

## Diversity of the third form of complement, C3, in fish: functional characterization of five forms of C3 in the diploid fish *Sparus aurata*

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We have recently shown that *Sparus aurata*, the gilthead sea bream (a diploid species), similarly to rainbow trout (a quasi-tetraploid species), possesses multiple forms of the third form of complement (C3). In the present study we have evaluated the ability of the gilthead sea bream proteins to function as active C3 molecules. All five C3 isoforms could be fixed covalently to sheep erythrocyte ghosts and were able to bind to various complement-activating surfaces in the presence of MgEGTA. In the absence of MgEGTA their binding capacities generally increased, presumably as a result of classical-pathway activation by the natural antibodies present in the serum. The presence of EDTA abrogated the binding of all C3 isoforms to the various surfaces tested. The C3 isoforms differed in the efficiency of their binding to complement-activating surfaces: the two most abundant C3 isoforms

(C3-1 and C3-2) bound to zymosan as well as to sheep and rabbit erythrocyte ghosts, whereas C3-3, C3-4 and C3-5 were unable to bind to zymosan. Upon complement activation, all five C3 isoforms were cleaved to 'iC3b' by factor H and I-like proteins, generating fragments similar to those generated from C3 molecules of other species. Furthermore the degradation of methylamine-hydrolysed C3 isoforms to iC3b was significantly inhibited by EDTA. The structural and functional diversity that we have observed in the C3 isoforms of *S. aurata* would increase the capacity of this fish to recognize a broader spectrum of potential pathogens and reinforce a specific immune response, which in fish is delayed compared with that of higher vertebrates, and is based on a single Ig type (IgM).

### INTRODUCTION

C3, the third component of complement, plays a central role in all existing activation pathways [1]. C3 has been purified from a large number of animal species, ranging from the first vertebrates (such as lampreys and hagfish) to humans [2]. In all these species, C3 contains a thioester bond that is essential for one of its main functions: binding covalently to acceptor molecules on cell surfaces, complex carbohydrates or immune complexes [3,4].

Fish, which seem to have been the first vertebrates of the animal kingdom, are unusual in immunological terms: their low degree of complexity in terms of body structure is correlated with a less elaborated immune system. For example, the specific immune response in teleost fish is represented by a single Ig type (IgM), and the response time is longer than that of mammals [5]. Fish have nevertheless a well-developed complement system that has an important role in their innate immune response [2,6]. Unique features of the complement system in fish include the ability of some fish sera to lyse a variety of erythrocytes (sheep, goat, dog or rabbit erythrocytes) through the alternative pathway [7], in contrast with human serum, which can lyse only rabbit erythrocytes [8]; in addition, fish have very high alternative complement pathway titres (10-fold higher than those of higher vertebrates) [7].

In nearly all species that have been analysed, functionally active C3 seems to be the product of a single gene. Nevertheless we have recently described the presence in trout of multiple forms of functionally active C3 that differ in their binding efficiencies to various complement-activating surfaces [9]. Rainbow trout are tetraploid fish in the process of diploidization [10]. Therefore the existence of different forms of C3 in trout might be attributed to their quasi-tetraploid condition. To ascertain whether the presence of multiple forms of C3 is specific to this species, we recently investigated the presence of multiple forms of

C3 in the gilthead sea bream (*Sparus aurata*), a modern diploid fish [11] with well-developed alternative and classical complement pathways [6,7]. We reported that the gilthead sea bream possesses five C3 isoforms that differ in their immunological and biochemical properties [12]. In the present study we analysed the functional properties of these C3 isoforms and we found that although they function as C3, they differ from each other in: (1) their efficiency in binding to various complement-activating surfaces, (2) their pattern of degradation by sea bream factor H and I-like proteins and (3) the metal dependence of factor H- and I-like-mediated cleavage of C3b.

