Immune responses in sheep naturally infected with *Oestrus ovis* (Diptera: Oestridae) and gastrointestinal nematodes

B.F. Silva*, C.C. Bassetto, A.F.T. Amarante

UNESP – Univ Estadual Paulista, Departamento de Parasitologia, Instituto de Biociências, Caixa Postal 510, CEP 18618-000, Botucatu, SP, Brazil

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**A B S T R A C T**

This study was carried out to evaluate the immune response in young Ile de France (IF) and Santa Ines (SI) sheep naturally infected by *Oestrus ovis* and gastrointestinal nematodes (GIN). Mast cells, eosinophils and globule leucocytes were enumerated in the upper respiratory tract (septum, middle meatus and ventral nasal conchae) and in the mucosa of abomasum and small intestine. Immunoglobulin G (IgG) levels in serum samples and immunoglobulin A (IgA) levels in mucus from the nasal, abomasum and small intestinal mucosae were determined against *O. ovis*, *Haemonchus contortus* and *Trichostrongylus colubriformis* antigens. Significant positive correlation coefficients were observed in both breeds between the number of *O. ovis* larvae × IgG against *Oestrus* crude extract (IF: *r* = 0.58; SI: *r* = 0.66; *P* < 0.05), and between *O. ovis* larvae × IgG against *Oestrus* excretory and secretory products (IF: *r* = 0.59; SI: *r* = 0.63; *P* < 0.05). Apparently, the presence of antibodies in the serum or nasal mucus, as well as inflammatory cells, was not able to efficiently protect against *O. ovis* infestation. With regard to GIN, the levels of immunoglobulins and the inflammatory cell numbers in the gastrointestinal mucosa presented a significant inverse relationship with *H. contortus* worm burden in SI animals and this may have contributed to the fact that these animals presented the lowest FEC and worm burden compared to IF. In conclusion, the immune responses against *O. ovis* and GIN are very similar and involve the recruitment of inflammatory cells and production of immunoglobulins against the parasites.

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1. Introduction

Mixed infections by dipteran larvae and helminthes are quite common in ruminants. Sheep are frequently parasitized simultaneously by gastrointestinal nematodes (GIN) and by *Oestrus ovis* larvae. These parasite infections stimulate immune mechanisms of defense that can be mediated by antibodies or cells, but the efficiency of this immune response depends on the animal genotype, age, gender, physiological status, prior exposure to the pathogen, capacity to recall the antigen, health and nutritional status, parasite and the infection stage (Colditz, 2008).

Proinflammatory immune reactions are characteristic of *O. ovis* infection and involve the recruitment of cells (mast cells, eosinophils, macrophages, T and B lymphocytes) and the secretion of immunoglobulins, suggesting a type Th2 immune response (Angulo-Valadez et al., 2011) that is similar to the immune response against gastrointestinal parasitism by nematodes (Anthony et al., 2007; Rowe et al., 2008). Studies of the relationship between *O. ovis* and helminth co-infections have revealed that there are antagonist interactions between *O. ovis* larvae and the...
Strongyle nematodes, *Trichostrongylus colubriniformis* and *Haemonchus contortus* (Yacob et al., 2004; Terefe et al., 2005). The infection of the digestive tract with nematodes did not modify the biology of *Oestrus* populations, but in contrast, infections with *O. ovis* were related to significant reductions in nematode egg excretion and worm burdens. These changes are associated with significant modifications in populations of mast cells, globule leukocytes and eosinophils in the respiratory and digestive tracts. They also indicate that parasitic infection in one particular anatomical site induces “at distance” inflammatory reactions of the whole mucosal system (Dorchies et al., 1997; Yacob et al., 2002; Terefe et al., 2005).

This study was carried out to evaluate the humoral and cellular immune response in young Ile de France and Santa Ines sheep that were naturally infected with *O. ovis* and gastrointestinal nematodes. We used samples from a previously published study (Silva et al., 2012) that demonstrated no breed difference regarding *O. ovis* infestation, but that revealed that animals with more nasal bot fly larvae tended to display a smaller worm burden. In the present study, we investigated which inflammatory cell populations and immunoglobulins are involved in the protection against these parasites.

