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Antigen-specific antibody isotypes, lymphocyte subsets and cytokine profiles in cattle naturally infested by *Hypoderma* sp. (Diptera: Oestridae)

L. Vázquez, V. Dacal, C. López, P. Díaz, P. Morrondo, P. Díez-Baños, R. Panadero*

Departamento de Patología Animal: Sanidad Animal, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27071 Lugo, Spain

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ABSTRACT

Antigen-specific antibody responses, T cell subsets and cytokine profiles were studied in 7 heifers naturally infested by *Hypoderma* sp. in Northwestern Spain. Immunoglobulin G (IgG) levels increased significantly at the end of the endogenous life cycle of the parasite (Mr). Similarly, IgG1 subclass increased considerably when first instars (L1) started their migration towards the back (Nv-Ja), whereas IgG2 increased earlier, coinciding with the arrival of L1 to the resting sites (Jn-Jl). Both subclasses decreased significantly when L3 began to leave the host. IgM levels and CD4 and CD8 profiles hardly oscillated throughout the life cycle of the parasite into the host. The CD4/CD8 ratio showed helper T cell predominance. Serum interferon-γ (IFN-γ) concentrations decreased from October to the end of the study. Interleukin 4 (IL-4) concentrations decreased in January and increased in February and May. There were a significant positive relationship between IL-4 and IgG2 subclass and a negative correlation between IFN-γ, IgG and IgG1 and also between IgM and CD2 and CD8 counts. These results suggest that in the early phases of natural primoinfestations by *Hypoderma* there is a slight predominance of a Th1 response, characterized by high IgG2 and IFN-γ levels, which is followed by a Th2 response with a clear predominance of IgG1 and IL-4.

1. Introduction

Hypoderma sp. (Diptera: Oestridae) larvae cause a subcutaneous myiasis mainly in grazing cattle. First stage larvae (L1) penetrate into the skin and migrate through connective tissues to reach the oesophageal submucosa (*H. lineatum*) or the epidural fat (*H. bovis*), where they overwinter for some months before migrating to sub-dermal tissues of the back. Second and third instar development takes place within subcutaneous granulomas (warbles). Mature third instars (L3) exit the host and pupate within a short period of time. Larvae migrating within the host and in sub-dermal warbles provoke production losses and increased susceptibility to diseases (Drummond, 1987).

Both cattle species (*Hypoderma bovis*, L. and *Hypoderma lineatum*, De Villers) coexist in Spain, but *H. lineatum* is observed much more frequently than *H. bovis*, whose populations are very scarce (Panadero et al., 1998; Reina et al., 1998).

In Northwestern Spain, two main types of husbandry are practiced; one aimed at the production of meat, using Rubia Gallega and crossbreeds, where cattle is maintained on pasture in an extensive or semi-extensive system, favoring their contact with the parasite, and other at the production of milk, using Frisians and Brown Swiss that are maintained in an intensive system.

Hypoderma sp. biology, as occurs with most of insects, is very much conditioned by weather conditions; climate

* Corresponding author. Tel.: +34 982822125; fax: +34 982822001.
E-mail address: rosario.panadero@usc.es (R. Panadero).

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directly influences the development of free stages of the parasite, flies and pupae, affecting the chronology of this myiasis and the intensity of infestation. In North-western Spain the period of fly activity of *H. lineatum*, usually starts in March–April, first instars (L1) can be found in the oesophageal submucosa from June to February and the appearance of warbles in the back (warble season) begins in January–February (Panadero, 1996; Panadero et al., 2007).

First stage larvae of *Hypoderma* penetrate intact skin aided by enzymatic secretions (hypodermins) stored in their midgut (Nelson and Weintraub, 1972) and undertake a long migration through the deep connective tissue of their host, where they are continuously exposed to immune effector mechanisms. Infestation of cattle with *Hypoderma* sp. has been shown to induce an immunological response characterized by an increase in serum immunoglobulin G levels with a peak when the first warbles become detectable on the back and drop once L3 have emerged (Sinclair and Wassall, 1983; Boulard, 1985; Pruett and Barrett, 1985; Panadero et al., 1997, 2002). However, there is not apparent correlation between antibody titres and resistance to *Hypoderma* infestation in cattle (Pruett et al., 1989; Panadero, 1996). Although detailed analyses have been performed on humoral responses involving total IgG responses to *Hypoderma* there is no information available with regard to the production of different immunoglobulin isotypes and subclasses.

