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Interaction between eicosanoids and the complement system in salmonid fish

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ABSTRACT

Both eicosanoid generation and the complement system have long evolutionary histories predating the emergence of the vertebrates over 500 myr ago. This study investigated the interplay between these two systems in an example of a bony fish, the rainbow trout (*Oncorhynchus mykiss*). Specifically, it examined whether purified complement fragments including C3a-1 and zymosan-activated serum, stimulate the biosynthesis of any of these eicosanoids by trout macrophages. Incubation of macrophages with zymosan pre-incubated with normal trout serum resulted in the phagocytosis of such particles and the generation of both intra- and extra-cellularly located lipoxygenase and cyclooxygenase products. Both eicosanoid generation and phagocytosis levels were significantly elevated following incubation of zymosan in trout serum in comparison with heat-inactivated (60 °C for 30 min) trout serum and saline alone. A combined mass spectrometry/high performance liquid chromatography approach was employed to conclusively demonstrate the presence of the cyclooxygenase product, prostaglandin E (PGE) in the culture supernatants of ionophore-challenged macrophages. Incubation of trout macrophages with zymosan-activated trout serum (i.e. no zymosan present) failed to stimulate PGE generation. Similarly, incubation of these cells for up to 60 min with C3a-1 (4 or 50 nM) failed to generate significant amounts of PGE or lipoxygenase products such as leukotriene $B_{4/5}$ or lipoxin $A_{4/5}$. Longer term (6 & 24 h) incubation of macrophages with C3a-1 (4 nM) resulted in a time dependent increase in the generation of PGE but not leukotriene B in culture supernatants. No conclusive evidence that the increase in PGE generation was caused by changes in the expression of either cyclooxygenase-1 or -2 was found.

1. Introduction

Both eicosanoids and complement products are key players in inflammation. For example, leukotriene (LT) B_4, a lipoxygenase (LO) product of the fatty acid, arachidonic acid, is a chemoattractant for mammalian leukocytes including neutrophils and mononuclear phagocytes (Funk, 2001; Serhan and Savill, 2005). Similarly, C3a and particularly C5a, cause the chemotaxis and activation of granulocytes as well as stimulating the release of other mediators of inflammation from mast cells and basophils (Morgan, 2001).

The interaction between products of the complement system and eicosanoids is potentially an important amplification stage leading to heightened inflammatory responses. Püschel et al. (1993) found that short-term (> 10 min) incubation of rat Kupffer cells with C3a resulted in the *de novo* synthesis of prostaglandins (PG) including PGD_2, $PGF_{2\alpha}$ and thromboxane (Tx) B_2 (the stable breakdown product of TxA_2). Similarly, others have also found that C5a causes a rapid time-dependent synthesis of PGE_2, PGD_2 and TxB_2 by hepatic cells including Kupffer cells and stellate cells (Hesperling et al., 1995; Schieferdecker et al., 1998) while Kodani et al. (2000) observed that intra-tracheal administration of C5a in rats resulted in cysteinyl LT production. Longer term incubation of human monocytes with C3b and C3bi also causes a time dependent accumulation of PGE in culture medium but this was only apparent after 18 h incubation (Rutherford and Schenkein, 1982). More recently, the mechanism by which sub-lytic C5b-9 membrane attack complexes induce PGE_2 and TxB_2 generation in rat

Abbreviations: COX, cyclooxygenase; EIA, enzyme immunoassay; HEPE, hydroxy eicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; RP-HPLC, reverse phase high performance liquid chromatography; LO, lipoxygenase; LT, leukotriene; LX, lipoxin; PL, phospholipase; PG, prostaglandin; Tx, thromboxane; GCECMS, gas chromatography electron capture mass spectrometry.

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glomerular epithelial cells has been elegantly investigated (Takano and Cybulsky, 2000). C5b-9 stimulates cytosolic phospholipase (PL) A_2 activity in the kidney leading to the production of eicosanoids (Liu et al., 2001). This activation of PLA_2 involves an increase in intracellular Ca^{2+} and the transactivation of receptor tyrosine kinases (Cybulsky et al., 1999). An additional mechanism of elevated PG generation appears to be the induction of cyclooxygenase-2 (COX-2), an enzyme central in the biosynthesis of these compounds (see review by Funk, 2001). Normal (unstimulated) rat glomerular epithelial cells mainly express the COX-1 isoform but after 2 h exposure to C5b-9, these cells show COX-2 mRNA and COX-2 protein that is sensitive to selective COX-2 inhibitors (Takano et al., 2001). Similarly, it has been reported that other mammalian cell types exposed to complement proteins show enhanced production of PGs via the direct or indirect induction of COX-2 (e.g. Bustos et al., 1997).

Both complement components, such as C3a and C5a (Boshra et al., 2006), and eicosanoids (Rowley, 1996) are thought to be key pro-inflammatory molecules in fish. Bony fish, in general, appear to possess classical, alternative and lectin pathways of complement activation (Boshra et al., 2006). Differences do exist, however, between fish and mammals in terms of their complement systems in that some species of fish have multiple isoforms of C3. For example, rainbow trout, *Oncorhynchus mykiss* have four C3 isoforms (C3-1, C3-2, C3-3 and C3-4) (Sunyer et al., 1996). These authors also found that the most abundant form is C3-1 and that this isoform avidly binds zymosan, an activator of the alternative pathway. The mechanism of biosynthesis and biological activities of eicosanoids is well studied in fish particularly in *O. mykiss* (Pettitt et al., 1991; Rowley et al., 2004; Hong et al., 2005; Ishikawa and Herschman, 2007). For example, trout macrophages contain 5- and 12-LO activities leading to the generation of trihydroxy fatty acid derivatives, the lipoxins (LX) as well as dihydroxy (e.g. LTB_4) and monohydroxy fatty acids (Pettitt et al., 1991). LTB_4 and LXA_4 are chemokinetic and chemotactic respectively towards trout mononuclear phagocytes (Sharp et al., 1992). Nothing is known, however, about possible interactions between complement proteins and eicosanoids in fish. Therefore, the aim of this study was to determine if purified complement fragments (C3a-1), zymosan-activated serum and zymosan (opsonised or non-opsonised) are capable of causing the generation of eicosanoids by rainbow trout macrophages. Since fish, like mammals, express both isoforms of COX (i.e. constitutive COX-1 and inducible COX-2) (e.g. Zou et al., 1999; Grosser et al., 2002; Ishikawa and Herschmann, 2007; Havird et al., 2008) this study also aimed to determine if C3a-1 could induce the expression of COX-2 leading to heightened prostaglandin (PG) generation.

