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Complement diversity: a mechanism for generating immune diversity?

J. Oriol Sunyer, Ioannis K. Zarkadis and John D. Lambris

Unlike mammalian species, several cold-blooded species have been shown to possess multiple forms of complement components. The multiple forms of C3 characterized in several fish species can bind with different specificities to various complement-activating surfaces. Here, Oriol Sunyer, Ioannis Zarkadis and John Lambris explore the possible advantages conferred by having multiple forms of individual complement proteins in a single organism.

Immune responses in animal species have evolved from innate to more-elaborate and adaptive mechanisms of nonself recognition[1,2]. All of the invertebrate phyla studied to date show a wide range of nonadaptive immune responses that allow them to recognize and neutralize foreign material (Fig. 1)[3,4]. Fish, the first of the true vertebrates, have also developed adaptive immune responses similar to those of higher vertebrates. Immunoglobulin (Ig) molecules are found in cartilaginous and bony fish but are absent from the most ancient group of fish, the agnathans (lampreys and hagfish) (Fig. 1)[5,6]. The adaptive immune response in fish is inefficient, but they appear to compensate for their rudimentary antibody response by making use of a well-developed complement system. Recent evidence has shown that the complement system of fish and other poikilothermic (cold-blooded) vertebrates is structurally and functionally more diverse than that of higher vertebrates because it contains components with multiple isoforms. We hypothesize that this diversity allows these animals to expand their innate immune recognition capabilities.

Phylogeny and evolution of the complement system

Complement components [C3, factor B and mannose-binding-lectin-associated serine protease 1 (MASP-1)] have been described in invertebrate species such as equinoderms[7,8] and tunicates[9], both of which are deuterostomes, the ancestors of all vertebrate species. The complement system is therefore a very old defense mechanism that emerged at least 600–700 million years ago, long before the appearance of the Igs (Fig. 1). The presence of C3 and factor B-like molecules in sea urchins suggests that the alternative pathway is at least as ancient as the lectin pathway, which is present in tunicates[9] but has not yet been found in sea urchins. Interestingly, it has been shown that MASP molecules are present in lamprey, shark and *Xenopus*, suggesting that the lectin pathway is present in all of these species too[10]. Complement activity (alternative pathway) and complement components (C3 and factor B) have been characterized in the most ancient vertebrates, the jawless fish (agnathans), although these fish lack the classical pathway and the membrane attack complex (MAC)[11–13]. Cartilaginous fish (the chondrichthyes) appear to have molecules functioning in all

