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The dynamics of neutrophils in zebrafish (*Danio rerio*) during infection with the parasite *Ichthyophthirius multifiliis*

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ABSTRACT

Ichthyophthirius multifiliis is a ciliated protozoan parasite infecting the skin and gills of freshwater fish. Neutrophils are attracted to the infection sites, as a part of the innate immune response. In this study a transgenic line of zebrafish (Tg(MPO:GFP)^{i114}) with GFP-tagged neutrophils was infected with *I. multifiliis* and the neutrophil influx in the caudal fin was quantified. Twenty-four hours post infection (pi) the neutrophil count had gone up with an average of 3.4 fold. Forty-eight h pi the neutrophil count had dropped 12% and 72 h pi it had dropped to 21% compared to 24 h pi. At 72 h pi the neutrophil count was 2.7 times higher than prior to infection. A few dead parasites were observed, which were disintegrated and covered internally and externally with neutrophils. Live parasites, both surrounded by neutrophils and with no neutrophils in the near vicinity, were found during the infection. Neutrophils interacted directly with the parasites with pseudopod formation projecting towards the pathogen. These results indicate a strong innate immune response immediately following infection and/or a subsequent immune evasion by the parasite.

1. Background

Ichthyophthirius multifiliis is a ciliated protozoan parasite infecting freshwater fish worldwide. It infects mainly skin and gills and to a lesser extend the nasal and the buccal cavity [1]. It penetrates through the epidermal layer and is subsequently able to move around underneath this layer. Its host specificity is low and it has an obligate life cycle with 4 defined life stages: the infective theront, the feeding trophont, a free living tomont/tomocyst and the daughter cells called tomites [2].

Research on immune reactions against *I. multifiliis* has primarily been conducted on carp [3–8], rainbow trout [9–13] and channel catfish [14–16] due to the fact that this parasite has enormous economically consequences for the fish food industry. The ornamental fish industry also experiences massive economical losses due to this disease, called white spot disease or ichthyophthiriasis. Fish are able to acquire immunity against the disease and antibodies are believed to play a major role in this protection [3,13,14,17]. However, no vaccine is available and treatment includes harmful and carcinogenic chemicals [18]. Immune reactions against the parasite have been investigated for more than 50 years [19] however, importance of different immune factors are still relatively unknown.

Ichthyophthiriasis causes severe tissue damage, proliferation, histolysis and local infiltration of leucocytes at the infection sites [1]. The role of the leucocytes has not been fully elucidated, but it has been observed that the cells do not damage the parasites and mainly ingest debris from the destruction caused by the parasite [4,20]. Following penetration by theronts, the parasites develop into trophonts, which have constant circulation inside an intra-epithelial space [21,22].

Neutrophils are the most abundant cells of the innate immune system and react fast towards infection or damage. During an inflammatory response, which in teleosts are predominated by neutrophils [23], the neutrophils are delivered quickly to the site of damage/infection through vasodilation and vascular permeability [24]. Neutrophils play a critical role in the initial defence against pathogens through phagocytosis, secretion of granule proteins and other antimicrobials, productions of reactive oxygen species (ROS) and release of neutrophil extracellular traps (NETs) [25]. Furthermore, neutrophils mediate the inflammatory response by recruiting and activating other immune cells. During infections with *I. multifiliis* in carp, it has been observed that neutrophils arrived at the site of infection within one day and that these cells surrounded the parasites 2–3 days post infection in the skin [4].

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Only a few studies have been conducted with *I. multifiliis* infections and immunology in zebrafish [26,27]. Transgenic zebrafish present a novel method of elucidating immune responses against the parasite. With specialised lines it becomes possible to follow the dynamics of specific cell populations during an *I. multifiliis* infection or an adaptive immune response of the host during a secondary infection.

In this study an elucidation of the dynamics and number of neutrophils in the caudal fin of zebrafish during a primary *I. multifiliis* infection was investigated. A transgenic zebrafish line (Tg(MPO:GFP)^{i114}) [28] was used with GFP-tagged neutrophils.

2. Materials and methods

2.1. Ethics

All experiments were conducted according to a permission obtained by the Animal Experiments Inspectorate under the Danish Ministry of Environment and Food (License number 2016-15-0201-00902).

2.2. Fish

Tg(MPO:GFP)^{i114} zebrafish were reared in a recirculated system (Aquaschwarz, Germany) at 27 °C with a pH of 7.4 and conductivity at 550 μS. Ten percent of the water was changed every day and the fish were fed with live Artemia and pelleted dry feed (ZM Fish Food, England) one to three times per day. Adults of 3.5 months were used for this study. They were acclimatised to room temperature for two days before the experiment started.

