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Histological and immunohistochemical characterization of *Hypoderma lineatum* (Diptera: oestridae) warbles

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ABSTRACT

Hypoderma larvae are tissue invading parasites which spend several months migrating within the host tissues before completing their development in the sub-dermal tissues of the back. Subcutaneous stages of the parasite produce an inflammatory reaction in the skin called "warbles", as well as holes through which larvae breathe. In order to elucidate the microscopical structure of the warbles, three hides from warbled cows were collected in a slaughterhouse in Lugo (NW, Spain) between March and May 2012. A total of 60 skin samples, including warbles at different phases of development, were chosen for histopathological and immunohistochemical examination. Microscopic lesions were classified into three groups, according to the predominance and distribution of different cell populations. In warbles containing living or recently dead larvae with apparently well preserved cuticle (type 1), plasma cells were observed in high number. However, macrophages and lymphocytes were the predominant cells in granulomas (type 2) formed in relation to remnants of the dead parasite, containing or not remains of the altered cuticle. Scars (type 3) were characterized by granulation tissue. Immunohistochemistry showed that B lymphocytes and IgG^+ cells were predominant in the lesions, as long as the cuticle of the larvae is intact. On the other side, $CD3^+$ lymphocytes increased once cuticle is destroyed and a granuloma is formed. Macrophages, revealed by $CD68^+$, $MAC387^+$ and lysozyme immunolabelling, were detected in all types of lesions, but they were more abundant in type 2 and scarce in scars. These cells appeared isolated around the intact larvae or forming aggregates around its remains in the granuloma. Moreover, a strong immunolabelling against MAC387 antibody was registered in the squamous epithelium covering the breathing pore. This finding may be associated with the expression of calprotectin, a molecule involved on the healing process of the skin after larvae outcome. Our results suggest the predominance of a humoral response inside the warble as long as larvae are intact. Once they are destroyed, cellular response occurred, isolating and destroying the remains of the larvae until healing process completes and scars with low numbers of inflammatory cells appear.

1. Introduction

Hypoderma larvae are obligate parasites that migrate and feed for several months in host's tissues. Newly hatched larvae penetrate unbroken bovine skin thanks to enzymatic secretions produced by their salivary glands (Boulard, 1970). Once the penetration is complete, the larva begins migration in subcutaneous tissue (Nelson and Weintraub, 1972), which leads to the oesophageal submucosa for *Hypoderma lineatum* and epidural fat for *Hypoderma bovis*. After that, larvae continue their migration towards subcutaneous tissue in the back, where they turn to second and third instar larvae successively. These larvae are aerobic and breathe through a hole bored in the skin (Hadwen and Bruce, 1916).

After first instar molt, the expulsion of its content triggers an inflammatory response to encapsulate second and third instar larvae with connective tissue (Berkemkamp and Drummond, 1990),

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Table 1
Primary antibodies used in immunohistochemistry.

Specificity	Type or clone	Origin	Pretreatment	Dilution	Source
Myeloid/Histiocyte antigen (calprotectin) (Macrophages)	MAC 387	Mouse	Trypsine	1:75	Dako
CD68 (Macrophages)	PG-M1	Mouse	Trypsine	1:100	Dako
Lysozime	Policlonal	Rabbit	Trypsine	1:1000	Dako
CD79 (B cells)	JCB117	Mouse	Microwave	1:25	Dako
CD3	Policlonal	Rabbit	Steamer	1:200	Sigma
IgG Lambda Light Chains	Policlonal	Rabbit	Triton X-100	1:6000	Dako

forming the characteristic swellings or subcutaneous furuncles called "warbles" (Beesley, 1974). According to Boulard (1975), fibroblasts are very active and produce lots of collagen fibers to isolate larvae, which stay in stable environment. Mature third instars 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).

Larvae are continuously exposed to host immune effector mechanisms trying to isolate and kill them. Gingrich (1980) reported that immunity mechanisms are especially active in early phases of larvae development, in which larvae mortality is higher. Nevertheless, Pruett and Kunz (1996) pointed out that larvae destruction is very intense in the last stages of development.

