Summary: In mammals the complement system plays an important role in innate and acquired host defense mechanisms against infection and in various immunoregulatory processes. The complement system is an ancient defense mechanism that is already present in the invertebrate deuterostomes. In these species as well as in agnathans (the most primitive vertebrate species), both the alternative and lectin pathway of complement activation are already present, and the complement system appears to be involved mainly in opsonization of foreign material. With the emergence of immunoglobulins in cartilaginous fish, the classical and lytic pathways first appear. The rest of the poikilothermal species, from teleosts to reptilians, appear to contain a well-developed complement system resembling that of homeothermic vertebrates. However, important differences remain. Unlike homeotherms, several species of poikilotherms have recently been shown to possess multiple forms of complement components (C3 and factor B) that are structurally and functionally more diverse than those of higher vertebrates. It is noteworthy that the multiple forms of C3 that have been characterized in several teleost fish are able to bind with varying efficiencies to various complement-activating surfaces. We hypothesize that this diversity has allowed these animals to expand their innate capacity for immune recognition.

Introduction

Recognition of non-self has been a fundamental trait for assuring survival in all forms of living organisms. A wide range of mechanisms of non-self recognition have emerged and evolved to fulfill the needs of each individual species. Such mechanisms have evolved and diversified in response to many factors including the environment in which the organism lives, its body complexity and physiology, and its life span. Hence, primitive organisms with simple body structures and with a short life span have developed and relied to a greater extent upon innate mechanisms of non-self recognition, whereas more evolved organisms have generally developed so-called adaptive-type responses.

Most invertebrate species studied to date rely on non-adaptive mechanisms of immune recognition, such as lectins, the prophenoloxidase activating system (ProPO), and a wide range of peptides and proteins with fungicidal, bactericidal, and anti-
viral properties (1–4). Surprisingly, invertebrates have also been shown to possess a complement system with components structurally and functionally similar to those from vertebrate species (Fig 1) (5–8).

With the appearance of vertebrates, immune recognition responses gradually evolved from innate to more adaptive mechanisms of non-self recognition, particularly after the emergence of immunoglobulins (9–11), major histocompatibility molecules (MHC) (12), and T-cell receptor (TCR) (13, 14) molecules in cartilaginous fish. However, within vertebrates, poikilothermic (cold-blooded) and homeothermic (warm-blooded) species have generated differing strategies of immune recognition. It appears that the warm-blooded vertebrates have shown more diversification in terms of their immune mechanisms involved in adaptive immune responses, whereas the cold-blooded vertebrates have relied upon and shown more diversification in their innate mechanisms. This difference is especially true in terms of the complement system. Recent evidence has shown that the components of the complement system in some cold-blooded species occur in multiple forms, and we have hypothesized that this complement diversity has been used by these animal species as a strategy to expand their innate immune recognition capabilities (15).

This paper will focus on the phylogeny of the complement system of poikilothermic vertebrates, with special emphasis upon the complement system of teleost fish and recent studies of the functional and structural diversity of some of its components, in particular the proteins C3 and factor B.

The mammalian complement system

The mammalian complement system consists of a complex group of about 35 soluble proteins and receptors that play an important role in host defense against infection and in various immunoregulatory processes. Activation of the complement system results in the initiation of many biological processes, including phagocytosis, lysis, inflammation, and regulation of the adaptive immune response (16–18). Indeed, one of the most important roles of the complement system is to bridge the innate with the adaptive immune response (19, 20).

Recent evidence has conclusively established a critical role for complement in the generation of the normal antibody response to T-cell dependent and T-cell independent antigens. It is clear now that animals deficient in either C3 or complement receptor type 2 (CR2) are incapable of generating a normal immune response (21).

The complement system is divided into three different pathways of activation, termed the classical, alternative, and lectin pathways. Each of the complement pathways produces a C5 convertase that cleaves C5 to C5b and C5a, with C5b initiating the self-assembly of the membrane attack complex (MAC) (22).

Alternative pathway

The alternative pathway is activated directly by a large number of microorganisms (viruses, bacteria, fungi, and protozoans, among others) (29) and its activation is antibody independent (18, 23). However, aggregated antibody has been shown to enhance the activation of this pathway (24). The alternative pathway is at a low level of steady-state activation due to the hydrolysis of the thioester group of native C3 to form C3(H2O), also termed C3 inactive. Once formed, C3(H2O) is able to bind to factor B in a Mg2+-dependent manner. Factor B, the catalytic subunit of the alternative pathway, is proteolytically activated and cleaved to Bb (66-kDa) and Ba (33-kDa) fragments by another serine protease, factor D, generating the enzyme C3 convertase (C3b,Bb). C3b,Bb is able to cleave native C3 to C3a and C3b; C3b through its thioester bond can then be covalently bound on the surface of nearby particles via ester or amide linkages. Most of the C3b generated in this manner is instantaneously inactivated by factor I in the presence of co-factor regulatory molecules (CR1, factor H, MCP, DAF) (25). However the C3b that is not inactivated, if deposited on activating surfaces, participates in an amplification loop of the activation process, a key feature of the activation of the alternative pathway. Consequently, after binding of factor B to the newly generated C3b, a new C3 convertase (C3b,Bb) is formed, which is further stabilized by properdin. The C3b,Bb complex becomes the enzyme C5 convertase (C3b,Bb,3b) when another C3b molecule binds to the C3 convertase (C3b,Bb), allowing the newly formed complex to cleave C5 into C5a and C5b. Activation of the pathway is very much dependent upon the microenvironment that surrounds the C3b-bound molecule, which will...
Time to present (million years)

Protochordates
- No antibody
- Complement: (ACP, LCP, C3)
- MHC: (allograft rejection)
  proPO, lectins

Arthropoda
- No antibody
- Complement: (ACP, LCP, C3)
- MHC: (allograft rejection)
  proPO, lectins

Echinodermata
- No antibody
- Complement: (ACP, LCP, C3)
- MHC: (allograft rejection)
  proPO, lectins

Ancestral protist
- No antibody
- Complement?
- MHC: (allograft rejection)
  proPO, lectins

Porifera
- No antibody
- MHC: (allograft rejection)
  lectins

Acoelomates
- Coelomates

Mollusca
- Annelida

Aves
- Reptilia

Agratha
- No antibody
- Complement: (ACP, LCP, C3)
- MHC: (allograft rejection)
  thymus, spleen

Chondrichthyces
- No antibody
- Complement: (ACP, LCP, C3)
- MHC: (allograft rejection)
  thymus, spleen

Osteichthyces
- No antibody
- Complement: (ACP, LCP, C3)
- MHC: (allograft rejection)
  thymus, spleen

Mammalia
determine whether amplification (binding of factor B to C3b) or abrogation of the pathway (binding of a regulator molecule to C3b) occurs (23, 26).