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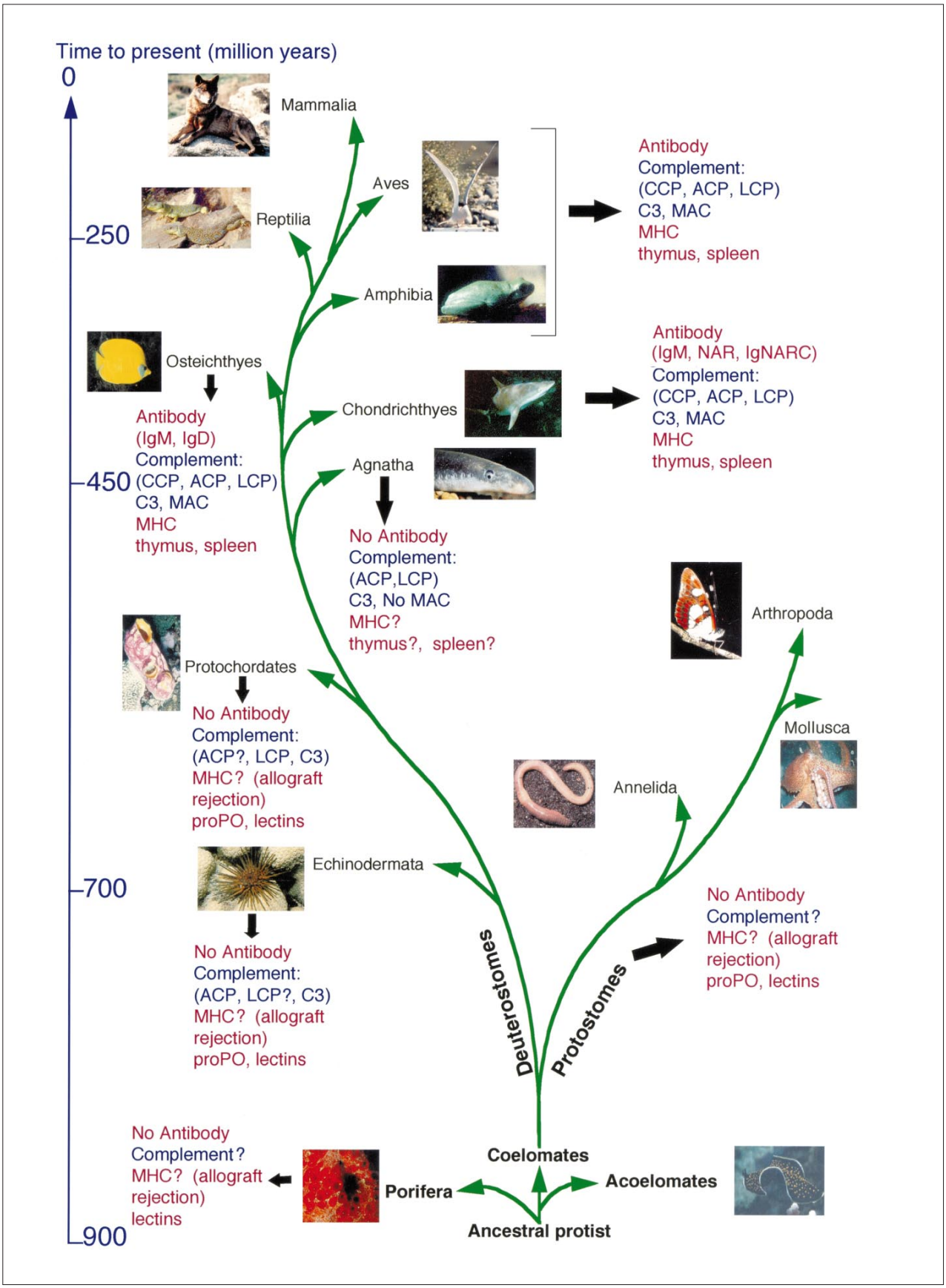
Time to present (million years)
0
250
450
700
900
Mammalia
Aves
Reptilia
Amphibia
Antibody
Complement:
(CCP, ACP, LCP)
C3, MAC
MHC
thymus, spleen
Osteichthyes
Antibody
(IgM, IgD)
Complement:
(CCP, ACP, LCP)
C3, MAC
MHC
thymus, spleen
Chondrichthyes
Antibody
(IgM, NAR, IgNARC)
Complement:
(CCP, ACP, LCP)
C3, MAC
MHC
thymus, spleen
Agnatha
No Antibody
Complement:
(ACP,LCP)
C3, No MAC
MHC?
thymus?, spleen?
Arthropoda
Protochordates
No Antibody
Complement:
(ACP?, LCP, C3)
MHC? (allograft
rejection)
proPO, lectins
Mollusca
Annelida
Echinodermata
No Antibody
Complement:
(ACP, LCP?, C3)
MHC? (allograft
rejection)
proPO, lectins
Deuterostomes
Protostomes
No Antibody
Complement?
MHC? (allograft
rejection)
proPO, lectins
No Antibody
Complement?
MHC? (allograft
rejection)
lectins
Porifera
Coelomates
Acoelomates
Ancestral protist

Fig. 1. *A simplified tree of the evolutionary relationships of the major phyla of living animals and their immune system features. Complement features are shown in blue. Abbreviations: ACP, alternative complement pathway; CCP, classical complement pathway; LCP, lectin pathway; MAC, membrane attack complex; MHC, major histocompatibility complex; proPO, prophenoloxidase activating system.*

pathways of complement activation, including the MAC (Ref. 14); however, the nature and similarity of these molecules to mammalian complement components are ill defined. All other vertebrates (including teleost fish, amphibians, reptiles, birds and mammals) have a well-developed complement system that involves all pathways of complement activation and the MAC (Fig. 1).

