Immunohistochemical study and mRNA cytokine profile of the local immune response in cattle naturally infected with *Calicophoron daubneyi*.

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Abstract

In order to recognize the local immune response of the definitive host to *Calicophoron daubneyi* natural infection, an immunohistochemical study was carried out in the reticulum and rumen in 49 naturally infected cattle. The role of cytokines (IL-4 and IL-10 interleukins and IFN- γ) in the activation of specific defence mechanisms was evaluated by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) assays to study cytokine mRNA expression. In all infected animals, CD3⁺ T lymphocytes seemed to be the main element of the inflammatory infiltrate in the reticular and ruminal lamina propria at the point of the parasite adhesion. Intraepithelial globule leukocytes also showed immunolabelling for CD3. Most CD3⁺ cells also expressed CD4 (T cell helper) antigen although sporadic CD8⁺-cytotoxic lymphocytes were observed. Local expression of IFN- γ was observed in damaged papillae at the site of parasite attachment and in scattered

cells in the lamina propria. B cells (CD79 α cy+, CD45+ and IgG+) were found constantly in relation to lymphoid aggregates. MAC387 was expressed in squamous epithelium and in macrophages of the lamina propria of affected papillae. Macrophages in this location also stained positively for CD163 and CD68. Intraepithelial Langerhans cells and macrophages located in the lamina propria showed immunopositivity for MHCII in the affected areas. RT-qPCR analysis confirmed a statistical significant increase of IFN- γ , and IL-10 expression ($p < 0.01$) in the rumen associated with the presence of flukes. These findings suggest a predominant Th1 polarized local immune response with the probable involvement of Th regulatory cells in cattle *C. daubneyi* natural infection.

Keywords

Calicophoron daubneyi, local immune response, cytokines, cattle.

Introduction

Paramphistomosis is a ruminant digestive parasitosis caused by trematodes of the family Paramphistomidae (Trematoda, Digenea), belonging to different genera (*Paramphistomum*, *Calicophoron* and *Cotylophoron*), which is considered, at present, as an important emerging disease of livestock still highly underestimated (Tandon et al, 2014). In two recent studies, the importance of this infection in Spain, where *Calicophoron daubneyi* was the only agent found, has been reported (González-Warleta et al., 2013; Ferreras et al., 2014).

There is a scarcity of information on the immunophenotypical characterization of the inflammatory cells present in the lesions or the local immune responses associated with trematodes infections in the large animal definitive hosts. The majority of these studies have been carried out in *Schistosoma* spp, *Fasciola* spp and *Dicrocoelium* spp infections. Broadly, they have shown that inflammatory cells are composed of a mixed population of B and T lymphocytes, mainly CD4+ and CD8+, together with macrophages, globular leukocytes and IgG+ plasma cells (Ferreras et al., 2000, 2007; Pérez et al., 2002; Molina and Skerrat, 2005; Zafra et al., 2009). None of these studies have evaluated the local expression of cytokines. Concerning paramphistomes, these trematodes were confirmed as the cause of inflammatory lesions in the rumen and reticulum whose severity was directly related to the parasite burden, and characterized morphologically by lymphocytes together with some macrophages and eosinophils (Fuertes et al., 2015). However,

the phenotypical characteristics of the inflammatory infiltrates against this trematode or the cytokine genic expression in infected tissues have not been documented to date.

In general, the adaptive immune response of the host to extracellular parasites, such as helminths, involves the development of T helper cells with characteristic T-helper type 2 (Th2) anti-inflammatory cytokine profiles (Interleukins 4, 5 and 10: IL-4, IL-5, IL-10). In contrast, intracellular parasites induce a T-helper type 1 (Th1) response characterized by production of pro-inflammatory mediators such as IL-12 and Interferon-gamma (IFN- γ). However, certain extracellular helminths (*Trichuris muris*) and intracellular protozoan (*Leishmania major*) parasites are capable to induce both Th1 and Th2 components (Jankovic et al, 2001). In *Fasciola hepatica* infections, it has been observed that infected cattle show an early Th1 type response which may be polarized in chronic infection to that of a Th2 type (Clery et al, 1996).

The main objective of this study is to immunohistochemically investigate the host local cellular immune response in natural infections of cattle with *C. daubneyi*. Moreover, the mRNA expression of three cytokines (IFN- γ , IL-4 and IL-10), representative of the Th1 and Th2 T cell responses, were evaluated with the aim of understanding their possible role in this parasitosis. The knowledge of the host local immune response against *C. daubneyi* would be of interest to understand the pathogenesis of cattle paramphistomosis.