Classical pathway

Typically, antigen–antibody immune complexes activate complement through the classical pathway (17). However, various viruses and bacteria have been shown to activate the classical pathway in the absence of antibodies by directly interacting with the C1q subunit of the C1 complex (18, 27, 28). In addition, in the absence of antibodies, the classical pathway is also activated by the complex formed between C-reactive protein (CRP) and its ligand (29). Activation of the system by antigen–antibody complexes is primarily dependent on the IgM or IgG present in the immune complexes, which binds to the C1 complex via the Clq subunit (Fig. 2). This binding induces conformational changes in the C1 complex and leads to the activation of its C1r and C1s serine protease subunits.

Activation of the C1 complex enables it to associate with C4, which is cleaved to C4a and C4b by C1s. As occurs with C3b, C4b contains a thioester bond through which it can attach covalently to surfaces via their hydroxyl or amino groups (30). Thus, an antibody which has activated the C1 complex will have various C4 molecules surrounding the antibody–C1 site. Moreover, one molecule of activated C1s can cleave multiple molecules of C4, which represents an amplification step of the classical pathway. The next protein to bind is the serine protease C2. C2 binds to C4b in a Mg²⁺-dependent manner where it is cleaved by C1s to C2a and C2b. The C2a fragment (containing the catalytic site), together with C4b, forms what is called the classical pathway C3 convertase, C4b,2a (Fig. 2). This enzyme contains the catalytic site on the C2a fragment and is capable of binding and cleaving C3 to C3b, which is deposited in large amounts onto the target surface. This deposition of C3 serves two main purposes: to opsonize the target surface and facilitate its phagocytosis and to initiate the terminal reaction sequence by forming a C5 convertase (C4b,2a,3b). With the C2a fragment again functioning as the catalytic subunit of the convertase, C5 is cleaved to C5b, which is added to the complex.

Lectin pathway

The complement system can be directly activated by the binding of mannans to the complex composed of mannose-binding lectin (MBL) and the MBL-associated serine protease (MASP). Upon binding of MBL to mannans localized on the surface of various microorganisms, the MASP is able to cleave and activate C4 and C2 (Fig. 2) (31, 32). Nevertheless, there are two MASP isotypes, MASPl and MASP2. In contrast to C1s, the MASP1 isoform is also able to cleave C3 directly to C3b, which in turn activates the alternative pathway. MASP2, unlike MASPl, is able to cleave C4 (33). However, the distinct roles of MASPl and MASP2 are still unresolved.

Fig. 2. A scheme of the three pathways of complement activation.
Lytic pathway

All pathways of complement activation converge with the production of C5 convertase, which cleaves C5 to C5b and C5a (22). Thereafter, C5b initiates the self-assembly of the MAC. The MAC is a supramolecular organization of molecules that contains C5b, C6, C7, C8, and numerous molecules of C9 and is responsible for creating the transmembrane channels that lead to cell lysis (Fig. 2). The assembly of MAC and its insertion into the cell membrane occurs by the following sequence of events. The C5b-C6 complex binds C7 and exposes hydrophobic sites that are hidden within the molecule. This transition allows insertion of the C5b-C7 complex into a discrete site on the target membrane and it now serves as a receptor for C8. The C5b-C8 complex can then bind multiple molecules of C9 (n=1-18), and the polymerization of C9 molecules produces membrane lesions that are characteristic of the MAC (22). Although polymerization of C9 is not essential for lysis of erythrocytes and nucleated cells, it is believed to be required for the killing of bacteria. Like the classical and alternative pathways of complement activation, channel formation by the MAC is also under the control of serum and membrane regulatory proteins such as CD59 (34), which acts by inhibiting the C5b-8-catalyzed insertion of C9 into the lipid bilayer of the cell.

Phylogeny and diversity of the complement system in cold-blooded vertebrates

Complement-like activity has been detected in a variety of invertebrates (35, 36). However, the first and most ancient invertebrate species that has been demonstrated to contain complement molecules (factor B (L. C. Smith, personal communication) and C3 (5)) is the sea urchin (Fig. 1), an echinoderm. More evolved invertebrates such as tunicates have also been shown to contain complement molecules such as C3 and MASP (6-8). The presence of MASP in tunicates led to the hypothesis that the lectin pathway was the first complement pathway. However, since a factor B gene has been cloned from sea urchins (a more primitive species), it now appears that the alternative pathway may have predated the appearance of the lectin pathway. Nevertheless, the order in which the pathway emerged is still an open question, and more primitive animal species need to be studied for the presence of both the alternative and lectin pathways.

In invertebrates, the complement system may have emerged as a simple system comprising a small number of components (perhaps C3, factor B and D and/or C3, MASP, and MBL) with limited functions, possibly only involved in the opsonization of foreign material. Gene duplication in combination with exon shuffling, including sequential addition or deletion of several types of modules or domains from various proteins, has given the functional and structural complexity that complement embraces in vertebrate species.

Complement activities have been demonstrated in the most ancient group of vertebrates (the jawless fish), with the alternative and lectin pathways being the only pathways detected thus far in this group; these fish lack the classical pathway and MAC (37, 38). The cartilaginous fish (sharks) appeared after the jawless fish, and seem to have molecules functioning in both complement pathways (39); however, the nature and the similarities to mammalian complement components are ill defined. All other vertebrates (including teleost fish, amphibians, reptiles, birds, and mammals) present a well-developed complement system that includes both the alternative and classical pathways of activation, including the MAC (Fig. 1). The lectin pathway has not been extensively studied in all species, although the presence of MASP in tunicates, lampreys, sharks, carp, and frogs suggests that this pathway is present in all of these species (40, 41).

One of the most fascinating features of the complement system in cold-blooded animal species is the structural and functional diversity of some of its components, such as C3 and factor B. We have suggested that this complement diversity is a strategy to expand the innate immune recognition capabilities of these animals (15).

Below we will examine the most important properties of the complement system in poikilothermic vertebrates giving special emphasis to the complement system in teleost fish and the structural and functional aspects of their duplicated complement components.

Cyclostomata

The jawless fishes of the class Agnatha are the most primitive of all living vertebrates. The only surviving members of this class, the cyclostomes, are the lampreys and the more primitive hagfishes. An alternative pathway in the lamprey, similar to that in mammals, was initially identified on the basis of zymosan's ability to activate this pathway.

A protein homologous to mammalian C3/C4, which has been described as a C3 relative, has been isolated and cloned from the lamprey (Lampreja japonica) (42). This protein has been shown to have opsonic activity, thus implying the presence of complement receptors in lampreys. The lamprey C3 is similar to C4 in having a three-chain structure (α, β, and γ) (43) but is more homologous to C3 in terms of amino acid sequence (Table 1). It has been observed, however, that a) the C3 convertase cleavage site in lamprey C3 is similar to that of C4 and not
would appear that the hagfish C3 cannot be cleaved at the RRR-
sequence corresponds to the isolated two-chain C3, then it
may include two different forms of C3. If the available cDNA
These findings suggest that the hagfish complement system
ature, but it was also later purified as a two-chain structure.
and y chains (46), an arrangement that would suggest a three-
cluster of three basic residues (KRR) between the putative a
and p chains and another
residues (RKKR) between the putative
hagfish C3 cDNA, however, shows one cluster of four basic res-
with lytic activity has been described in lamprey serum.
complement activation (37), although a single component
found to lack the MAC, which is responsible for cell lysis upon
complement activation (37), although a single component
with lytic activity has been described in lamprey serum.

