The ontogeny of complement component C3 in Atlantic cod (Gadus morhua L.)—an immunohistochemical study

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Abstract

The complement system in fish is well developed and plays an important role in the immune response. Very little is known about the ontogeny of C3 in fish and no study has previously been done on the development of C3 in teleosts. In this study we have detected the presence of C3 in cod larvae from the age of 1 day post hatching (p.h.) till 57 days p.h., using immunohistochemistry. The specific primary antibodies used, were produced against the β-chain of cod C3. Immunostaining on cod larvae sections revealed that C3 is detectable in the yolk sac membrane from day 1 p.h., and in liver, brain, kidney and muscle from day 2 p.h. C3 was also detected in other organs such as eye, notochord, stomach, intestines, pancreas, heart and gills at different stages of cod larval development. These findings suggest that complement is not only important in immune defence against invading pathogens but may also play a role in the formation and generation of different organs.

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1. Introduction

The complement system is one of the first lines of immune defence as well as a modifier of acquired immunity [1]. Complement consists of a group of at least 20 serum proteins that cooperate with other defence mechanisms. The complement system is an important element of both the innate and adaptive immune system and is activated through three pathways: the antibody-dependent classical pathway, the antibody-independent alternative pathway, and the lectin pathway triggered by the interaction of mannose-binding lectin (MBL) with mannose-rich polysaccharides [2]. C3 is the best characterised complement component and interacts with many proteins, including some that participate in or control cell adhesion and cell-to-cell communication [3].
Cod C3 has been isolated and found to be a two-chain (α-chain, 115 kDa; β-chain, 74 kDa) glycoprotein with an intrachain thioester bond in the α-chain [4]. These characteristics are similar to those of mammalian C3, which through its thioester bond can covalently bind to target cells [5]. Mammalian C3 is primarily synthesised in the liver but it has been shown that other cells and tissues also produce complement components. This includes monocyte/macrophages, fibroblasts, endothelial cells, leucocytes, cells of the central nervous system and cells of the renal glomerulus [6]. The local synthesis of C3 as well as of other complement components in tissues other than the liver may play a very important role in local inflammatory processes [7].

Accumulating evidence suggests that complement might have important roles in diverse biological processes, ranging from early haematopoiesis to skeletal and vascular development and normal reproduction [8,9]. Furthermore, it is now becoming evident that the complement-regulated pathways interact with other signalling networks and influence the outcome of complex developmental processes, such as limb regeneration in lower vertebrates and organ regeneration in mammals [10].

Fish larvae are normally exposed to microorganisms immediately after hatching, and to survive in such an environment, the possession of an effective immune system in fish larvae is of particular importance [11]. It has been shown that the ontogenetic development of the immune system varies between different fish species, and thus it is probably incorrect to apply the data for one species to another [12]. The morphological development of Atlantic cod larvae and juveniles is well described [13,14] and the ontogeny of the lymphoid organs and the immunoglobulin production has been studied [15].

In this paper the results from an immunohistological study, examining the presence of complement component C3 in cod larvae from the age of 1 day p.h. until 57 days p.h. are presented.

2. Materials and methods

2.1. The fish

Cod (Gadus morhua L.) larvae were obtained from the Marine Institute’s Experimental fishfarm Staður, Grindavik, Iceland. Fertilised eggs from either wild or cultured cod were hatched at 7 °C in 25 l silos. Following hatching the larvae were kept for 3 days in the dark in 150 l silos at 7 °C, with flow through water supplied from day 2. One ml of nannochloropsis per silo twice a day was used for darkening and feeding. From day 3 feeding was with rotifers twice a day until day 15 when gradually they were replaced by Artemia nauplii. By day 24 A. metanauplii were used. From day 40 dry food pellets were gradually introduced. The temperature was increased gradually from 7 °C, being 10 °C on day 3, 12–13 °C on day 24 and thereafter kept at 14 °C ± 2 °C. Twenty- to 30-day-old larvae were transferred to circular tanks, diameter 3 m and water depth 0.8 m and the water temperature was gradually reduced to 7 °C.

2.2. Sampling

Samples collected for immunohistology were fixed in 4% formalin in phosphate buffered saline (PBS) and kept at 4 °C until embedded in paraffin within 2 days. The paraffin embedded blocks were stored at room temperature until cut. Samples were taken at 1, 2, 4, 10, 15, 22, 29, 36, 43, 50 and 57 days post hatching. Four larvae were collected for each date and 2 to 4 larvae of each age stage were used for immunostaining. The environmental temperature and the relationship between days after hatching and body length (mm) of the cod larvae are shown in Fig. 1.