### MATERIALS AND METHODS

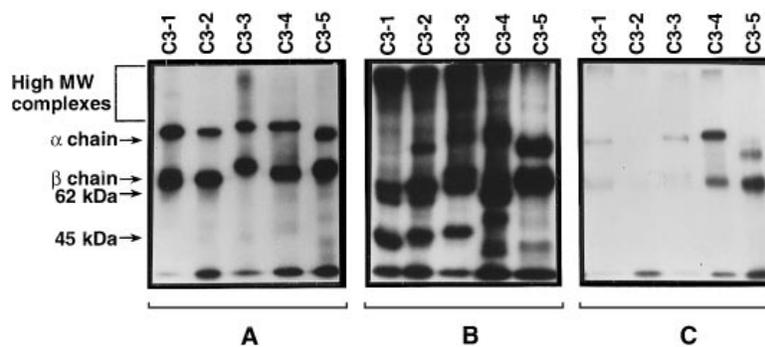
#### Plasma

Gilthead sea bream (*Sparus aurata*) were obtained from Aquadelt fish farm in the Ebre delta (East Spain). Blood was obtained by syringe from the caudal artery, plasma was separated from cells by centrifugation at 2000 *g* for 5 min and serum was obtained by incubating the blood at 4 °C for 4 h. The serum was then separated by centrifugation at 2000 *g* for 10 min. Plasma and serum were stored at –70 °C.

#### Purification of the C3 isoforms

The strategy used to purify the various proteins has previously been described elsewhere [12]. Pooled plasma (20 ml) from sea bream (10 fish) was precipitated with 4% (w/v) poly(ethylene glycol) at 4 °C for 30 min in the presence of 20 mM EDTA/10 mM benzamidine/1 mM PMSF, then centrifuged (15000 *g* for 20 min). The resulting supernatant was brought to

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**Figure 1** Covalent binding of  $^{125}\text{I}$ -labelled sea bream C3 isoforms to sheep erythrocyte ghosts

Sheep erythrocyte ghosts were incubated with  $^{125}\text{I}$ -labelled sea bream C3-1, C3-2, C3-3, C3-4 or C3-5 and serum, as described in the Material and methods section. Panel (A) shows the radiolabelled C3 isoforms. Samples were incubated with (C) or without (B) 20 mM EDTA.  $^{125}\text{I}$ -labelled C3 bound to erythrocyte membranes was separated from free iodinated protein by centrifugation through 20% (w/v) sucrose. The pellets obtained were solubilized by boiling in SDS sample buffer and run on SDS/PAGE [7.5% (w/v) gel] under reducing conditions. The gel was then dried and subjected to autoradiography. Abbreviation: MW, molecular mass.

16% (w/v) in poly(ethylene glycol), stirred for 30 min at 4 °C and centrifuged (15000 *g* for 20 min). The pellet obtained was then resuspended in 10 mM phosphate buffer, pH 7.5, and loaded on a Mono Q HR 10/10 anion-exchange chromatography column (Pharmacia Biotech, Piscataway, NJ, U.S.A.). Thereafter the pools containing the various proteins (C3-1, C3-2, C3-3, C3-4 and C3-5) were subjected to several additional chromatographic steps. All the pools containing the various proteins were passed through a Mono S HR 5/5 cation-exchange chromatography column (Pharmacia Biotech) that had been equilibrated in 10 mM phosphate buffer, at pH 6 for C3-1 and at pH 5.8 for the other proteins. C3-1 and C3-2 were obtained after the previous chromatographic step of cation-exchange chromatography. C3-3 and C3-4 were further purified on a Mono Q HR 5/5 column (Pharmacia Biotech) previously equilibrated in 10 mM phosphate buffer, pH 7.5, although C3-4 needed an additional chromatographic step of gel filtration on Superose 12 (Pharmacia Biotech) previously equilibrated in 10 mM phosphate buffer (pH 7.5)/150 mM NaCl. The pool from cation-exchange chromatography containing C3-5 was then subjected to gel-filtration chromatography on a Superose 12 column (Pharmacia Biotech) previously equilibrated in 10 mM phosphate buffer (pH 7.5)/150 mM NaCl. Before being applied to a column, the samples were exchanged into the buffer used to equilibrate that column. Purification of the various proteins was monitored by gel electrophoresis and Western blotting by using an antibody specific for the  $\beta$ -chain of trout C3-1 [12]. This antibody was used to distinguish the fractions containing the C3-1, C3-2, C3-4 and C3-5 proteins from those containing C3-3.