### 2. Material and methods

#### 2.1. Animals

The immune response was evaluated in the upper respiratory tract (septum, middle meatus and ventral nasal conchae) and in the digestive tract (abomasum – fundic region and small intestine – 1 m from the pylorus) of the Ile de France (IF) and Santa Ines (SI) young sheep, which were naturally infected with *O. ovis* larvae and GIN. The experimental design of this procedure has been described previously (Silva et al., 2012). Briefly, 12 IF and 12 SI lambs were purchased from different farms located in Sao Paulo State. Four lambs were acquired from each farm to assure a minimum of genetic variability in each breed. All lambs were born in June 2009, except for four IF lambs, which were born in May. Lambs, weaned at two months of age, were moved in late August to University facilities. The animals were kept exclusively in pasture during the experimental period (September to early December 2009, spring season) in a paddock (0.3 ha) with *Brachiaria decumbens* grass, where they had free access to tap water.

At the beginning of the trial, in order to start the study with animals in the same conditions, all lambs were treated with anthelmintics (levamisole phosphate + albendazole). Fifteen days after this treatment, mean faecal egg counting (FEC) were 60 and 158 eggs per gram of faeces (EPG) of Strongyle and 20 and 75 EPG of *Strongyloides papillosus* in SI lambs and IF lambs, respectively. Two SI lambs died early in the trial of unknown causes and the data for these animals were excluded from analyses. At six months of age, in early December 2009, the animals were euthanized. Blood serum, tissue and mucus samples were collected for immunological and histological analysis.

#### 2.2. Hematology

Blood samples were collected by jugular vein puncture into vacuum tubes without EDTA and serum samples were stored at −20°C until use for immunoglobulin G (IgG) measurements.

#### 2.3. Histology

Immediately after death, tissue samples were taken from three anatomical regions in the upper respiratory tract, i.e., septum, middle meatus and ventral nasal conchae and from two sites of the digestive tract, i.e., abomasum (fundic region) and small intestine (1 m from the pylorus) for counting of mucosal mast cells, eosinophils and globule leukocytes. All tissue samples were fixed in 10% buffered formaldehyde for 48 h. The samples were then dehydrated with alcohol and embedded in paraffin wax. Sections, 2 μm thick, were stained with 1% toluidine blue or haematoxylin and eosin (H&E).

Mast cells were counted in sections stained with toluidine blue and eosinophils and globule leukocytes in sections were stained with H&E. Cells were enumerated under a 10x eye piece containing a calibrated graticule and 100x objective lens viewing an area of 0.01 mm². Thirty fields, which were randomly selected, were observed per animal for each histological region and the mean numbers of cell/surface were calculated and compared between the groups. The counts were expressed as number of cells per mm² of mucosa.

#### 2.4. Mucus

Mucus was taken from the nasal cavities, abomasum and small intestine mucosas to determine the levels of immunoglobulin A (IgA). While the larvae of *O. ovis* were collected, mucus from nasal mucosa was extracted by lightly scraping the mucosal surface with a glass slide and mucus was stored in a falcon tube at −20°C until processing. A 5 cm piece of abomasum and small intestine were sampled for the extraction of mucus and stored at −20°C until processing. Tissues were thawed and mucus was scraped off with a glass slide. The scrapings were collected in a falcon tube on ice. Three millilitres of ice cold PBS supplemented with protease inhibitors (Complete®, Roche) was added to each sample. The samples were shaken for 1 h at 4°C and centrifuged for 30 min at 4°C and 3000 × g. The supernatant was collected and centrifuged again for 30 min at 4°C and 15 000 × g (Kanobana et al., 2002).

Protein concentrations were determined using a kit (Protal método colorimétrico® – Laborlab, Brazil) and the samples of abomasum mucus were adjusted to a protein concentration of 0.4 g/dl: small intestine to 0.1 g/dl and nasal mucus to 0.7 g/dl using PBS supplemented with protease inhibitors.

#### 2.5. Enzyme-linked immunosorbent assay (ELISA)

IgG levels in serum samples were determined against excretory and secretory products (ESP) and crude extract (CE) antigens from second instar (L2) *O. ovis* larvae; and
against third stage larvae (L3) and adults (L5) of *H. contortus* and *T. colubriformis* antigens. IgA levels in nasal mucus were tested against excretory and secretory products (ESP) and crude extract (CE) from L2 O. ovis larvae; abomasal mucus was tested against L3 and L5 of *H. contortus* and small intestine mucus against L3 and L5 of *T. colubriformis."

2.6. Parasites used in antigen production

Second instar (L2) of *O. ovis* were collected from naturally infected sheep heads and were washed several times in phosphate-buffered saline (PBS pH 7.2) with 240 000 UI of penicillin and 100 mg/ml of streptomycin and the viability of larvae were checked under a stereomicroscope.