2.3. Antibodies

The primary antibody used, was a mono-specific polyclonal mouse antibody prepared in our laboratory against cod C3 β-chain as described earlier [4]. The specificity was amongst others tested in western blotting
on cod serum [4]. As a negative control, normal mouse ascitic fluid was used, which contained IgG1, IgG2a, IgG2b, and IgG3 as verified with the ISOStrip Mouse Monoclonal Antibody Isotyping Kit, following the manufacturer’s instructions (Boehringer Mannheim, Germany).

The secondary antibody used was biotinylated goat anti-mouse Ig from DAKO, Denmark.

2.4. Immunohistochemistry

Tissue sections (5 µm) were placed on SuperFrost*/Plus microscope slides (Manzel Gläser, USA) and stored at room temperature until used. Immunohistochemistry was performed with the ABComplex/AP solution kit (DAKO) following the manufacturer’s instructions with some modifications, as described below, for optimal immunostaining of cod tissue.

Tissue sections were kept at 60 °C for 45 min, followed by deparaffinising and rehydrating. The sections were incubated in 1% NaBH₄ in 0.1 M phosphate buffer (pH 8.0) for 20 min and blocked in 20% skimmed milk, 3% BSA, 0.3% Tween 20 and heparin 500 IU ml⁻¹ in Tris buffered saline ((TBS) 0.5 M Tris, 150 mM NaCl₂, pH 7.6) for 20 min at room temperature. The sections were treated with biotin–avidin blocker (DAKO) according to the manufacturer’s instructions and then incubated with primary antibody (diluted 1/100 or 1/500 in TBS) in a humidity chamber at 4 °C overnight. Control mouse ascitic fluid (diluted 1/100 or 1/500 in TBS) was used as a negative control. The sections were washed in TBS and incubated with secondary antibody (diluted 1/200 in TBS) in a humidity chamber at room temperature for 30 min. Following washing in TBS, the sections were incubated for 30 min at room temperature with ABComplex/AP solution, mixed according to the manufacturer’s instructions. The sections were washed again in TBS and colour detection was performed with fast red solution (DAKO) for 3 to 5 min at room temperature. The sections were rinsed with distilled water, counterstained with 1% methylene green for 2 min and mounted with Glycerol gelatine (Sigma, USA).

3. Results

3.1. Detection of C3 in cod larvae

Immunohistochemistry revealed that C3 is detectable in cod in different organs at all developmental stages from 1 day p.h. till 57 days p.h.
On day 1 p.h. C3 was detected in the periblast of the yolksac (Fig. 2 A). At this age a faint response was also found in some parts of the muscle, mainly in between muscle fibres.

On day 2 p.h. C3 was detectable for the first time in small amounts in endothelial cells in the sinus of the liver (Fig. 2 B). A positive reaction was also seen in the endothelial cells of the capillaries in the glomerulus part of the kidney (Fig. 2 C). The brain showed a strong reaction with anti-cod C3 as did nerve cells in the plexiform layer of the retina of the eye (Fig. 2 D), and this was the maximum reaction detected in these organs in all the developmental stages analysed. C3 was also detected in the columnar epithelium of the stomach. At this age the response in muscle was greatly increased, showing a clear positive reaction in the muscle fibres in the tail section of the larvae.

On day 4 p.h. C3 was detected in the cell membranes of vacuolated cells in the notochord (Fig. 2 E). C3 was also found in the columnar epithelium in rectum as well as in mucosal cells in the intestines.

On day 10 p.h. the C3 response in the liver had increased, being clearly detectable in most hepatocytes (Fig. 2 F) and a positive reaction was seen for the first time in the gills.

On day 15 p.h. C3 was observed in the heart (Fig. 2 G). A positive reaction was seen in the squamous epithelial cells of the epicardium and in the endothelial cells of the endocardium of the heart. Blood cells in the lumen of the atrium of the heart were also positive. At this stage C3 was found in epithelial cells and fibroblasts in the perifery of the epithelial folds of the oesophagus (Fig. 2 H). C3 was widely distributed in the columnar epithelium of the stomach and was also detected in acinar cells in the exocrine part of pancreas (Fig. 2 I). At this stage the presence of C3 in the liver had increased and C3 was widespread in the brain.

On day 22 p.h. a strong response against C3 was seen in the primary and secondary lamellae of the gills (Fig. 2 J) and in the pseudobranch. At this stage C3 was frequently seen in the hepatocytes in the liver.

