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Short communication

The effect of β-glucan on formation and functionality of neutrophil extracellular traps in carp (*Cyprinus carpio* L.)

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ABSTRACT

The formation of neutrophil extracellular traps (NETs) has been characterised as a novel antimicrobial host defence strategy of neutrophils besides phagocytosis and degranulation, which may lead to entrapment and subsequent immobilisation and/or killing of bacterial pathogens. Here we studied the effect of the feed additive β-glucan, namely MacroGard®, on the formation and functionality of NETs in carp. Therefore, common carp (*Cyprinus carpio*) head kidney and kidney cells were isolated and treated with or without β-glucan over time. The formation of NETs was analysed by immunofluorescence microscopy and revealed a distinct increase of NET-formation with β-glucan. Furthermore the subsequent entrapment of *Aeromonas hydrophila*, an important fish pathogen, was increased after stimulating the cells with β-glucan. However, β-glucan did not lead to a stimulation of antimicrobial activity of neutrophils against *A. hydrophila*. In conclusion, the data underline the fact that the feed additive β-glucan is able to modulate carp neutrophil functions.

1. Introduction

Innate immune cells such as neutrophils are an important component of the carp (*Cyprinus carpio*) innate immune defence against a range of invading pathogens (Scharsack et al., 2003). They are mainly produced in the head kidney, then migrate and mature in the kidney, where they are exposed to antigens (Zapata et al., 2006). Neutrophils mediate their antimicrobial activities by intracellular uptake and killing (phagocytosis), degranulation of antimicrobial substances and/or release of neutrophil extracellular traps (NETs). NET-formation has recently been characterised as a strategy to entrap and subsequently immobilise and/or kill microbial pathogens in fish and mammals (von Köckritz-Blickwede and Nizet, 2009). They consist of nuclear DNA fibres linked to antimicrobial peptides and stabilising proteins such as histones, and are released upon stimulation by proinflammatory signals or pathogens themselves (Remijsen et al., 2011). Interestingly the feed additive β-glucan has been shown to induce NET formation in zebrafish kidney cells (Palic et al., 2007). However, until now, the functionality of NETs in fish remains to be investigated and only little is known about β-glucan-dependent NET-formation in carp (Brogden et al., 2012).

Our own previous work has already shown that the feed additive β-glucan is able to stabilise the formation of NETs against degradation by *Aeromonas hydrophila*. *A. hydrophila* is a ubiquitous Gram-negative bacterium associated with mass mortalities in carp aquaculture (Hazen et al., 1978). Recently, we demonstrated that *A. hydrophila* is able to degrade NETs via nuclease activity (Brogden et al., 2012) and that β-glucan is able to stabilise NETs against this nuclease degradation (Brogden et al., 2012). However it was still unclear if β-glucan is able to induce the formation of NETs and subsequently increase entrapment and/or killing of bacteria in carp. Thus, here we investigated the effect of β-glucan on NET formation and functionality of NETs against *A. hydrophila* derived from carp kidney and head kidney cells.

2. Materials and methods

2.1. Isolation of neutrophils from head kidney and kidney of carp

For all experiments, head kidneys and kidneys were isolated from a minimum of $n = 5$ common carp (*Cyprinus carpio*), which were kept at 22 °C (±1 °C) at the University of Veterinary Medicine Hannover, Germany. All experiments have been approved by the

Abbreviations: NETs, neutrophil extracellular traps.

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respective Committee on the Use and Care of Animals and have been performed using internationally accepted veterinary standards and federal guidelines. The fish were euthanized by narcosis with MS-222 (Tricaine Methane Sulphonate Powder, Pharmaq, United Kingdom) and exsanguination. Isolated head kidneys and kidneys were kept in RPMI (PAA, Germany) on ice. The tissues were processed by being passed through a 100 μm cell strainer, centrifuged at 250*g* for 15 min at 4 °C and resuspended and kept in unsupplemented RPMI without phenol red. The viability of the cells was tested using trypan blue exclusion and confirmed more than 95% to be viable. Flow cytometry using an Epics XL (Beckman Coulter, USA) was used to quantify the percentage of neutrophils in each individual tissue based on granularity and cell size and revealed an average of 42.66 ± 3.00% and 41.20 ± 0.67% of cells to be neutrophils in the head kidney and kidney, respectively.

2.2. Visualisation of NETs

For immunofluorescent microscopic visualisation of NETs, 2×10^6 of the total cells were seeded on poly-L-lysine coated glass slides in a 24 well-plate. To allow attachment of cells to glass slides, the cells were incubated for 30 min at 26 °C. Then, the cells were incubated in the presence or absence of β-glucan (MacroGard©, Biorigin) at indicated concentrations. For immunostaining, cells were fixed with 4% PFA and kept at 4 °C. Immunostaining of NETs was done as previously described (Berends et al., 2010). Briefly, fixed and washed cells were treated with mouse anti H2A-H2B-DNA complex antibody (Losman et al., 1992) at a concentration of 0.95 mg/ml overnight at 4 °C followed by an Alexa 488-conjugated secondary goat anti-mouse immunoglobulin antibody (1:500-diluted; Invitrogen, Germany) for 45 min at room temperature. After washing, samples were embedded in DAPI-Prolong-Gold (Invitrogen, Germany) to stain all nuclei blue. Microscopic evaluation of NETs was done using a Leica DMI6000CS confocal microscope with a HCXPLAPO 40× 0,75–1,25 oil objective oil objective. The gain settings used remained constant throughout each individual experiment. A minimum of 5 random images were taken from each sample (individual fish) and the number of neutrophils showing histone protrusions were counted. The number of positive NET cells was then adjusted to the percentage of neutrophils in each tissue, which was determined by flow cytometry, to standardise differences between individual fish and tissues.