Materials and methods

Sampling

This study has been carried out in 49 cattle naturally infected by *C. daubneyi* and 15 healthy animals as controls, all of them slaughtered in an abattoir (Ferrerias et al., 2014). The characteristics of the animals and the inflammatory lesions present in these animals were described in detail in previous works (Ferrerias et al., 2014; Fuertes et al., 2015).

For each animal, different tissue samples of the reticulum and different anatomical parts of the rumen (at least 12 per animal) were fixed in 10% neutral-buffered formalin and were dehydrated through graded alcohols before being embedded in paraffin wax for histopathological and immunohistochemical studies. Besides, tissue samples of the ruminal atrium of infected and uninfected cattle were snap frozen and stored at -80 °C until used.

In 11 parasitized cattle and 4 uninfected control cattle (without gross or microscopical ruminal lesions) two samples of the ruminal atrium and other two from the ruminal dorsal sac, approximately 5 mm³ each, were obtained for each animal, placed in RNA^{later} (Sigma-Aldrich, Saint Louis, MO, USA) and stored at -20 °C until they were used for cytokine mRNA expression analysis. No flukes were detected in the ruminal dorsal sac in the 11 parasitized animals.

Immunohistochemistry

Selected sections from the rumen and reticulum were immunohistochemically labelled with a panel of antibodies. Table 1 lists the primary antibodies and immunostaining protocols used. In all the cases, a polymer-based detection system (EnVision® System Labelled Polymer-HRP; Dako, Glostrup, Denmark) was employed and immunolabelling was developed with a solution of 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA, USA). The slides were counterstained with Harris' haematoxylin and mounted in hydrophobic medium. Technique specificity and sensitivity were controlled by omitting of the primary antibody or Envision® and using tissue samples of bovine lymph node as positive control for all the primary antibodies.

Quantification of cytokine mRNA expression levels in the rumen

Total RNA for cytokine expression analysis was extracted from the above mentioned ruminal tissues by a combined method based on the TRIzol Reagent (Life Technologies, Pasley, UK) and Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was carried out using SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies, Pasley, UK). Primers used for bovine IFN- γ , IL-4 and IL-10 cytokines and the housekeeping gene β -actin were designed using the Primer-3Plus software (Untergasser et al, 2007) and are described in Regidor-Cerrillo et al., (2014). Real-time PCR reactions were performed in 20 μ l using Power SYBR®PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 10 pmol of each primer and 5 μ l of diluted cDNA

samples in an ABI 7300 Real Time PCR System (Applied Biosystems). The cytokine mRNA level was expressed in fg of cytokine mRNA/mg of tissue. The relative quantification of cytokine mRNA expression levels (x-fold change in expression) was carried out by the comparative $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak, 2008).

Statistical analysis

Statistical analysis was conducted with the aid of SAS package (SAS Institute, 2008). All data were analysed for normality and homogeneity of variances using the Saphiro-Wilk and Levene tests, respectively. Within each anatomical area (atrium and dorsal sac), comparison of IFN- γ , IL10- and IL4-citokine mRNA expression between infected and non-infected animals were performed using the Mann-Whitney U-test.

Results

Immunohistochemical findings

In all infected animals, CD3+ T lymphocytes, diffusely scattered or forming lymphoid aggregates, were seen as the principal element of the inflammatory infiltrate in the reticular and ruminal lamina propria mainly at the point where the parasite was attached to conical papillae. Furthermore, labelling of CD3 epitopes revealed grouped cells in the periphery of the lymphoid follicles and few cells inside them (Fig. 1a). Intraepithelial CD3+ lymphocytes (IELs) were observed in suprabasal location. Cytoplasmatic granules of the intraepithelial globule leukocytes (GLs) also stained positively for CD3 (Fig. 1b). Most of CD3+ cells also expressed CD4+ T-helper cells forming mantles around the lymphoid follicles and within the epithelium (Fig. 1c). Sporadic CD8+ cytotoxic T cells were observed surrounding lymphoid follicles. CD79 α cy B lymphocytes were observed constantly scattered in small groups but were also demonstrated in relation to lymphoid aggregates and follicles as well as the IgG+ plasma cells. The CD45 mAb stained groups of lymphocytes located in the lamina propria, in subepithelial location, only in the papillae on which the parasites were attached.

