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The coagulation system helps control infection caused by the ciliate parasite *Philasterides dicentrarchi* in the turbot *Scophthalmus maximus* (L.)

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ABSTRACT

Many studies have shown that coagulation systems play an important role in the defence against pathogens in invertebrates and vertebrates. In vertebrates, particularly in mammals, it has been established that the coagulation system participates in the entrapment of pathogens and activation of the early immune response. However, functional studies investigating the importance of the fish coagulation system in host defence against pathogens are scarce. In the present study, injection of turbot (*Scopthalamus maximus*) with the pathogenic ciliate *Philasterides dicentrarchi* led to the formation of macroscopic intraperitoneal clots in the fish. The clots contained abundant, immobilized ciliates, many of which were lysed. We demonstrated that the plasma clots immobilize and kill the ciliates *in vitro*. To test the importance of plasma clotting in ciliate killing, we inhibited the process by adding a tetrapeptide known to inhibit fibrinogen/thrombin clotting in mammals. Plasma tended to kill *P. dicentrarchi* slightly faster when clotting was inhibited by the tetrapeptide, although the total mortality of ciliates was similar. We also found that kaolin, a particulate activator of the intrinsic pathway in mammals, accelerates plasma clotting in turbot. In addition, PMA-stimulated neutrophils, living ciliates and several ciliate components such as cilia, proteases and DNA also displayed procoagulant activity *in vitro*. Injection of fish with the ciliates generated the massive release of neutrophils to the peritoneal cavity, with formation of large aggregates in those fish with live ciliates in the peritoneum. We observed, by SEM, numerous fibrin-like fibres in the peritoneal exudate, many of which were associated with peritoneal leukocytes and ciliates. Expression of the CD18/CD11b gene, an integrin associated with cell adhesion and the induction of fibrin formation, was upregulated in the peritoneal leukocytes. In conclusion, the findings of the present study show that *P. dicentrarchi* induces the formation of plasma clots and that the fish coagulation system may play an important role in immobilizing and killing this parasite.

1. Introduction

The coagulation system plays an important role in homeostasis and also in the defence against pathogens in invertebrates and vertebrates. The blood clots formed generate a competent barrier that prevents the spread of pathogens into the circulation (Sun, 2006). This system, which appeared early on in evolution and has been demonstrated in invertebrates such as the horseshoe crab (*Limulus polyphemus*) (Iwanaga and Kawabata, 1998), is considered one of the major defence systems in invertebrates (Iwanaga and Lee, 2005). The mammalian coagulation system consists of a cascade of enzyme activation events that culminate in the formation of a fibrin clot. The blood clotting cascade can be triggered by two major routes, known as the tissue factor pathway and the contact pathway (Smith et al., 2015). The former of these, also known as the extrinsic pathway, functions in normal haemostasis and probably also in many types of thrombosis. The contact pathway, also known as the intrinsic pathway, is triggered when plasma comes into contact with certain types of surfaces (Smith et al., 2015). Tissue factor is the major initiator of the extrinsic pathway in mammals, which is mainly expressed by cell vessel walls and is released when the vessel wall is disrupted (Gaertner and Massberg, 2016). The contact system can be activated by non-physiological and physiological compounds such as kaolin, dextran sulphate, polyphosphate, neutrophil extracellular traps (NETs), nucleic acids and collagen, and also by pathogens

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such as bacteria and viruses (Nickel and Renné, 2012; Long et al., 2016). Interestingly, deficiencies in the contact pathway factors lead to prolonged clotting times *in vitro*, but do not cause bleeding complications *in vivo* (Maas and Renné, 2012).

During bacterial infections, the coagulation system cooperates with the immune system to eliminate the invading pathogens. Studies in the horseshoe crab have shown that the coagulin blood clot immobilizes microbes and acts together with plasma components to destroy clot-entrapped microbes (Isakova and Armstrong, 2003). In mammals, activation of the tissue factor pathway is considered part of the host defence to infection, and a protective role against certain pathogens has been described (van der Poll and Herwald, 2014). Many bacterial species have been shown to activate the contact pathway by different mechanisms (Nickel and Renné, 2012). The contact pathway acts synergistically by entrapping bacteria with fibrin and enhancing proinflammatory signalling, which suggests that coagulation is important in preventing the spread of the invading pathogen (Frick et al., 2006; Loof et al., 2014; Nordahl et al., 2005).

Knowledge of the coagulation system in fish is rather limited. Although most coagulation factors found in mammals appear to occur in teleosts such as zebrafish (*Danio rerio*) and puffer fish (*Fugu rubripes*) (Davidson et al., 2003; Doolittle, 2015; Weyand and Shavit, 2014), some genes involved in the contact system, such as factor XII and prekallikrein, have not been found in fish (Doolittle, 2011). It has been suggested that the cascade equivalent to the plasma kallikrein-kinin system in mammals is absent in teleosts (Wong and Takei, 2013). Nonetheless, both the intrinsic and extrinsic coagulation pathways have been described in zebrafish (Jagadeeswaran and Sheehan, 1999). However, as far as we know, there is no information available about the role of the coagulation system in defence against pathogens in fish.