Hagfish (Eptatretus stouti) C3, originally identified as hagfish
Ig (44), is apparently a two-chain structure, containing a
thioester site in the α chain (37). Like lamprey C3, it also acts
as an opsonin (45). The primary structure deduced from the
hagfish C3 cDNA, however, shows one cluster of four basic res-
duies (RRRR) between the putative α and β chains and another
cluster of three basic residues (RRR) between the putative α
and γ chains (46), an arrangement that would suggest a three-
chain structure, similar to that of lamprey C3.

Hagfish C3 was initially reported to be a three-chain struc-
ture, but it was also later purified as a two-chain structure. These findings suggest that the hagfish complement system
may include two different forms of C3. If the available cDNA
sequence corresponds to the isolated two-chain C3, then it
would appear that the hagfish C3 cannot be cleaved at the RRR-
linker region between the putative α and γ chains. In further
support of the two-chain structure for hagfish C3 are data
showing that the N-terminal sequences of the α chain and
β chains of the purified three-chain C3 correspond to the α and
the γ chain minus the first residue; in addition, data have shown
this three-chain molecule to be the product of enzymatic
degradation. A similar situation might occur in the case of lam-
prey C3 protein, which has been purified as a three-chain mol-
cule. Unfortunately, a lack of N-terminal sequence data for the
individual chains of the isolated lamprey C3 does not make it
possible to conclude that the lamprey C3 is indeed a three-
chain molecule.

From all the data reported thus far it appears that cyc-
losteome complement is representative of an early intermediate
stage in complement phylogeny. In addition, the antibody-
mediated classical pathway activity is absent in cyclostomes,
since these animals do not have antibody molecules. Also rep-
resentative of an early intermediate stage in complement phy-
logeny is the lamprey factor B molecule. The sequence of this
molecule has been shown to be equally similar to those of mouse factor B and C2, suggesting that it represents a stage
before the Bf/C2 gene duplication (47). It is interesting that in
contrast to sea urchin factor B (which has five short consensus
repeat (SRC) domains (L. C. Smith personal communica-
tion)), lamprey factor B contains three SCR domains, as do all
remaining factor B and C2 molecules characterized to date.

### Table 1. Amino acid sequence conservation between C3 and other related proteins

<table>
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<tr>
<th></th>
<th>Hu C3</th>
<th>Rb C3</th>
<th>Ra C3</th>
<th>Mo C3</th>
<th>GP C3</th>
<th>Ch C3</th>
<th>Xe C3</th>
<th>Co C3</th>
<th>Tr C3</th>
<th>La C3</th>
<th>Hu C5</th>
<th>Hu αm</th>
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<td>44/59</td>
<td>32/48</td>
<td>31/47</td>
<td>28/43</td>
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<td>79/86</td>
<td>78/89</td>
<td>52/67</td>
<td>51/65</td>
<td>50/66&lt;sup&gt;2&lt;/sup&gt;</td>
<td>43/58</td>
<td>33/48</td>
<td>29/45&lt;sup&gt;3&lt;/sup&gt;</td>
<td>27/42</td>
<td>29/45&lt;sup&gt;3&lt;/sup&gt;</td>
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<tr>
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<td>81/88</td>
<td>54/70</td>
<td>53/68</td>
<td></td>
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<td>33/48</td>
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<td>11/27</td>
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</table>

Abbreviations: Hu, human; Mo, mouse; Ra, rat; Rb, rabbit; Xe, Xenopus; Co, cobra; GP, guinea pig; Tr, trout; Po, pig; Ch, chicken; La, lamprey; Ha, hagfish

<sup>1</sup>Partial amino acid sequence

<sup>2</sup>Identity/identity plus similarity (%)

<sup>3</sup>All alignments were performed using Clustal program of PCGENE with open gap cost 7 and unit gap cost 2 except the ones indicated with r where open gap cost 15.

All numbers are rounded to nearest integer.
Chondrichthyes

The complement of cartilaginous fishes has been best studied in the nurse shark, Ginglymostoma cirratum. An early study of the nurse shark (39) revealed six functionally pure complement-like molecules that interact sequentially and provide classical equivalent and lytic pathways. Given the variety of immunoglobulin types in the shark (IgM, NAR, IgNARC (10, 11, 13)) it would be of great interest to determine which types are able to activate the classical pathway. Evidence for the presence of the lectin pathway in cartilaginous fish comes from the cloning of a MASP gene in sharks (40). As in the case of lampreys, only one MASP molecule has been cloned from sharks, a molecule that resembles the MASP2 of other animal species. Further studies on the nurse shark complement have described the presence of a functional alternative pathway as well as the characterization of molecules resembling C3, C4, factor B, and factor H (48-50).

Sharks occupy a privileged phylogenetic position since they are the most primitive species containing part of the molecular machinery required to mount an adaptive immune response (i.e. antibodies (13), TCR (14), and MHC class I and II molecules (12)). In addition, cartilaginous fish would be expected to possess the most simple stages of the classical and lytic pathways, since these two pathways are not present in anagathan fish. Therefore, further studies of specific components of the complement system of sharks are required to shed new light on the phylogeny of the complement system.

Osteichthyes

Of all the fish, the complement system of the teleost (bony) fishes has been the best studied; both the classical and alternative pathway activities have been characterized. The only evidence for the presence of a lectin pathway in teleost fish comes from the cloning of a gene encoding a MASP-like molecule in carp (Cyprinus carpio) (41). In addition, a molecule similar in structure and function to mammalian MBL has been described in the Atlantic salmon, Salmo salar (51). Complement function in these fish differs in a number of important ways from that in mammals: the optimal activation temperature for fish complement is 20-25°C (52, 53), whereas that for mammalian complement is 37°C. Moreover, the inactivation temperature of fish complement is 40-45°C (53, 54), whereas in mammals it is 50°C for the alternative pathway and 56°C for the classical. These differences may reflect the ambient environmental temperature of the water in which the fish live. Classical pathway titers in teleost fish are similar to those in mammals (52, 55), but alternative pathway titers in fish are 5-10 times higher (52, 53). Furthermore, in contrast to human complement, complement from a variety of fish can lyse the erythrocytes of sheep, goats, dogs, and humans with high efficiency through the alternative pathway (53). Because in fish the antibody response is quite rudimentary and not as well developed as in mammals, it is postulated that complement plays the leading role in protecting fish from potential pathogens.

