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The complement system in teleosts

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Complement, an important component of the innate immune system, is comprised of about 35 individual proteins. In mammals, activation of complement results in the generation of activated protein fragments that play a role in microbial killing, phagocytosis, inflammatory reactions, immune complex clearance, and antibody production. Fish appear to possess activation pathways similar to those in mammals, and the fish complement proteins identified thus far show many homologies to their mammalian counterparts. Because information about complement proteins, regulatory proteins, and complement receptors in fish is far from complete, it is unclear whether all the complement functions that have been identified in mammals also occur in fish. However, it has been clearly demonstrated that fish complement can lyse foreign cells and opsonise foreign organisms for destruction by phagocytes. There are also indications that complement fragments participate in inflammatory reactions. Fish possess multiple isoforms of several complement proteins, such as C3 and factor B. It has been hypothesised that the function of this diversity in complement proteins serves to expand their innate immune recognition capacity and response. Understanding the functions of complement in fish and the roles the individual proteins, including the various isoforms, play in host defence, is important not only for understanding the evolution of this system but also for the development of new strategies in fish health management.

Key words: complement, innate, immunity, fish.

I. Introduction

The vertebrate immune system consists of an adaptive and a non-specific, or innate, component. Adaptive immunity first appeared about 450 million years ago in cartilaginous and bony fishes. It plays an important role in the protection against recurrent infections by generating 'memory' cells and specific soluble- and membrane-bound receptors, such as immunoglobulins (Ig) and T cell receptors (TCR), which allow for the fast and efficient elimination of the specific pathogens. The development of vaccines relies on the principle of adaptive immunity, and vaccination technologies have revolutionised the fish farming industry, as they previously have domestic animal and human medicine. The presence of an adaptive immune system, however, has not made

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innate immunity obsolete. On the contrary, by functioning as a first line in host defence, innate immune responses can ward off many microbial attacks or keep them in check until an efficient adaptive immune response has been developed.

Complement is an essential part of the innate immune system and involves about 35 soluble and membrane-bound proteins. The functions of complement are numerous but it is most well known for its capacity to kill pathogens by creating pores in their surface membranes. Complement-mediated killing occurs when complement is activated either directly by microorganisms or by antibody-antigen (Ag-Ig) complexes. This activation by Ag-Ig complexes, makes complement an important effector mechanism for the adaptive immune response. In addition, complement plays a role in immune complex clearance and participates in inflammatory reactions by attracting phagocytic cells to the site of injury. By opsonising pathogens, complement proteins can stimulate phagocytosis, a process that is mediated by complement receptors on the surface of phagocytic cells. One fascinating aspect of complement is its role in modulating the adaptive immune response by binding to specific receptors on mammalian lymphocyte surfaces and follicular dendritic cells [1–3]. Complement thereby provides an important link between adaptive and innate immune responses.

Although the functions just described are based on the mammalian complement system, several parallels can be drawn between the complement systems of mammals and fish and these will be discussed in later sections. Fish are unique in the sense that they display a large diversity in complement components, especially in the case of C3, which can have at least five isoforms in a single species [4–6]. It has been hypothesised that this diversity in complement proteins is to expand their innate immune recognition repertoires [7]. It is still unclear at what time in evolution the gene duplications and divergence that gave rise to the different isoforms actually took place. Several invertebrates have been shown to possess a complement-like system that may be involved in phagocytosis of foreign material [8, 9].

Studying the complement system of invertebrates and that of lower vertebrates, such as fishes, can help us discern which complement functions have been conserved throughout evolution and which are newly acquired with the origin of the mammalian lineage. The phylogeny of the complement system has been recently reviewed [10, 11] and will not be addressed in the present article. However, we will examine the chemistry and function of individual complement proteins in teleost fishes and compare this information with what is known about their mammalian counterparts. Moreover, we will review some of the functions of fish complement in the defence against bacteria, viruses and parasites. For more detailed information on the complement system and its functions in mammals see references [12–14].

II. The Complement Activation Pathways

Complement received its name because it was first identified as a heat-labile component of serum that ‘complemented’ the role of antibodies in the killing of bacteria. Most of the complement proteins are synthesised as inactive

precursors, that function either as enzymes or as binding proteins upon activation by certain foreign substances. Complement activation can take place through three pathways: the classical complement activation pathway (CCP), the alternative complement pathway (ACP) and the lectin complement pathway (LCP). All three activation pathways have been identified in fish, with the exception of the jawless fishes, which appear to lack the CCP and the lytic pathway, i.e. the formation of the membrane attack complex (MAC) [15, 16].

The CCP, the first pathway to be discovered, is triggered by the binding of antibody to a cell surface (see Fig. 1). It can also be activated by acute-phase proteins such as ligand-bound C-reactive protein [17] or directly by certain viruses, bacteria and virus-infected cells [18–20]. The proteins involved in this pathway are numbered in the sequence in which they were discovered, and not necessarily in the order in which they are activated in the pathway.

