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Immunological features of LPS from *Ochrobactrum intermedium* on sheep experimentally infected with *Fasciola hepatica*

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ABSTRACT

The effects of the lipopolysaccharide (LPS) from *Ochrobactrum intermedium* in sheep with fasciolosis was reported previously, resulting in lower fecal egg counts and fluke burden. In the current study, we analyzed its immunological effects in two groups of sheep, treated (T) and controls (C). Fasciolosis induces a T helper (Th) type-2 response, characterized by IL-4 and IL-10 production; however, at the beginning of the infection, the IFN-γ production predominates (Th type-1 response). Although we did not find differences in IL-4 production or in the expression level of this gene in the hepatic lymph nodes, the expression level of IL-10 was higher ($P < 0.05$) in the T group at 4 wpi. The IFN-γ production was higher ($P < 0.01$) at 12 wpi as well as its level of expression at 4 wpi ($P < 0.05$) in the T group. We found a higher expression level of TGF-β at 4 wpi in the T group ($P < 0.05$), associated with the previous report of thicker fibrous tracks in a treated group. Immunoglobulin G1, related with a Th type-2 response, was higher ($P < 0.01$) in the T group at 4 and 12 wpi. In conclusion, the effects of LPS from *O. intermedium* could have resulted from a predominant Th type-2 immune response.

The prevalence of fasciolosis is currently rising possibly due to several factors: change in climatic conditions, man-made modifications of the environment and/or increment of anthelmintic resistance (Martínez-Valladares et al., 2013). Although the administration of flukicides is essential, there has been a reported increase in the prevalence of anthelmintic resistance not only by its widespread use, but also due to misuse (Martínez-Valladares et al., 2010). Therefore, new alternatives to anthelmintic therapy should be tested, such as vaccines and immunostimulatory products. The adjuvants are an integral part of most vaccines and can enhance their immunogenicity by stimulating the protective immunity through antibodies and T cell production (Coffman et al., 2010). Immunomodulators (IMMs) are immunostimulating and/or immunosuppressive compounds used to modify the host defense mechanisms against disease causative agents. An IMM which induces endogenous production of cytokines by monocytes and macrophages is the lipopolysaccharide (LPS) (Veremeichenko and Zdorovenko, 2008). Recently, the administration of LPS from *Ochrobactrum intermedium* in sheep infected by *Fasciola hepatica* led to a lower fecal egg count (FEC) and a reduction of the fluke burden, suggesting an increase in the nonspecific resistance to the infection (Martínez-Pérez et al., 2013). Under this context, the aim of this study was to evaluate the immunological effects of LPS from *O. intermedium* on sheep experimentally infected with *F. hepatica*.

Immunological response was analyzed from a group of 36 Merino sheep experimentally infected with 200 *F. hepatica* metacercariae on day 0 of the trial. The experimental design has been previously described by Martínez-Pérez et al. (2013). Briefly, infected animals were divided into two groups, i) the treated group (T), constituted by 18 sheep treated on day 0 and 15 post infection (pi) with 2 ml of an IMM (6 μg/ml LPS from *O. intermedium*, strain LMG3306, Laboratorios Ovejero S.A., León, Spain) and ii) the control group (C), 18 sheep treated with a placebo at the same days as the T group. Blood samples were collected from day 0 until the end of the experiment every 4 weeks. Necropsies were carried out at 4 weeks post infection (wpi), in the half of each group, and at 12 wpi, in the half left, to recover the hepatic lymph nodes (HLNs) in all sheep.

Peripheral blood mononuclear cells (PBMC) were isolated from all sheep every 4 weeks (Haçariz et al., 2009a). Cells were stimulated by duplicate with 25 μl of Concanavalin A (ConA) and/or with *F. hepatica* excreted–secreted (ES) antigen, both at 10 μg/ml, as well as with 25 μl of complete medium as control. Culture supernatants were collected after incubation at 37 °C in 5% CO_2 for 72 h. IFN-γ and IL-4 were measured with commercial kits "Bovine IFN-γ Screening Set and Bovine IL-4 Screening Set" (Thermo Fisher Scientific, UK). Results were expressed as the OD ratio between samples stimulated with ConA or *F. hepatica* ES antigen and the complete medium.