The excretory and secretory products (ESP) were obtained from five L2 maintained in a culture medium in vitro. The L2 were placed in a tube containing 10 ml RPMI-1640 (Sigma; 8758) with penicillin and streptomycin and were incubated in darkness for 24 h in a 5% CO₂ atmosphere at 37 °C. Supernatant extracts were collected, centrifuged at 15 000 × g for 30 min at 4 °C and supernatants were collected and centrifuged immediately and stored at −80 °C until use.

To obtain the crude extract (CE), 10 L2 were fragmented/homogenized, using a homogenizer (T10 basic, IKA), in 5 ml of PBS pH 7.2 supplemented with protease inhibitor (Complete®, Roche). The extract was centrifuged at 15 000 × g for 30 min at 4 °C and supernatants were collected and centrifuged immediately. Protein concentrations of *O. ovis* antigens were determined using a kit (Bicinchoninic Acid Protein Assay Kit – Sigma) and absorbance was read at 562 nm. The antigen extracts were stored in aliquots at −80 °C until further use.

The production of antigens of infective third stage larvae (L3) and adults (L5) of *H. contortus* and *T. colubriformis* have been previously described by Amarante et al. (2009) and Cardia et al. (2011), respectively.

2.7. Parasite-specific serum IgG

Polystyrene micro-titre plates (F96 MicroWell plate – Maxisorp® – NUNC, USA) were coated with 100 μl of the different antigens (5 μg/ml) diluted in carbonate-bicarbonate buffer (pH 9.6); plates were incubated overnight at 4 °C. All subsequent incubations were carried out for 1 h at 37 °C using, in each well, a total of 100 μl of reagents. Plates were washed three times between each step with ultra pure water (EASYpure II UV, Barnstead, USA) containing 0.05% Tween 20 (ProPure® – Amresco).

After coating, blocking was carried out with 0.1% Gelatin (Amresco, USA) and 0.05% Tween 20 (ProPure® – Amresco) in PBS 7.2 (PBS-GT). Serum samples were diluted in PBS-GT (1:500) and applied in duplicate. Plates were then incubated with peroxidase-conjugated rabbit-anti sheep IgG diluted at 1:10 000 (A130-101P, Bethyl Laboratories, Inc., USA). Finally, OPD substrate solution (1,2-phenylenediamine dihydrochloride, Dako, Denmark) was added to each well and the enzymatic reaction was allowed to proceed at room temperature, in the dark for 15 min and stopped with 5% sulphuric acid solution; plates were immediately read using an automated ELISA reader (Biotrak II, Amersham-Biosciences, UK) at 492 nm.

The positive standard serum for *O. ovis* was obtained from a sheep evaluated by titration of all serum samples tested from this experiment and, as negative control, serum samples were obtained from young animals kept indoors that had no contact with adult bot flies. The standard positive serum for *H. contortus* and *T. colubriformis* were obtained from a sheep that was repeatedly infected with these nematodes. Results were expressed as the percentage of the optical density value (OD) of the positive standard serum and employing the following formula: % OD = [(OD mean of the tested serum − OD mean of blank)/(OD mean of the positive standard serum − OD mean of blank)] × 100 (Kanobana et al., 2001).

2.8. Parasite-specific mucus IgA

The ELISA reactions for parasite-specific mucus IgA were as previously described for serum analysis with 1:10 mucus dilution to abomasum and nasal mucus and with 1:2 mucus dilution to small intestine. Peroxidase-conjugated rabbit-anti sheep IgA was diluted at 1:10 000 (A130-108P, Bethyl Laboratories, Inc., USA). Finally, OPD substrate solution (1,2-phenylenediamine dihydrochloride, Dako, Denmark) was added to each well and the enzymatic reaction was allowed to proceed at room temperature, in the dark for 15 min and stopped with 5% sulphuric acid solution; plates were immediately read using an automated ELISA reader (Biotrak II, Amersham-Biosciences, UK) at 492 nm. The results were expressed as the percentage of OD of sample minus OD of blank (Kanobana et al., 2001).

2.9. Statistical analyses

The percentages of infective larvae of each genus of Strongyle obtained from cultures were used to estimate the FEC of each nematode genus.

Significant differences between groups for cell counts and IgA in mucus were assessed by one-way analysis of variance using SAS (release 9.2). To test whether there was any effect of time on serum IgG levels and FEC, repeated measures analysis was performed using the same software. Group means were considered different when P < 0.05. All data were transformed using log₁₀(x + 1) prior to analysis. Spearman’s correlation coefficient between variables was assessed. Figures and table present data as arithmetic means (±standard error of the mean).