The first complement component, C1, is a Ca^{2+} dependent protein complex consisting of two molecules each of C1r and C1s bound to one molecule of C1q. Complement activation is initiated when surface-bound IgM or IgG binds C1q, which results in the activation of C1s, the serine protease units of the C1 complex. Activated C1s first cleaves C4 into C4a and C4b, which binds covalently to the cell surface through a free thioester site, and then C2 into C2a and C2b. Many molecules of C4 can be cleaved by one C1s molecule, thus providing an amplification loop of the CCP. The C4bC2a enzymatic complex, known as C3 convertase, cleaves C3 into C3a and C3b.

Complement factor 3 is one of the most abundant proteins in the serum and plays a central role in complement activation. Like C4b, C3b is deposited on the cell surface through a covalent bond between an activated thioester site on the C3b molecule and an hydroxyl or amino group on the cell surface. When bound, C3b serves as an opsonin, i.e. increases phagocytosis by binding to specific receptors on the surface of phagocytic cells, and can form a complex with C4bC2a to give rise to C5 convertase. Upon cleavage of C5 by the C5 convertase, the fragments C5a and C5b are generated. The smaller fragment, C5a, is an anaphylatoxin that contributes to the inflammation process by attracting phagocytic cells to the site of infection. The larger fragment, C5b is deposited on surface-bound C3b and initiates the self-assembly of the MAC by subsequent binding of C6, C7, C8 and C9. Formation of MAC creates a channel or pore in the cell membrane, leading to cell lysis and death.

In contrast to the CCP, the ACP is activated directly by viruses, bacteria, fungi or even tumour cells and is independent of antibody. Activation occurs when C3b generated from the cleavage of C3 binds to hydroxyl or amine groups of carbohydrates or proteins on a foreign cell surface. Factor B, a protein homologous to C2, binds to the deposited C3b, and is activated and cleaved into Bb and Ba by another plasma serine protease, factor D. The resulting C3bBb complex functions as the C3 convertase of the alternative pathway and is stabilised by the serum glycoprotein, properdin. The C3 convertase causes an amplification of the ACP by cleaving more C3 into C3a and C3b and becomes the C5 convertase when an additional molecule of C3b

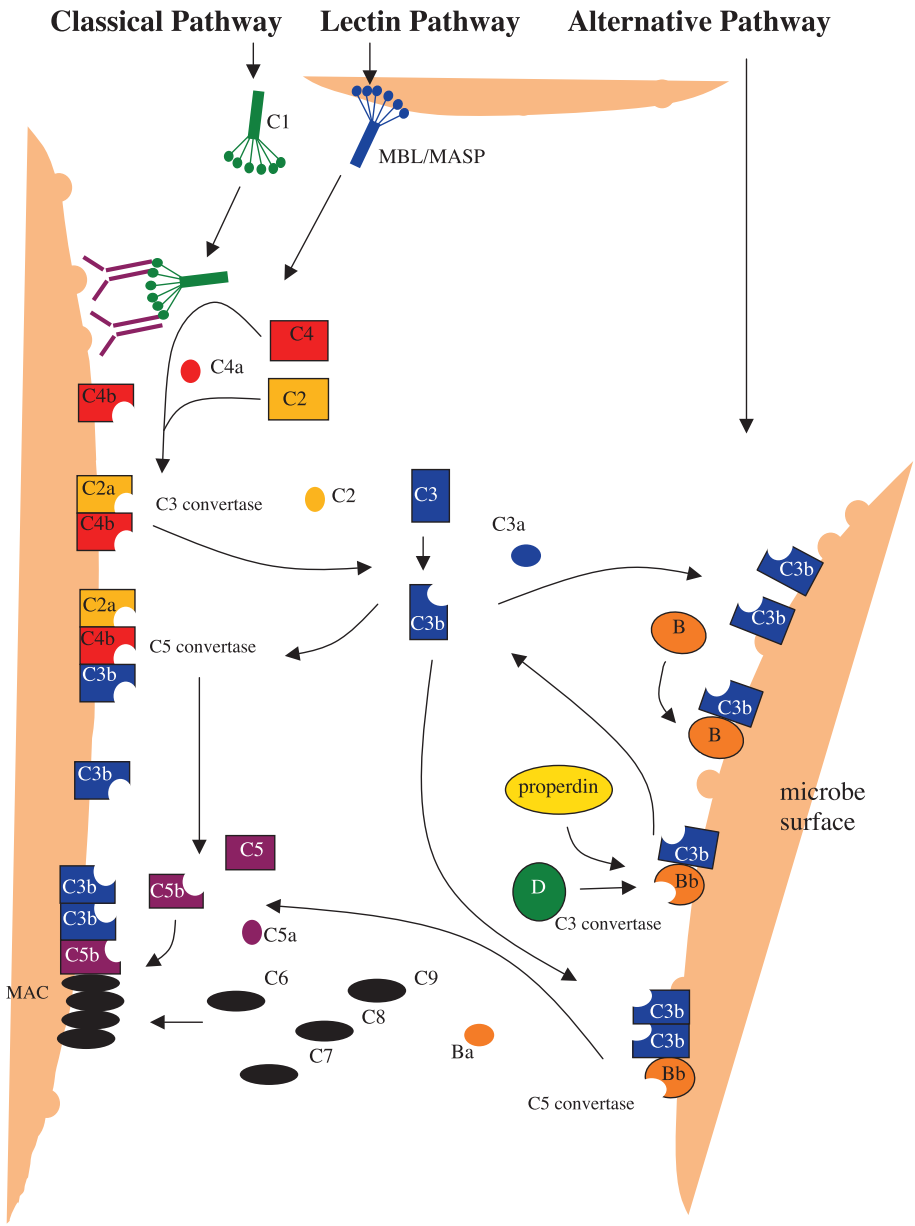

Fig. 1. Schematic representation of the three complement activation pathways in mammals. The structure and activities of the individual complement proteins are discussed in the text.