#### Binding efficiencies of sea bream C3-1, C3-2, C3-3, C3-4 and C3-5 to various complement-activating surfaces

To investigate the relative binding abilities of the various sea bream C3 isoforms, the amount of C3 bound to various surfaces (sheep or rabbit erythrocyte ghosts, or zymosan) was determined by incubating 20  $\mu\text{l}$  of each activator (a 50% suspension of cell ghosts or zymosan) with 50  $\mu\text{l}$  of sea bream serum containing 30  $\mu\text{l}$  (2  $\mu\text{Ci}$ ) of  $^{125}\text{I}$ -labelled sea bream C3 protein in 10 mM PBS, pH 7.4, in the presence or absence of 2 mM MgEGTA or 10 mM EDTA. The reaction mixture was incubated at 20 °C for 30 min. Bound  $^{125}\text{I}$ -labelled C3 was separated from free protein by centrifugation through 20% (w/v) sucrose in 10 mM phos-

phate buffer, pH 7.4, containing 500 mM NaCl and 20 mM EDTA. The specific radioactivity of the C3 proteins varied from 5 to 8.4  $\mu\text{Ci}/\mu\text{g}$ . To demonstrate that the binding to the surfaces was covalent in nature, the pellets obtained containing the sheep erythrocyte ghosts were solubilized by boiling in SDS sample buffer and run on SDS/PAGE [7.5% (w/v) gel] under reducing conditions. The gel was then dried and subjected to autoradiography.

#### Degradation of C3 isoforms by sea bream factor I and H-like proteins and assessment of its metal-dependence

Untreated or methylamine-treated 30  $\mu\text{l}$  samples of the various radiolabelled sea bream isoforms (2  $\mu\text{Ci}$ ) were incubated with 20  $\mu\text{l}$  of a 1:1 mixture of rabbit and sheep erythrocyte ghosts (50% suspension) and 50  $\mu\text{l}$  of sea bream serum, in the presence or absence of 10 mM EDTA; treatment with methylamine was performed as previously described [13]. The reaction mixture was incubated at 20 °C for 30 min. The erythrocyte ghosts were pelleted by centrifugation, and 2  $\mu\text{l}$  of supernatant from each reaction mixture was mixed with SDS sample buffer and run on SDS/PAGE [9% (w/v) gel] under reducing conditions. The gel was then dried and subjected to autoradiography.

## RESULTS

### Covalent binding to sheep erythrocyte ghosts

All five sea bream C3 isoforms were able to incorporate [ $^{14}\text{C}$ ]methylamine into their  $\alpha$ -chains, indicating the presence of a thioester bond in each isoform [12]. After incubation with sheep erythrocyte ghosts and sea bream serum, all five C3 isoforms showed similar high-molecular-mass complexes migrating more slowly than their  $\alpha$ -chains on SDS/PAGE (Figure 1B). When complement activation was inhibited by EDTA, the binding of the various  $^{125}\text{I}$ -C3 isoforms to the sheep erythrocyte ghosts was greatly decreased (Figure 1C).

The appearance of fragments similar in size to those generated by the degradation of C3 to iC3b (Figure 1B) suggests that the C3 isoforms are most probably cleaved by proteins similar to factors I and H. The sizes of these fragments varied significantly

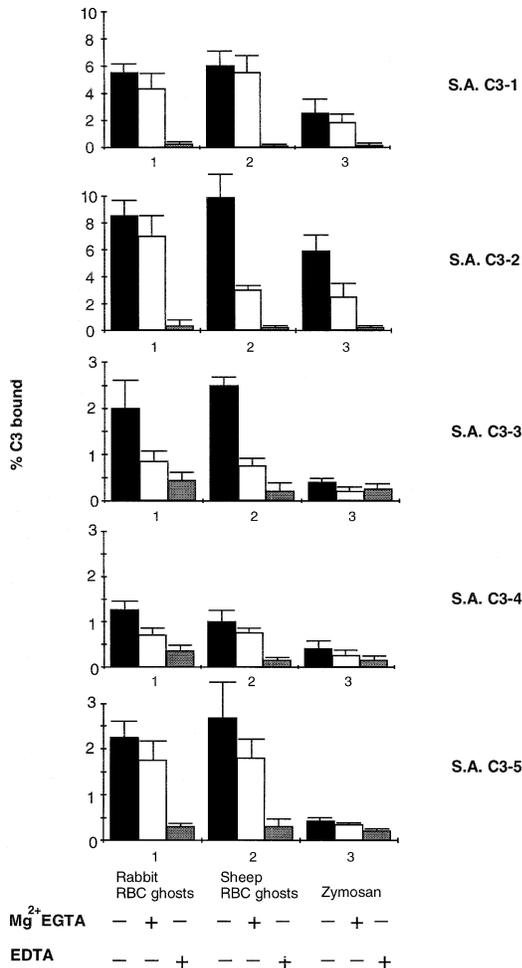
**Table 1** Fragments generated by factor H- and I-like cleavage of human C3 and sea bream C3 isoforms

