Increased levels of cysteinyl leukotrienes and prostaglandin E_2 in gastrointestinal tract mucus are associated with decreased faecal dry matter in Merino rams during nematode infection

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Short title – faecal moisture in worm-infected rams

Abstract

Immune-mediated scouring in sheep is a mucosal hypersensitivity response incited by infective larvae of gastrointestinal nematodes in the southern half of Australia and in New Zealand, regions characterised by a Mediterranean, winter-rainfall climate. To gain insights into the underlying mechanism(s), this study investigated the relationship between nematode infection of sheep in pens, faecal dry matter and release of inflammatory mediators in the intestinal tract. Six worm-free, immune rams received a dose of 3000 *Trichostrongylus* third stage larvae (L_3) and 3000 *Ostertagia* L_3 per week for six weeks, after which they were euthanased. Three rams acted as uninfected controls. Faecal dry matter was significantly lower in the infected group from week one of infection until the end of the trial. Concentrations of cysteinyl leukotrienes and prostaglandin E_2 were higher in mucus from both the abomasum and the small intestine of infected rams compared to controls. Within the infected group, concentrations of both mediators were higher in mucus from the small intestine than from the abomasum. There were greater numbers of eosinophilic granulocytes and mucosal mast cells in abomasal and small-intestinal tissue sections from infected rams compared to the controls. The results are consistent with the hypothesis that nematode infection causes an inflammatory response in the gastro-intestinal mucosa, characterised by increased fluid secretion.

Introduction

Nematode parasitism costs the Australian sheep industry over $370 million annually, a figure which is certain to increase with the threat of widespread drench resistance

48 (Sackett *et al.,* 2006). Controlling nematodes by improving the genetic resistance of the
49 host appears an attractive, long-term solution and researchers have demonstrated the
50 feasibility of a breeding program aimed at breeding worm-resistant sheep (Bisset *et al.,*
51 1996; Woolaston and Windon, 2001; Karlsson and Greeff, 2006). However, in areas with
52 a Mediterranean, winter-rainfall climate there is an increased tendency for resistant sheep
53 to scour as a result of nematode infection (Karlsson *et al.,* 2004). Larsen *et al.* (1994)
54 showed that scouring in sheep with low worm egg counts (WEC) during the winter
55 rainfall season is most likely due to a hypersensitive immune response to ingested larvae
56 ('immune-mediated scouring'). Scouring was an "over-response" for worm immunity as
57 other sheep within the same flock had low WEC without exhibiting scouring (Larsen *et*
58 *al*., 1994). Increased numbers of eosinophilic granulocytes, a key indicator of
59 inflammation and hypersensitive immune responses (Rothwell, 1989), were detected in
60 the intestinal mucosa of scouring individuals and the phenomenon appears to have some
61 genetic component (Larsen *et al*., 1994).
62
63 The inflammatory response is central to effector mechanisms against
64 gastrointestinal nematode parasites (Emery, 1996). Following ingestion of worm larvae,
65 inflammatory changes are observed in the intestinal and abomasal mucosa, including the
66 accumulation of eosinophilic granulocytes, mast cells and globule leukocytes (Jones *et*
67 *al.,* 1994). These cells are able to release potent inflammatory mediators such as the
68 prostaglandins and the cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4). These lipid
69 mediators may aid in removing both larvae and possibly adult worms from the intestinal
70 tract, as well as stimulating fluid secretion and smooth muscle contraction. It is possible

that some sheep bred for worm resistance have an increased, inflammatory- immune response to worm larvae compared to un-selected sheep, leading to increased scouring. We wish to study immune-mediated scouring but, before doing so, need to develop a reliable experimental model where the condition can be reproduced under controlled conditions in the animal house. Relying on seasonal conditions in the paddock to generate experimental animals is too variable and an inefficient use of resources. We hypothesised that immune-mediated scouring could be satisfactorily mimicked in the animal house by administering an artificial infection of worm larvae to parasite-resistant sheep. Specifically, we hypothesised that a trickle infection of worm larvae would decrease faecal dry matter in penned, worm- resistant sheep compared to uninfected sheep of the same genotype kept under identical conditions. Furthermore, we expected that there would be higher numbers of inflammatory cells and increased concentrations of Prostaglandin E_2 and cysteinyl leukotrienes in the intestinal and abomasal mucosa at post-mortem examination.