is bound to the complex (C3bBbC3b). Cleavage of C5 by C5 convertase initiates the formation of the MAC and cytolysis.

The LCP varies from the CCP in the way it is activated. Instead of being activated by Ag-Ig complexes, this pathway is initiated by binding of a protein complex consisting of mannose-binding lectin (MBL) and the serine proteases,

mannose-binding lectin associated proteases 1 and 2 (MASP-1 and -2) to mannans on bacterial cell surfaces; thus, its activation is independent of antibody. The MBL/MASP complex acts similarly to C1r/C1s in cleaving C4 and C2, leading to the formation of the C3 convertase of the CCP.

III. Structure and Characteristics of Fish Complement Components

Complement activation is a multifaceted and complex process, involving a multitude of factors. However, many of the components have similar features and are believed to be products of gene duplication. For instance, it is generally accepted that C3, C4 and C5 are derived from a common ancestor gene, probably the plasma proteinase inhibitor α-2 macroglobulin, which is also present in invertebrates. Which of these three complement proteins was the first to evolve and at what distance from their common ancestral molecule this divergence occurred are still matters for debate [11, 21]. However, the fact that proteins with high similarity to C3 have been isolated from several invertebrate species [8], suggests that the gene duplication that led to C3 preceded those that gave rise to C4 and C5. The C1s/C1r/MASP, Bf/C2 and the members of the lytic pathway (C6/C7/C8/C9), are also believed to share common ancestry. The number of complement proteins and corresponding gene sequences identified in a variety of teleost fishes are listed in Table 1.

C1Q/MBL-LIKE MOLECULES

Few reports have described the C1q molecule in fishes. This sparsity of data is quite surprising, considering that C1q is a key component of the CCP and thus part of an important antibody effector mechanism. Mammalian C1q is composed of six globular heads linked via six collagen-like stalks each comprised of three different types of chains (A, B and C), to a fibril-like central region. A C1q-type molecule, composed of at least two chain types, has been isolated from the nurse shark, *Ginglymostoma cirratum* [22]. In addition, a C1q-type protein in channel catfish, *Ictalurus punctatus*, has been described [23] but full sequence information for this protein is not yet available. Mammalian MBL is structurally analogous to C1q, and an MBL-like molecule has been described in the Atlantic salmon, *Salmo salar* [24].

THE C1R/C1S/MASP FAMILY

Members of the C1r/C1s/MASPs family clearly diverged before the mammalian lineage, giving rise to the CCP and LCP. In teleosts, a MASP molecule has been identified only in carp, *Cyprinus carpio* [25]. Complementary DNA sequences of two homologous isotypes have recently been obtained, suggesting a second member of the C1r/C1s/MASPs family in this fish [26]. Whether these two isotypes function as a C1q-or MBL-binding complex still needs to be addressed, and therefore it remains unclear whether the point of divergence of this protein family occurred before or after the branching-off of the main vertebrate lineage from the ray-finned fishes.

Table 1. Number of complement proteins and cDNA sequences (between parentheses) isolated from different teleost species

	C1q/MBL	C1r/C1s/MASP	Bf/C2	C3	C4	C5	C6	C7	C8	C9	Factor D	References
Catfish	1(0)			1(0)	1(0)							[23, 123]
Carp		0(2)	0(3)	5(8)	*	1(0)	1(0)	1(0)	1(0)	1(0)	1(0)	[25, 26, 30, 39, 43, 46, 49]
Pufferfish										0(1)		[50]
Flounder	*	*		*				*	0(1)	0(1)		[51]
Medaka	*	*	0(1)	0(2)	0(1)				*	*		[34, 41]
Rainbow trout			2(3)	4(3)	2(0)	1(1)		*	*	0(1)	1(1)	[4, 27, 29, 31, 37, 38, 44, 45, 53]
Seabream				5(0)		1(0)						[5]
Zebrafish	*		0(2)	0(3)						*		[32]

*Unpublished cDNA sequences that are available in the DataBase.

