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Characterisation of rainbow trout peripheral blood leucocytes prepared by hypotonic lysis of erythrocytes, and analysis of their phagocytic activity, proliferation and response to PAMPs and proinflammatory cytokines

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Highlights

- A method for hypotonic lysis of fish erythrocytes has been optimised to isolate PBL.
- PBL isolated by hypotonic lysis and density gradients have a similar leucocyte marker gene profile.
- The PBL proliferate in response to mitogen stimulation.
- Phagocytic activity of PBL is enhanced by cytokine stimulation.

- PBL cytokine gene expression is modulated in response to PAMPs and cytokines.

Abstract

Rapid and high quality preparation of peripheral blood leucocytes (PBL) is important in fish immunology research and in particular for fish vaccine development, where multiple immune parameters can be monitored on the same fish over time. Fish PBL are currently prepared by density separation using Percoll or Hispaque-1.077, which is time consuming, costly and prone to erythrocyte contamination. We present here a modified PBL preparation method that includes a 20 s hypotonic lysis of erythrocytes and a subsequent separation of PBL from cell debris by a cell strainer. This method is simple, rapid and cost effective. The PBL obtained are similar in cellular composition to those prepared by density separation but have less erythrocyte contamination as demonstrated by FACS analysis and the expression of cell marker genes. Marker gene analysis also suggested that PBL prepared by hypotonic lysis are superior to those obtained by the gradient method in that some high-density cells (certain B cell types and neutrophils) might be lost using the latter. The PBL prepared in this way can proliferate in response to the T cell mitogen PHA, and both lymphoid and myeloid cells can phagocytose fluorescent beads and bacteria, with the latter enhanced by treatment with pro-inflammatory cytokines (IL-1β and IL-6). Furthermore, the PBL can respond to stimulation with PAMPs (LPS, poly I:C) and cytokines (IL-1β and IFNγ) in terms of upregulation of proinflammatory cytokine gene expression. Such data demonstrate the utility of this approach (hypotonic lysis of erythrocytes) for PBL isolation and will enable more studies of their role in disease protection in future immunological and vaccine development research in fish.

Introduction

Fish immunology research has attracted much interest in recent years for theoretical and practical reasons. Fish immune systems provide important comparative outgroups for understanding the evolution of disease resistance. As a large vertebrate group, fish may have evolved novel mechanisms to tackle infections, and research into their responses should eventually lead to an increased understanding of the general principles of immune system adaptability in vertebrates (Feng and Martin, 2015; Flajnik, 2018). At the same time, the expanding aquaculture industry and associated

disease risks requires fish immunology research to identify ways to manipulate the immune response and allow development of novel/efficacious vaccines (Secombes, 2008; Van Muiswinkel, 2008; Lafferty et al., 2015; Little et al., 2016). From a functional perspective, this research needs methods to rapidly prepare leucocytes from immune tissues such as head kidney, spleen and blood that are rich in erythrocytes. Peripheral blood leucocytes (PBL) are particularly relevant to vaccine development work, since samples can be obtained multiple times from the same individual during an immune response without killing the fish.

Classically, mammalian PBL have been purified by lysis of the non-nucleated erythrocytes that they possess with hypotonic ammonium chloride solutions that are commercially available. However, teleost erythrocytes are nucleated and resistant to ammonium chloride lysis (Rowley, 1990). Fish PBL have been routinely prepared by continuous or discontinuous density gradient centrifugation through separation media such as Percoll and Histopaque (Reitan and Thuvander, 1991; Korytar et al., 2013; Maisey et al., 2016; Takizawa et al., 2016; Zhang et al., 2017). Density gradient preparation of leucocytes is time consuming, expensive, and prone to erythrocyte contamination. Crippen et al. (2001) reported a simple, rapid and inexpensive leucocyte purification method by hypotonic lysis of erythrocytes. In their method, blood was diluted (1:2) and erythrocytes lysed in a hypotonic solution by addition of distilled water for 20–40 s. The osmotic pressure was then brought back to isotonicity by addition of 10x phosphate-buffered saline (PBS). The cell suspension was centrifuged (750 g, 10 min) leaving a viscous mass containing cell debris and nuclear material on top of the cells, that could be removed and discarded. Whilst the resultant PBL were comparable to PBL prepared by gradient methods (Crippen et al., 2001), this method has not gained popularity in fish immunology research. This is partly due to the difficulty in separating the PBL from a viscous mass of cell debris and nuclear material in their method, and partly the lack of demonstrated functionality of the PBL prepared.

We report here an improved hypotonic method to prepare leucocytes from fish blood using rainbow trout as a model. The blood was collected from the caudal vein and erythrocytes lysed by direct addition of cold water for 20 s (i.e. without dilution). 10x PBS was then added and the resultant PBL preparation kept on ice for 5–10 min to allow cell debris and nuclear material to clump and settle. The PBL are then easily separated from cell debris by passing through a cell strainer. The method is simple, rapid and inexpensive. The cell composition of the PBL isolated in this way is comparable to PBL prepared by use of density gradients and is free from erythrocyte contamination. Furthermore, we demonstrate that these PBL can proliferate, phagocytose and respond to pathogen associated molecular patterns (PAMPs) and

cytokine stimulation.

Section snippets

Fish

Juvenile rainbow trout were purchased from College Mill Trout Farm (Perthshire, U.K.) and maintained in aerated fibreglass tanks supplied with a continuous flow of recirculating freshwater at 14 °C. Fish were fed twice daily on a commercial pellet diet (EWOS), and were reared to 200–500 g prior to use. All the experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals, and were carried out under UK Home Office project licence

The improved hypotonic method

PBL prepared by the hypotonic lysis of erythrocytes described above were free of red blood cell contamination, as determined by microscopy, and had >99.9% viability as assessed by trypan blue exclusion. The yield from healthy fish was ~30×10^6 PBL/ml blood. The yield and viability of PBL prepared by the gradient method were similar but the preparations were typically contaminated with some red blood cells.

Compared to the method introduced by Crippen et al. (2001), our method firstly eliminated

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2019, Fish and Shellfish Immunology

> *Citation Excerpt :*
>
> ...Similarly, the hypotonic lysis technique originally developed by Crippen et al. [37] has been recently improved by Hu et al. [38] to facilitate fast and affordable isolation of lymphoid tissue and PBL for fish immunological studies. The technique has recently gained use [38,75–78] and reported to give superior cellular composition, with limited erythrocyte contamination [38] allowing a holistic assessment of fish immune parameters at innate and adaptive levels in immune and vaccine studies [38,171]. Within the 114 reviewed articles, over 200 immunological tests were conducted to study isolated lymphoid tissue and PBL in salmonids (Table 3)....

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