Aquaculture 292 (2009) 180–188

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Differing cell population structure reflects differing activity of Percoll-separated pronephros and peritoneal leucocytes from barramundi (*Lates calcarifer*)

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ARTICLE INFO

Article history:
Received 13 February 2009
Received in revised form 19 April 2009
Accepted 20 April 2009

Keywords:
Barramundi
Macrophage
Respiratory burst
Phagocytosis
Flow cytometry
Chemiluminescence

ABSTRACT

Aquaculture of barramundi or Asian sea bass (*Lates calcarifer* L) is expanding throughout the Asia-Pacific region in both marine and freshwater systems. Incidence of bacterial and viral diseases is high in this species throughout the region yet little is known about the immune system of this highly adaptable euryhaline fish. Ultimately, most pathogens are eradicated by the phagocytic cells, however there is great diversity and plasticity amongst these cell populations in mammals and in fish. To better understand disease processes in barramundi, Percoll-purified leucocyte populations from haematopoietic tissues of the head kidney were compared with populations isolated from the peritoneal cavity morphologically, cytochemically and in terms of the ability to respond to stimulation, using flow cytometry, light microscopy and fluorimetric/luminometric assays. The peritoneal cells comprised predominantly of macrophages and putative mature monocytes whilst the head kidney cells comprised lymphocytes, including immunoglobulin-positive B-lymphocytes, some small monocytes and macrophages. The differing population structures were reflected in the ability of the cells to respond to stimulation with either lipopolysaccharide or phorbol myristate acetate, as the chemiluminescence response of peritoneal cells was 7 to 9-fold higher than head kidney cells. Both populations were capable of being primed by LPS, but the kinetics differed, with optimal priming in peritoneal cells occurring after 6 h exposure whilst head kidney cells required at least 24 h exposure to LPS for optimal priming. Both head kidney and peritoneal populations produced nitric oxide in response to stimulation with LPS and interferon gamma, but again, response was higher in peritoneal cells. The implications of the differing population structures and activities amongst these cells should be considered when developing models for further study of host–pathogen interactions in this increasingly important fish.

1. Introduction

Barramundi (*Lates calcarifer*) is an iconic fish with high cultural significance and economic value in Australia. Commonly known as Asian sea bass, barramundi are widely farmed throughout the Asia Pacific region and in Australia barramundi represent the second largest finfish aquaculture sector after Atlantic salmon (Love and Langenkamp, 2003). The barramundi industry has shown strong growth in Australia lately with a 21% increase in production in 2003/2004 and more than 20% increase over 2005/2006 (Lobegeiger and Wingfield, 2007). Farmed barramundi are prone to a number of infectious diseases including bacterial infections by *Aeromonas* sp, *Flexibacter columnaris*, *Vibrio harveyi* and *Streptococcus iniae*. Nodavirus represents the most prevalent viral disease of farmed barramundi (Munday et al., 1994; Bromage et al., 1999; Bromage and Owens, 2002; Agnew and Barnes, 2007). In spite of the industry's value and the restrictions imposed upon its growth by disease, little is known about the barramundi immune system. Research to date has focused predominantly on the adaptive immune system of barramundi, with immunoglobulins having been purified and characterised on several occasions (Bryant et al., 1999; Crosbie and Nowak, 2002; Bromage et al., 2004), and both serum and mucosal antibody responses to killed vaccines determined in freshwater and seawater (Delamare-Deboutteville et al., 2006). However, the innate immune system of barramundi remains uncharacterised.