C2/BF-LIKE MOLECULES

In mammals, the C2 and Bf genes are located in the major histocompatibility (MHC) class III region, together with the complement components C4A and C4B, and they share the same domain and genomic organisation. They are believed to have arisen by gene duplication from a single gene encoding an ancestral molecule. It appears that in teleosts a common molecule carries out the functions of Bf and C2. In the rainbow trout, *Oncorhynchus mykiss*, two factor B molecules with about 75% sequence similarity (Bf-1 and Bf-2) have been identified [27]. They share many characteristics with mammalian factor B and C2 proteins, among which are the three short-consensus repeats (SCRs) at the N-terminus. SCRs are tandem structural units of approximately 60 amino acids that are found in most plasma and membrane complement-regulatory proteins [28]. Recently, the cDNA sequence of a third type of factor B was identified in trout, which showed closer similarity to carp B/C2-B than to trout Bf-1 or Bf-2 [29]. Trout Bf-2, the dominant of the two isolated forms in plasma, has been shown to act as an intermediate in both the CCP and ACP, and both Bf molecules demonstrate C3-convertase activity in the ACP [27]: cleavage of C3 by Bf-1 or Bf-2, in the presence of factor D (Df) and Mg^{2+}EGTA, generates the C3 fragments C3a and C3b and the Bf cleavage products Ba and Bb. Factor D, a relatively low molecular weight protein that is essential to the generation of the alternative pathway C3 convertase, has been purified from carp [30] and rainbow trout [27]. N-terminal sequencing of 25 residues of rainbow trout factor D showed 100% homology with a kallikrein-like molecule isolated from brook trout, *Salvelinus fontinalis* [27, 31]. This structural similarity, together with the finding that antibodies directed against the kallikrein-like molecule reacted strongly with rainbow trout factor D (Lambvis *et al*., unpublished observations), suggests that the two proteins are the same molecule that is highly conserved among salmonids.

Rainbow trout is not the only teleost from which multiple Bf isoforms have been isolated. Two sequences have been cloned from zebrafish, *Danio rerio* [32], and three Bf/C2 genes with different expression patterns are present in carp [6]. One of the carp Bf/C2 isoforms, termed Bf/C2-B has an amino acid substitution at a site critical for its protease activity, indicating that this isoform may not be able to cleave C3 [33]. Thus far, only one gene with factor B-like sequences has been found in medaka, *Oryzias latipes* [34].

THE C3/C4/C5 FAMILY

The C3, C4 and C5 molecules are synthesised as single chains with an approximate mass of 180 kDa. From these pro-molecules they are processed into the mature proteins, consisting of two (C3 and C5) or three (C4) chains linked together by a disulphide bond and non-covalent forces [35, 36]. The C3 and C4 molecules contain an active thioester site in the *α* chain, which allows them to bind covalently to activating surfaces. This thioester is buried in the native molecule, but upon activation, either by the C3-convertase, which cleaves off the C3a fragment, or by a small nucleophile, the molecule undergoes a conformational change that exposes the thioester site and allows it to react with amino or hydroxyl groups present on foreign cell surfaces.

Four C3 isoforms have been isolated from rainbow trout, C3-1, C3-2, C3-3 and C3-4 [4], all of which have the characteristic thioester site. C3-2 does not appear to play a role in complement-mediated hemolysis, and only N-terminal sequence information is available of the α- and β-chains of this isoform [37]. The sequence identity/similarity of the other trout C3 isoforms are 55/67% of C3-1 to C3-3, 54/67% of C3-1 to C3-4 and 76/81% of C3-3 to C3-4 [38]. The C3 convertase cleavage site (Arg-Ser) is conserved in these isoforms, while the factor I cleavage sites are different from those of human C3: instead of Arg-Ser at position 1281 of human C3, C3-1 and C3-4 have the residues Arg-Ala and C3-3 has Arg-Thr. At position 1298 of human C3, the second factor I cleavage site, C3-4 has the same residues as human C3 (Arg-Ser), while the sequences of C3-1 and C3-3 align with Arg-Thr [38].

From carp, eight C3 cDNA sequences and five proteins, termed C3-1, C3-2, C3-3, C3-4 and C3-5, have been isolated, which share about 80–86% amino acid sequence identity [39]. Five C3 isoforms have also been isolated from the gilthead seabream, *Sparus aurata* [5]. Like those of trout, the seabream C3 isoforms varied in molecular weight, glycosylation, and binding specificities to zymosan, sheep and rabbit erythrocytes [40]. In medaka two C3 isoforms have been identified, Orla C3-1 and Orla C3-2, with 90% similarity to each other. However, the thioester site is conserved in Orla C3-1 only [41].

The C3 isoforms present in teleosts are structurally and functionally similar to mammalian C3. Interestingly, the catalytic His residue that is conserved in the C3s of all other animals studied thus far is not present in all teleost C3 isoforms, and neither is the Glu at a site close to the catalytic His residue (His^{1126} and Glu^{1128} of human C3). These differences in critical amino acids may account in part for their difference in binding specificity for various target surfaces [10, 33, 38], since the His and Glu residues have been shown to be important in determining the thioester-binding specificity of mammalian thioester-containing molecules [35, 42]. Nevertheless, carp C3-1, which lacks the His residue, has a three-fold higher haemolytic activity than C3-2, suggesting that the catalytic His group is not a main determinant for C3 activity [39]. In mammals, C3 is one of the most abundant proteins in the blood, with serum concentrations in the range of 1–2 mg/ml. Similar concentrations have been found for C3-1 in trout and C3-1 and C3-2 in seabream, while the levels of the other C3 isoforms in these species were at least five-fold lower [4].