Materials and Methods

Experimental Design

Six, parasite-resistant Merino rams were drenched and then received a dose of 6000 nematode L_3 weekly for six weeks. Three additional parasite-resistant rams were kept worm free to act as controls. Faecal samples were taken regularly before and after infection started to determine faecal moisture. After six weeks all rams were killed and total worm counts determined. Tissue and mucus samples were taken from the

93 abomasum and small intestine and analysed for numbers of inflammatory cells and
94 concentrations of inflammatory mediators.
95
96 *Location and Animals*
97 Nine, 20-month-old Merino rams were selected from the parasite-resistant, Rylington
98 Merino line based at the Mount Barker research station owned by the Department of
99 Agriculture & Food Western Australia. Sheep from this line have been bred for worm
100 resistance since 1987. Worm resistance is determined by BLUP estimated breeding
101 values (EBV) for WEC at hogget age following a natural, moderately- high, parasite
102 challenge consisting mainly of *Trichostrongylus* spp. and *Ostertagia* spp. For further
103 details on the management and structure of this flock see Karlsson and Greeff (2006).
104 The rams for this experiment were selected on the basis of EBV for WEC, as well as
105 phenotypic indicators of scouring (dag score and faecal consistency score - FCS) taken at
106 hogget age (Table 1). The rams that were used had low WEC but had high dag scores
107 and FCS, i.e. they were prone to scouring but had low WEC. The rams had not been
108 grazing green pastures for at least four months before the experiment, due to the dry
109 Western Australian summer. Before the experiment they were drenched (Q-drench,
110 Jurox, active ingredients – 40 g/L levamisole, 37.5 g/L closantel, 25 g/L albendazole and
111 1 g/L abamectin), and then transported to the Large Animal Facility at the University of
112 Western Australia where they were housed indoors in individual pens. The rams were
113 fed a daily diet consisting of 800 g oaten chaff, 400 g lupins and 25 g mineral
114 supplements (Siromin^{TM}).
115 **Insert Table 1**

Infection regime

Infective, third-stage larvae (L3) of *Trichostrongylus colubriformis* (Tc) and *Ostertagia circumcincta* (Oc) were cultured at the Animal Health Laboratories of the Department of Agriculture & Food Western Australia. After a two-week period of acclimatisation in the animal house, six rams were selected at random for infection. The remaining three rams acted as un-infected controls. Only three control animals were used as we anticipated that there would be little deviation from baseline faecal moisture in the absence of nematode infection and any other intestinal inflammation (e.g. coccidia). In addition we thought we would be able to detect differences in post-mortem histopathology with a small control group as long as they were maintained worm-free. The dose consisted of 6000 L_3 (1:1, TcL3 and OcL3) per week, given daily in equal proportions. This dose is comparable to the larval availability on winter pastures grazed by sheep from the parasite-resistant Rylington line (Williams *et al*, unpublished data). The larvae were suspended in water and administered orally using a drench gun throughout the six weeks of the trial.

Sampling procedures

Faecal samples were taken from rams upon arrival at the animal house and WEC performed using the modified McMaster technique (Whitlock, 1948) to ensure sheep were worm- free. During the acclimatisation period, three faecal samples were taken from each sheep at regular intervals four days apart and dried overnight in an oven at 90°C to determine faecal dry matter. These three values were averaged to determine a

baseline, pre-infection value of faecal moisture content for each sheep. Following the commencement of larval dosing, faecal samples were taken weekly for dry matter determination. Three weeks after dosing commenced, weekly faecal samples were also taken for WEC.