2.3. Entrapment assay

A. hydrophila subsp. *Hydrophila* (Chester 1901; DSMZ No. 30187) was grown in LB medium at 26 °C shaking at 150 rpm and was harvested during the log-phase of growth (at an optical density at 600 nm of 0.5) by centrifugation at 3000*g* for 10 min. The supernatant was removed and the bacteria were washed with PBS. The bacteria were centrifuged again and resuspended in 5 ml PBS and adjusted to an OD_{600} = 0.5. After this, 15 ml of the bacterial solution was labelled with 0.33 mg/ml of FITC (Invitrogen) and incubated for 30 min on ice in the dark. After incubation the bacteria were centrifuged again at 3000*g* for 10 min. After removing the supernatant and washing with PBS, the bacteria were centrifuged again and finally resuspended in RPMI without phenol red.

For the entrapment assay, the cells of the head kidney and kidney of the fish were isolated as described above. 4×10^5/ml cell in 100 μl were seeded into a 96 well plate and incubated for 30 min in the presence or absence of 200 μg/ml β-glucan at room temperature. Then, FITC-labelled bacteria were added to the cells at a multiplicity of infection of 20 or 200. The cells and bacteria were incubated for 30 min in the dark after centrifugation once at 370*g* for 5 min. After this, the wells were washed twice with RPMI and centrifuged again to remove unbound bacteria. Finally, entrapment of FITC-labelled bacteria was measured using green fluorescence at 485/538 nm (exc./em.). Percentage of entrapment was calculated compared to total fluorescence of total bacteria per well (incubated under the same conditions but in the absence of cells). Furthermore, extracellular fluorescence was quenched via the addition of Trypan blue at a final concentration of 0.08%. Control wells of bacteria in RPMI alone revealed an average quenching efficiency of 96.4%. Each sample was run in triplicate to exclude technical artifacts.

2.4. Visualisation of bacterial entrapment

The kidney and head kidney cells were isolated as described above. 2×10^6 of the total cells were seeded on poly-L-lysine coated glass slides in a 24 well-plate. To allow attachment of cells to glass slides, the cells were incubated for 30 min at 26 °C. Then, the cells were incubated in the presence or absence of 200 μg/ml β-glucan (MacroGard©, Biorigin) and subsequently infected with FITC-labelled *A. hydrophila* at a multiplicity of infection of 50, 100 or 200 bacteria per cell for 30 min. For immunostaining, cells were fixed with 4% PFA and kept at 4 °C. Immunostaining of NETs was done as previously described (Chow et al., 2010). Briefly, fixed and washed cells were treated with mouse anti H2A–H2B–DNA complex antibody (Losman et al., 1992) at a concentration of 0.95 mg/ml overnight at 4 °C followed by an Alexa 633-conjugated secondary goat anti-mouse immunoglobulin antibody (1:500-diluted; Invitrogen, Germany) for 45 min at room temperature. After washing, samples were embedded in Dapi-Prolong-Gold (Invitrogen, Germany) to stain all nuclei blue. Microscopic evaluation of NETs was done using a Leica DMI6000CS confocal microscope with a HCXPLAPO HCXPLAPO 40× 0,75–1,25 oil objective.

2.5. Bacterial killing assay

A colony forming units (cfu) plating assay was used to quantify the bactericidal ability of carp NETs. *A. hydrophila* was cultured as described above. Head kidney and kidney cells were isolated as previously described and seeded in 96 well plates at a density of 5×10^6 in a total volume of 250 μl. The cells were then stimulated with or without 200 μg/ml β-glucan for 30 min at 23 °C. Bacteria at MOI 2 were added, centrifuged for 5 min at 370*g* and incubated for a further 25 min at 23 °C. After incubation, the extracellular surviving bacteria were collected, plated and enumerated on LB agar plates. Colonies were counted after 24 h incubation at 37 °C.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad (GraphPad Prism 5, 2011). Data are presented as means ± standard error of the mean (SEM) of a minimum of 3 individual fish. Statistical testing was done using paired one-tailed Student's *t*-test. Results with $p \leqslant 0.05$ were considered as significantly different.

3. Results and discussion

3.1. β-Glucan augments the formation of NETs in kidney and head kidney-derived neutrophils

Since Brinkmann et al. (2004) first discovered NETs in 2004, they have been additionally found in a wide range of vertebrate animals including 3 species of fish: zebrafish (Palic et al., 2007) fat head minnow (Jovanovic et al., 2011) and common carp (Brogden et al., 2012). Here in this study we investigated the NET formation from both carp kidney and head kidney derived neutrophils