Veterinary Parasitology 200 (2014) 104–110

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Perch liver reaction to *Triaenophorus nodulosus* plerocercoids with an emphasis on piscidins 3, 4 and proliferative cell nuclear antigen (PCNA) expression

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ARTICLE INFO

Article history:
Received 4 September 2013
Received in revised form
19 November 2013
Accepted 21 November 2013

Keywords:
Fish
Cestode larvae
Immunohistochemistry
Antimicrobial peptides
Cell proliferation
Mast cells

ABSTRACT

Histopathological lesions caused by plerocercoids of *Triaenophorus nodulosus* within the liver of perch, *Perca fluviatilis*, from Lake Trasimeno were studied. Livers harbored 1–3 parasite larvae and pathological alterations were more marked in those with 3 plerocercoids. In the liver, larvae were encysted, surrounded by a capsule of host tissue; two of 14 plerocercoids were necrotic. In infected livers, some hepatocytes showed degenerative changes, i.e. swelling and hydropic degeneration, notably those in close proximity to larvae. By comparison, hepatocytes in uninfected livers or in regions away from the point of infection appeared normal. The occurrence of macrophage aggregates (MAs) distributed among the mast cells (MCs) was observed around the encysted larvae. The cellular elements involved in the immune response within liver were assessed by immunohistochemical techniques and by the use of antibodies against the antimicrobial peptides piscidins 3 and 4, which revealed a sub-population of positive MCs. In infected livers, numerous MCs that were immunopositive to P4 and a few that were positive to P3 were found around *T. nodulosus* larvae. Histological sections of both uninfected and infected liver were immunostained with proliferative cell nuclear antigen (PCNA) antibody. Within the capsule and in close proximity to the parasite larvae, various cell types (i.e., MCs, fibroblasts and epithelioid cells) and a significantly higher number of PCNA-positive hepatocytes that were immunoreactive to PCNA were found compared to uninfected livers (ANOVA, $P < 0.05$). No parasites of any type were found in gill, spleen, kidney or gonad of *P. fluviatilis* and the intestine of 3 perch were infected with few specimens of *Acanthocephalus lucii*.

1. Introduction

The fish tapeworm *Triaenophorus nodulosus* has a worldwide distribution that is closely correlated with the distribution of its definitive host, the pike, *Esox lucius*. This parasite is currently recorded in almost all waters of Europe where pike normally occurs. *T. nodulosus* inhabits the intestine of pike, uses copepods as first intermediate hosts, and a broad range of fish species (>70 spp.) as second intermediate hosts (e.g., European perch, *Perca fluviatilis*, see Kuperman, 1973; Kuchta et al., 2007). In European perch, plerocercoids are localized in the liver but are occasionally seen in other organs such as spleen, gonad, kidney and musculature (Kuperman, 1973; Brinker and Hamers, 2007). Controversial data exists on the effects of *T. nodulosus* plerocercoids on European perch growth and condition

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http://dx.doi.org/10.1016/j.vetpar.2013.11.023

(Hoffmann et al., 1986; Brinker and Hamers, 2007). In European perch, the liver is the main site of infection; this organ is a nutrient-rich environment, an important reticulo-endothelial tissue, and metabolizes many drugs and other compounds (Hrckova et al., 2010). In mammals, such as mice, a fibrous capsule serves as a mechanical barrier to migration of liver-residing cestodes to other organs (Hrckova et al., 2010).

In European perch liver, *T. nodulosus* plerocercoids become encapsulated by a host tissue response (Brinker and Hamers, 2007), the walls of the capsule consisting of thick connective tissue and epithelioid cells (Hoffmann et al., 1986). In response to infection, a variety of cells become activated and cooperate in an effort to control and eliminate the invading pathogens (Makepeace et al., 2012). In fish, the innate defences responding to helminth infection commonly involve eosinophilic granular cells (Secombes and Chappell, 1996; Reite and Evensen, 2006; Buchmann, 2012; Dezfuli et al., 2012b, 2013) also named mast cells (MCs) (Ellis, 1985; Reite, 1997) and macrophage aggregates (MAs) or melanomacrophage centers (Agius and Roberts, 2003). A key factor of the piscine immune system is a group of antimicrobial peptides (AMPs) named piscidins, having potent, broad-spectrum antimicrobial activity against viruses, bacteria, fungi, water molds and parasites (Silphaduang and Noga, 2001; Park et al., 2011; Salger et al., 2011; Zahran et al., 2012).

Little is known about the immune response of fish to larval cestodes and no record exists on expression of proliferating cell nuclear antigen (PCNA) in liver of fish infected with a helminth. Alterations in the expression of PCNA have recently been applied to the fields of fish parasitology (Dezfuli et al., 2012a) and to fish health (Blas-Machado et al., 2000; Kong et al., 2008; Chikwati et al., 2013). Indeed, only few papers have been published on piscidins in fish-metazoan systems (see Dezfuli et al., 2010a, 2011a). Thus, the current study represents the first record of piscidins (P3, P4) and PCNA-positive cells within the liver of fish infected with a tapeworm. Emphasis will be placed on the role of MCs as an important component of the host's innate immune system.

