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Expression of genes in gastrointestinal and lymphatic tissues during parasite infection in sheep genetically resistant or susceptible to *Trichostrongylus colubriformis* and *Haemonchus contortus*

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ABSTRACT

Resistance to an acute gastrointestinal nematode (GIN) infection is dependent on the ability of the host to recognise the parasite and mount a protective Th2 response. It is hypothesised that lambs which are genetically susceptible to GIN will differentially up-regulate Th1-type genes and therefore remain susceptible to chronic parasitism compared with genetically resistant lambs which will differentially up-regulate Th2-type genes and clear the parasite infection. Two selection flocks, in which lines of Merino sheep produced lambs genetically resistant or susceptible to GIN, were acutely challenged once or thrice with either *Haemonchus contortus* or *Trichostrongylus colubriformis*. Faecal-egg counts (FECs), and plasma and tissue anti-parasite (*H. contortus* or *T. colubriformis)* antibody isotype responses showed that resistant animals challenged three times with *T. colubriformis* established a protective Th2 response (negligible FEC, IgG1 and IgE) whereas susceptible animals required multiple challenges to establish a significant IgG1 response despite FECs remaining high. *Trichostrongylus colubriformis* elicited a more pronounced host response than *H. contortus*. RNA extracted from tissues at the site of each parasite infection and associated lymph nodes were interrogated by microarray and quantitative PCR analyses to correlate host gene expression to FECs and antibody responses. The IFN-γ inducible gene cxcl10 was up-regulated in the susceptible line of the *Trichostrongylus* selection flock sheep after a tertiary challenge with the parasites *H. contortus* and *T. colubriformis*. However, a uniform pattern of genes was not up-regulated in resistant animals from both selection flocks during both parasite infections, suggesting that the mode of host resistance to these parasites is different, although some similarities in host susceptibility were apparent.

1. Introduction

Gastrointestinal nematode parasites (GINs) of sheep and cattle represent a major health issue for Australian livestock production systems. It has been estimated that GINs, of which *Trichostrongylus colubriformis* and *Haemonchus contortus* are the major species, cost the Australian sheep industry over AU$400 million annually (Sacket et al., 2006). Insight into the regulatory processes involved in establishing an appropriate gastrointestinal immune response is essential for the development of effective and sustainable control strategies to reduce the current reliance on anthelmintic chemicals. These anthelmintics are rapidly becoming ineffective against all commercially important GINs due to selection pressure on parasite populations that has led to increasing proportions of individual nematodes being able to withstand their single mode of action (Gill, 1991).

In rodents, immunological protection against GINs is associated with the efficacy of the T-helper (Th) response as well as tissue repair systems. Resistance to nematode parasites in these animals usually requires a dominant, polarised Th2 response, whereas susceptible animals usually evoke an inappropriate Th1-type response (Bellaby et al., 1996). To this end, it is known that an effective and appropriate mucosal immune response is essential for the development of resistance to GINs in sheep. Like rodents, resistance to GINs by sheep usually requires a dominant Th2 immune profile (Gill et al., 2000; Lacroux et al., 2006; Pernthaner et al., 2005, 2006; Terefe et al., 2007); however the mechanism of host resistance to *T. colubriformis* and *H. contortus* at the molecular level remains poorly understood. The characteristics of a "typical" Th2-biased host response established by sheep resistant to GIN challenge include the production of elevated levels of parasite-specific IgA, IgE and IgG_1 antibodies (Gill, 1991; Pernthaner et al., 2006) eosinophilia (Gill, 1991; Rothwell et al., 1993) mucosal

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mastocytosis and goblet cell hyperplasia (Douch et al., 1996). However some host responses are also parasite species-specific. For example, part of the host response to *T. colubriformis* is characterised by an increase in the number of crypt goblet cells in the jejunum (Angus and Coop, 1984).

Determining the genes which are activated for protective immune responses in sheep to different GIN species (*T. colubriformis* and *H. contortus*) is a critical step in understanding the biological pathways and augmenting the biology of the host response to these parasites. A valuable model for such studies is the experimental comparison between genetically resistant and susceptible lambs. To this end, lambs from the resistant and susceptible lines of both the *Haemonchus* (Woolaston et al., 1990) and *Trichostrongylus* selection flocks (Dawkins et al., 1988), located at CSIRO Livestock Industries, Armidale, Australia, were used in this study. We have used a genomics approach involving high throughput microarray technology, together with the two parasite selection lines of sheep involving animals with genetically defined resistance and susceptibility phenotypes, in an attempt to identify common gastrointestinal gene expression responses employed by these animals in protection against either a *T. colubriformis* or *H. contortus* challenge. As such, this work reported here is a companion to and complementary with an earlier study (Ingham et al., 2008) where a candidate gene approach was carried out using the same tissue samples.

