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Effect of reinfestations on systemic immune responses in cattle naturally infested by *Hypoderma* sp. (Diptera: Oestridae)

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ABSTRACT

Systemic humoral and cellular immune responses were studied during natural infestations by *Hypoderma lineatum* in cattle at their first (G-1) and second exposure (G-2). Four out of seven animals in G-1 were palpation positive, with a mean intensity of 11.2 (12.81SD) warbles; the same proportion (4/7) presented warbles in G-2 but the intensity was 3.7 (2.21SD). The evolution of total IgG levels was characterized by a noticeable increment coinciding with the presence of warbles on the back, especially in G-2. The IgG1 isotype displayed a parallel evolution in both groups, with peak values prior to the appearance of first warbles. The IgG2 subclass followed an irregular pattern in both groups and IgM maintained low and constant levels throughout the study, mainly in G-1. CD4/CD8 ratios showed a predominance of $CD4^+$ throughout the infestation, principally in G-2 during the warble season. The evolution of IFN-γ in G-2 was constant, whereas in G-1 there was a gradual descent until warble emergence. The dynamics of the IL-10 differed between G-1 and G-2, although both groups showed a significant drop after the exit of the larvae that could be implicated in the termination of the inflammatory response. IL-4 and TNF-α levels did not show differences between groups. Our results suggest that the resistance mechanisms would become more apparent at the latest stages of the infestation by *Hypoderma*, supporting the hypothesis that considerable larval destruction in sensitized animals might take place after their arrival to the back.

1. Introduction

Hypoderma spp. larvae are tissue invading parasites which spend 9–10 months migrating within the host tissues where they are continuously exposed to host immune effector mechanisms (Sandeman, 1996; Otranto, 2001). To complete their development, the larvae must accomplish three stages that can be critical to their survival: (i) penetration of newly hatched first instars (L1) through the skin, (ii) migration of L1 within the host tissues, and (iii) arrival and moult into L2 and L3 in the subcutaneous tissues of the back where they are surrounded by a granulomatous host reaction, referred as a "warble".

It is well known that cattle develop acquired resistance after repeated exposures to *Hypoderma* larval antigens (Gingrich, 1980, 1982). This resistance is recognized as an important factor in controlling grub populations and depends on the host's age and the number of larvae invading the host (Baron and Weintraub, 1987); however, at present there is still little information available about the mechanisms responsible for protection against *Hypoderma* in cattle. According to Panadero et al. (2009) the proteases (hypodermins) secreted by first instars of *Hypoderma lineatum* are highly immunogenic but they also tend to modulate the host responses in favour of parasite survival, consequently, larval survival depends on the modulation of

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the immune system to avoid harmful immune responses. Some authors, like Gingrich (1980), reported that the effect of immunity occurs during the early stages of the parasite life-cycle with most larvae dying shortly after entering the host. On the contrary, Pruett and Kunz (1996) stated that host acquired resistance is expressed with larval mortality occurring predominantly late in the life-cycle.

Currently, control measures against bovine hypodermosis are mainly focused on the administration of macrocyclic lactone compounds, involving some potential environmental risks (Strong, 1993). Despite effective chemotherapy, warble flies continue infecting cattle in North America (Colwell, 2002; Quintero-Martínez et al., 2007), some countries in Europe (Papadopoulos et al., 1997; Haine et al., 2004; Panadero et al., 2007), northern Africa (Saidani et al., 2011) and Asia (Guan et al., 2005; Ahmed et al., 2012). Moreover, the apparent lack of efficacy of experimental vaccines, based on different antigenic fractions of first instars or their recombinant equivalents (Baron and Colwell, 1991; Chabaudie et al., 1991), suggests the involvement of other molecules and/or more complex mechanisms in the development of protection by the host.