One of the most fascinating properties of the complement system of teleost fish is that some of its components (C3 and factor B) are present in multiple isoforms that are the products of different genes and differ in their structure and function. Teleost fish are the first living organisms in which multiple forms of functionally active C3 have been characterized; and these isoforms are the products of various genes. The most important feature of these C3 isoforms is that they differ in their binding efficiencies to a number of complement-activating surfaces. Specifically, four C3 isoforms (C3-1, C3-2, C3-3, and C3-4) have been characterized in the rainbow trout, a quasi-tetraploid fish (56, 57). In the carp (a tetraploid teleost fish), as many as eight different PCR clones showing 85-98% amino acid identities with one another have been characterized and found to be highly homologous to C3 from all other species. Whether all these isoforms are allelic variants or products of different genes is still unknown; however, all the clones were amplified from a cDNA library derived from a single fish (58, 59). The existence of multiple forms of C3 in trout and carp might be attributed to their tetraploid condition. However, five different forms of C3 have also been purified and characterized in the gilthead sea bream (Sparus aurata) (C3-1, C3-2, C3-3, C3-4, and C3-5) (60, 61), a diploid teleost fish (Fig. 3), indicating that multiple forms of C3 are not unique to tetraploid animals. Medaka fish (Oryzias latipes), another diploid fish, have recently been shown to contain three C3 genes (62). Therefore, multiple genes for C3 appear to be a general feature of all teleost fish.

Biochemical characterization of multiple C3 isoforms

The trout and sea bream isoforms were first identified at the protein level by looking for proteins with a chain structure similar to that of C3 and by testing the reactivity of these molecules with anti-C3 antibodies. All the purified C3 isoforms were found to contain an α and a β chain (Figs 3 & 4) and to have a thioester bond in the α chain. However, they differed in their electrophoretic mobility, glycosylation, reactivity with monospecific C3 antibodies, and the N-terminal amino acid sequences of their chains (Table 2) (56, 60). Cloning of the genes encoding for the trout C3 isoforms has recently been completed (63). A comparison of the deduced amino acid sequences showed that the sequence identity/similarity of C3-3 to C3-4 is 76.5%/81% while that of C3-3 and C3-4 to C3-1
Fig. 3. Characterization of the sea bream C3 isoforms. A. Purified gilthead sea bream C3 proteins resolved on 7.5% SDS-PAGE under reducing conditions and stained with Coomassie blue.
B. Concanavalin A-binding carbohydrates of the α and β chains of the various C3 proteins. C. Incorporation of [4C]-methylamine in the α chain of the various C3 isoforms (except for the C5 molecule, all C3s incorporated [4C]-methylamine in their α chains, indicating the presence of an active thioester bond).


is 54.6/66.6% and 54.4/67% respectively. Interestingly, the β chain of C3-4 contains two insertions of 65 (residues 504-569), and 23 amino acids (residues 123-146), while the β chain of C3-1 contains a 14 amino acid insertion (residues 143-157). The sequence of the thioester site is conserved in all three trout C3s, corroborating previous evidence for the presence of such a thioester at the protein level (Fig. 4) (56). The C3 convertase cleavage site (Arg-Ser) is conserved in the three trout isoforms, whereas the factor I cleavage sites are Arg-Ala (C3-1, C3-4) and Arg-Thr (C3-3), instead of Arg-Ser at position 1281 of human C3, and Arg-Thr (C3-1, C3-3) instead of Arg-Ser at position 1298 of human C3, as well as trout C3-4. It is of special interest that the His residue (His133 of the human C3d molecule), which is involved in catalyzing the binding reaction of the thioester (64, 65), is only present in trout C3-1 and C3-3, whereas the corresponding residue in C3-4 is Thr. Furthermore, the Glu residue closest to His133 (Glu135 in the human C3d) is thought to be responsible for the higher rate of acylimidazole formation in human C3 than in C4b (66); human C4b has a Ser in equivalent position, and that is why its thioester has a slower reactivity. Trout C3-3 and C3-4 have a Thr and an Ile residue, respectively, in the equivalent position, whereas trout C3-1 (like human C3) has a Glu. All these amino acid differences in the residues of trout C3-3 and C3-4 that appear to relate to the reactivity of the thioester may be responsible, at least in part, for the differences observed in the reactivity of the trout C3s with a variety of complement-activating surfaces (56).

Trout C3-2 yields a tryptic peptide map that differs significantly (a 20% mismatch in the peptides) from that of C3-1; this molecule is apparently hemolytically inactive, although it contains a thioester bond (57).

Functional characterization of C3 isoforms

Without a doubt, the most significant feature of the C3 isoforms isolated from trout and sea bream is the difference in their efficiencies of binding to various complement-activating surfaces (zymosan, E. coli, and rabbit and sheep erythrocytes) (56, 61). Although from a biochemical point of view the trout and sea bream C3 isoforms are apparently not related, in terms of binding efficiencies there seem to be some homologies among the C3 isoforms from both fish species. For example, the most abundant C3 isoforms from both fish species (trout C3-1 and sea bream C3-1 and C3-2, each 1-2 mg/ml in serum) bind with high efficiency to zymosan particles (a potent activator of the alternative pathway). In contrast, the less abundant C3 isoforms (trout C3-3 and C3-4 and sea bream C3-3, C3-4, and C3-5 (0.2-0.4 mg/ml)) do not bind to zymosan particles.
Fig. 4. Characterization of the trout C3 isoforms. A. Purified trout C3 proteins resolved on 7.5% SDS-PAGE under reducing conditions and stained with Coomassie blue. B. Concanavalin A-binding carbohydrates of the α and β chains of the various C3 proteins. C. Incorporation of 14C-methylamine in the α chain of the various C3 isoforms (all the C3s incorporated 14C-methylamine in their α chains, indicating the presence of an active thioester bond).

Table 2. Properties of the gilthead sea bream C3 and C5 proteins

<table>
<thead>
<tr>
<th>C2 isoform</th>
<th>C3-1</th>
<th>C3-2</th>
<th>C3-3</th>
<th>C3-4</th>
<th>C3-5</th>
<th>C5</th>
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<tbody>
<tr>
<td>Chain structure</td>
<td>α/β</td>
<td>α/β</td>
<td>α/β</td>
<td>α/β</td>
<td>α/β</td>
<td>α/β</td>
</tr>
<tr>
<td>M, αβ x 10^4</td>
<td>112/65</td>
<td>112/70</td>
<td>112/70</td>
<td>120/60</td>
<td>115/60</td>
<td>112/70</td>
</tr>
<tr>
<td>Thioester in</td>
<td>α</td>
<td>α</td>
<td>α</td>
<td>α</td>
<td>α</td>
<td>Not present</td>
</tr>
<tr>
<td>Con A binding to</td>
<td>α</td>
<td>α</td>
<td>α</td>
<td>α</td>
<td>α</td>
<td>α/β</td>
</tr>
<tr>
<td>Binding to:</td>
<td>Anti-CVF Ab</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Anti-trout C3-1 Ab</td>
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<td>+++</td>
<td>+</td>
<td>+</td>
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<td>Anti-trout C3-3 Ab</td>
<td>-</td>
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<td>-</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Anti-trout C3-4 Ab</td>
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<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
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<td>No. of amino acids (identical/different) to</td>
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<td>(36/1)</td>
<td>(20/17)</td>
<td>(31/6)</td>
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<tr>
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<td>(31/7)</td>
<td>(35/1)</td>
<td>(5/22)</td>
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<td>Sea bream C3-3</td>
<td>(38/0)</td>
<td>(19/19)</td>
<td>(19/17)</td>
<td>(6/21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sea bream C3-4</td>
<td>(38/0)</td>
<td>(30/6)</td>
<td>(4/22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sea bream C3-5</td>
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<td>(42/23)</td>
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<tr>
<td></td>
<td>Sea bream C5</td>
<td>(27/0)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

The reactivity of the sea bream C3 isoforms with the various antibodies was assessed by ELISA.