Post-mortem procedures

Six weeks after infection, all nine rams were killed with an overdose of pentobarbitone, immediately eviscerated and the abomasum and first five metres of the small intestine tied off and removed. The organs were opened, the contents collected and the mucosal surface of the organ then gently washed with water and the washings added to the contents. The contents were then sieved through a 43 μm sieve (abomasum) or 150 μm sieve (small intestine) and the contents of the sieve preserved in 5% formalin for worm counts. The abomasum was retained for a pepsin/hydrochloric acid digest to remove arrested worms from the mucosa (Herlich, 1956). Small tissue pieces were taken from the abomasum and jejunum and preserved in 4% buffered formaldehyde solution for histology. Mucus was collected by gently scraping the surface of the abomasum and first metre of the small intestine with a scalpel handle and immediately placed on ice. An aliquot of mucus was removed for protein determination, the remainder was then homogenised (5x sample volume) in a 2:1 mixture of methanol/phosphate buffered saline (PBS), centrifuged at 2500 rpm for 15 minutes and the supernatants stored at -80°C.

Isolation and assay of mediators

161 Leukotrienes and prostaglandins in mucus were isolated using 300 mg SPE C18
162 cartridges (Alltech Associates Australia). The samples were acidified (pH 4) with acetic
163 acid. For leukotrienes, the cartridges were rinsed with 5 ml tetrahydrofuran, 5 ml of
164 methanol and 5 ml of water. The samples were applied and the cartridges then washed
165 with 5 ml of water, 5 ml of petroleum ether and the leukotrienes eluted with 3 ml
166 methanol. For prostaglandins, the cartridges were rinsed with 5 ml methanol and 5 ml
167 water. The samples were applied and the cartridges washed with 5 ml water, 5 ml
168 petroleum ether and the prostaglandins eluted with 3 ml ethyl acetate containing 1%
169 methanol. The eluting solvents were then evaporated to dryness using compressed air
170 and the residues re-suspended in EIA buffer for assay. Leukotriene and Prostaglandin E_2
171 (PGE_2) concentrations were determined using ELISA kits (Cayman Chemical, Ann
172 Arbor, USA). The leukotriene kit has 100% reactivity for LTC_4 and LTD_4, and 67%
173 reactivity for LTE_4. Because of the rapid metabolism of PGE_2 in tissue, we elected to
174 measure instead PGE_2 metabolites as an indirect measure of PGE_2 production in mucus.
175 The kit used converts all PGE_2 metabolites to a stable derivative, which is then directly
176 quantified by ELISA.
177 Mediator concentrations were expressed per mg of protein, and protein concentrations
178 were determined using a modification of the Lowry method (Hartree, 1972), with bovine
179 serum albumin serially diluted in PBS as the protein standard.
180
181 *Histology*
182 Samples from each ram were embedded in paraffin wax blocks, sectioned at 5 µm and
183 stained with haematoxylin and eosin (eosinophils and globule leukocytes) or toluidine

184 blue (mast cells). Cell counts were estimated by scanning a section of tissue from the
185 base of the lamina propria through to the mucosal epithelium. At least ten sections were
186 counted for each tissue sample. Cell counts were expressed as the mean number of cells
187 per mm^2 of tissue.
188
189 *Statistical Analysis*
190 Repeated measures ANOVA was used to assess the main effects and interaction of
191 treatment (control or infected) and week of infection on faecal dry matter. The mean dry
192 matter value from three pre-infection faecal samplings (baseline faecal moisture) was
193 initially fitted as a covariate for each sheep in the analysis, however the effect of the
194 covariate was not significant and was deleted from the final model. Student's t-tests were
195 used to determine differences in mediator concentrations in mucus and mean cell counts
196 in tissue samples between control and infected groups. Within the infected group, the
197 difference in mediator concentrations and cell counts between each organ was also
198 compared with a paired t-test. Analyses were conducted using Genstat 5 for Windows
199 (Second Edition, Lawes Agricultural Trust, Rothamsted Experimental Station, UK). The
200 level for significance was $P<0.05$.
201
202 **Results**
203 *Worm infection*
204 Worm egg counts (WEC) in the infected group were low throughout the experiment and,
205 although WEC in one ram reached 500 eggs per gram of wet faeces (epg) the week
206 before slaughter, the remaining WECs were <100epg with 2 rams having zero epg over

207 the 6 weeks. The resistance of the rams to worms was confirmed by the total worm
208 counts at slaughter, with mean counts of 656 ± 280 and 40 ± 27 worms, for *Ostertagia*
209 and *Trichostrongylus*, respectively. Only adult worms were found – no immature worms
210 were found for either species despite the sheep receiving a dose of L_3 the day before
211 slaughter. One ram dosed with larvae had no worms of either species.
212
213 *Faecal dry matter*
214 The percentage of faecal dry matter was consistently lower in the infected group
215 throughout the experiment. One week after infection, faecal dry matter was lower in the
216 infected group ($P<0.05$; Fig. 1) and remained significantly lower throughout the
217 experiment. After the first week, there were no significant differences between weeks in
218 the infected group. There was an interaction ($P<0.05$) between week and treatment group
219 because the control group had a higher faecal dry matter percentage 36 days after
220 infection compared to 0-15 days after infection. Three weeks after infection started, two
221 rams in the infection group had faecal dry matter values of 13-14% and their faeces were
222 noticeably fluid until slaughter. Faeces in the control group remained firm throughout.
223
224 **Insert Figure 1**
225
226 *Mediators*
227 Concentrations of leukotrienes and prostaglandin E_2 were higher ($P<0.05$) in the infected
228 group than in the control group in both the abomasum and small intestine (Tables 2 & 3).
229 Within the infected group, concentrations of leukotrienes were higher ($P<0.05$) in the

small intestine than in the abomasum. Concentrations of PGE_2 in the small intestine were higher than in the abomasum (P = 0.096).

Insert Table 2

Insert Table 3

Histology

Mean counts of eosinophilic granulocytes and mast cells were higher ($P<0.05$) in abomasal and intestinal tissues from infected rams compared to control tissues (Table 4). Degranulation of mast cells was common but this was not quantified. Globule leukocytes were higher in infected rams but this was not significant in the abomasum (P=0.7) or small intestine (P=0.19; Table 4).

Insert Table 4

Discussion

The results support the hypothesis that nematode infection causes an inflammatory response in the intestinal tract and this manifests as increased moisture in the faeces. The results also show that immune-mediated scouring can be mimicked in the animal house as all six rams responded to the trickle L_3 infection over six weeks. Although only two animals could be considered to be clinically scouring, all six infected rams had wetter faeces than the controls. In addition, the small numbers of animals used in this trial were sufficient to detect significant differences between control and infected animals for inflammatory cells and associated lipid mediators.

253

254 Leukotrienes and prostaglandins are potent mediators of the immune system,
255 causing increased fluid and electrolyte secretion and smooth muscle contraction. Their
256 involvement in asthma (Montuschi and Barnes, 2002) and inflammatory bowel disease in
257 humans (Bueno and Fioramonti, 2002) has been well documented. Exogenous injections
258 of PGE_2 in previously healthy pigs have been shown to impair intestinal transport and
259 lead to diarrhoea, which was caused by increased mucosal secretion and reduced
260 absorption of water and electrolytes (De Saedeleer *et al.,* 1992), This is consistent with
261 the pathology of immune-mediated diarrhoea due to parasitic infection in humans
262 (Farthing, 2003).

263

264 The release of leukotrienes and PGE_2 is probably induced by IgE binding to mast
265 cells, and the consequent metabolism of liberated arachidonic acid. Leukotriene C_4 is
266 also released in large amounts by eosinophils (Shaw *et al.,* 1985). Larsen *et al.* (1994)
267 reported increased eosinophilic infiltration in the jejunum and ileum as the major
268 difference between scouring and non-scouring Merino sheep. Researchers have reported
269 increased levels of IgE in both sheep serum and lymph following parasite infection, with
270 parasite-resistant sheep displaying increased levels compared to unselected sheep (Shaw
271 *et al.,* 1998; Bendixsen *et al.,* 2004; Pernthaner *et al.,* 2005). Shaw *et al.* (1999) also
272 noted a positive genetic correlation between IgE and dag score in Romney sheep.

273

274 Gray *et al. (*1992) and Jones *et al.* (1994) have also demonstrated that levels of
275 leukotrienes increase in parasite-resistant sheep compared to control animals following

nematode infection, as well as increased numbers of eosinophils and mast cells (Bisset *et al.,* 1996). The results from this current study provide further support that elevated levels of inflammatory mediators generated following ingestion of worm larvae are causally linked to increased scouring in resistant sheep. However, this will need to be validated further in studies involving sheep that are both susceptible and not susceptible to immune-mediated scouring.