The humoral immune response against this parasite is characterized by an increase in serum immunoglobulin G levels, reaching peak levels when the first grubs became detectable on the back and dropping once grubs had emerged (Sinclair and Wassall, 1983; Boulard, 1985). However, the lack of correlation between circulating antibody levels and the number of mature larvae (Pruett and Barrett, 1985) suggested that cell-mediated mechanisms of immunity are more important for resistance to infection.

Different studies (López et al., 2005; Dacal et al., 2009, 2011) have determined cellular immunity, including cytokine responses and lymphocyte subsets, during the early stages of the infestation in experimental infestations by *H. lineatum*, suggesting a Th0 response. In a recent study, Vázquez et al. (2012) have determined the dynamics of cellular and humoral responses during the course of natural first infestations by *H. lineatum* in cattle. However, there is no information describing what would happen in subsequent infestations. Therefore, clarification of host immune response in reinfested animals would provide important information to design new control strategies based on the reinforcement of natural mechanisms of host defence.

The aim of this study was to determine the effect of successive infestations on the development of humoral and cellular immune responses during the course of natural infestations by *Hypoderma*, in order to establish the mechanisms implicated in the development of resistance against *H. lineatum*.

2. Materials and methods

2.1. Animals and samplings

The trial was carried out on a beef farm (Rubia Gallega breed) with a history of hypodermosis. On this farm, located in Northwestern Spain (area endemically infested by *H. lineatum*), cattle are raised in a semiextensive grazing system, enabling natural infestations by *Hypoderma*. The herd received semestral benzimidazole treatments, but no fly control products were used during the summer months. Routine faecal examinations, using flotation and sedimentation techniques were negative. Identification of some grubs (L3) obtained in previous infestations on the farm, showed the presence of *H. lineatum*.

A total of 14 animals were selected for inclusion in this study on the basis of the presence of anti-*Hypoderma* antibodies and the parasite antigen hypodermin C (HyC), determined by an indirect (Panadero et al., 1997) and sandwich ELISA (Panadero et al., 2002), respectively. The animals were distributed in two groups according to the number of previous infestations: first exposed group (G-1), composed by seven 1-year-old heifers in their 1st grazing year, thus undergoing their first infestation by *Hypoderma*; reinfested group (G-2), integrated by 7 cows older than 2 years, which had presented warbles in the year preceding the study, so they had one previous contact with the parasite.

From May 2007 all animals were bled monthly for one year, by caudal venipuncture, covering the entire endogenous cycle of *H. lineatum* in Northwestern Spain (Panadero et al., 2007). Acid citric dextrose (ACD) blood was collected and immediately processed for flow cytometric analysis. Blood samples without additive were centrifuged 800G for 10 min and the serum was separated and frozen in aliquots at −20 °C until assayed by ELISA tests.

All larvae that survived to form warbles were counted by monthly palpation of the back of cattle. Results are expressed as the mean number of warbles per infested animal (±SD).

2.2. ELISA protocols

Antibody detection. Serum samples were processed by an indirect ELISA test described by Panadero et al. (1997) for IgG detection against the antigenic fraction HyC. Horseradish peroxidase-conjugated sheep anti-bovine-IgG1 (ShAB IgG1-HRP, 1/5000), ShAB IgG2 (1/500) and ShAB IgM (1/20,000) were purchased from Serotec (Oxford, UK). A positive and negative control serum was introduced in every plate to normalize absorbance values.

Cytokine detection. IL-4, IL-10, TNF-α and IFN-γ serum levels were determined by sandwich ELISAs that specifically detect soluble cytokine proteins according to the protocols described in Dacal et al. (2009). Standard curves to calculate cytokine concentrations (ng/ml or pg/ml) were generated using recombinant bovine IL-4, TNF-α and IFN-γ (Serotec). Because of the lack of commercial recombinant bovine IL-10, the results of this cytokine are presented as OD values.

2.3. Flow cytometric analysis of lymphocyte subsets

Peripheral blood mononuclear cells were analysed for expression of cell-surface differentiation antigens by direct immunofluorescence labelling as described in Vázquez et al. (2012). Data are expressed as a percentage of positive cells.