In contrast to C3 and C5, which are single gene products in mammals, the C4 gene is present in duplicate. The two human C4 isoforms, C4A and C4B, differ in their binding specificity for hydroxy or amino groups. In teleosts, two C4 isoforms have been isolated from carp [43] and, recently, from trout [44]. So far, only one C4 has been cloned from medaka [41].

The cleavage fragments of C5 play critical roles in the initiation of the lytic pathway (C5b) and in inflammation (C5a). In trout, one C5 gene has been cloned which appears to correspond to an individual protein [45]. At the amino acid level, this protein is 40% and 39% identical to human C5 and mouse C5, respectively, and several of the functionally important residues have been conserved. The C5a fragment consisted of 77 amino acids, and included the six Cys residues that are also present in human and mouse C5.

Complement factor 5-like molecules have also been isolated from carp [46] and gilthead seabream [5], and they all share the typical α and β chain structure and lack the active thioester site.

THE COMPONENTS OF THE LYTIC PATHWAY

The complement components C5b to C9 together form the MAC. Assembly of this complex is initiated by the attachment of C5b to surface-bound C3b, which results in the exposure of the C5 binding site for C6. Sequential binding of C7, C8 and multiple C9 molecules to this preliminary C5bC6 complex leads to the formation of a porous transmembrane structure (MAC) that is inserted into the lipid membrane and causes cytolysis. Complement factors C6 to C9 are ancestrally related and are structurally similar to perforin, a lytic protein of natural killer cells and cytotoxic T cells [47]. The human components share common structural motifs, such as thrombospondin, low-density lipoprotein receptor, and epidermal growth factor precursor domains [48]. In addition, C6 and C7 possess two SCRs and factor I modules in the C-terminal domain. These domains have been conserved and are also present in their teleost homologues [49–51].

In teleosts, the MAC have been microscopically observed as small pores in the cell surface [52, 53]. Several of the MAC components have been cloned and/or purified from rainbow trout [53], common carp [46, 49], Japanese flounder, *Paralichthys olivaceus* [51], and pufferfish, *Fugu rebripes* [50]. As in humans, carp C8 is composed of three polypeptide chains with a molecular mass of 62 kDa (α-chain), 62 kDa (β-chain), and 22 kDa (γ-chain), linked together by a disulphide bond (α and γ) and non-covalent forces [49]. However, the N-terminal amino acid sequences of the three chains in carp have no homology to those of human C8. Carp C9 is produced as a 91 kDa single polypeptide. The serum concentration of carp C8 is lower than that of humans (17 v. 55 μg/ml), while that of C9 is similar in the two species (42–59 μg/ml). Functional studies have shown that carp C8 is required for the haemolysis of foreign erythrocytes, while lysis of rabbit erythrocytes can, in some cases, take place in the absence of C9 [49]. A similar situation has been observed involving human C9-depleted serum.

IV. Regulatory Proteins of Complement Activation

Complement activation results in serious damage to cells, and it is of crucial importance that self-cells are protected from autologous attack. Mammals are equipped with a number of regulatory proteins that control the extent of complement activation by, e.g. (1) degrading C3b and C4b into fragments that are incapable of participating in the complement amplification cascades, (2) disassembling the C3-convertases, (3) disrupting MAC assembly and (4) inactivating the C3a and C5a anaphylatoxins. Several of the C3 and C4 fragments generated by degradation, such as iC3b and C3dg, are recognised by complement receptors (CR) and have specific biological activities (discussed in the next section). Cleavage of C3b into iC3b, C3c and C3dg is carried out by the plasma protease factor I, in the presence of the appropriate co-factor.

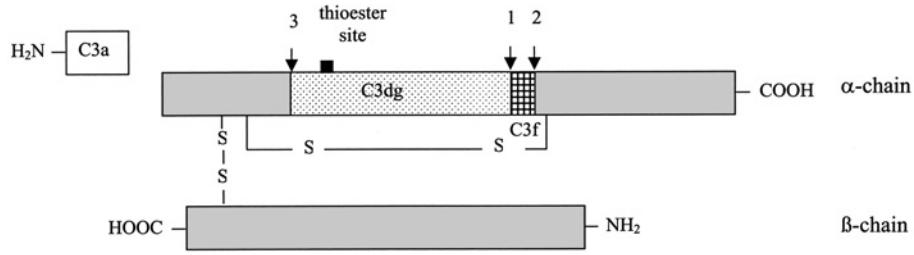

Fig. 2. Schematic representation of a C3 molecule. Cleavage of native C3 by the C3 convertase releases the C3a fragment. Cleavage of C3b by factor I and cofactors, at the two indicated sites (arrows 1 and 2), results in the excision of the C3f fragment (hatched area) leaving a fragment termed iC3b. Further cleavage of iC3b at the third factor I cleavage site (arrow 3) results in the C3dg (dotted area) and C3c (shaded area) fragments.