The expression level of IFN-γ, TGF-β, IL-4 and IL-10 genes was carried out in HLNs at 4 and 12 wpi. ATPase and GAPDH

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Table 1
Primers for real-time PCR amplification.

Cytokine	Accession no.	Size (bp)	Forward 5′-3′	Reverse 5′-3′
ATPase	X02813	96	TTCCTACTGCCCTGGAATG	CACGAAGATGAGAAGCGAGT
GAPDH	NM_001190390	125	ACCCAGAAGACTGTGGATGG	TTGAGCTCAGGGATGACCTT
IL-4	NM_001009313	69	GGGAGGACTTGACAGGAA	ACTCGTCTTGGCTTCATTCAC
IL-10	NM_001009327	75	CCAGGATGGTGACTCGACTAG	TGGCTCTGCTCTCCCAGAAC
IFN-γ	NM_001009803	60	CTGCAGATCCAGCGCAAA	TGGCGACAGGTCATTCATCA
TGF-β	NM_01009400	133	GAAGTCTAGCTCGCACAGCA	CACGTGCTGCTCCACTTTTA

housekeeping genes (HKG) were used as controls (Table 1). The RT-PCR and the analysis of the Ct values were carried out following the specifications of Martínez-Valladares et al. (2012).

Detection of IgG1 was carried out at 0, 4, 8 and 12 wpi. The *F. hepatica* ES antigen from adults was obtained in accordance with the specifications described by Ortiz et al. (2000). Indirect ELISA was carried out according to the protocol described by Sánchez-Andrade et al. (2000).

Data were analyzed using the statistical package SPSS for Windows and were subject to one-way analysis of variance (ANOVA) with the treatment as source of variation. A post-ANOVA analysis (Tukey's honestly significant difference test) was applied when the overall differences were determined.

The aim of the current study was to evaluate the role of LPS from *O. intermedium* on the immune response of sheep experimentally infected with *F. hepatica*. The effects of LPS from *O. intermedium* on fasciolosis in sheep were previously described by Martínez-Pérez et al. (2013). These authors reported that infected sheep treated with this IMM had lower FEC, a reduction of the fluke burden and flukes with smaller sizes at necropsy than untreated infected animals. IMMs can modify the immune system by enhancing the host immune response and inducing endogenous production of cytokines against pathogens. It is known that Th type-2 humoral-mediated immune or anti-inflammatory response (Berger, 2000) is typically described in the resistance to parasitic helminth infections (Pleasance et al., 2011; Rojo-Vázquez et al., 2012). Liver fluke infection induces a strongly polarized Th type-2 response associated with an early production of pro-inflammatory cytokines, which contributes to the overall pathophysiological pattern in the host. In this sense, IL-4 is a cytokine functionally associated with a Th type-2 response (Viallard et al., 1999) which favors at the same time the IL-10 production (Schmidt-Weber et al., 1999).

In the present study, after the stimulation with ConA, the IL-4 production was significantly higher in the C group than in the T group ($P < 0.01$) at 12 wpi (Fig. 1c). However, when PBMC were stimulated with *F. hepatica* ES antigen, the IL-4 production was similar between the two groups (Fig. 1d). In a similar way, we did not find differences between groups when the expression level of the gene was analyzed in the HLNs at 4 and 12 wpi (Fig. 1e and 1f). In relation to the IL-10, the level of expression of the gene was significantly higher ($P < 0.05$) in the T group at 4 wpi (Fig. 1e).