This anti-trout C3-1 antibody recognized only the β-chain of C3.

This anti-trout C3-1 antibody recognized the native C3-1 molecule.

The residues were obtained by sequencing the NH2-termini of the α- and β-chains of the various C3 isoforms and C5.

Table 3. Binding of sea bream and trout C3 isoforms to various complement activating surfaces

<table>
<thead>
<tr>
<th>Surface</th>
<th>Binding*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. bream C3-1</td>
<td>S. bream C3-2</td>
</tr>
<tr>
<td>Sheep RBC ghosts</td>
<td>++++</td>
</tr>
<tr>
<td>Rabbit RBC ghosts</td>
<td>+++</td>
</tr>
<tr>
<td>Zymosan</td>
<td>+++</td>
</tr>
</tbody>
</table>

Abbreviations: RBC, red blood cells; EGTA, [ethylenebis(oxyethylenenitrito)]-tetraacetic acid

* Symbols represent the extent of binding to the tested surface in the presence of Mg2+EGTA

- no binding; +, low binding; ++, intermediate binding; ++++, good binding; ++++, very good binding

Table 2. Properties of the gilthead sea bream C3 and C5 proteins

Table 3. Binding of sea bream and trout C3 isoforms to various complement activating surfaces
Another similarity is the higher binding efficiency of all the C3s to the various surfaces in the absence of Mg\textsuperscript{2+}-EGTA, an observation which suggests that all of the C3 isoforms can be activated through the classical pathway (56, 61). A significant difference, however, can be observed between the binding efficiencies of the trout and sea bream isoforms to sheep erythrocyte ghosts in the presence of Mg\textsuperscript{2+}-EGTA, a situation which allows only the activation of the alternative pathway: while the trout C3s did not bind to sheep erythrocyte ghosts (except for the weak binding of C3-3 and C3-4), all the sea bream C3 isoforms bound with high efficiency to that surface (Table 3). This difference in the binding to sheep erythrocyte ghosts correlates with the very low hemolytic activity observed for trout serum against sheep erythrocyte and the high hemolytic activity of sea bream serum against sheep erythrocyte through the alternative pathway (56, 61). This suggests that the specificity of the C3 for the surface may be responsible, at least in this case, for determining the cytolytic action of the complement system when activation occurs through the alternative pathway. At this point, it is unclear whether these differences in binding efficiency are the result of a change in affinity between the C3 isoforms and the regulatory proteins of the complement system (such as factor B and H) or a change in the surface specificity of the C3s.

Not only does C3 exist as the product of several genes but it appears that some of these genes in trout (67) and carp are quite polymorphic (58). This polymorphism may have important functional consequences: for example, the product of a particular C3 allele could react with a specific pathogen, conferring resistance on the fish carrying that allele. A combination of polymorphism and gene duplication could together generate a large C3 “repertoire”. In the sea bream a surprising degree of specificity of the C3 convertase for its respective C3 isoforms has been observed (J. O. Sunyer, J. D. Lambris, unpublished observations); this specificity might be a mechanism to avoid activation of all the C3 isoforms each time a specific C3 convertase is activated. Such specificity may have important biological consequences for the survival of the fish.

Thus far, this structural and functional diversity of C3 has been demonstrated only in fish. Nevertheless, a related situation has been observed for human C4, which exists in two different isoforms, C4A and C4B. Although the two molecules have very few amino acid differences (13 substitutions in 1,722 residues), C4A binds preferentially to surfaces carrying amino groups, whereas C4B binds with higher affinity to those containing hydroxyl groups (68, 69). The existence of multiple forms of C3 that differ in their binding properties may well serve to augment the number of potential pathogens that the animal can recognize, and thus help expand the immune recognition capabilities of fish (15).

Presence of a unique molecule with factor B and C2 functions

Another unusual feature of the complement system of teleost fish concerns the function of the factor B protein. In mammals, the factor B (Bf) and C2 complement genes are closely linked within the MHC class III region, and they show a similar domain structure and genomic organization (70). Therefore, both factor B and C2 are thought to have arisen by gene duplication from a single gene encoding an ancestral molecule; the animal phylum in which this duplication event took place is unknown. Two teleost fish, the zebrafish (Brachydanio rerio) and medaka fish, have each been shown to possess a single molecule that shows an equivalent degree of similarity to mammalian Bf and C2 (71, 72). In contrast to the situation in these teleosts, trout have recently been found to have not one but two factor B molecules (Bf-1 and Bf-2) that show ~9% more sequence similarity to mammalian factor B than C2 (73). Trout Bf-1/Bf-2 showed amino acid identities of 38/39% to human factor B, 30/31% to human C2, 37/38% to mouse factor B, 28/31% to mouse C2, 34/33% to Xenopus factor B-A, 33/32% to Xenopus factor B-B, 50/51% to medaka fish factor Bf/C2, 42/43% to zebrafish factor B, and 16/22% to lamprey factor B. Trout Bf-1 was 75% identical to Bf-2. The full-length Bf-1 and Bf-2 cDNAs are 2,509 and 2,560 bp respectively. Similarly to factor B and C2 molecules from other species, the Bf-1 and Bf-2 molecules consisted of three SCR at the N-terminus, a von Willebrand domain, and a serine protease domain at the C-terminus. The distribution of the cysteine residues was highly conserved. Residues His, Asp and Ser, which are located at the active center of the serine protease domain, were also conserved, as well as the amino acids involved in magnesium binding. Both trout Bf genes are mainly expressed in liver (Fig. 5) and appear to be single-copy genes. A phylogenetic tree generated from all available factor B and C2 sequences showed that, regardless of the higher sequence similarity of trout Bf-1 and Bf-2 to mammalian factor B than to C2, both trout factor B molecules clustered with the mammalian C2 sequences (Fig. 6).