Depending on the type of cleavage, this cofactor can be factor H, membrane co-factor protein (MCP), or complement receptor (CR) 1. A diagram of a C3 molecule showing the position of the three factor I cleavage sites is shown in Fig. 2. Factor I, in the presence of C4 binding protein (C4bp) also degrades C4b. All these regulatory (co) factors are comprised mainly of SCRs.

It is interesting that in many species, cleavage of C3 into C3c- and C3dg-like fragments can be mimicked by trypsin [54, 55]. The use of this protease, however, has not proved successful in generating these C3 fragments in teleosts (Lambris *et al.*, unpublished data), despite the conserved amino acids at the C3 convertase and factor I cleavage sites of some C3 isoforms [38]. It is possible that the factor I cleavage sites of the fish C3 molecules are inaccessible to trypsin. Binding of factor I, and appropriate co-factors to C3b may be required to first expose the cleavage sites before the molecule can be successfully cleaved.

The C3 convertases C4bC2a and C3bBb can be effectively dissociated by decay-accelerating factor (DAF), a membrane glycoprotein present on virtually all blood cells. MAC assembly can be disrupted by S-protein and clusterin, which restricts binding of the C5b-7 complex to the cell membrane. CD59, a GPI-anchored membrane protein present on a wide range of cells, inhibits binding of C9 to C8 and consequently prevents formation of the MAC. In addition, the serum carboxypeptidase N inactivates the anaphylatoxins, C3a and C5a by removing the C-terminal Arg residue, thus preventing excessive inflammation.

Relatively little is known about the complement regulatory proteins in lower vertebrates. Contrary to the findings in higher vertebrates, only a single protein has been found to serve as a co-factor for factor I in the barred sand bass, *Paralabrax nebulifer* [56, 57]. This protein, SBP1, has both C4bp- and factor H-like activities, and its cDNA sequence encodes 17 SCRs. Another protein with high similarity to SBP1, SBCFR-1, has been cloned from the same species and is comprised of 3 SCR [58]. Two cDNA clones of similar molecules have been identified from the Japanese flounder [59, 60]. Factor H-like clones have been isolated from trout and sequence analysis has shown high homology with the SBP1 protein from the barred sand bass [61]. A molecule similar to

mammalian CD59 has been cloned from hagfish, *Eptatretus stouti*, a jawless fish, and from the brook trout [62, 63].

V. Complement Receptors

Several of the C3 fragments generated upon C3 activation are ligands of distinct sets of receptors. Since the fragments C3b, iC3b and C3dg still carry the thioester site, they remain attached to the activating surface, and this polymeric presentation of C3 fragments may be an important determinant for efficient binding. Several complement receptors have been identified in mammals, and these receptors differ in their affinity for the different C3 fragments. Human complement receptor 1 (CD35), present on leucocytes and erythrocytes, preferentially binds C3b and iC3b, while CR2 (or CD21), found on lymphocytes and follicular dendritic cells, primarily binds iC3b and C3dg. Both receptors are comprised of SCRs and are, among others, modulators of adaptive immune responses [64]. A receptor for C1q has been identified on human platelets, monocytes and macrophages and has been implicated in the regulation of phagocytosis [65] and immune complex clearance [66]. Thus far, no homologues of these receptors have been identified in lower vertebrates. However, another type of CR, CR3, has been shown to be present in amphibians [67] as well as in an invertebrate species [9]. Complement receptor 3 is a member of the integrin family and preferentially binds iC3b. Activation of CR3 in mammals by iC3b-opsonised particles stimulates phagocytosis by macrophages, monocytes, and neutrophils [68, 69]. Two integrin α-subunits have been cloned and characterised from the solitary ascidian, *Halocynthia roretzi*, and antibodies against these subunits bind to molecules on the surface of a subpopulation of phagocytic hemocytes and blocked C3-dependent phagocytosis [9]. Although a CR3-type molecule still remains to be identified in teleosts, a beta 2 integrin molecule has been cloned from channel catfish [124], and C3-opsonised particles have been shown to increase phagocytic activity of macrophages and neutrophils in several fish species [70, 71]. These findings suggest the presence of a similar molecule in fish.

In mammals, anaphylatoxin receptors have been found on various cell types and binding by C3a (to C3aR) or C5a (to C5aR) stimulates various inflammatory responses, such as histamine release by mast cells, contraction of smooth muscle cells and increase of vascular permeability [72, 73]. Binding of trout C3a fragments to a subpopulation of head kidney cells has been described [74], but the nature of the C3a-binding proteins present on these cells is still unknown.

VI. Complement and Host Defence

The complement system plays an important role in the killing and neutralisation of micro-organisms. These effects are mediated by activation of the lytic pathway and by opsonisation. Because assays to measure the levels of specific complement proteins are not yet available for fish (due to lack of purified proteins and specific antisera), information concerning the involvement and mechanisms of the various complement pathways and individual

proteins is sparse. However, over the years, complement activity in fish has been studied by measuring, (1) the haemolytic activity of serum, which is indicative of activation of the lytic pathway, and (2) the phagocytic activity of monocytes/macrophages in the presence of normal and heat-inactivated serum, demonstrating the involvement of opsonisation by complement.