Complement diversity in fish and other poikilothermic vertebrates

C3 is the best characterized and the most versatile of all complement proteins, interacting with numerous serum, cell surface and foreign proteins[15], and playing a central role in all three pathways of complement activation. Activation of the complement system results in C3 cleavage to C3b, which exposes a highly reactive thioester group that can react with foreign material (viruses, bacteria, fungi), complex carbohydrates or immune complexes.

Until recently, the functionally active form of C3 was thought to exist in all vertebrate species as the product of a single gene. However, it has been demonstrated[16–19] that teleost fish possess multiple forms of functionally active C3 that are the products of several genes. More specifically, rainbow trout, a quasi-tetraploid species, contains three C3 isoforms (C3-1, C3-3 and C3-4) (Fig. 2a). The amino acid sequence identity/similarity of C3-3 to C3-4 is 78/82%; that of C3-3 to C3-1 is 57/69%; and that of C3-4 to C3-1 is 53/65% (Ref. 16). An additional isoform C3-2 yields a tryptic peptide map that differs (a 20% mismatch in the peptides) from that of C3-1 – this molecule is apparently functionally inactive[20]. In the carp (a tetraploid teleost fish), as many as eight different polymerase chain reaction (PCR) clones showing 85–98% amino acid identity 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[19]. The existence of multiple forms of C3 in trout and carp might be attributed to their tetraploid condition. However, five different forms of C3 (C3-1–5) have also been purified and characterized in the gilthead sea bream[17,18], a diploid teleost fish (Fig. 2b), indicating that multiple forms of C3 are not exclusive to tetraploid animals.

This exceptional C3 diversity observed in three fish families that are distant from one another in evolutionary time suggests that