The similarity of numbers of globule leukocytes between control and infected rams is puzzling, considering the large difference in mast cell numbers between the two groups. It may be that the staining techniques used in this study were insufficient to estimate the number of globule leukocytes accurately or that turnover of globule leukocytes in immune sheep was too rapid. Degranulation assays have been used to quantify the release of sheep mast cell proteinase (SMCP) from isolated mast cells incubated with soluble protein extract from nematode L_3 (Huntley *et al.,* 1987; Jones *et al.,* 1992), but have not been sensitive enough to establish a correlation with worm counts. However, this assay may be useful in further studies on immune-mediated scouring.

The pattern of faecal dry matter percentages detected in this pen trial is interesting, given field observations of scouring in Merino sheep. Under field conditions in a Mediterranean environment, signs of clinical scouring usually occur 2-3 months after the break of season. The exact conditions that lead to severe scouring are likely to be complex and difficult to replicate experimentally in pens. They may include the length of

299 time and dose of larval infection that challenges the primed immune system (i.e. a sheep
300 continuously grazing throughout the day and thus constantly ingesting larvae) and a large,
301 undefined component from lush green pasture. So the significant decrease in faecal dry
302 matter after one week of infection with this regime of L3 infection implies strongly that
303 infective larvae induce conditions associated with diarrhoea in the absence of significant
304 worm infection. This is also supported in field studies where albendazole capsules, that
305 killed ingested nematode larvae, ameliorated or prevented scouring (Larsen, *et al.,* 1994).
306
307 This increased fluid secretion is likely to be a powerful method of rejecting
308 worms by the sheep. The fact that no immature worms or larvae were found at post-
309 mortem examination, despite receiving a dose of L_3 the day before slaughter, supports
310 this concept. Assuming no experimental error, it can only be concluded that the sheep
311 had been expelling their entire worm challenges for at least several days before slaughter.
312 Wagland *et al*. (1996) and Harrison *et al.* (1999) reported immune sheep expelled an
313 entire challenge of 20,000 *Trichostrongylus* L_3 within two hours of challenge and worms
314 residing in the same location were also expelled (Emery *et al*., 1993). This rapid
315 expulsion is a feature of the immune response of sheep with a well-developed immune
316 response, and is characterised by the increased secretion of fluid and mucus physically
317 trapping and flushing larvae from the intestinal lumen (Rothwell, 1989).
318
319 The increased concentrations of mediators in the small intestine, compared to the
320 abomasum, may indicate that the sheep were more effective at expelling *Trichostrongylus*
321 than they were at expelling *Ostertagia.* It is also possible that the lower mediator

322 concentrations may be because of rapid destruction of the lipid compounds due to the
323 reduced pH in the abomasum, rather than decreased synthesis in the abomasal mucosa.
324 However, the higher numbers of *Ostertagia* at post-mortem examination support the
325 hypothesis that there is an increased immune response in the small intestine. Whether
326 *Trichostongylus* or *Ostertagia* L_3 are more or less responsible for the induction of
327 immune-mediated scouring in winter rainfall regions of Australia needs further
328 investigation. The unique contribution of this study is to provide an experimental regime
329 to decrease faecal dry matter in pens, relieving the dependence on limited, seasonal
330 investigations in the field.
331
332 In future experiments, we will examine phenotypic measures of worm resistance,
333 including inflammatory reactivity, WEC and faecal moisture content, when sheep of
334 differing genetic resistance to worms and scouring potential are given a similar infection
335 of one or both nematodes. Tissue samples will be analysed by genomic and proteomic
336 techniques to understand the mechanisms of hypersensitive scouring in sheep, and to
337 provide genetic and biochemical markers that may be used to select sheep that are
338 resistant to worms but not susceptible to immune-mediated scouring.
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Table 1. Mean EBV for worm egg count (WEC) and mean dag scores in July and September 2006 for experimental rams and flock averages.