Recently it has become clear that, as in the case of mammals, complement factors in fish can be upregulated in an acute phase response [75]. Using suppression subtractive hybridisation to identify upregulated genes, Bayne *et al.* [29], demonstrated that both C3-1 and Bf-2 act as acute phase response proteins in rainbow trout in response to a *Vibrio* bacterin injection. Although it is not yet known whether other isoforms can be upregulated using different challenges, this type of study may provide important information concerning the regulation of complement proteins in fish.

THE HAEMOLYTIC ACTIVITY OF COMPLEMENT

Complement-mediated haemolysis occurs when the lytic pathway is activated by foreign erythrocytes. In contrast to human complement, complement from a variety of fish can lyse, with high efficiency, the erythrocytes of sheep, goats, dogs, and humans through the alternative pathway [76]. Because the haemolytic activity of serum is a function of complement levels, it has also been used to study the effects of infection on complement levels as well as the impact of various environmental, nutritional, and genetic factors on complement activity.

The titres of the CCP and ACP are indicated by CH50 and ACH50, respectively, and are defined as the reciprocal of the serum dilution giving 50% haemolysis of antibody-sensitised (activation through the CCP) or non-sensitised (activation through the ACP) foreign erythrocytes. The presence of two bivalent ions, Ca^{2+} and Mg^{2+}, are required for the CCP, while activation through the ACP relies on the presence of Mg^{2+} only [77, 78]. As a result of this dependence, both pathways can be blocked by the addition of the chelating agent EDTA.

The ACH50 of fish is many-fold higher than that of mammals, a difference that may indicate greater importance of ACP in the innate response compared to mammals, or a species-specific difference in the sensitivity of target molecules. Other differences between fish and mammals include the temperatures that are required for complement activation or inactivation [79]. Fish complement displays its highest activity at 15–25° C and is still active at 0–4° C, whereas the optimum temperature for mammalian complement is 37° C. Complement of cold-water fishes can be inactivated when held at 40–45° C for 20 min. [80, 81]; in warm-water fishes temperatures of 45–54° C (values closer to that of mammalian complement) are required for inactivation [79]. The broader and lower optimum temperature range of fish complement probably reflects the ambient water temperatures the fish may encounter, and it shows that at lower temperatures, when the adaptive immune response slows down, the complement system is still highly functional.

It has been well documented that fish display genetic variations in the resistance or susceptibility to diseases [82, 83]. This variation may be partly

mediated through differences in complement levels, since the haemolytic activity of serum appears to vary among families, as has been shown for Atlantic salmon [81] and carp [84]. Serum haemolytic activity could therefore be considered a genetic marker for disease resistance and a trait that could possibly be selected for in breeding programmes. Farmed fish are exposed to various stressors, ranging from environmental pollution to handling. Although not all the effects of these stressors on humoral immunity have been investigated, it is clear that some of them result in a rapid depletion of serum complement activity. Short-term or long-term crowding, for instance, depresses serum haemolytic activity in the gilthead seabream [85, 86] and carp [87]. In seabream the stress-induced reduction in haemolytic activity is antibody-independent and thus ascribed to the ACP [88]. Also, diet and dietary supplements such as vitamins C and E have been shown to affect complement titres [85, 89–92]. Taken together, these data demonstrate the need for a well-balanced diet and optimised culturing conditions in aquaculture in order to avoid or minimise the risks associated with immunodepression.

CYTOLYSIS

Various studies have demonstrated the bactericidal activities of fish complement [89, 93–96]. Generally, non-virulent Gram-negative bacteria are highly susceptible to complement lysis, while virulent Gram-negative or Gram-positive bacteria are less so. This difference in sensitivity to complement attack is due to the presence of certain molecular components in the membranes of these bacteria. Ourth and Bachinski [97] have demonstrated that pathogens with a high sialic acid content, such as *A. salmonicida* and *F. columnaris*, are less able to activate complement than non-pathogenic Gram-negative bacteria, which possess low levels of this acid. That sialic acid content is a determinant for bacterial virulence has also been observed in mammals, in which surfaces with a high sialic acid content are considered host cells, whereas those lacking this acid are considered foreign [98]. Also LPS and the A-layer have been shown to be virulence-determining structures on the surface of certain Gram-negative bacteria which may render bacterial cells resistant to complement-mediated killing [96, 99, 100]. Both the CCP and ACP can be activated in response to bacterial infection. Depending on the type of host and pathogen, activation can occur through the CCP [96], the ACP [93, 95] or both pathways [94]. A potential involvement of the LCP has not been addressed in these studies.

The complement system of mammals plays an important role in the neutralisation and killing of viruses and virus-infected cells [101]. Although less attention has been devoted to the involvement of fish complement in viral infections than in bacterial infections, the neutralising activity of rainbow trout serum in response to viral haemorrhagic septicaemia virus (VHSV) has been shown to be dependent on a heat-labile component [102]. This activity was inhibited by both EDTA and EGTA, suggesting the involvement of the CCP. Moreover, fry of trout and masu salmon, *Oncorhynchus masou*, whose serum did not yet possess complement activity, were more susceptible to infectious haematopoietic necrosis (IHN) and infectious pancreatic necrosis

(IPN) viruses than were chum salmon fry, whose serum did have complement activity [103].