Immunolabelling for the anti-IFN- γ mAb was observed in the lining epithelial cells, scattered or forming clusters, at the site of parasite attachment in the base of the affected papillae (Fig. 1e).

Sparse IFN- γ + cells, consistent with lymphocytes, were also located in the lamina propria of the rumen and reticulum in relation to damaged conical papillae.

MAC387 mAb was expressed in squamous epithelia and in the macrophages located in the lamina propria of the rumen and reticulum in the areas of parasite attachment (Fig. 1d). Within the epithelium of the affected papillae, epithelial cells showed diffuse cytoplasmic immunoreactivity for this mAb which was more intense in epithelial cells located in the base of the papillae encircled by the parasite ventral sucker. In the lamina propria, positively stained MAC387 macrophages, scattered or in groups, were located in the inflammatory infiltrate. The distribution of CD68 and CD163 positive macrophages was similar. These cells were found scattered in the lamina propria between lymphoid aggregates and follicles at the site of parasite attachment. MHCII expression was detected in dendritic shaped Langerhans cells located within the squamous cell layer of ruminal epithelium, mainly in the conical papillae in which *C. daubneyi* parasites were attached (Fig. 1g). Numerous MHCII reactive cells with abundant cytoplasm and pale ovoid nuclei compatible with macrophages were also observed intermingled with lymphoid cells in the lamina propria in affected areas (Fig. 1f).

Cytokine mRNA expression levels in the ruminal tissues.

Within the group of parasitized animals, the gene expression levels of IFN- γ , IL-4 and IL-10 showed a significant increase ($p < 0.01$) in ruminal atrium samples when they are compared with those in the dorsal sac, an area free of parasites. In the control, unparasitized cattle, the gene expression of IL-4 and IL-10 was also significantly higher ($p < 0.05$) in the ruminal atrium than in the dorsal sac. However, no differences were observed for IFN- γ levels in this group.

When comparing parasitized and control cattle, significant increases in the IL-10 and IFN- γ gene expression levels ($p < 0.01$ and $p < 0.05$ respectively) were found in the ruminal atrium of the parasitized animals. In the dorsal sac, all three cytokine levels were raised in the infected group but no statistical differences could be established with the unparasitized animals (Figure 2).

Discussion

In this study we have used the same tissue samples to immunohistochemically characterize the cellular inflammatory infiltrates and the cytokine mRNA expression levels in selected ruminal

tissues, where lesions associated with natural *C. daubneyi* infection in cattle were previously evaluated (Fuertes et al., 2015). Phenotypic analysis of the inflammatory cellular infiltrates in the reticular and ruminal mucosa in *C. daubneyi* naturally infected cattle has shown that they are formed by both T and B lymphocytes. The T cell response was composed predominantly of CD4+ T helper cells, distributed as lymphoid aggregates and follicles in the lamina propria and epithelium (Intraepithelial lymphocytes or IELs) of damaged conical ruminal and reticular papillae. An intense infiltration of T and B lymphocytes in hepatic lesions has also been reported also in other trematodoses like experimental fasciolosis and dicrocoeliosis in ruminants (Pérez et al, 2002; Ferreras et al., 2007; Zafra et al, 2009). These data demonstrate that *C. daubneyi* is also able to trigger a local immune response against flukes and/or their secretion products. The scant amount of CD8+ cells in the present study can be linked to the cytotoxic activity of these cells (Tizard, 2009), that would not be needed in extracellular parasitosis such as *C. daubneyi* infection.

A remarkable finding was the presence of CD3+ intraepithelial globule leukocytes (GLs) in high amount in some parasitized cattle. This result supports a lymphocytic origin for GLs in cattle in agreement with previous studies in cats (Konno et al, 1994; Roccabianca et al, 2006). The presence of CD79 α cy+ B lymphocytes and IgG plasma cells intermingled with T cells in the ruminal and reticular lamina propria may be indicative of a local humoral immune response against *C. daubneyi* that could be linked with the serum antibody response in natural paramphistome infections (Díaz et al, 2006).