Since phylogenetic and sequence analysis did not indicate whether the trout molecules were factor B or C2, the only other viable approach was to analyze their functions. As factor B-like molecules, the purified Bf-1 and Bf-2 (Fig. 7) proteins are able to form the alternative pathway C3 convertase and are cleaved (in the presence of purified trout C3, trout factor D, and Mg\textsuperscript{2+}-EGTA) into Ba- and Bb-like fragments (Fig. 7, lane 6), in a manner similar to that for mammalian factor B. The formation
Fig. 5. Tissue-specific expression of Bf-1 (A), Bf-2 (B), and EFTu-1 (C) by Northern blot. Fifteen micrograms of total RNA from specified tissues of 1 year-old rainbow trout were electrophoresed, blotted to nylon and sequentially hybridized and stripped with probes corresponding to trout Bf-1 (A), Bf-2 (B), and EFTu-1 (C). Strong expression of Bf-1 (~3 kb) and Bf-2 (~2.7 kb) was found primarily within the liver (1 day’s exposure), but with prolonged exposure (7 days) weak intestinal expression of Bf-1, but not of Bf-2, was observed.


of the C3-convertase was Mg$^{2+}$ dependent, and in the presence of EDTA (Fig. 7, lane 5) C3 and Bf-2 were not cleaved. The most remarkable feature of trout Bf-2 (in contrast with its position in the phylogenetic tree) is its requirement for both the classical and alternative pathways of complement activation, implying a dual function for Bf-2 as a C2 and Bf. Most particularly, Bf-2 was shown to completely restore the hemolytic activity of trout Bf-depleted serum through both the alternative and classical pathways (Fig. 8). Whether Bf-1 possesses a similar activity is unclear at present. This finding suggests that before the divergence of C2 and factor B from a common ancestor, a molecule existed that was able to function in both alternative and classical pathways. Although the presence of functional alternative and classical pathways in teleosts might suggest the presence of a C2 molecule, these recent results imply that in teleost fish some of the components may be shared by the two pathways.

Factor D, C1 and C4 molecules
Factor D has been purified from carp (74) and trout (73); in both cases the amount in serum (6 µg/ml in carp and 25–50 µg/ml in trout) was higher than that in humans. A factor D gene has been cloned in the brook trout (Salvelinus fontinalis), and its sequence shows a high degree of homology to factor D from other species (75).

A putative C1-like molecule has been described in carp on the basis of its molecular weight and functional properties (76); however, a definitive characterization of C1 at both the protein and DNA level awaits further assessment. Proteins with similarity to mammalian serum amyloid P (SAP) and CRP have been identified in various teleost fish (77–80). Both SAP and CRP are members of the pentraxin family and are able to interact with C1q, activating the classical complement pathway. In addition, SAP and CRP are known to be acute phase (AP) proteins, and their concentration in serum increases dramatically after inflammation. The pentraxin-like molecules isolated from various teleost fish seem to play a role in activation of the classical pathway (81). However, there are contradictory reports describing either a dramatic increase or a decline in these molecules in fish serum after challenging the fish with an

Fig. 6. Phylogenetic tree of factor B and C2 protein sequences. The tree was generated by the neighbor-joining method, based on the entire sequences and the alignment of Fig. 1. Numbers on the branches show the percentage recovery in 1,000 bootstrap replications.

stimulus. Two different pentraxin molecules have apparently been isolated from rainbow trout, one being designated CRP and the other SAP (80). Whether these pentraxin proteins are in fact SAP or CRP is a matter of debate. However, on the basis of a cDNA sequence of a pentraxin molecule from salmon and trout it has been suggested that teleost fish, as well as frogs, contain an ancestral molecule from which SAP and CRP arose (80). Evidence for the existence of a C4 molecule comes from the cloning of a C4-like component with high homology to mammalian C4 from the medaka fish (62), suggesting that the gene duplication that gave rise to C4 may have happened before the divergence of teleost fish from the main line of vertebrate evolution.

**Terminal complement components**

Molecules homologous to C5 have been demonstrated in trout (82, 83), sea bream (60), and carp (84, 85). As in the case of C5 from other species, trout and sea bream C5 molecules do not contain an active thioester bond, and they show an α and β chain structure. In the sea bream the N-terminal amino acid sequence of both chains shows a greater resemblance to mouse C5 than to any of the various sea bream C3 isoforms. The MAC has been characterized in trout (83) and carp (84) and is very similar in structure to the mammalian MAC. Components C5-C9 have been identified from the carp MAC, and C8 and C9 have been purified to homogeneity (85). As in humans, carp C8 was found to be composed of three polypeptide chains, of 62, 62, and 22 kDa, whereas carp C9 has a molecular mass of

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**Fig. 7.** Formation of fluid phase alternative pathway C3 convertase with purified trout C3-1, Bf-2 and D proteins. Trout C3-1 (2 μg), Bf-2 (1 μg), and trout factor D (0.02 μg) were incubated together in the presence of EDTA or Mg²⁺-EGTA. Reaction mixtures were incubated for 40 min at room temperature (~20°C) and electrophoresed on 7.5% SDS-PAGE under non-reducing conditions and stained with Coomassie blue. Lanes 2, 3, and 4 show the purified trout C3-1, trout Bf-2, and trout factor D respectively. [Reproduced with permission of Sunyer JO, Zarkadis IK, Sarrias MR, Hansen JD, Lambris JD. Cloning structure and function of two rainbow trout Bf molecules. J Immunol 1998;161:4106–4114. Copyright 1998. The American Association of Immunologists.]

**Fig. 8.** Lysis of sensitized sheep erythrocytes (A) and rabbit erythrocytes (B) with Bf-depleted trout serum reconstituted with purified Bf-2. Trout serum was depleted of Bf-1 and Bf-2 by affinity chromatography, and its hemolytic activity through the classical pathway (CCP) or alternative pathway (ACP) was then restored by adding various amounts of purified Bf-2. The brown circles represent the amount of Bf-2 present in normal trout serum (X-axis) plotted against the hemolytic activity of normal trout serum (Y-axis) through the alternative (B) or classical pathway (A). CH50, ACH50: the reciprocal of the serum dilution causing 50% lysis of erythrocytes; results are presented as CH50 and ACH50 arbitrary units/ml for the classical and alternative pathways respectively. [Reproduced with permission of Sunyer JO, Zarkadis IK, Sarrias MR, Hansen JD, Lambris JD. Cloning structure and function of two rainbow trout Bf molecules. J Immunol 1998;161:4106–4114. Copyright 1998. The American Association of Immunologists.]
Fig. 9. Degradation of various C3 by factor I and H-like proteins from sea bream serum and metal dependence of this activity. Supernatants resulting from the incubation of sheep erythrocyte ghosts with 
\[^{125}\text{I}\]labeled sea bream C3-1, C3-2, C3-3, C3-4, or C3-5 and sea bream serum in the presence (panel B) or absence (panel A) of EDTA were analyzed on
a 9% SDS-PAGE gel under reducing conditions. The different C3s were
hydrolyzed with methyamine and then incubated with sea bream serum
in the presence (panel C) or absence (panel D) of EDTA and analyzed on
9% SDS-PAGE as outlined above. The gels were then dried and subjected
to autoradiography.

93 kDa, higher than that of human C9 (71 kDa). Carp serum
depleted of C8 failed to hemolyze both carp antibody-sensi-
tized sheep erythrocytes and non-sensitized rabbit erythro-
cytes. C9-depleted carp serum was able to hemolyze only the
antibody-sensitized sheep erythrocytes, as is true of C9-
depleted human serum. C9 is the only component from the
teleost fish MAC (rainbow trout) that has so far been cloned
(86). Unlike C9 from mammalian species, trout C9 was found
to contain an additional carboxyterminal thrombospondin
domain. This domain (present in all the other C6-C8 terminal
components in mammals) apparently is not restricting poly-
merization, since circular membrane attack complement
lesions were clearly observed on the surface of rabbit erythro-
cyte membranes upon activation of the trout serum through
the alternative pathway (87).