The innate immune response of fish is the first line of defence against invading pathogens and comprises a variety of immune defence mechanisms involving both cellular and humoral components. The cellular defences include a diverse population of cells that act both independently and collaboratively and fulfill numerous roles in both adaptive and innate immunity, including pathogen detection, signalling, phagocytosis and destruction. Ultimately, most invading

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doi:10.1016/j.aquaculture.2009.04.030

bacterial species are eradicated by phagocytic cells, including the macrophages and neutrophils (Ellis, 1977b). Fish phagocytic cells undergo a series of responses to specific stimuli and enter a state of activation resulting in increased microbicidal capability. Activated phagocytic cells such as macrophages and neutrophils also have the potential to manifest an increase in respiratory burst activity, a process where one electron of molecular oxygen is reduced into highly reactive superoxide anion (O_2^-) by a membrane enzyme, NADPH oxidase which is generated from glucose via the hexose monophosphate shunt (Babior, 2000). In unstimulated phagocytic cells like neutrophils, this enzyme complex is inactive and only assembled upon cell activation by a variety of factors including opsonized bacteria or zymosan, aggregated IgG, and protein kinase C activators such as phorbol myristate acetate (PMA) (Meseguer et al., 1994; Tahir and Secombes, 1995; Babior, 2000; Sarmento et al., 2004). Superoxide anion serves as the precursor for the formation of other ROS (Hampton and Winterbourn, 1999). Hydrogen peroxide (H_2O_2) is rapidly formed from superoxide anion by spontaneous dismutation or enzymatic dismutation by superoxide dismutase (SOD). In the presence of transition metal such as free iron (Fe^{2+}), the formation of hydroxyl radicals ($\cdot OH^-$) can be spontaneously degenerated from superoxide anion and hydrogen peroxide via iron-mediated Haber–Weiss type Fenton reactions (Bullen, 1987). The hydroxyl radicals are more reactive than peroxide and may interact with nitric oxide (NO) to form peroxynitrite. In addition, superoxide anion and hydrogen peroxide are used as precursors for the production of the bactericidal oxidants, the oxidized halogens group (e.g. ClO^-) and the oxidizing radicals group (e.g. $\cdot OH^-$).

The quantity of ROS produced, therefore, has been used as an indicator of the intensity of the innate immune response and the health status of the organism. Indeed, respiratory burst activity of phagocytes in response to various stimuli such as infection, environmental contaminants, growth factors and other immunomodulators has been extensively used as a reliable measurement of the immune response of a variety of fish species.

In higher vertebrates haematopoietic stem cells from which many of these leucocyte populations are derived originate in the bone marrow (Cooper, 2003; Ellis, 1982). Teleosts do not have bone marrow, but haematopoietic tissues are evident in the head kidney, spleen and liver (Ellis, 1977b). Haematopoiesis is first seen in the head kidney early in ontogeny (Ellis, 1977a) and it is believed that the low-pressure renal portal system coupled with environmental radiation shielding resulting from the large amounts of melanin in the head kidney provide an ideal microenvironment for progenitor stem cells of the leucocyte population (Randall, 1970; Cooper et al., 1980).

Progenitor cells originating in the haematopoietic tissues give rise to a number of specific functional lineages via the myeloid lineage precursor including the granulocytic lineage which includes neutrophils, and the monocytic/macrophage lineage. These professional phagocytic cells are of great interest in the study of health and immunity as their differing antimicrobial activities and ability to mobilise into infected territories has been clearly established in a number of fish species (Afonso et al., 1998; do Vale et al., 2003).

Inflammatory response in fish is considered to be biphasic, with an initial recruitment of neutrophils to the site of infection followed by the arrival of monocytes and macrophages (Reite and Evensen, 2006). Thus leucocyte populations isolated from haematopoietic tissues may be expected to differ in population structure and in their interactive function from those isolated from sites of infection or inflammation.

