge may have been significantly lower, and no increase in serum antibody was detected.

In a number of trials in which the recombinant 17 kDa antigen was given with adjuvant by i.p. or s.c. injection, the reduction in *T. colubriformis* worm counts was higher, if inconsistent (40–67%, Emery et al., 1999; Emery and McClure, unpublished observations), than that observed in these rectal delivery trials. However, the protection obtained in Experiment 1, Group 2, approached this level, suggesting that immunisation of the distal (rectal) site induced a slower response than did local immunisation.

In Experiment 4, rectal vaccination induced neither immunity nor protection, despite the fact that these lambs were 2–3 months older than those in the other experiments and would thus be expected to develop immunity more rapidly. This raises the possibility that the 5 week induction period in this trial was inadequate for the generation of an effective mucosal response, even though it appeared sufficient if the antigen were delivered to the jejunum itself. In 25-week-old lambs receiving a trickle infection of 3000 or more *T. colubriformis* larvae per week, rejection of adults commences at about week 7, at which time larval establishment is 1% (Dobson et al., 1990b). It is possible that the time taken for mucosal effector responses to develop following vaccination is similar, and that it increases if the site of induction is distant from the jejunum. If this were the case, we should not expect a vaccination protocol to achieve an optimal protective jejunal response any more rapidly than does natural infection. This may be another contributing factor in the inconsis-

tency of protection data from past CSIRO vaccine trials with recombinant larval antigens from *T. colubriformis* or *H. contortus*, many of which used s.c. or i.p. vaccination followed by challenge at 6 weeks after the first vaccination. Future trials should allow for this possibility.

In these mucosal trials no adjuvant was used, but it is to be expected that the inclusion of an appropriate adjuvant would improve the immune response induced. The usual mucosal adjuvants are toxins of cholera or *E. coli*, but in our experience cholera toxin did not enhance the protection following vaccination of sheep with other recombinant helminth antigens (Emery, D.E. and McClure, S.J., unpublished observations). The antigen(s) employed are also presumably critical, and other antigens or combinations of antigens may give better responses.

These trials did not directly compare the efficacy of cellulose and chitosan as formulants, however, both resulted in immunity. While the chitosan sponge showed considerable promise as a means of delivering antigen to the mucosal surface, the duration of retention of the sponge was variable, and in both Experiment 5 and in another trial using a similar system for delivery of ovalbumin (Hauptli, 2006), some animals receiving the chitosan sponge did not mount significant immune responses. A more practicable formulation is required for rectal delivery, to overcome both the ease and the success of application.

McNeilly et al. (2008) have suggested that an alternative to the delivery of vaccines across the gastrointestinal mucosal for the induction of mucosal immunity, is the s.c. or i.m. delivery of antigen in combination with strategies to up-regulate the mucosal production of Th2 cytokines. Viable worms, even in the abnormal site, more effectively induced the IgG1/IgE type of immunity typical of natural infections (Douch et al., 1994; Shaw et al., 1998), and identification of the reason for this may provide a means of improving vaccines against luminal pathogens.

These results demonstrate the validity of the mucosal vaccination approach for the induction of protective immunity against browsing nematodes, and warrant further, more systematic, investigation of formulation and delivery of helminth vaccines for trans-epithelial application in ruminants. Mucosal delivery of vaccines was recently identified as a requirement for the induction of optimal mucosal responses in humans (Hinkula, 2007), and a successful and practicable formulation and delivery system for young ruminants may have major implications for the successful vaccination of children against gastrointestinal diseases.

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