Human C3	Sea bream				
	C3-1	C3-2	C3-3	C3-4	C3-5
64.3	62	62	64	64	64
39.5	45	46	48	48/38	43

**Table 2** Binding of sea bream and trout C3 isoforms to various complement-activating surfaces

Values represent the percentage binding to the tested surface in the presence of MgEGTA minus the percentage binding to the same surface in the presence of EDTA.

Surface	Degree of binding (%)								
	Sea bream					Trout			
	C3-1	C3-2	C3-3	C3-4	C3-5	C3-1	C3-2	C3-3	
Sheep erythrocyte ghosts	5.5	3	0.6	0.8	1.5	-0.02	0.2	0.3	
Rabbit erythrocyte ghosts	5.2	6.2	1.5	1	2	4.2	0.9	0.9	
Zymosan	2	2.3	-0.1	0.05	0.05	2.2	0.01	0.001	

**Figure 2** Binding of the sea bream C3 isoforms to various complement-activating surfaces

Each bar represents the mean  $\pm$  S.D. for three different experiments.

from one isoform to another (Table 1), indicating important differences in their primary structures, as we have previously shown [12].

#### Binding efficiencies of C3 isoforms to complement-activating surfaces after complement activation

The efficiency with which these C3 isoforms bind to various complement-activating surfaces was investigated by using iodine-

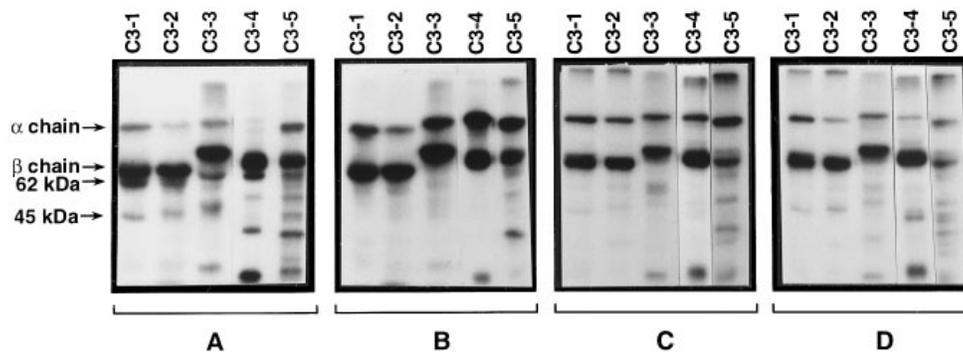
ated proteins, with zymosan and erythrocyte membranes as complement activators (Figure 2). In all cases, the degree of C3 binding to various surfaces was lower in the presence of MgEGTA than in its absence, most probably as a result of the inhibition of the classical pathway by EGTA. The observed pattern suggests that all five C3 isoforms participate in the activation of both the classical and alternative pathways.