On the other hand, a Th type-1 cell-mediated or pro-inflammatory response (Berger, 2000) is associated with the presence of IFN-γ (Viallard et al., 1999). In fasciolosis, this immune response is present at the beginning of the infection until 3 wpi (Moreau et al., 1998). In this study, IFN-γ values were similar between groups after the *in vitro* stimulation of PBMC with ConA (Fig. 1a), although significant higher levels were shown in the T group after the stimulation with *F. hepatica* ES antigen at 12 wpi ($P < 0.01$) (Fig. 1b). Regarding the analysis of the expression level of the gene in the HLNs, IFN-γ showed higher values in the T group at 4 wpi ($P < 0.05$) (Fig. 1e). In the present study, the higher IFN-γ production in the T group after the *in vitro* stimulation of PMBC with *F. hepatica* ES antigen could be due to the induction by LPS of higher proportions of natural-killer (NK) cells expressing IFN-γ (Kanevskiy et al., 2013). Besides, both LPS and IFN-γ induce nitric oxide (NO), which promotes the cytotoxic and microbicidal activities of macrophages (Brunet, 2001). O'Neill et al. (2000) reported that the magnitude of the response of pro-inflammatory and anti-inflammatory cytokines depends on the fluke burden; the low fluke burden resulted in the study by Martínez-Pérez et al. (2013) coincides with higher IFN-γ values and might be caused by a toxic effect of NO upon the parasite because of its oxidant properties and its ability to react with iron-containing compounds (Beytut et al., 2011).

Comparing the expression levels of all cytokines, the values were higher for IL-4 than for IFN-γ at 4 and 12 wpi, supporting that a Th type-2 cytokine profile persists during fasciolosis, in accordance with Pleasance et al. (2011) and Haçariz et al. (2009a).

The immune response is also controlled by other specialized populations of T regulatory cells, Th type-3 and $CD4^+/CD25^+$ cells (Shevach, 2002). These ones regulate the immune response by several mechanisms, such as the production of IL10 and TGF-β or via cell contact (Mauri and Ehrenstein, 2008). These two cytokines play a central role in minimizing pathology and enhancing tissue repair during helminth infections acting in synergy (Lee et al., 2002). IL-4 is known to stimulate both IL-10 (Schmidt-Weber et al., 1999) and TGF-β (Kohyama et al., 2001). This fact is shown in the current study after analyzing the higher and similar expression levels of these three cytokines, especially in the T group, in comparison with the lower values in IFN-γ (Fig. 1e and 1f). It is noteworthy that TGF-β also promotes collagen production (Cutroneo, 2007), co-participating with IL-10 in the induction of fibrosis (Flynn et al., 2010). In fasciolosis, the movement of the flukes during the pre-patent period of the infection can be restricted after the inflammation of the bile duct epithelia (Behm and Sangster, 1999). This fact could have been favored by the administration of the LPS from *O. intermedium* in the study by Martínez-Pérez et al. (2013) since the treated group had smaller flukes, lower fluke burden and in liver higher hepatic damage scores characterized by fibrous tracks. The higher expression levels of IL-10 and TGF-β ($P < 0.05$) at 4 wpi (Fig. 1e) in the T group confirmed the way of action of LPS from *O. intermedium* during the pre-patent period of the infection. According to Haçariz et al. (2009a), low levels of TGF-β may cause insufficient collagen production and fibroblastic activity in the liver leading to a higher fluke burden.

Fasciolosis is also characterized by an increase in the production of IgG, especially IgG1 (Clery et al., 1996), which is associated with a Th type-2 immune response (Estes and Brown, 2002). Indeed, the IgG1 response is highly correlated with an early protective immune response in the gut mucosa against newly excysted juveniles (Van Milligen et al., 1999). Our results showed higher IgG1 levels in the T group ($P < 0.01$), principally at 8 wpi. In fact, the higher IgG1 values reported in the T group (Fig. 1g) are in agreement with an upper IgG1 title in cattle infected by *F. hepatica* (Hoyle et al., 2003) and sheep infected by *F. gigantica* (El-Ahwany et al., 2012), both treated with cysteine proteinases (CP).

Therefore, the LPS from *O. intermedium* could induce a Th type-2 immune response and could act against the parasitic forms in a similar way as adjuvants such as Freund's incomplete adjuvant (FIA), Titer MaxGold (Haçariz et al., 2009b) or CP (El-Ahwany et al., 2012) in sheep or FIA in cattle (Mulcahy et al., 1998).