While lesions caused by first instar larvae during penetration and migration in the host are well described (López et al., 2005; Dacal et al., 2009, 2011), not detailed descriptions on the different pathological responses associated with the presence of the larvae in the subcutaneous tissue have been carried out. Moreover, little information is also available on the cellular reactions responsible for larval isolation or destruction at sub-dermal sites.

The aim of this study was to define and classify the microscopic lesions and to characterize, by using histopathology and immunohistochemistry, the cells that participate in the cutaneous reaction against subcutaneous larvae, including the response of macrophages, T cells, B cells and immunoglobulin G-producing plasma cells, during the stay of second or third instar *H. lineatum* in the back of the cattle.

2. Material and methods

2.1. Animal samples

The hides from three intensely warbled cows were collected in a local slaughterhouse in Lugo (NW, Spain). The outer side of the hides was examined to confirm the absence of other relevant lesions (mange, ticks, lice, etc.) and the inner side was inspected recording warbles at different stages of development. The first hide (H1) was collected in March 2012 and presented 16 viable warbles and 13 non-viable warbles; the second hide (H2) was collected in April 2012 and exhibited 4 viable warbles and 38 non-viable warbles; the last hide (H3) was obtained in May and presented 19 non-viable warbles.

A total of 45 skin samples (15 from H1, 31 from H2 and 9 from H3), including warbles containing living ($n = 10$), dead larvae ($n = 20$) and scars ($n = 15$) were taken for histopathological and immunohistochemical (IHC) examination. Samples were fixed in 10% formaldehyde and embedded in paraffin wax using standard protocols.

2.2. Histopathological and immunohistochemical examination

For histopathological examination samples were processed by routine methods and sections, 4 μm thick, were stained with haematoxylin and eosin (HE).

Table 2
Evaluation of immunolabelled cell population numbers in the three histological lesions described.

	$CD3^+$	$CD79^+$	$CD68^+$	$MAC387^+$	Lysozyme	IgG^+
1	+	+++	+	++	++	++
2	++/+++	+	+++	+++	++	0
3	+	+	+	+	+	0

0: nil; +:mild; ++:moderate; +++:intense.

For IHC studies skin sections, 4 μm thick, were placed onto poly-L-Lysine coated slides. Endogenous peroxidase activity was blocked in deparaffinised sections by incubation with 3% hydrogen peroxide for 30 min in darkness at room temperature (RT). Rehydrated slides were rinsed twice in PBS pH 7.4. To optimize the immunoreaction, the antigen retrieval was performed using enzymatic or heat-based methods, depending on the primary antibody (Table 1).

Sections were incubated with the primary antibodies diluted in PBS overnight at 4 °C in a humidified chamber. After extensive washing with PBS, sections were incubated for 40 min at RT with EnVision®+/HRP solution (Dako North America Inc., Carpinteria, USA) for the appropriate monoclonal or polyclonal antibodies. After washing in PBS, antibody localization was determined using 3,3-diaminobenzidine (DAB, Sigma–Aldrich Corp.) as chromogenic substrate for peroxidase. Sections were counterstained with haematoxylin for 30 s.

Evaluation of immunostaining was performed under a light microscope with final magnification of 500×. Labelled cells taking part into the lesion were examined and a qualitative classification of the number of positively immunostained cells, from nil (0) to intense (+++), was established (Table 2).

3. Results

3.1. Gross lesions

Macroscopically, 4 mm diameter breathing holes ($n = 20$) and 2 mm diameter scars ($n = 70$) (Fig. 1a) were visible in the outer side of the skin. In the inner side, warbles at different stages of development were observed in the hides examined: viable warbles ($n = 20$) were composed of a layer of fibrous connective tissue that creates a cavity containing the larvae and necrotic material (Fig. 1b); in non-viable warbles ($n = 40$), larvae seemed to be dark, crushed and dehydrated (Fig. 1c). The remaining 30 samples showed healing tissue without visible larvae remains.

3.2. Histopathology

In all the examined samples, the superficial dermis showed a mild to moderate perivascular inflammatory infiltrate, composed mainly of lymphocytes, plasma cells and occasional polymorphonuclear eosinophils.

In the deep dermis lesions were variable and were classified into three different groups, according to the presence and stage of preservation of the larvae, the characteristics of the inflammatory infiltrate and the predominant cells present in the lesions: