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Short communication

Immuno-histochemical determination of humoral immune markers within bacterial induced granuloma formation in Atlantic cod (*Gadus morhua* L.)

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ABSTRACT

In this study the involvement of several humoral immune parameters of Atlantic cod (*Gadus morhua* L.) were studied in granuloma formed as a result of infection by *Aeromonas salmonicida* ssp. *achomogenes*. The results showed a clear association of immune parameters within the granuloma, in particular the localization of complement component C3, and including evidence for the presence of IgM, APoLP-A1 (Apolipoprotein), CRP-PI and CRP-PII (pentraxin).

1. Introduction

Bacterial infections, especially due to *Vibrio anguillarum* (Va) and *Aeromonas salmonicida* ssp. *achomogenes* (Asa), have been associated with cod aquaculture in Iceland and other countries [1–3]. Vaccination against *Vibrio* sp. in cod has resulted in limited protection while vaccination against Asa has not been successful to date. Immunization of cod with protein antigens has similarly resulted in poor or no specific antibody response [4,5]. Unusual features associated with the immune system of cod have recently come to light including the absence of the major histocompatibility complex class II (MHC class II) gene, generally considered essential for classical humoral antibody response [6,7]. It is likely, therefore, that cod rely to a large extent on cellular immune defence and innate humoral parameters, including natural antibodies [8], to combat pathogens. Granuloma formation is a characteristic feature of the immune response in cod following bacterial infection. For infections due to Asa, for example, granulomatous infiltrates are seen in all organs harbouring this pathogen within four weeks following an experimental infection [9]. The histopathology of granuloma formation in cod has been described previously [9,10], but little or no information exists which details humoral components of this cellular response. In this short communication we describe the detection of Asa and five humoral immune parameters in a classical granuloma in the spleen of Asa infected cod using immunohistochemistry.

2. Materials and methods

Tissue sections were obtained from cod that were naturally infected with Asa as verified by bacterial isolation from the kidney and gross findings at the time of necropsy. Sections were prepared as previously described [11]. Tissue sections (5 μm) were placed on SuperFrost*/Plus microscope slides (Manzel Gläser, USA) and immunohistochemistry was performed using the Dako REAL Detection System K5005, AP/Red Rabbit/Mouse (Dako, Dk.) following the manufacturer's instructions. Minor modifications for optimal immunostaining of cod tissue were applied [11]. The resulting immuno-histochemical preparation showed antibody staining red, and the background tissue in blue due to application of a haematoxylin counterstain. The specific antibodies and preparation protocols used for the study are listed in Table 1. All antibodies were diluted 1/200 prior to application.

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Table 1
The antibodies used in the study.

Antibody	Production[a]
Control rabbit serum 1 (results not shown)	Prepared at our Institute using standard procedure, absorbed with Asa without A-layer protein [18].
Control rabbit serum 2	Pre-immunization serum from rabbit A (see below)
Mouse control ascites	Normal mouse acites, containing IgG1, IgG2a, IgG2b, and IgG3, verified with ISOStrip Mouse Monoclonal Antibody Isotyping Kit, following the manufacturer's instructions (Boehringer Mannheim, Germany).
Rabbit anti Asa serum	Prepared at our Institute using standard procedure, absorbed with Asa without A-layer protein [18].
Mouse anti-cod IgM ascites	Prepared at our Institute using protocol according to Overkamp et al., 1988 [19], using purified cod IgM as an antigen [20].
Rabbit anti cod C3 serum	Prepared by GenScript (USA) based on complete peptide polyclonal antibody package SC1015: Rabbit A, sequence: LNNKHRLTQKKVWDC
Mouse anti-cod ApoLP A1 ascites	Prepared at our Institute using protocol according to Overkamp et al., 1988 [19], using purified cod ApoLP A1 as an antigen [21].
Mouse anti-pentraxin CRP PI ascites	Prepared at our Institute using protocol according to Overkamp et al., 1988 [19], using purified CRP-PI as an antigen [16].
Mouse anti-pentraxin CRP PII ascites	Prepared at our Institute using protocol according to Overkamp et al., 1988 [19], using purified CRP-PII as an antigen [16]

[a] In all instances, the specificity of the anti-cod antibodies was verified by SDS-PAGE separation of purified cod protein and cod serum followed by Western blotting analysis using the relevant antibody.

3. Results

Sections from spleen, liver and kidney were included in this study, all organs showing granuloma at various stages of development. In the spleen 3–6 granuloma were seen per 10× magnification field when examined by light microscopy, 0–1 in the liver and 2–4 in the kidney. The results shown here, in Fig. 1A–H, are of advanced granuloma formations for the splenic tissue sections. The immuno-histochemical reaction associated with the granulomas from other organs (liver and kidney), using the same set of antibodies, was identical to the results presented for the spleen.

Neither the control rabbit serum 1 (not shown) nor 2 (Fig. 1A), nor the control mouse ascites (Fig. 1B) showed any positive reaction within the cod tissue. The necrotic debris with bacteria in the centre of the granuloma are indicated by white arrows in the figures, and the associated inflammatory infiltrate (histiocytes/macrophage, leukocytes, epithelioid and fibroblast cells) are indicated by black arrows.

When stained with the anti-Asa antibody (Fig. 1C) primarily the bacteria at the core of the granuloma stained positive, as did some of the histiocytes (mononuclear phagocytes) and granulocytes surrounding bacterial core. The intensity of the staining did, however, vary between granulomas. Application of the anti-IgM antibody (Fig. 1D) showed that the bacteria within the granuloma were negative, while individual leukocytes (a combination of lymphocytes, monocytes, and histiocytes) both within the granuloma and the surrounding interstitium stained positive. This antibody stained the highest number of leukocytes within the granuloma. The anti-C3 antibody stained both the bacteria and some of the surrounding histiocytes and leukocytes (Fig. 1E). The anti-ApoLP − A1 antibody did not stain the bacteria but did react with a moderately high percentage of the surrounding histiocytes/leukocytes cytoplasma (Fig. 1F). In general, larger or more vacuolated leucocytes were positive for this antibody. The anti-pentraxin CRP-PI antibody did not stain the bacteria but the surrounding lymphocytic leukocytes were weakly positive (Fig. 1G). Some staining of the bacterial core was seen with the anti-pentraxin CRP-PII antibody (Fig. 1H) and the surrounding leukocytes were more densely stained than with the anti-CRP-PI antibody.

4. Discussion

The aim of this study was to examine the possible involvement of several humoral immune parameters in granulomas frequently seen in cod following bacterial infection. It was not within the scope of this study to identify individual cells of the complex mixture of cells within the granuloma or to follow the developmental stages of the granuloma formation.

Apart from IgM being known to be a surface marker on cod B-lymphocytes [12] the exact cellular expression (or secretion) of the other parameters tested in this study have not previously been identified in cod tissue.

The antibody against the complement protein C3 was the only antibody (apart from the specific anti-Asa antibody) to react strongly with the bacteria. A more modified reaction was seen with the anti-CRP-PII. The association of these two parameters with the central bacterial debris suggests an active involvement of the complement classical or/and alternative pathway in the killing or degradation of the bacteria, bearing in mind that one of the roles attributed to the pentraxins is the activation of the classical complement pathway [13]. In this context it is also interesting to remember that cod C3 has been shown to be insensitive to the enzyme degradation by the extra cellular proteins of Asa [14].

The involvement of IgM (presumably B-lymphocytes) and ApoLP-A1 may be a more subsidiary one. IgM may, however, also be involved in the activation of the classical complement pathway and there is some evidence that ApoLP-A1 can act as a control or inhibitory element of the complement lytic pathway of cod [15].

The reaction of the anti-pentraxin CRP-PI antibody was relatively weak but yet associated with the granuloma and not the surrounding tissue. Recent studies on the expression of the two pentraxins of cod, first described by Gisladottir et al. [16], seem to indicate that CRP-PII is more active in immune defence than CRP-PI while CRP-PI may be more involved in ontogenic development [17] (and unpublished data on cod larval development). It may, hence, be speculated whether CRP-PI could be involved in the structural formation of the granuloma, drawing in the epithelioid and fibroblast cells for its structural boundary.