The presence of strong immunoreactivity for the cytosolic protein complex L1 or calprotectin (MAC387+) in the epithelium and in the macrophages within the lamina propria of altered papillae was another noteworthy finding. It is known that the L1 antigen is a calcium binding protein expressed by squamous epithelium of mucous membranes and injured epidermis, but not by normal epidermis and other cells harboured in the skin (Brandtzaeg et al, 1988; Paquet and Piérard, 1999). Besides expression of the MAC387 antigen in the inflamed epidermis is directly associated with cell-mediated activity in the papillary dermis (Kirkham et al, 1990). In the present study modifications of MAC387 labelling in the rumen and reticulum mucosa were observed at the point of fluke attachment in parasitized cattle in comparison to uninfected animals. Rumen and reticulum mucosa are then considered as reactive pointing out squamous cell damage due to this parasitosis.

The MAC387⁺ monocytes/macrophages constitutes as a recently infiltrated cell subpopulation in local inflammatory response so they are considered active inflammation markers (Soulas et al., 2011). The presence of these cells in the inflammatory infiltrate of ruminal and reticular lamina propria in paramphistomosis could be associated to a continuous antigenic stimulus by fluke excretion products or tegumental molecules.

In this study intraepithelial dendritic cells, morphologically compatible with Langerhans cells, as well as macrophages within the lamina propria in lesion sites showed immunolabelling for MHCII antigens. The role of Langerhans cells in the presentation of antigens to T cells through dendritic cell MHCII antigen complex during inflammation and the induction of a Th response is well known (Tizard, 2009) and these results emphasize that *C. daubneyi* infection is able to mount a local immune response triggered by MHCII expression in ruminal antigen presenting cells. .

In other trematodosis, an initial proinflammatory reaction mediated by Th1 cytokines, related to the presence of immature flukes, is followed by a Th2 response (Flynn et al., 2010; Chuah et al., 2014). In the present study the local expression of IFN- γ (main Th1 proinflammatory cytokine associated with classical macrophage activation) was detected in infected cattle using immunohistochemistry and qPCR techniques. This result would indicate that *C. daubneyi* infection is able to trigger a cell mediated immune response at the local level, as it has been pointed out in other trematodosis such as fasciolosis in the hepatic lymph nodes (Zafra et al, 2009).

Besides the immunolabelling associated to T cells, in the present study the presence of IFN- γ was observed focally in the epithelial lining cells of those papillae encircled by the parasite ventral sucker. Although IFN- γ is produced mainly by lymphocytes, recently the ability of respiratory epithelial cells to produce IFN- γ after being infected by human parainfluenza virus type 3 has been described (Lewandowska-Polak et al., 2015). This finding would indicate that epithelial cells in areas in contact with the parasite could contribute to the cellular response to trematode antigens or the mechanical damage due to fluke attachment.

Concerning mRNA cytokine level results, our findings showed that IFN- γ and IL-10 mRNA expression was significantly increased (upregulated) in ruminal atrium mucosa of parasitized animals in comparison to the control group and with the ruminal dorsal sac levels in parasitized

cattle, leading to the conclusion that their expression is directly related to the presence of parasites. In contrast, no significant differences were found concerning the IL-4 mRNA levels in the same locations, although comparatively high amount of this type 2 cytokine were produced in the ruminal atrium of infected cattle. The significant local production of IFN- γ by lymphocytes related to the presence of parasites, may indicate the establishment of a cell-mediated immunity which, however, was ineffective in the elimination of the parasite, as already stated for extracellular parasites (Jankovic et al., 2001).

The simultaneous and constant high gene expression levels of for IL-10 found in this study would indicate that in *C. daubneyi* naturally infected cattle this cytokine probably downregulates or represses the expression of proinflammatory cytokines such as IFN- γ and protects against the tissular epithelial damage caused by inflammation as it has been suggested (Ouyang et al, 2011; Chuah et al., 2014). The finding that IL-10 regulates both Th1 and Th2 responses at mucosal surfaces has previously been reported in infected mice with *Trichinella spiralis* (Helmby and Grencis, 2003).

Finally, in unparasitized animals both immunomodulating IL4 and IL-10 cytokine gene expression was upregulated in the ruminal atrium when compared with the dorsal sac. This could be due to a necessary immunomodulation on ruminal mucosa consequence of constant antigenic stimulation by exogenous and endogenous microbiota of ruminal content (Koboziev et al., 2014). This condition is not present in dorsal sac where ruminal content in direct contact with mucosa is composed by gases and flora is scant or absent.