3. Results

The data on FEC, nematode and *O. ovis* burdens of IF and SI lambs have been presented in detail by Silva et al. (2012) and are summarized in Table 1 and Figs. 1 and 2.

No significant differences between groups were found in the number of inflammatory cells counted in nasal and digestive mucosa, except for eosinophils/mm² average in the nasal conchae and globules leucocytes/mm² average in the abomasums, which were significantly higher in IF than in SI lambs (P < 0.05) (Fig. 3).
The levels of serum IgG against Oestrus were similar between breeds, except for the IgG against Oestrus CE in the last sampling (2nd December 2009), which was found to be significantly higher in IF lambs ($P < 0.05$) (Fig. 4A). During the first month of the experiment (September 2009), the IgG against Oestrus levels were close to zero (Fig. 4A and B), but started to increase on 7th October 2009, simultaneously with the appearance of clinical signs of oestrus in both breeds. The levels of serum IgG against Oestrus increased significantly in both breeds throughout the experiment until reaching the highest mean value on the last day of collection ($P < 0.05$). The mean levels of serum IgG against Oestrus CE were higher than the mean values of IgG against Oestrus ESP.

At the beginning of the experiment, all lambs were parasitized with GIN; as such, they already displayed relatively high levels of serum IgG against L5 and against L3 of H. contortus (Fig. 1) and T. colubriformis (Fig. 2). There was no difference between the breeds ($P > 0.05$) and there was a low variation in serum IgG levels against GIN antigens tested throughout the experiment, except for the levels of IgG against L5 for T. colubriformis and IgG against L3 for H. contortus, which increased significantly until the end of the experiment for both breeds ($P < 0.05$). No significant interactions were observed between time x group regarding parasite specific IgG levels or FEC ($P > 0.05$). The IgA levels in nasal, abomasal and intestinal mucus were similar in both breeds (Fig. 5).

Although the experimental groups were composed of a limited number of animals, a significant ($P < 0.05$) positive
correlation was observed in both breeds between the number of *O. ovis* larvae × IgG against *Oestrus* CE in IF ($r = 0.58$) and SI ($r = 0.66$), between *O. ovis* larvae × IgG against *Oestrus* ESP in IF ($r = 0.59$) and SI ($r = 0.63$).

IF lambs showed a significant positive correlation between the number of *O. ovis* larvae × globule leucocytes in the nasal meatus ($r = 0.71$; $P < 0.05$). With regard to GIN burden and immune response, significant correlations were observed just in SI lambs: abomasum mast cells × *H. contortus* burden ($r = 0.73$; $P < 0.05$); IgG against L3 Hc × *H. contortus* burden ($r = 0.72$; $P < 0.05$); IgA against L5 Hc × *H. contortus* burden ($r = 0.61$; $P = 0.07$); and mast cells from small intestine × *T. colubriformis* burden ($r = 0.60$; $P = 0.07$).

No significant correlation coefficients were observed between inflammatory cells from nasal tract and from GIN tract, with the exception of globule leucocyte values of the nasal conchae and small intestine in IF lambs ($r = 0.63$; $P < 0.05$).

**4. Discussion**

Parasitism with GIN and *O. ovis* causes an increase in inflammatory cell numbers of the upper respiratory and gastrointestinal tract mucosas and the production of anti-parasite specific immunoglobulins (Yacob et al., 2002; Bricarello et al., 2005; Terefe et al., 2005; Cardia et al., 2011), changes that were observed in the present study. Such an immune response was similar in the animals of both breeds and resulted in no breed difference regarding *O. ovis* infestation or GIN worm burdens. However, SI lambs showed a higher proportion of L1 of *O. ovis* compared to IF, indicating a possible delay in larval development caused by a more intense immune response in the former breed (Silva et al., 2012). The immune response is involved in the regulation of *O. ovis* populations (Jacquiet et al., 2005), and may have an inhibitory effect on *O. ovis* larval growth, delaying development (Frugère et al., 2000; Angulo-Valadez et al., 2007b).

At the beginning of this experiment the serum IgG levels against *O. ovis* were close to zero in SI and IF lambs, probably because the lambs had never had previous contact with this parasite; but after the first experimental month, most of the lambs started to show clinical signs of oestrosis, i.e., dyspnoea and nasal discharge, coinciding with the significantly gradual rise of serum IgG levels. In *O. ovis* infestation, the humoral systemic response of IgG usually reaches seroconversion at 2–4 weeks post-first infection and the highest levels are observed during the development of L2 and L3 larvae (Alcaide et al., 2005; Angulo-Valadez et al., 2011).