Cattle develop acquired resistance after repeated exposures to larval antigens (Gingrich, 1982). This resistance is recognized as an important factor in controlling *Hypoderma* populations and depends upon the number of previous exposures and the number of larvae infesting the host (Baron and Weintraub, 1987).

Numerous studies have implicated the isotype of antibodies in the effective protection against diseases (Brown et al., 1999; Avramidis et al., 2002; Hagiwara et al., 2005; Almería et al., 2009; Piper et al., 2009). IgG subclasses in both the murine and human systems have been reported to be differentially regulated by cytokines, which act as switch and growth factors for B cells at different stages of maturation (Stavnezer, 1996). Cytokine control of antibody production in cattle may differ from the regulatory pathways, which have been described in the mouse and human (Estes, 1996). It is clear from studies on the T lymphocyte response that the Th1/Th2 paradigm, postulated by Mosmann et al. (1986) in a murine model, is less well defined in ruminants (Estes and Brown, 2002). It is also well established that Th1 and Th2 cytokines have an influence on the antibody production and isotype expression; for instance, recombinant bovine IL-4 up regulates the expression of IgM, IgG1 and IgE *in vitro* (Estes, 1996).

Cytokine responses and lymphocyte subsets during larval penetration in cattle experimentally infested by *H. lineatum* have been determined in recent studies (Dacal et al., 2009, 2011). At this early phase of the infestation there is an increase of both Th1 (IFN-γ) and Th2-type cytokines (IL-4 and IL-10) suggesting a Th0 response. However, there is no information describing the subsequent phases of larval migration, especially during their residence in the oesophageal submucosa and after their arrival at the subcutaneous tissue of the back.

The aim of this study was to study the dynamics of antigen-specific antibodies (IgM, IgG, IgG1 and IgG2), T lymphocyte subsets (CD2, CD4 and CD8) and cytokine profiles (IFN-γ and IL-4), and their possible relationships during the course of natural infestations by *Hypoderma* in cattle.

2. Materials and methods

2.1. Animals and blood samplings

The trial was carried out on a beef farm with a history of hypodermosis. Identification of third instars (L3) obtained in previous infestations on the farm, showed the presence of *H. lineatum*. On this farm, located in A Coruña (Northwestern Spain), cows are raised in a semiextensive grazing system, allowing natural infestations by *Hypoderma* sp., whereas calves are maintained indoors until weaning (8–11 months of age) them they are destined for the slaughterhouse. They fed milk from their mothers and concentrates are gradually introduced in their diet. The herd received benzimidazole treatments twice a year, but drugs for lice or *Hypoderma* control were not used routinely and no fly control product was used during the summer months. Routine faecal examinations, using flotation and sedimentation techniques were negative to parasitic forms.

Seven 1-year-old Rubia Gallega heifers were selected for this study on the basis of the presence of both anti-*Hypoderma* antibodies and the parasite antigen hypodermin C (HyC), as determined by an indirect (Panadero et al., 1997) and sandwich ELISA (Panadero et al., 2002), respectively. Those animals were in their 1st grazing year, thus it was considered that they were undergoing their first infestation by *Hypoderma*. All the animals were bled monthly (May 2007–May 2008) by caudal venipuncture, covering the entire endogenous cycle of *H. lineatum* in our region (Panadero, 1996; Panadero et al., 2007). Warbles were counted by manual palpation at monthly intervals. Results are expressed as the mean number of warbles per infested animal.

Infested animals were treated subcutaneously with ivermectin (Ivertin® injectable, Laboratorios Calier, Spain) at the recommended dose (0.2 mg/kg p.v.) 2 months before the end of the study (March 2008).

An uninfested control group, comprised by four Rubia Gallega calves, was also bled at monthly intervals. Control calves were born at the farm and were maintained indoors, avoiding their contact with the parasite. They were 3-month-old at the beginning of the study and they remained at the farm until weaning (11 months of age). In consequence, control calves were only available for the first 8 months of the trial and their results are expressed as mean (SD) of the individual values obtained from May to December 2007.

2.2. ELISA protocols

2.2.1. Antibody detection

Serum samples were processed by an indirect ELISA test described by Panadero et al. (1997) for IgG detection against the antigenic fraction hipodermin C (HyC).