On day 29 p.h. no change in response was observed.

On day 36 p.h. a very clear response was seen in the skeletal muscle fibres (Fig. 2 K). At this stage the response in liver was still very strong and C3 was detected in the same organs as before (stomach, pancreas, glomerulus of kidney and heart) as well as being seen in the lymphomyeloid tissue in the kidney. The response in the brain was reduced from earlier stages while the response in gills had increased, also being detectable in the chondrocytes in the gill arch.

On 43 and 57 days p.h. C3 was detected in the same organs as before. A schematic view of the detection of C3 in the various organs of cod larvae from day 1 p.h. until day 57 p.h. is shown in Fig. 3.

Fig. 2. Immunohistochemical detection of C3 in different organs of cod from the age of 1 to 36 days post hatching using monospecific anti cod C3 β-chain antibody as the primary antibody. (A) Yolksac on 1 day p.h. A positive response was seen in the periblast of the yolksac. The magnification is 100×. (B) Cod liver on 2 days p.h. A slight positive reaction in the endothelial cells of the hepatic sinus is indicated by an arrow. The magnification is 40×. (C) Cod kidney on 2 days p.h. A positive reaction detected in the endothelial cells of the glomerulus capillaries of kidney is pointed out by an arrow. The magnification is 100×. (D) Cod brain and eye at 2 days p.h. A positive reaction was found in nerve cells in the retina of the eye (re) as well as in the brain (br). The magnification is 40×. (E) Cod notochord at 4 days p.h. A positive reaction in the cell membranes of vaculated cells of the notochord is indicated by arrows. The magnification is 40×. (F) Cod liver at 10 days p.h. A positive reaction is seen in the endothelial cells of the liver sinosides and the hepatocytes. The magnification is 40×. (G) Cod heart at 15 days post hatching. Squamous epithelial cells of the epicardium (ep) and endothelial cells of the endocardium (en) show a positive reaction. Note a positive reaction of blood cells in the lumen of the atrium (bl). The magnification is 40×. (H) Cod oesophagus at 15 days p.h. A positive reaction in the epithelial cells and the fibroblasts on the perifery of the epithelial folds of the oesophagus is indicated by arrows. The magnification is 40×. (I) Cod stomach and pancreas at 15 days p.h. A positive reaction was detected in the columnar epithelium of the stomach (st) and in the acinar cells of the pancreas (pa). The magnification is 40×. (J) Cod gill arch at 22 days p.h. A positive reaction was seen in the ventral aorta and the gills aorta. The magnification is 40×. (K) Cod muscle at 36 days p.h. A positive reaction in the skeletal muscle fibres is indicated with the arrows. The magnification is 100×.
In the present study the presence of C3 was detected at different stages of cod development, from day 1 till day 57 post hatching. Complement component C3 was detected in cod liver from the age of 2 days p.h. and increased with age. Thus C3 was present in most hepatocytes of cod liver at 10 days p.h. The primary site of biosynthesis for the majority of the fluid-phase complement components in mammals is the hepatocyte and more than 90% of the plasma complement is derived from the liver\textsuperscript{[6]}. Recent evidence suggests that complement components might be essential for liver regeneration in settings of acute toxic injury in mice \textsuperscript{[16]}. Complement component C3 was detected in the glomerulus of cod kidney from the age of 2 days p.h. During all the developmental stages examined, it was mainly detected in the glomerulus except for on 36 days p.h. when it was also seen in the lymphomyeloid tissue of the kidney. In teleosts, the kidney is an important lymphoid organ and plays an important role in the trapping of antigens and the production of antibody \textsuperscript{[17,18]}. In a study by Leivo and Engvall \textsuperscript{[19]} on human tissues, it was found that C3d, a biologically active breakdown product of C3, can be found in the glomerular basement membrane in the kidney.

The first sign of complement component C3 associated with the formation of the notochord was found in the cell membranes of vaculated cells of the notochord of cod at the age of 4 days p.h. This indicates that complement probably plays a role in the development of the spine. From the age of 10 days p.h. C3 was present in the gills and on day 36 p.h. C3 was seen in the chondrocytes of the gill arch. The gills are an important site for immune defence, and complement detected in the lamellae most likely plays a role in the host’s immune defence. In a study by Andrades et al. \textsuperscript{[8]}, the expression of several complement components was seen localised in distinct zones of the developing endochondral bone of foetal rats, suggesting that complement proteins are involved in the normal development of cartilage–bone transformation. Complement detected in the chondrocytes might thus be involved in the formation of the gill arches.