The major symptoms of infestation, nasal discharge and frequent sneezing, are immune mediated, i.e., depend
on the acquisition of an immune response against the parasite. These symptoms are more intense in some animals, indicating hypersensitivity. In animals with these clinical manifestations, larvae, especially L1 in the nasal cavities, are at high risk of becoming trapped in dense mucus, asphyxied and expelled from the host (Angulo-Valadez et al., 2011). Studies suggest that O. ovis uses immunosuppressive strategies, such as the reduction of specific lymphocyte proliferation and the degradation of immunoglobulins, to evade defensive attacks from the host (Tabouret et al., 2003; Jacquet et al., 2005) and L1 plays an important role in the regulation of inflammatory reactions (Duranton et al., 1999). It is well known that larvae stimulate mucus production, which is utilized in their nutrition. Salivary gland products of O. ovis contain thermostable proteases, which appear to be important in larval nutrition and host–parasite interaction (Angulo-Valadez et al., 2007a).

In the present study, animals with the highest levels of IgG and IgA against O. ovis had the highest numbers of O. ovis larvae, while inflammatory cell numbers did not present any consistent association with O. ovis larval burden. The opposite is observed in gastrointestinal infections, where the levels of immunoglobulins and the inflammatory cell numbers in gastrointestinal mucosa present an inverse relationship with the worm burden and FEC (Amarante et al., 2005; Cardia et al., 2011; Shakya et al., 2011). Apparently, the presence of antibodies in serum or nasal mucus, as well as inflammatory cells, did not efficiently protect against O. ovis infestation. However, it has been shown that mucus IgA, associated with humoral and cellular immune response, possibly promote the regulation of O. ovis burden in the host (Jacquet et al., 2005) and may also have an influence on larval weight and consequently on the viability of adult flies (Cepeda-Palacios et al., 2000), i.e., although the immune response is not enough to limit the establishment of parasites, this can at least affect the O. ovis population.

With regard to GIN, the levels of immunoglobulins and the inflammatory cell numbers in gastrointestinal mucosa presented a significant inverse relationship only with H. contortus worm burden in Santa Ines animals. This was one of the reasons why these animals showed the lowest FEC and worm burden compared to IF. In other studies, inflammatory cells and parasite-specific IgA in the abomasum were also inversely associated with H. contortus worm burden and FEC, indicating that they may impair parasite development or fecundity (Strain and Stear, 2001; Amarante et al., 2005; Bricarello et al., 2005).

Animals with more nasal bot fly larvae tended to display a smaller worm burden (Silva et al., 2012). It has been previously demonstrated that nematode egg production, worm burden and clinical signs of GIN infections are significantly depressed in mixed infections with O. ovis (Dorchies et al., 1997; Terefe et al., 2005; Yacob et al., 2006). O. ovis infestation stimulates the immune response, which may have a negative influence on GIN parasitism via the enhanced recruitment of activated inflammatory cells (eosinophils, mast cells and globule leucocytes) and/or their products towards the gut mucosa. Eosinophils are considered to be important in the response against helmith infections and are frequently associated with the expression of resistance to parasites (Dawkins et al., 1989; Stear et al., 2002; Balic et al., 2006; Shakya et al., 2011). These alterations might create an unfavourable environment to the nematodes, thereby reducing worm length and fecundity (Terefe et al., 2005), an occurrence that could explain the low FEC and worm burden in animals of both breeds in this study.

In conclusion, the immune responses against O. ovis and GIN were very similar and involved the recruitment of inflammatory cells and production of immunoglobulins against the parasites. However, the host–parasite interaction may be more well balanced for O. ovis, allowing parasites infestation without acute disease; while is less balanced for Haemonchus, that frequently cause acute disease and death in sheep.

**Fig. 5.** Mean levels of mucus IgA against crude extract (CE) and excreatory and secretory products (ESP) of Oestrus ovis (Oo) second larval instar (A); against third stage larvae (L3) and against adults (L5) of Haemonchus contortus (Hc) (B); against third stage larvae (L3) and against adults (L5) of Trichostrongylus colubriformis (Tc) (C) in Ile de France and Santa Ines males lambs naturally infected with O. ovis and gastrointestinal nematodes. Bars are standard errors. There was no significant difference between means ($P>0.05$).
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