The two most abundant C3 isoforms, C3-1 and C3-2, were able to bind to rabbit and sheep erythrocyte ghosts and zymosan particles in both the presence and the absence of MgEGTA. Nevertheless the efficiency of C3-2 binding (in the absence of MgEGTA) was almost twice that of C3-1; however, in the presence of MgEGTA the efficiencies of C3-1 and C3-2 binding were similar, suggesting a greater ability of C3-2 to be activated through the classical pathway. Unexpectedly, and in contrast with the results obtained for C3-1 and C3-2, the other three isoforms were unable to bind to zymosan particles (Figure 2 and Table 2). In addition, the binding efficiencies of C3-3, C3-4 and C3-5 to rabbit and sheep erythrocyte ghosts were one-half to one-quarter of those of C3-1 and C3-2. There were no significant differences in the binding efficiencies of C3-3, C3-4 and C3-5 to rabbit and to sheep erythrocyte ghosts, although the efficiency of C3-5's binding to rabbit and sheep erythrocyte ghosts in the presence of MgEGTA was twice that of C3-3 and C3-4.

#### Degradation of the C3 isoforms by sea bream factor I- and H-like proteins and metal dependence of this activity

To study the fluid-phase cleavages that occurred on complement activation, each of the radiolabelled C3 isoforms was incubated with sea bream serum and sheep or rabbit erythrocyte ghosts, in the presence or absence of EDTA, and the unbound C3 fragments were analysed by SDS/PAGE. For all C3 isoforms the amount of unbound  $\alpha$ -chain in the absence of EDTA (Figure 3A) was much smaller than when EDTA was present (Figure 3B). In addition, for all C3 isoforms we obtained fragments that were very similar in molecular size to those produced by the cleavage of human C3b to iC3b (Figure 3A and Table 1). This cleavage pattern was similar to that observed previously for the bound C3 (Figure 1B). It is noteworthy that we did not detect the presence of an  $\alpha'$ -chain, and its absence suggests that complement activation led to complete cleavage of C3b to iC3b.

It has been reported that the degradation of human C3 by sand bass (a teleost fish) factor I- and H-like proteins, in contrast with the mammalian factor I and H, is metal-dependent [14,15]. To investigate whether this is also true for the degradation of sea bream C3 by sea bream factor I- and H-like molecules, we analysed the degradation of all C3 to 'iC3b' during complement



**Figure 3** Degradation of C3 isoforms by factor I- and H-like proteins from sea bream serum and the metal-dependence of this activity

Supernatants resulting from the incubation of sheep erythrocyte ghosts with  $^{125}\text{I}$ -labelled sea bream C3-1, C3-2, C3-3, C3-4 or C3-5 and sea bream serum in the presence (B) or absence (A) of EDTA were analysed by SDS/PAGE [9% (w/v) gel] under reducing conditions. All five C3 isoforms were hydrolysed by treatment with methylamine, as described in the Materials and methods section. The hydrolysed C3 isoforms were then incubated with serum in the presence (C) or absence (D) of EDTA and analysed by SDS/PAGE [9% (w/v) gel] as outlined above. The gels were then dried and subjected to autoradiography. Figure 1(A) shows the radiolabelled C3 isoforms.

activation. For this purpose, the C3 isoforms were treated with methylamine to generate hydrolysed C3. With the exception of C3-1, EDTA partly inhibited the disappearance of the  $\alpha$ -chain from all the C3 isoforms (Figure 3C). However, in the absence of EDTA the  $\alpha$ -chain was degraded to a great extent (Figure 3D). For C3-1, the 62 kDa fragment generated by the degradation of C3 to iC3b (Figure 3A) was not present after incubation of the hydrolysed C3-1 with serum; instead, a 74 kDa fragment (Figures 3C and 3D) appeared (because of the C3a portion, which is still in the  $\alpha$ -chain of the hydrolysed C3). In a similar manner, the 62 kDa fragment was not present when C3-2 was hydrolysed, and under these circumstances the  $\alpha$ -chain fragment had an apparent molecular mass that was similar to that of the  $\beta$ -chain (Figures 3C and 3D). The fact that this 62 kDa  $\alpha$ -chain fragment was not generated in the absence of EDTA with hydrolysed C3-2 (Figure 3D) excludes the possibility that the marked increase in  $\alpha$ -chain disappearance in the absence of EDTA was due to complement activation. This finding suggests that the presence of EDTA does indeed partly inhibit the cleaving activity mediated by molecules equivalent to factors I and H. C3-3, C3-4 and C3-5 behaved in a similar manner to C3-2.