2. Materials and methods

In July 2012, a total of 54 European perch, *P. fluviatilis* (19.74 ± 2.68 cm, mean total length $\pm$ standard deviation, SD; 107.94 ± 30.45 g, mean weight $\pm$ SD), were processed from Lake Trasimeno (Province of Perugia, Central Italy). The fish were caught by gill net that was deployed on one occasion by professional fishermen belonging to a local fishing consortium. Immediately upon landing, the fish were transferred alive to the consortium's facility where they were euthanized using an overdose of 125 mg L^{-1} MS222 (tricaine methanesulfonate, Sandoz, Basel, Switzerland). Thereafter, the spinal cord was severed and the fish measured and weighed. Upon post mortem, the fish were sexed before the digestive tract and other organs were removed in search of helminths. Pieces of infected liver (15 mm $\times$ 15 mm) were fixed in Bouin's fluid for 10 h and thereafter, were rinsed in several changes of 4 °C 70% ethanol before being stored in the same medium until they were processed for histology. The fixed tissues were dehydrated through an alcohol series and then paraffin wax embedded using a Shandon Citadel 2000 Tissue Processor. After blocking out, 5 μm thick sections were taken from each tissue block, stained with haematoxylin and eosin (H&E) and/or alcian blue 8 GX pH 2.5 combined with periodic acid Schiff's reagent (AB/PAS).

For tapeworm species and larval stage identification purposes, some live larvae were fixed in hot 10% formalin, examined *in vitro*, and identified on the basis of relevant literature (Kuchta et al., 2007).

Some histological sections were subjected to an indirect immunohistochemical method (peroxidase–anti-peroxidase immunocomplex) using anti-piscidin 3 (anti-HAGR) and anti-piscidin 4 (anti-5.3-02-3A) antibodies. The two primary antibodies against piscidins were produced by a commercial laboratory (Bethyl Laboratories, Montgomery, Texas, USA) using the company's standard procedures which are detailed in Dezfuli et al. (2010a) and Corrales et al. (2010). Briefly, sections (5 μm) were de-paraffinised in xylene, rehydrated through a graded alcohol series, then endogenous peroxidase activity and non-specific staining were blocked in 3% H_2O_2 for 10 min and then in normal goat serum (1:20, Elite Rabbit IgG Vectastain ABC Kit, Vector, Burlingame, USA) for 30 min. After incubation with the primary antibodies (anti-HAGR diluted 1:400 and anti-5.3-02-3A 1:8000) for 3 h at room temperature (RT), the sections were incubated for 30 min with a biotinylated goat anti-rabbit serum (Elite Rabbit IgG Vectastain ABC Kit, Vector), and then for 30 min with avidin-conjugated horseradish peroxidase (Elite Rabbit IgG Vectastain ABC Kit, Vector). The sections were then developed using DAB (3,30-diaminobenzidine 0.04% w/v in TBS 0.05 M, pH 7.4) and H_2O_2 (0.005%), rinsed and then counterstained with alcian blue and Harris's haematoxylin. Non-immune serum and diluent-only sections were used as negative controls. The positive control tissue was hybrid striped bass (*Morone saxatilis* $\times$ *M. chrysops*) intestine. The specificity of the reaction was confirmed by pre-absorption of each antiserum with the corresponding antigen.

Additional sections were subjected to the IHC method using a commercially available anti-PCNA antibody (PC10 sc-56 mouse monoclonal antibody, Santa Cruz Biotechnology, Inc.). After dewaxing in xylene and rehydrating through a graded alcohol series, the sections were treated for antigen retrieval in a citrate buffer (pH 8.0) for 20 min in a steam bath at 95 °C; thereafter, the slides were left for 10 min to cool to RT. Endogenous peroxidase activity and non-specific staining were blocked, respectively, in 3% H_2O_2 for 10 min and then in horse normal serum (1:20, Elite Mouse IgG Vectastain ABC Kit, Vector, Burlingame, USA) for 30 min. Sections were then incubated with the primary antibody (anti-PCNA diluted 1:500) for 2 h at RT. After washing with PBS, the slides were incubated for 30 min with biotinylated horse anti-mouse serum (Mouse IgG Vectastain ABC Kit, Vector) followed by avidin-conjugated horseradish peroxidase (Mouse IgG Vectastain ABC Kit, Vector). The enzyme activity was detected using DAB. Non-immune mouse serum and diluent-only sections were used as negative controls. The sections were then dehydrated, counterstained with alcian blue and Harris's haematoxylin.