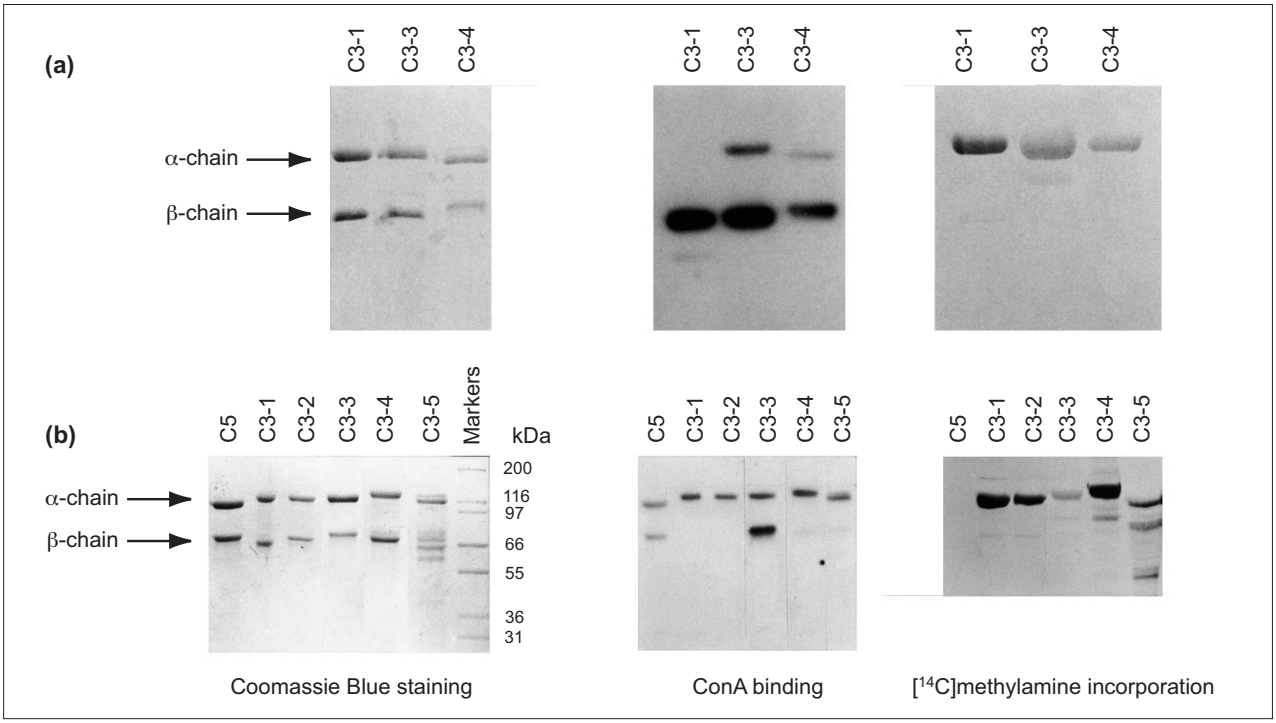

Fig. 2. *Characterization of (a) the trout and (b) gilthead sea bream C3 isoforms. Purified trout and gilthead sea bream C3 proteins resolved on 7.5% SDS-PAGE under reducing conditions and stained with Coomassie Blue (left). Concanavalin A (ConA)-binding carbohydrates of the α- and β-chains of the various C3 proteins (center). Incorporation of ^{14}C-methylamine in the α-chain of the various C3 isoforms (right). It can be observed that all C3 isoforms from both fish differed in the sizes of their chains as well as in their glycosylation patterns. In addition, all the C3s incorporated ^{14}C-methylamine in their α-chains, indicating the presence of an active thioester bond.*

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

Surface	Binding[a]							
	S. bream C3-1	S. bream C3-2	S. bream C3-3	S. bream C3-4	S. bream C3-5	Trout C3-1	Trout C3-3	Trout C3-4
Sheep RBC ghosts	++++[b]	++	++	++	+++	−	+	+
Rabbit RBC ghosts	++++	++++	++	++	+++	++++	+++	+++
Zymosan	+++	++	−	−	−	++++	−	−

Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; RBC, red blood cells.
[a]Symbols represent the extent of binding to the tested surface in the presence of Mg^{2+} EGTA.
[b]−, no binding; +, low binding; ++, intermediate binding; +++, good binding; ++++, very good binding.

regardless of the mechanisms that have generated these C3 isoforms, and despite the great amino acid differences among them, the genes encoding these isoforms have become fixed into the genome of these animals. Most importantly, their gene products have remained functional. This is particularly significant because many genes in tetraploid fish and other tetraploid animals have become pseudogenes, have been diploidized or their products have acquired new functions[21,22]. Therefore, the maintenance of these duplicated C3 genes must have important functional implications.

Although they are not yet fully characterized at the DNA and protein levels, multiple forms of C3 also appear to be present in other vertebrates[23]. Three C3 genes are present in cobra, one encoding functionally active C3 (Ref. 24) and two encoding cobra venom factor (CVF)[25], a C3c-like molecule present in the venom glands that forms a very stable C3-convertase in the presence of factor B. C3 is not the only complement protein to occur in multiple forms in these lower vertebrates. Factor B, the catalytic subunit of the C3 convertase, has been found in several forms in amphibians and fish. Two molecules identified in Xenopus, Bf-A and Bf-B, show high sequence similarity to factor B from other species[26,27]. In teleost fish [trout[28] and carp (M. Nakao, unpublished)], two factor B/C2 molecules have also been identified.