2. Materials and methods

2.1. Selection lines

Sheep and tissues used in this study are those described by Ingham et al. (2008). A $2 \times 2 \times 2 \times 2$ factorial design was employed. Factors and levels are presented in Table 1. Helminth naïve *Trichostrongylus* Selection Flock (TSF) and *Haemonchus* Selection Flock (HSF) lambs (Table 1) in treatment groups of between five to seven lambs per group, were reared in an animal house facility on wooden slats. They were weaned between 5 and 11 weeks of age and fed 900 g/day pelleted feed ration. Lambs receiving three infections were first infected at 16 weeks of age (day 1). Infections were terminated 6 weeks later by treatment with 8.25 mg/kg levamisole hydrochloride (Rotate; Novartis Animal Health Australasia Ltd). At 27 weeks of age, these lambs received a second infection which was again terminated 6 weeks later by drenching with levamisole hydrochloride. Final infections for these lambs and primary infections for lambs receiving only a single infection were administered 4 weeks later. Infections were synchronised so that lambs were approximately 37 weeks old when killed on the third day of a primary or tertiary infection for collection of tissues (day 108). Further details are provided by Ingham et al. (2008). These animal experiments were approved by and performed under guidelines of the F.D. McMaster Animal Ethics Committee, CSIRO Livestock Industries.

Table 1
The $2 \times 2 \times 2 \times 2$ factorial experimental design used in this study.

Factor	Level	Descriptor
Selection flock	*Trichostrongylus* selection flock	TSF
	Haemonchus selection flock	HSF
Line	Resistant	TSF-R, HSF-R
	Susceptible	TSF-S, HSF-S
Parasite	*Haemonchus contortus* (5000 Kirby strain)	*H. contortus*
	Trichostrongylus colubriformis (30,000 McMaster strain)	*T. colubriformis*
Infection protocol	1	Primary
	3	Tertiary

2.2. Faecal-egg counts and sample collection

Faecal-egg counts (FECs) (expressed as eggs/g faeces, EPG) following infections 1 and 2 in the tertiary infected groups were determined at the times and using the methods previously described (Ingham et al., 2008). Plasma samples were obtained by centrifugation of blood taken by jugular venipuncture during primary, secondary and tertiary infections. Sections of jejunum or abomasum for tissue antibody determination were obtained at the time of slaughter and frozen at −20 °C. These were then weighed while frozen, and placed in an equal volume (w/v) of ice cold PBS containing protease inhibitors and homogenised on ice, centrifuged (405*g* for 5 min at 4 °C), and the supernatant aliquoted and stored at −20 °C until required. Jejunum sections, mesenteric and abomasal lymph nodes were snap-frozen in liquid nitrogen and stored at −80 °C until required for RNA extraction. The abomasums of infected animals were scraped into RNAlater (Ambion, USA) and stored at −20 °C until required.

2.3. Parasite antigen-specific antibody profiles determined by ELISA

The levels of parasite-specific antibodies in plasma and tissue of lambs were measured using ELISA. Briefly, these assays measured the anti-parasite responses of total Ig, IgG1, IgG2, IgM, IgA and IgE. The antigen used in these studies consisted of soluble protein obtained from homogenised *T. colubriformis* or *H. contortus* L3s (Haig et al., 1989). Microtitre plates (NUNC MaxiSorb, 96-well) were coated with 1 μg/well of soluble proteins diluted in carbonate buffer (pH 9.6) overnight at 4 °C. Plates were washed with PBS supplemented with 0.05% Tween 20 (PBST) and blocked with 0.1% Na-casein in PBST for 1 h at room temperature (RT). Sheep plasma was serially diluted from 1:100 in tripling dilutions in 1% Na-casein/PBST for total Ig determinations in plasma, and from undiluted homogenates to in tripling serial dilutions in 1% Na-casein/PBST for total Ig, IgG1, IgG2, IgM, IgE and IgA in jejunum and abomasum tissue homogenates and incubated for 1 h at RT. The isotype-specific anti-sheep monoclonal antibodies IgG1, IgG2, (Beh, 1987) IgM (Beh, 1988) and IgE (Bendixsen et al., 2004), were in culture supernatants and diluted 1:10 in 1% Na-casein/PBST and mouse anti-bovine IgA (VMRD Inc., Australia) diluted 1:10,000 in 1% Na-casein/PBST and incubated for 1 h at RT. Donkey anti-sheep/goat-horseradish peroxidase (HRP) (Chemicon, Australia) was used to measure total Ig levels (diluted 1:5000), and sheep anti-mouse-HRP (Chemicon, Australia) (diluted 1:2000) was used to determine antibody isotypes in tissue and plasma. The conjugated antibodies were diluted in 1% Na-casein/PBST and incubated for 1 h at RT. ELISA plates were developed using tetramethyl benzidine (TMB) substrate (Sigma Aldrich, USA) for 30 min at RT, stopped with 0.5 M H_2SO_4 and read at 450 nm using a Thermomax Microplate Reader (Molecular Devices, USA). Individual responses, expressed as titres, were determined by calculating the mid-point, on a double log scale, of the straight line section of the curve using linear regression of the absorbance resulting from the serial doubling dilution of each sample. Each titre represented the inverse of the dilution at this point. A Repeated Measures Analysis of Variance was then carried out for each antibody isotype with the effects of flock (HSF versus TSF), line (resistant: R versus susceptible: S) and treatment (primary versus tertiary infection) in the main stratum, and time and its interactions in the sub-stratum.

2.4. Total RNA isolation and cDNA synthesis

Total RNA from jejunum sections, abomasal scrapings, mesenteric lymph node sections and whole abomasal lymph nodes of lambs infected with GINs were isolated using the RNeasy purification (Qiagen) kit using an on-column DNase digestion step. The