Our results suggest that a regulatory parasite antigen-specific mechanism exists in natural *C. daubneyi* infection in cattle at the local site of attachment of this trematode towards the production of a polarized Th1 immune response, with the involvement of Th regulatory cells, which may be associated with a mixed cell population activity in this infection. The differences in the cytokine responses may be due to the antigen molecules actively secreted by the surface tegument of the adult rumen fluke in direct contact with the host tissues. Further studies are required to understand host defence against these parasites.

Competing interests

The authors declare that they have no competing interests.

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Figures.

Figure 1. a) Rumino-reticular orifice. Conical papilla encircled by fluke ventral sucker showing inflammatory infiltrate composed by CD3+ T cells. Parasite burden= 93 (12 worms recovered from the rumino-reticular orifice). Envision® System. Haematoxylin counterstain. Bar= 200µm. b) Reticulum. Cytoplasmatic granules of Globular Leukocytes (GLs) intensely immunostained by pAb CD3 (arrows). Parasite burden= 908 (400 worms recovered from the reticulum). Envision® System. Haematoxylin counterstain. Bar= 30µm. c) Ruminal atrium. Inflammatory infiltrate in the lamina propria of a mushroom-shaped affected papilla composed mainly by CD4+ T helper cells. Parasite burden= 253 (82 worms recovered from the ruminal atrium). Envision® System. Haematoxylin counterstain. Bar= 200µm. d) Rumino-reticular fold. Papillar epithelium expressing mAb MAC387 especially in areas in close contact with the tegument of fluke ventral sucker. Sparse immunolabelled macrophages in lamina propria. Parasite burden= 8005 (571 worms recovered from the rumino-reticular fold). Envision® System. Haematoxylin counterstain. Bar= 200µm. e) Rumino-reticular fold. IFN-γ Immunolabeling of intraepithelial lining cells in the papillar areas in close contact with the parasite. Parasite burden= 215 (324 worms recovered from the rumino-reticular fold). Envision® System. Haematoxylin counterstain. Bar=100µm. f) Reticulum. MHC II expression in dendritic cells in the organized inflammatory infiltrate of the lamina propria. Parasite burden= 908 (400 worms recovered from the reticulum). Envision® System. Haematoxylin counterstain. Bar= 50µm. g) Reticulum. Detail of f). Expression of MHC II in Langerhans cells within the lining epithelium. Envision® System. Haematoxylin counterstain. Bar= 10µm.

Figure 2. Gene expression of IFN- γ , IL-4 and IL-10 in ruminal atrium and dorsal sac of uninfected and infected cattle. Statistical significances amongst animal groups and locations are expressed as *a* ($p < 0.01$) and *b* ($p < 0.05$).

Table 1. Antibodies, specificity and immunohistochemical procedure used.

Antibody	Clone	Type	Marker for	Tissue	Antigen retrieval	Dilution	Source
CD3	-	Rabbit, Polyclonal	T cells	Paraffin embedded	Heat and pressure; citrate buffer, pH6	1:100	Dako, Denmark
CD4	CC30	Mouse, Monoclonal	T helper cells	Frozen	None	1:100	Serotec, USA
CD8	CC63	Mouse, Monoclonal	Cytotoxic T cells	Frozen	None	1:50	Serotec, USA
CD79 α cy	HM57	Mouse, Monoclonal	B cells	Paraffin embedded	Microwave; citrate buffer pH9	1:100	Dako, Denmark
CD45R	C363.16A	Mouse, Monoclonal	B cells	Paraffin embedded	Microwave; citrate buffer pH9	1:100	South Biotech, USA
IgG	-	Biotinylated antibody	Plasma cells	Paraffin embedded	Trypsin	1:200	Vector Laboratories, USA
Calprotectine	MAC387	Mouse, Monoclonal	Macrophages and activated epithelial cells	Paraffin embedded	Protease XIV	1:100	Dako, Denmark
CD68	KP1	Mouse, Monoclonal	Macrophages	Paraffin embedded	Trypsin	1:50	Dako, Denmark
CD163	EDHu-1	Mouse, Monoclonal	Macrophages	Paraffin embedded	Heat and pressure; citrate buffer, pH6	1:200	BioRad, USA
MHCII	MCA2224	Mouse, Monoclonal	Antigen-presenting Cells	Paraffin embedded	None	1:100	Serotec, USA
IFN- γ	CC330	Mouse, Monoclonal	IFN- γ	Paraffin embedded	Microwave; citrate buffer pH9	1:250	Serotec, USA