Dag scores are on a subjective 1-5 scale where 1 is no dag and 5 is severe dag.

	WEC EBV	July Dag Score[1]	September Dag Score
Experimental rams (n=9)	-96.1	3.3	2.5
Flock Average (n=60)	-87.4	2.1	2.2

[1] Then crutched

Table 2. Concentrations of cysteinyl leukotrienes in mucus from the abomasum and small intestine of control and infected rams (mean ±s.e.m).

Different letter subscripts within columns indicate significant difference (P<0.001).

* Indicates that within this group concentrations were different between organs (P<0.005).

	Concentration LTC4/D4/E4 (pg/mg protein)	
	Abomasum	Small intestine
Control	89.6 ± 18.2^{A}	122.3 ± 26.2^{A}
Infected*	430.5 ± 63.6^{B}	812.9 ± 89.4^{B}

Table 3. Concentrations of prostaglandin E_2 metabolites in mucus from the abomasum and small intestine of control and infected rams (mean ± s.e.m).

Different letter subscripts within columns indicate significant difference ($P<0.05$)

	Concentration PGE_2 metabolites (pg/mg protein)	
	Abomasum	Small Intestine
Control	97 ± 33.9^{A}	64.5 ± 13^{A}
Infected	249.6 ± 43.6^{B}	479.6 ± 120.9^{B}

Table 4. Inflammatory cell counts (mean number of cells / mm^2 from 10 fields, ± s.e.m for group mean) in small intestine and abomasum tissue samples from control and infected rams.

Values within rows followed by a different subscript are different at P=0.05.

		Control	Infected
Eosinophils	Abomasum	22.9 ± 3.2^{A}	44.9 ± 9^{B}
	Small Intestine	16.9 ± 2.8^{A}	55.2 ± 10.2^{B}
Globule Leukocytes	Abomasum	15.3 ± 5.3	19.6 ± 10.5
	Small Intestine	8.8 ± 4.6	21 ± 7.6
Mast Cells	Abomasum	22.9 ± 4.8^{A}	63 ± 15.7^{B}
	Small Intestine	37.4 ± 10.4^{A}	139.5 ± 21.6^{B}

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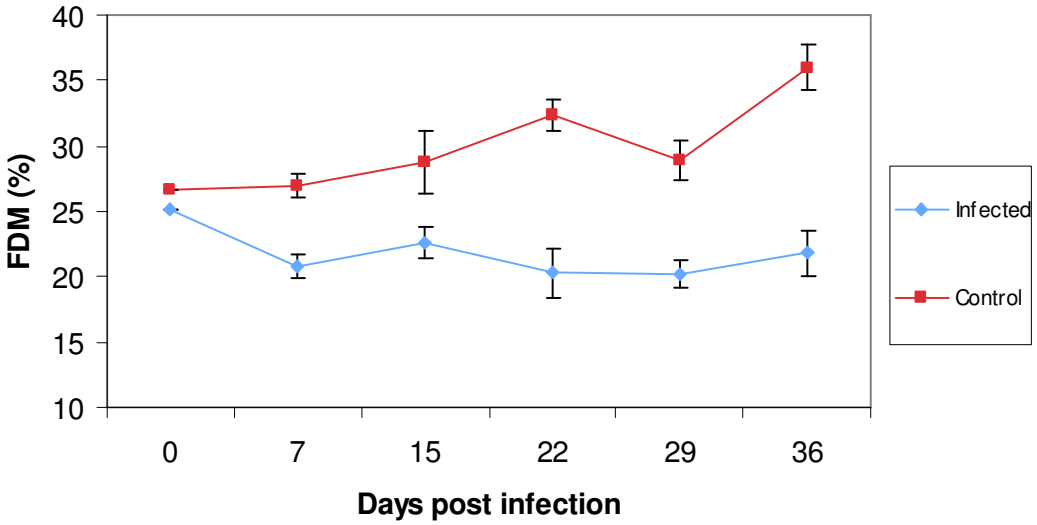

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517 **Figure 1.**

518 Faecal dry matter percentage (FDM%, means ±s.e.) in control (n=3) and infected (n=6)

519 rams following infection at day 0.

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