2. Materials and methods

2.1. Animals and serum preparation

Rainbow trout, *O. mykiss* (25–30 cm length), were obtained from Lliw Mill Trout Farm, Swansea and maintained in large external tanks fed by a nearby stream. They were fed *ad libitum* on an expanded trout diet. Trout were anaesthetised by immersion in 3-aminobenzoic acid ethyl ester methanesulfonate salt (0.1 g ml^{-1}, Sigma, UK) and killed. Blood (*ca.* 5 ml) was then removed from the caudal vessel, left to clot and centrifuged (300 g, 5 min) to obtain serum. This was stored at −20 °C for later use.

2.2. Generation and purification of trout C3a-1

C3a was produced *in vitro* by incubating C3 in the presence of factor B and factor D as described elsewhere (Sunyer et al., 1998; Rotllant et al., 2000, 2004). Briefly, C3-1 (5 mg), factor B (250 μg) and factor D (30 μg) were incubated for 1 h at room temperature (RT), in the presence of 5 mM Mg^{2+}. After incubation, the reaction mixture was injected to a Superdex 200 gel filtration column (Pharmacia). C3a-1 was eluted at a flow rate of 0.4 ml/min. Mass spectrometric analysis and SDS-PAGE were used to verify the purity and mass of the C3a molecule as previously reported (Rotllant et al., 2004).

2.3. Macrophage isolation

The hemopoietic head kidney of trout was removed from recently killed fish, pressed through a nylon mesh into ice-cold Leibowitz (L-15) medium (Sigma Aldrich UK) centrifuged (1000 g, 10 min at 4 °C) and cells were re-suspended in fresh L-15 medium (5 ml) and loaded onto a 54% continuous Percoll gradient (formed by centrifugation at 22,000 g for 15 min at 4 °C) in Ca^{2+}/Mg^{2+} free Hank's balanced salt solution (HBSS; final concentration: 8.2 g l^{-1}NaCl, 0.4 g l^{-1} KCl, 0.1 g l^{-1} KH_2PO_4, 1.27 g l^{-1} $NaHCO_3$, 2.0 g l^{-1} glucose, pH 7.2). The loaded gradient was centrifuged (2,800 g for 15 min at 4 °C) and the upper white cell band collected. The cells were then washed twice in Ca^{2+}/Mg^{2+} free HBSS (1000 g, 10 min at 4 °C) and re-suspended in RPMI-1640 medium (Sigma Aldrich, UK) or L-15 medium. Judged by morphology, these preparations contained *ca.* 95% macrophages with the remaining 5% of cells consisting of neutrophils and lymphocytes.

2.4. Zymosan preparation for phagocytosis assay

Zymosan (100 μl, 2.5×10^7 particles ml^{-1}) was pre-incubated with normal trout serum, heat-inactivated serum (30 min, 60 °C) or Ca^{2+}/Mg^{2+} free HBSS for 1 h at RT. The zymosan was pelleted (300 g, 5 min), washed twice in Ca^{2+}/Mg^{2+} free HBSS then re-suspended in fresh HBSS containing 2 mM $CaCl_2$ and 3 mM $MgSO_4$. When necessary, clumps were removed by passing through a 23-gauge needle.

2.5. Macrophage phagocytosis assay

Macrophages (1×10^6 cells/slide) from Percoll gradients were allowed to adhere on glass slides for 20 min at RT and incubated with zymosan particles (macrophage:zymosan ratio 1:2.5; zymosan previously incubated in normal trout serum, heat-inactivated trout serum or HBSS) for 30 min at 16 °C. Slides were briefly immersed in Ca^{2+}/Mg^{2+} free HBSS then fixed for *ca.* 30 min in Baker's formol calcium, air-dried and stained in Giemsa stain (Merck, UK). The slides were rinsed in HBSS and left unmounted. 200 cells/slide were randomly counted to establish the % of cells phagocytosing zymosan. The experiment was repeated with slides produced from four fish.

2.6. Effect of zymosan and zymosan-incubated serum on extracellular eicosanoid release by trout macrophages

Zymosan (5 mg ml^{-1}) was incubated for 1 h at RT with normal trout serum, heat-inactivated serum (30 min, 60 °C) or Ca^{2+}/Mg^{2+} free HBSS. Subsequently, the zymosan was pelleted (300 g, 5 min) from each incubation condition and the supernatants retained for later use in experiments designed to determine if such exposure of macrophages yields eicosanoids. All zymosan pellets were washed twice by centrifugation (300 g, 5 min) to remove unattached serum factors, re-suspended in HBSS containing 2 mM $CaCl_2$ and 3 mM $MgSO_4$ and subsequently incubated for 30 min in flasks containing 2×10^7 adherent macrophages (previously left to adhere for 1 h). Calcium ionophore, A23187 (5 μM final concentration) was used as a positive control for eicosanoid