Complement regulatory proteins
Factor H- and I-like activities have been demonstrated in the
sera of various teleost fish, implying the presence of homolo-
gous factors in these animals. The five different sea bream C3
isoforms have been shown to be cleaved in serum to "iC3b" by
factor H and I-like proteins, generating fragments similar to
those generated from C3 molecules of other species (Fig 9); in
contrast to the situation in higher vertebrates, the generation of
such fragments is highly dependent on metal ions (61). SBP1,
a protein with factor H and/or C4bp-like activity, has been
purified and cloned from sand bass (Pomaleus nebulifer), a teleost
fish (88-90). The protein has three subunits: two identical
polypeptides of 110 kDa and a 42-kDa subunit that is antigen-
ically different from the 110-kDa polypeptide. The gene encod-
ing the larger subunit has been cloned, and its cDNA sequence
indicates a molecule containing 17 SCRs. The gene encoding an
additional SCR-containing molecule (SBCFR-1) has been
cloned from the same fish species; this molecule contains three
SCRs showing high sequence similarity to the SBPl SCRs. A
novel cDNA obtained through differential-display PCR of phor-
bol ester-stimulated ovarian tissue has been characterized in the
brook trout (Salvelinus fontinalis). This cDNA encodes a protein of
88 amino acids with high sequence similarity to CD59 (a
membrane protein whose functions include inhibiting C9
insertion into the target membrane); this protein has been pos-
tulated to be involved in complement regulation (91). Compl-
ent-dependent phagocytosis has been demonstrated in vari-
ous fish (92), and therefore, complement receptors are pre-
sumed to be present in teleost fish. The integrin \(\beta_3\) chain, a sub-
unit of CR3, has recently been cloned in carps (93) and con-
firms the functional data showing complement-mediated
phagocytosis in these animals.

The complement system in aquaculture
Since the complement system in teleost fish plays a leading role
in immune defense, a better knowledge of this system has an
immediate impact on health-related aspects of the rapidly
growing fish-farm industry. One of the major problems in
farming is the stress that fish suffer in these farms. Stressful conditions have been shown to lead to immunodepression in various fish species, and the fish therefore become more susceptible to infection (94). Under such conditions, complement activities in fish are seriously affected and can decrease to 50% of normal levels (95, 96). Therefore, all of the immune activities related to the complement system (including the bacterial activity of serum, viral inactivation, phagocytosis, and lysis) also become depressed. This immune depression might explain, at least in part, the higher susceptibility of stressed fish to infection by potential pathogens. In fact, complement activity has been shown to be a suitable indicator of immunocompetence in fish (97).

One of the strategies for infection prevention that is currently being studied is the use of immunostimulants. β-Glucans are immunostimulants that have been shown to increase the resistance of fish to several pathogens. An effect that has been observed after the administration of such immunostimulants is a significant increase in the activity and titers of the complement system (98, 99), emphasizing once more the very important role of the complement system in maintaining good health status in fish.

To date, these studies have only taken into account phagocytic, hemolytic, and bactericidal activities of complement. Further studies of specific components of the complement system are necessary if we are to understand the mechanisms that lead to the depression of these complement-mediated activities in stressed fish and the influence of immunostimulants on specific complement components.

**Amphibia**

Frogs have been shown to have alternative and classical pathway activities as well as the lytic pathway (100). Evidence for the existence of the lectin pathway is based on the recently cloned Xenopus MASp1 and MASp2 molecules (40, 41). C1 has been characterized from the bullfrog Rana catesbiana and has been shown to be functionally and structurally similar to C1 in mammalian species. This C1q component was similar in mass, chain structure, and amino acid composition to those of C1q in higher vertebrates (101). C3 has been purified from the African clawed frog, Xenopus (100), and the salamander (axolotl), Ambystoma mexicanum (102), and both have been found to resemble mammalian C3 in having a characteristic two-chain (α, β) structure and a thioester bond in the α chain. We have recently shown that the axolotl C3 is expressed in the regenerating tissue during urodele limb regeneration. C3 was found to be expressed in the dedifferentiated regeneration blastema and in the redifferentiated limb tissues. In the early stages of regeneration, C3 appeared to be equally present in all mesenchymal cells and in the wound epithelium, whereas in the later stages it was mainly expressed in the differentiating muscle cells. Since no expression was seen in the developing limb, it appears that the C3 expression was specific to the regeneration process. These findings implicate C3 in the dedifferentiation process and may indicate a new role for this molecule in muscle differentiation (103).

The cDNA sequence of the C-terminal α-chain region (residues 1299-1641 based on human C3 numbering) of Xenopus laevis C3 (104) and 95% of the C3 cDNA sequence of a Xenopus laevis/Xenopus gilli (Xenopus LG) hybrid have been obtained (105). The deduced amino acid sequences show that the C3 convertase and second factor I cleavage sites (Arg-Ser) are conserved in Xenopus C3; protein sequencing of the Xenopus C3 fragments fixed on zymosan during complement activation have demonstrated that Xenopus C3 is indeed cleaved by Xenopus C3 convertase and factor I at these sites. When a Xenopus LG liver cDNA library was screened with various C3 oligonucleotide probes, a C3 clone containing a deletion of 2,502 bases was identified, a finding which suggested the presence of a novel C3 transcript in Xenopus LG liver (105) that encoded a truncated C3. The molecule encoded by this C3 transcript contains the β chain of C3 and the first 59 residues and last 103 residues of the α chain with a molecular weight of 102 kDa. Thus, this truncated C3 is missing many of the C3-ligand binding sites that exist in the α chain of C3, and it may be the analog of a truncated human C3 molecule that has been isolated from the serum of patients with allergic dermatitis and has been shown to inhibit of eosinophil cytotoxicity and neutrophil adherence (106). As in the case of rainbow trout, two factor B genes have been cloned from Xenopus, Bf A and Bf B (107, 108). Both molecules share a domain structure that is similar to that of factor B/C2 molecules from other species. Thus, they are composed of three SCR domains at the N-terminus, a serine protease domain at the carboxy-terminus, and a von Willebrand domain in the middle of the molecule. The two Xenopus molecules are 82% identical in amino acid sequence and show more sequence similarity to mammalian factor B than to C2 (40% and 30% identity to mouse factor B and C2, respectively); in contrast to trout factor B molecules, in a phylogenetic tree they are grouped with the mammalian factor B molecules. Thus, the two Xenopus molecules were considered to be factor B molecules, although no functional characterization of either has been reported. A molecule with high similarity to mammalian C2 was initially reported in Xenopus, although a definitive demonstration of its identity has not yet been obtained. Demonstration of such a molecule in Xenopus is critical, since its presence would imply that the Bf/C2 gene duplication...
from a common ancestor occurred before the mammalian/amphibian divergence.