Functional diversity of the C3 isoforms

The products of the various C3 genes have been found to differ in structure as well as in function. Studies on fish have shown that the most important and unusual feature of these C3 molecules is that they bind with different efficiencies to various complement-activating surfaces. For example, all three C3 isoforms in trout bind, to varying degrees, to several erythrocyte surfaces or *Escherichia coli*, but only C3-1 (the most abundant isoform) binds to zymosan, a potent activator of the alternative pathway (Table 1)[16]. Similarly, in the gilthead sea bream, C3-1 and C3-2 (the most plentiful forms of C3 in serum) bind to zymosan, whereas C3-3, C3-4 and C3-5 do not bind (Table 1)[18]. These differences in binding to a specific substrate pertain not only to C3 isoforms from the same fish species but also to the C3 isoforms of two different species. For instance, all of the C3 isoforms in the gilthead sea bream bind with high efficiency to sheep erythrocytes (SRBC), whereas trout C3-3 and C3-4 bind very poorly, and trout C3-1 not at all (Table 1). It is interesting that this differential binding correlates with the high values of alternative complement pathway hemolytic activity (ACH50) displayed by sea bream serum against SRBC (66 ACH50 units ml^{-1})[29] compared with the very low activity in trout serum (2–4 ACH50 units ml^{-1})[16]. To date, this structural and functional diversity of C3 has only been shown 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 1722 residues), C4A binds preferentially to surfaces carrying amino groups, whereas C4B binds with higher affinity to those containing hydroxyl groups[30].

Not only does C3 exist as the product of several genes, but it appears that some of these genes in trout[31] and carp are quite polymorphic. This polymorphism may have important functional consequences, for example, the product of a particular C3 allele could react with a specific pathogen, conferring resistance to the fish carrying that allele. Accordingly, a combination of polymorphism and gene duplication could generate a large C3 repertoire.

Why do the various C3 molecules differ in their binding specificity for various surfaces? These differences may reflect *in vivo* differences in immune responsiveness. Selective pressure appears to be at work here. Perhaps the different environments in which trout and sea bream live (fresh and salt water, respectively) or the different microorganisms therein have driven, at least in part, the specificity of those C3s. For instance, salt water could harbor certain pathogenic microorganisms bearing surface molecules that are similar in structure to those on the surface of SRBCs (sea bream C3s in contrast to trout C3s bind to SRBC). If these microorganisms were not present in the fresh water in which trout live, there would be no selective pressure for trout C3 to bind to these molecules (or by analogy, to SRBC).

Advantages and implications of complement diversity

It seems reasonable to infer that the distinctive binding specificities of the various C3 isoforms might provide a mechanism for recognizing a broader range of microorganisms. Therefore, we hypothesize that the generation of structural and functional C3 diversity has evolved in teleost fish as a strategy for expanding their innate immunity. Having a C3 repertoire could not only enhance the natural immune recognition capabilities of these fish but also compensate for the limitations of their adaptive immune response. The acquired immune response and,

more specifically, the antibody response in fish is relatively restricted, being represented mainly by one IgM antibody[32]. Moreover, the antibodies generated in fish and most poikilothermic vertebrates are of low affinity and limited heterogeneity, and the response time is longer than that in mammals[5,33,34]. Even after repeated immunizations, fish show very little increase (if any) in the antibody affinity or titer. In addition, their antibody response is temperature dependent[35,36], and in winter, fish have been shown to have an impaired immune response[37].

Therefore, the complement system in these fish appears to provide the rapid, strong and varied innate immune capacity that is critical to their survival. Another beneficial feature of complement in these animals is that it can be activated at very low temperatures (as low as 0.5°C). Moreover, the activity of the alternative complement pathway is 5–10 times higher in fish than in higher vertebrates[29,38]. This combination of diversity, high titer and activity at low temperatures makes complement one of the most effective immune mechanisms in these animals. This potent defense system would be adapted to the aquatic environment with continuous exposure to large numbers of microorganisms.

Interestingly, the generation of diversity in C3 and Ig molecules has proceeded apparently in opposite directions in fish and mammals. This probably reflects the different immune needs of each animal group as determined by several factors including the local environment and the complexity of each animal taxon in terms of anatomy and physiology. These new findings suggest that complement in the lower vertebrates meets, in part, the needs that Ig molecules have evolved to meet in higher vertebrates: both providing greater diversity in the immune response.

Concluding remarks

It is clear that several species of lower vertebrates possess multiple forms of complement protein C3 having distinct functional properties. The structural and functional diversity of C3 alone or together with that of other complement components may provide a mechanism ensuring diversity of immune recognition in these animals and may represent an effective strategy for expanding their innate immunity.

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