Moreover, complement can play a role in the immune response against infections with parasites. Complement present in the skin and serum of rainbow trout can be easily activated by the carbohydrate-rich outer layer of the monogenean, *Gyrodactylus derjavini*, leading to rapid killing of the parasite [104, 105]. Killing of *G. derjavini* or *G. salaris* by complement of salmonids appears to be mediated through the ACP only [104, 105]. The ACP has also been suggested to play a role in the immobilisation and lysis of theronts of the ciliate, *Ichthyophthirius multifiliis* [106]. The immune response against other parasites, such as the flagellate *Cryptobia salmositica*, has been shown to involve activation of both the CCP and ACP [107].

OPSONISATION

Opsonisation of pathogens by complement or Ig, results in an enhanced uptake by phagocytes and is mediated through ligand-receptor interactions between the surfaces of the two cells. Opsonisation by either component can be studied by comparing the effects of heat-inactivated and normal serum on the phagocytic activity of the leucocytes. Complement-mediated opsonisation has been observed in various fish species [71, 108–111]. Whether a pathogen will be opsonised by complement or antibody appears to be dependent on the host and type of pathogen involved. Both opsonins have been shown to be capable of acting alone [112, 113] and in tandem [108]. Unfortunately, the mechanisms that regulate the opsonisation process in fish are still poorly understood. As is true for its cytolytic activity, fish complement appears to exhibit efficient opsonic activity primarily against non-pathogenic bacteria [110, 114, 115]. Consequently, virulence is linked to the ability of pathogens to evade complement activity by preventing deposition of complement proteins on their surface. Identification of the compounds that allow pathogens to escape complement attack will be important for the development of therapies against virulent pathogen strains.

INFLAMMATORY REACTIONS

Recently, C3a anaphylatoxin molecules have been generated from rainbow trout C3-1, C3-3 and C3-4 [74]. All three molecules stimulated respiratory burst activity in trout head kidney cells, but only C3-1a and C3-3a were found to bind to a sub-population of these cells. Complement activation in trout and Japanese eel, *Anguilla japonica*, generated factors that had chemotactic activity for leucocytes [116, 117]. Although the nature of these chemotactic fragments is not yet known, these findings show promise that a complement-dependent anaphylatoxin system similar to that of mammals exists in fish.

IMMUNE COMPLEX CLEARANCE

In mammals, complement participates in the elimination of immune complexes. Immune complexes consisting of C3b/iC3b-Ag-Ig attach to CR-bearing cells, such as erythrocytes, and are transferred to fixed macrophages in the

liver and spleen, where they are eliminated [118, 119]. In fish, antigen is trapped in melanomacrophage centres (MMCs) in the spleen and kidney, and it has been suggested that these centres, together with other structures in the spleen, play a role in the clearance of immune complexes [120]. Whether complement and its receptors are involved in the translocation of these complexes to the spleen is yet unknown.

VII. Conclusions and Future Directions

The complement proteins of fish are structurally similar to those of mammals, hence the conservation of the three activation pathways. The most striking difference is that fish possess multiple isoforms of various complement proteins. The fact that these isoforms are products of different genes, at least in the case of Bf and C3, indicates that a set of gene duplications must have taken place before the appearance of the bony fishes. In addition, C3 genes of fish, like to those of higher vertebrates, are polymorphic and this may provide another mechanism through which diversity is added to the C3 molecules [37, 121]. The function of the multiple isoforms of complement proteins is not yet clear; however, the observation that the various C3 isoforms of trout and seabream have different specificities for various activating surfaces suggests that they differ in their involvement in the immune response against certain pathogens. Perhaps by analysing the proteins that are upregulated in acute phase responses we may begin to understand the involvement of these isoforms in various types of infections and in the maintenance of homeostasis.

During the past decade considerable progress has been made in identifying individual complement proteins in fish. However, in order to be able to state unequivocally that fish and mammals have identical complement activation pathways, we will have to isolate the remaining complement components and regulatory proteins from fish and perform biochemical and functional characterisation studies. For example, binding studies with C3b and C5 should demonstrate which C3 isoform is involved in the formation of the C5-convertase. In addition, it will be interesting to determine whether C3 convertases formed from a particular C3 isoform will be able to recruit a different isoform for the formation of the C5 convertase.

Another fascinating area is that of the complement receptors. Based on the finding that complement is able to modulate the antibody response in mammals through CR2 [122], it will be of great interest to study the existence of a similar function in fish. Moreover, complement stimulates phagocytosis in mammals by interacting with CR3. Because of the importance of innate immune responses in fish, it will be important to identify this receptor in these animals and study its functions. Thus far, no CR have been identified in fish, but the finding that catfish neutrophils express a beta 2 integrin molecule offers promise that a CR3-homologue will soon be identified in fish. Identification of the various complement components, regulatory proteins, and complement receptors in fish, as well as their interactions, is important not only as a means of better understanding the evolution of innate immune mechanisms but also for the development of vaccines and therapeutics in aquaculture.

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