A C4 molecule has been purified from Xenopus serum and the gene encoding this component has been cloned and sequenced. Xenopus C4 is a 202-kDa protein that is similar to mammalian C4 in being comprised of three subunits of 96, 76 and 26 kDa. The protein is required for antibody-dependent lysis of sheep erythrocytes but not for the lysis of unsensitized rabbit erythrocytes (109). The amino acid sequence deduced from its cDNA is 39%, 30%, 25%, and 20% identical to the sequence of human C4, C3, C5 and α2-macroglobulin, respectively. In contrast to some mammalian species in which C4 is the product of two genes, genomic Southern blotting analysis has indicated that C4 is a single-copy gene in Xenopus (110). The Xenopus C4 and factor B genes are linked and reside within the Xenopus class III region (111). Factor I is the only complement regulatory protein whose gene has been cloned in Xenopus. This protein shows a conserved modular structure, except for the presence of an unusual highly charged segment of 29 amino acids that is encoded by a poly(dA)-rich mRNA insert not found in mammalian factor I (112). Xenopus complement receptors have not yet been characterized, although ingestion of sensitized sheep erythrocytes by Xenopus macrophages has been shown to be dependent on Xenopus C3 and a putative receptor (113).

Reptilia

In reptiles, the complement system has been studied almost exclusively in the cobra. The cobra complement system can be activated via either the classical or the alternative pathway (114), and it also includes the lytic pathway (115). Although there is no evidence for a lectin pathway in reptiles, it is most likely that these species do contain this pathway because it appears to be present in all other vertebrate species.

The cloning and cDNA sequencing of the cobra (Naja naja kaouthia) C3 gene showed that it encodes a two-chain (α, β) polypeptide of 1,651 amino acid residues with an internal thioester bond in the α chain; this C3 molecule has a high degree of sequence similarity to the C3 molecules of other species (116). Cobra venom factor (CVF), a complement-activating protein, that is structurally and functionally related to C3, has been isolated from the venom of several species of cobra. This molecule consists of three chains of approximately 70, 50, and 30 kDa, resembles C3c, and contains three oligosaccharide chains consisting of Galα1-3Galβ1-4(Fucα1-3)GlcNAcβ1 (117). When exposed to factor B and D, CVF forms a stable enzyme complex mimicking the C3bBb convertase, but it cannot be disassembled by factor H, and therefore confers a much longer half-life on the convertase. CVF continuously cleaves C3 and C5 in species other than cobra, leading to complement depletion in animals injected with this protein (118). Two mRNAs, with 90% sequence identity, have been identified for this protein, both of which show 90% sequence identity to cobra C3 (119); thus, it appears that there are at least three different C3-related genes in cobra genome. Although the sequence of the thioester site is conserved in these isoforms, the site is absent from the mature CVF protein, as a result of the removal of the C3d peptide during processing. The physiologic role of CVF, however, is as yet unclear.

Conclusions and future directions

During the last few years we have witnessed major discoveries that have had a drastic impact on our understanding of the phylogeny of the complement system. Definitive evidence for the presence of complement in invertebrates has come from the cloning of genes encoding C3 and factor B in sea urchins and the detection of C3 and MASP in tunicates. These findings indicate that the complement system is a very ancient immune mechanism that appeared at least 600–700 million years ago, predating by far the emergence of vertebrates. The question that remains to be answered now is in which animal species the complement system first appeared. To answer this question it will probably be necessary to look at the common animal ancestors of deuterostomes and protostomes.

Another important question that remains unresolved is whether the alternative or the lectin pathway was the first to appear. Since molecules homologous to C3 and factor B are present in the sea urchin, the alternative pathway may have predated the lectin pathway, which is already present in tunicates. However, the presence of both pathways will have to be examined in more primitive species before any definitive conclusions can be drawn.

An extraordinary feature of the complement system of some cold-blooded animals is the diversity of some of its components. All the teleost fish studied to date contain multiple forms of C3, and some contain multiple forms of factor B. It is fascinating that these multiple forms of C3 have the ability to bind with different efficiencies to various complement-activating surfaces; these distinctive binding specificities may provide a mechanism by which the fish can recognize a broader range of microorganisms. We have therefore hypothesized that the generation of structural and functional C3 diversity has evolved in teleost fish as a strategy for expanding their innate immune recognition capabilities. The C3 genes in trout and carp have been shown to be polymorphic, and gene duplication com-
combined with polymorphism could provide a C3 "repertoire" that would not only enhance the natural immune recognition capabilities of these fish but would also compensate for the limitations of their adaptive immune response. Indeed, the combination of diversity, high titer and activity at low temperatures makes complement one of the most effective immune mechanisms in teleost fish and probably other cold-blooded animals. The complement diversity found in these species opens a new area of research in which many questions are waiting to be addressed: Is this functional and structural C3 diversity present only in teleost fish, or is it also present in the rest of the cold-blooded animals? How were these multiple C3 isoforms generated? What is the mechanism(s) responsible for the differences in binding? Do these C3 isoforms perform other types of functions? Do they share the same complement receptor, or does each C3 have its own receptor? Relatively little progress has been made in characterizing the complement receptors of cold-blooded vertebrates, and much research remains to be done in elucidating the origins of mammalian complement receptors.

The structural composition of its components and the mechanism by which the classical pathway functions in cold-blooded vertebrates remain to be determined. How the classical pathway is defined is important in assessing whether these animal species indeed have this pathway. Thus far, what has been required to fulfill classical pathway activity in these animals is the dependence of such activity on the involvement of antibodies and on Ca^{2+} and Mg^{2+}. These two basic requirements may be the only ones that can define the classical pathway in such species, since preliminary results have suggested that, in these animals, the classical and alternative pathways share some components; in other words, some of the complement components of cold-blooded vertebrates may represent primordial molecules that in warm-blooded vertebrates have evolved to function in either the alternative or classical pathway. This situation appears to be true for factor B and C2: there is no evidence that cold-blooded species contain two distinct factor B and C2 molecules. Instead, trout have been shown to possess a factor B/C2 molecule (trout Bf-2) that functions in both alternative and classical pathways of complement activation. Functional studies have shown a lack of correlation between alternative and classical pathway hemolytic titers in various teleost fish, which suggests that the two activities follow different activation patterns. Taken together, these results suggest two possible scenarios: that if teleost fish contain a C1 molecule with a C1s subunit, it is required for cleavage of the C1s subunit may be able to cleave both the Bf/C2 molecule and C4, while factor D cleaves the Bf/C2 molecule, or b) the C1s subunit is only able to cleave C4 and factor D is then required for activation of the classical pathway. It is clear that, further characterization of classical and lectin pathway components in these species is necessary to allow us to choose between these scenarios.

Finally, a comprehensive understanding of complex biological systems such as the complement system requires a thorough knowledge of its origin and evolution, development, and diversity. These studies and forthcoming work have had and will continue to have an important impact on our understanding of complement in cold-blooded and warm-blooded animals, and should also contribute to solving health-related problems in species such as teleost fish that are of high economic value.

References


64. Sarrias MR, Zarkadis IK, Sunyer JO, Lambris JD. Structure and function of complement C3 in rainbow trout (Oncorhynchus mykiss). Mol Immunol (In press).


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