<table>
<thead>
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<th>Days post hatching</th>
<th>Volksack</th>
<th>Muscle</th>
<th>Liver</th>
<th>Kidney</th>
<th>Brain</th>
<th>Eye</th>
<th>Stomach</th>
<th>Notochord</th>
<th>Rectum</th>
<th>Intestines</th>
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Fig. 3. A schematic view of the detection of C3 in different organs of cod larvae, from 1 day p.h. until 57 days p.h.
The first positive response for C3 in cod muscle was found at day 1 p.h., mainly in the fibroblast cells in between the muscle fibres. On day 2 p.h. the response had increased, being very abundant in muscle fibres in the tail section of the larvae. The strong presence of C3 at this early stage suggests a role for complement in muscle formation. With increasing age, C3 was less abundant in muscle but clearly seen at day 36 p.h. in individual skeletal muscle fibres. C3 is expressed in normal human myoblasts [20,21] and associates with the regenerative process of limb formation in urodele amphibians [3,22].

The presence of C3 in heart at the age of 15 days p.h. emphasises the role of the heart in immune responses. Cod heart has been found to participate in uptake of soluble antigens like lipopolysaccharide from the bacterium *Aeromonas salmonicida* [23].

Complement component C3 was clearly detected in nerve cells in the retina of the eye in cod at 2 days p.h. The presence of C3 in these cells was not detectable after 10 days p.h. Whether complement play a role in the normal formation of the eye is not clear, but the fact that C3 is only present in the eye in the earliest stages of cod development strongly suggests that complement plays an important role in the formation and maturation of the eye. Complement has been found to participate in the regeneration process of the urodelian eye [22,24].

Complement component C3 was found to be evenly distributed in nerve cells of the brain of cod at the age of 2 days p.h. A strong response was still detected at the age of 15 days p.h. but thereafter C3 was less apparent in the brain tissue. Certain tissues, like the brain, are effectively shielded from plasma components by blood–tissue barriers, and local biosynthesis might be critical at these sites [6]. Studies have confirmed that complement components are synthesised in the mammalian brain [6,25–28] and are involved in the immune defence and brain tissue remodelling [28].

In cod, complement component C3 was detected in blood cells in the heart, in blood vessels and haematopoietic tissue in the kidney. The expression of various complement proteins, membrane regulatory molecules and receptors by a wide spectrum of blood-cell types has been well documented [29]. In addition a novel role of complement in haematopoietic development and stem-cell differentiation has recently been described (Reca et al. in [10]).

Other organs showing the presence of C3 in cod in this study were the yolk sac, oesophagus, intestines, stomach and the pancreas. Neither spleen nor thymus were found in these sections. The periblast of the yolk sac is closely associated with the liver and there appear to be continuities between them. The liver is involved in the resorption of yolk in several teleost species and in cod the liver invades the periblast and metabolises yolk products directly [30]. In oesophagus, intestines and stomach, C3 was mainly found in epithelial cells and fibroblasts, most likely acting in defence against invading pathogens ingested through the mouth. In a study by Andoh et al. [31] human intestinal epithelial cells were found to be local production sites of complement components C3, C4 and factor B, likely being independently regulated by several cytokines derived from monocytes/macrophages and T cells resident in the mucosal microenvironment. Interestingly, C3 was found in the acinar cells in the exocrine part of the pancreas in cod from 15 days p.h. The cells of the exocrine part of cod pancreas are similar to those of the adult cod, while it is not possible to classify the endocrine cells (such as islets of Langerhans) into the different types as seen in the adult cod [30]. Complement regulatory proteins have been found to be expressed in islets of Langerhans in mammals [32].

In this study the complement component C3 was demonstrated in cod development from 1 day post hatching until 57 days post hatching. C3 was detected in the yolk sac, liver, brain, kidney and muscle as well as eye, notochord, stomach, intestines, pancreas, heart and gills at different stages of cod larval development. Studies on the development of lymphoid organs and Ig-producing cells in cod [11] have shown that they are not detected until 58 days post hatching. This is considerably later than the first appearance of complement described in the present paper and indicates a relevant role of C3 in the early larval stages of cod. Our findings also suggest that complement is not only important in the immune defence but may play a role in the formation and generation of different organs.
The ontogeny of C3 has not been followed in fish before. This study will contribute to basic knowledge of the role of complement in foetal development and will be helpful in understanding of the diverse roles and actions of complement and associated immune responses.

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