2.3. Parasite population

The parasite was obtained from infected guppies in a pet shop. The parasite species verification was conducted morphologically using a stereomicroscope identifying the ciliated parasites with the unique horseshoe-shaped nucleus [29]. The infected fish were euthanized in an overdose of tricaine methanesulphonate (MS222) (Sigma, Denmark) and left for 4 h in clean fish facility water at room temperature, for the parasites to exit the fish. The dead fish were removed and the parasites were left overnight for further development into the infective stage, the theronts. The next day the formation of theronts was verified using a dissection microscope.

2.4. Infection of zebrafish

Theronts were counted using a dissection microscope. Two groups of 4 fish per group were held in one L of facility water at room temperature. The fish were infected by adding 100 mL water with 60 theronts/mL to each tank in order to infect the group of fish with 1500 theronts/fish. Two groups of 4 fish per group were sham infected by adding 100 mL of facility water without theronts to each tank. Twenty-four h post infection the fish received a complete water change.

2.5. Sampling points

At 24 h post infection (pi), 48 h pi and 72 h pi pictures were taken of the caudal fin of every fish using 3 different light settings on a dissection microscope (Leica MZ FLII). One setting included only background light, which is the best setting to perceive the parasites. Another setting included illumination by light with a wavelength of 488 nm in order to visualise the GFP-tagged neutrophils and the third setting included both normal light and GFP filter settings. During this procedure fish were anaesthetised in 100 mg/L MS222. Immediately, following the last sample point fish were euthanized in MS222 (500 mg/L).

2.6. Counting neutrophils

Neutrophils were counted in the dorsal posterior quadrant of the caudal fin from the centre of the fork (Fig. 1A). The program ImageJ was used for counting the cells. The pictures were prepared for counting by subtracting background (50 pixels), inverting and converting the images to 8-bit. A plugin in ImageJ was used called ITCN (Image-based Tool for Counting Nuclei, Centre for Bio-Image Informatics, University of California (version 1.6)) [30,31]. This is an algorithm based on a three-parametric model of cells: 1) cell diameter 2) minimal cell distance and 3) filter threshold. A procedure was used to set up ITCN algorithm's parameters to obtain the most accurate number of cells possible. The cell diameter was measured to 8 pixels and the recommended minimal cell distance was 4 pixels, which was used in this setup. The filter threshold had to be regulated from picture to picture to obtain the accurate cell

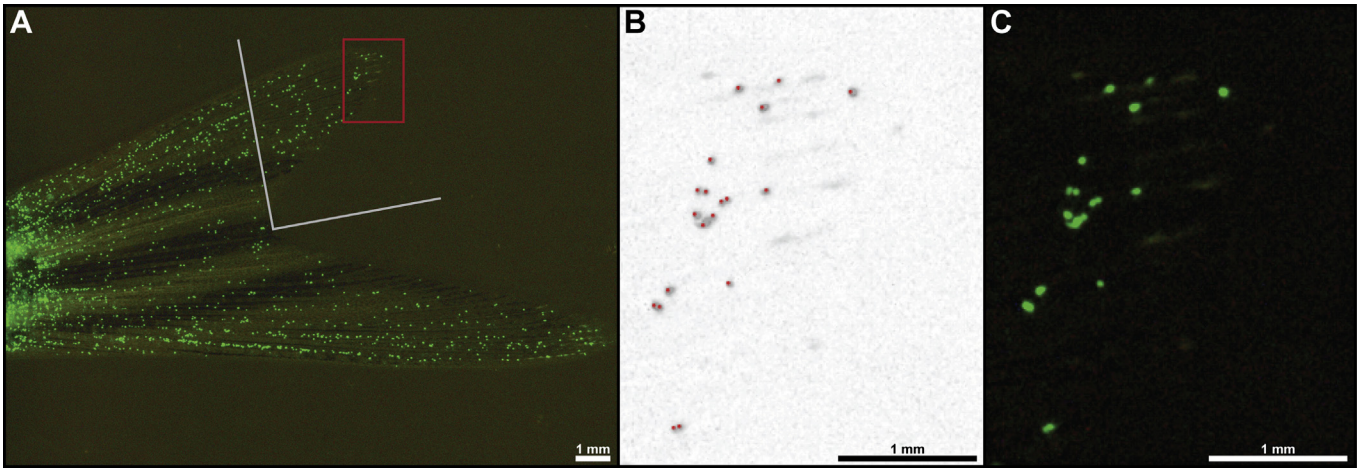

Fig. 1. Illustration of neutrophil counting methods on the caudal fin of zebrafish. The dorsal posterior quadrant of the caudal fin from the centre of the fork of a transgenic zebrafish (Tg(MPO:GFP)^{i114}) was used for the neutrophil count in this study (A, white bars). A red quadrant marks the area, which is used to demonstrate the counting technique (A) conducted by a computer program. The plugin ITCN in ImageJ counted nuclei and red dots mark counted cells (B). To verify the counting conducted by ITCN, background has been subtracted from A and the resulting image (C) was compared to B.