These diverse leucocyte populations have been described in a number of finfish species, including rainbow trout (Afonso et al., 1997), Atlantic salmon (Jorgensen et al., 1993), European sea bass (Do Vale et al., 2002), goldfish (Belosevic et al., 2006), Atlantic cod (Sorensen et al., 1997), gulf killifish (Roszell and Rice, 1998) and spotted wolfish (Norum et al., 2005). To date little is known of the population structure and function of the leucocytes of barramundi. The aim of the present study was, therefore, to characterise leucocytes from the head kidney (representative of haematopoietic tissue) and from casein-stimulated peritoneal cavity as a model site of inflammation. Populations were investigated structurally by light microscopy, flow cytometry and imaging flow cytometry. The activation kinetics of these populations in response to various stimuli was also determined by measuring luminol-enhanced chemiluminescent response. As head kidney macrophages are often chosen as models for assessing immunocompetence in fish (Cook et al., 2003; Bagni et al., 2005), or resistance of fish pathogenic bacteria to phagocytic attack (Sharp and Secombes, 1993; Barnes et al., 1999, 2002; do Vale et al., 2003; Zlotkin et al., 2003), an objective assessment of the structure and capabilities of head kidney leucocytes (HKL), compared to those arriving at a site of inflammation should provide further insight into their suitability as a representative models for the study of host–pathogen interactions.

2. Materials and methods

2.1. Experimental animals and maintenance

Barramundi, *L. calcarifer*, approximate weight 100 ± 20 g were purchased from a commercial farm (Barramundi Australia, Stapylton, Australia) and maintained in recirculating aerated water at a temperature of 25–28 °C and salinity of 10 ppt, in 500 L round plastic tanks. Water quality was maintained at pH 6.5–7.5, ammonia <2 ppm, nitrite <5 ppm and nitrate <40 ppm with mechanical and biological filtration, and periodic water changes as required. Before the experiment, the fish were acclimated for 2 weeks, and during this period, they were fed twice daily to apparent satiation with a commercial diet (Marine Float 4 mm, Ridley Aqua Feeds, Narangba, Australia).

2.2. Leucocyte isolation and cell culture

Macrophages from head kidney and peritoneal cavity were harvested, purified and maintained as described previously (Secombes, 1990). For stimulation of the peritoneum, fish were anaesthetised with Aqui-S (Aquatic Diagnostic Services, Wilston, Australia) in accordance with the manufacturer's instructions, and then injected with 1 mL of 12% casein (sterile, in phosphate buffered saline, PBS) into their peritoneal cavity 24 h before collection of macrophages. Prior to the isolation of peritoneal macrophages, fish were euthanised with overdose Aqui-S and then exsanguinated by cutting the ventral aorta. An aliquot (5 mL) L-15 medium containing 2% Foetal Bovine Serum (FBS, Invitrogen, Melbourne, Australia), 1% penicillin/streptomycin (P/S) (Invitrogen, Melbourne, Australia), and 10 U mL^{-1} heparin (Sigma, Castle Hill, Australia) was injected asceptically into the peritoneal cavity using a syringe fitted with a 25 G needle. The body cavity was then massaged for 30 s to disperse the medium and the lavage containing leucocytes was withdrawn using syringe fitted with a 19 G needle very carefully to prevent bleeding. Collection of head kidney macrophages was performed simultaneously with harvest of the peritoneal macrophages. The pronephros was dissected out aseptically and pushed through a 100 mm nylon mesh in L-15 medium containing 2% FBS, 1% P/S, and 10 U mL^{-1} heparin. The suspensions of head kidney and peritoneal cells were then layered onto a discontinuous (34%/51%) Percoll density gradient and centrifuged at 450 ×*g* for 25 min at 4 °C. The band lying at the interface was collected and washed twice with L-15 medium containing1% FBS and 1% penicillin/streptomycin (P/S). Concentration of viable cells was determined by Trypan blue exclusion. Cells (100 µL) were seeded in 96 well tissue culture treated microtitre plates (Greiner, Germany) at a concentration of 10^7 cells mL^{-1} in L-15 medium with1% FBS and 1% P/S. Both cell populations were allowed to adhere for 2 h at 28 °C and then washed twice with L-15 medium to remove the unattached cells. The adhered cells were maintained in L-15 with1% FBS and 1% P/S at 28 °C.