Philasterides dicentrarchi is an opportunistic histophagous ciliate that causes severe mortalities in cultured fish worldwide (Harikrishnan et al., 2010). Ciliates probably penetrate the fish through lesions in the gills or the skin and then proliferate in internal organs, causing systemic infection (Paramá et al., 2003). Fish infected experimentally with *P. dicentrarchi* show an intense inflammatory response that affects most organs (Puig et al., 2007) as well as upregulation of many genes involved in the immune response (Pardo et al., 2012). The ciliate and some of its components induce strong activation of turbot leukocytes; however, their role in defence against this pathogen seems to be minor, at least in comparison with humoral factors (Piazzon et al., 2011a, 2013). In this respect, fish complement appears to be a critical component in the defence against *P. dicentrarchi*, especially after activation of the classic pathway (Leiro et al., 2008; Piazzon et al., 2011a, 2013). In addition to complement, fish plasma also contains other soluble components, such as those forming part of the coagulation system, which may also have a role in controlling *P. dicentrarchi* infection. This study was undertaken to determine whether ciliates and their components can induce activation of the coagulation system and how coagulation affects the survival of *P. dicentrarchi*.

2. Materials and methods

2.1. Ciliates

Specimens of the ciliate *P. dicentrarchi* (isolate I1; Budiño et al., 2011) were aseptically isolated from ascites of naturally infected turbot. The ciliates were maintained at 18 °C in complete sterile L-15 medium (Leibovitz, Sigma-Aldrich, pH 7.2) containing adenosine, cytidine and uridine (90 mg/L), guanosine (150 mg/L), glucose (5 g/L), L-α-phosphatidylcholine (400 mg/L), Tween 80 (200 mg/L) and 10% heat inactivated foetal calf serum (Iglesias et al., 2003). The virulence of the ciliates was maintained by experimentally infecting samples of fish every 3 months by intraperitoneal injection, as previously described (Leiro et al., 2008). The ciliates were washed in L-15 medium by centrifugation (700 × g for 10 min) and resuspended in 3.5% NaCl, and the concentration was estimated with a haemocytometer. Alternatively, ciliates were resuspended in 3.5% NaCl containing 1 mM phenylmethylsufonyl fluoride (PMSF, dissolved in ethanol) and ultrasound was applied to the ciliate suspension on a bed of ice, in order to totally lyse the cells. Live and lysed ciliates were used in different clotting experiments.

To obtain the cilia, the ciliate suspension was centrifuged at 700 × g for 10 min, and the pellet was resuspended in L-15 medium containing 2.6 mM of dibucaine and incubated for 10 min (Thompson et al., 1974). Deciliation was monitored by observing the ciliate suspension under phase contrast microscopy. Cilia were separated from the other ciliate components by differential centrifugation, as indicated by Adoutte et al. (1980). The ciliate suspension was centrifuged at 700 × g for 10 min to eliminate other cell components derived from broken ciliates. The supernatant was centrifuged again at 15,000 × g for 15 min and the pellet was resuspended in distilled water and stored at −80 °C. The protein content of the samples was determined by the Bradford assay, as indicated by Piazzon et al. (2008).

The *P. dicentrarchi* DNA was purified using the DNAesy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. The quality, purity and concentration of DNA were estimated by A_{260} measurement in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA.).

Ciliate proteases were purified on a bacitracin–Sepharose affinity column, as described by Piazzon et al. (2011b). Briefly, ciliates isolated from 0.5 L of *P. dicentrarchi* culture (about 10^5 ciliates/mL) were washed with PBS, resuspended in 5 mL of equilibration buffer (100 mM CH3COONH4, pH 6·5), sonicated on ice until broken and filtered (0.22 μm). The ciliate samples were then applied to a bacitracin– CNBr-activated sepharose 4B (GE Healthcare, Madrid, Spain) XK 16/20 column connected to a protein purification system (ÄKTAprime™plus; GE Healthcare, Madrid, Spain). The non-retained fraction was washed with the same buffer until the absorbance at 280 nm returned to basal levels. The proteases bound to the column were eluted with 100 mM CH_3COONH_4, 1 M NaCl and 25% (v/v) 2-isopropanol, pH 6.5, until the OD at 280 nm was basal. Samples were then dialysed and concentrated by ultrafiltration in Amicon® Ultra 10 K centrifugal filter devices (Millipore, Billerica, MA, USA) and finally stored in 0·15 M PBS at −80 °C until use.

2.2. Fish

Specimens of the turbot *Scophthalmus maximus* (L.), of approximately 50 g body weight, were obtained from a local fish farm. The fish were maintained at 16 °C in 250-L tanks with aerated and recirculated sea water and fed daily with commercial pellets. The fish were acclimatized to the aquarium conditions for two weeks before the start of the experiments. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela (Spain). Before all procedures, the fish were anaesthetized with tricaine methanesulfonate (Sigma-Aldrich) (150 mg/L) and killed by pithing.

2.3. Collection of serum, plasma and leukocytes from fish blood

Fish blood was obtained by caudal venous puncture. Serum was obtained from blood that was allowed to clot for 2 h at room temperature before being centrifuged at 3000 × g for 10 min. To obtain the plasma, one ml of blood was mixed with 100 μl of 4% sodium citrate and centrifuged at 3000 × g for 10 min and at 4 °C. Plasma was carefully separated from the cells and used in the coagulation experiments. In some assays, serum and plasma were heated at 45 °C for 30 min. For separation of leukocytes, fish blood was diluted in L-15 medium (1:3, v:v) containing heparin (10 U ml^{-1}) (Castro et al., 1999). The cell suspension was then layered onto a 30%/49% v/v Percoll gradient (GE Healthcare), as previously described (Castro et al., 1999). After

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