## DISCUSSION

We have recently reported that rainbow trout, a quasi-tetraploid fish, contains three C3 forms of functionally active C3 [9]. If the multiple forms of rainbow trout C3 are the result of tetraploidization, one would expect the sea bream, which is a diploid fish, to contain only one or two different C3 isoforms. Instead, we found that sea bream contained even more C3 isoforms than did the trout [12]. In the present study we have analysed the functional properties of these proteins.

All five C3 isoforms contained a thioester bond in their  $\alpha$ -chains [12]. This thioester bond is functional because all five isoforms were able to bind covalently to sheep rabbit erythrocytes, as shown by the formation of high-molecular-mass complexes between  $^{125}\text{I}$ -labelled C3 fragments and the acceptor molecules on the surface of the erythrocytes (Figure 1). The banding pattern exhibited by the sea bream C3 isoforms was very similar to that seen for the trout C3 isoforms and for human C3 [9]. As also seen for human C3 and the trout C3 isoforms [9], a band of approx. 43 kDa was generated (probably by factors I- and H-like molecules) in all five sea bream C3 isoforms after

incubation with sea bream serum and erythrocyte ghosts (Figure 1B).

Perhaps the most intriguing and important feature of these C3 isoforms was the differences in the efficiencies of their binding to various complement-activating surfaces (Figure 2). Thus sea bream C3-3, C3-4 and C3-5 did not bind to zymosan particles (an activator of the alternative pathway), whereas sea bream C3-1 and C3-2 were able to do so. It is interesting to note that C3-1 and C3-2, the two major sea bream isoforms, were able to bind to all the surfaces tested, and their binding efficiencies were 2–4-fold greater than those displayed by C3-3, C3-4 and C3-5 under the same conditions. Similarly, the two minor C3 isoforms from trout, C3-3 and C3-4 (Table 2), were unable to bind to zymosan particles, and their binding efficiencies were one-third to one-quarter of those of trout C3-1 [9]. It is noteworthy that all five sea bream C3 isoforms bound to a high degree to sheep erythrocyte ghosts through the alternative pathway. This finding might explain the high haemolytic activity displayed by sea bream serum against sheep erythrocytes through the alternative pathway [66  $\text{ACH}_{50}$  units/ml (the reciprocal of the serum dilution causing 50% lysis of sheep erythrocytes)] [14]. In contrast, the major form of trout C3 (termed trout C3-1) did not bind to sheep erythrocyte ghosts, but trout C3-3 and C3-4 did bind to a very low degree (Table 2); this observation is also correlated with the low haemolytic activity displayed by trout serum against sheep erythrocytes through the alternative pathway (2–4  $\text{ACH}_{50}$  units/ml) (results not shown). In spite of the fact that the minor C3 isoforms of sea bream and trout seem to be functionally related, no correlation exists as far as immunological and sequencing data are concerned [12]. At present it is unknown whether these differences in the binding efficiencies of the sea bream C3 isoforms to the various complement-activating surfaces result from differences in affinity between each C3 isoform and the regulatory proteins of the complement system or from an alteration in the surface specificity of some of the C3 isoforms, or both. A similar binding property has been described for human C4, which exists in two different forms, C4A and C4B. Despite the fact that these two molecules have very few amino acid differences, they display distinct binding specificities: human C4A prefers to bind to surfaces carrying amino groups, whereas C4B binds preferentially to those containing hydroxy groups [15].

The cleavage of all five sea bream C3 isoforms in the fluid phase after complement activation yielded products that were

similar to those observed for surface-bound C3 (Figure 3A). Hence a fragment of approx. 62–64 kDa and another of approx. 38–48 kDa were present in all the C3 isoforms after incubation of the sea bream serum with rabbit or sheep erythrocyte ghosts (Figure 3A and Table 1). The factor H- and I-like cleavage of the hydrolysed C3 isoforms was partly inhibited by EDTA, unlike the situation in higher vertebrates, in which this activity is not metal-dependent [16]. This observation agrees with a study of Gigli and Kaidoh [17,18] in which they showed that the cofactor activity of serum or plasma from the sand bass (a teleost fish) towards human C3 is dependent on divalent cations such as  $\text{Ca}^{2+}$ , yet they were not able to observe cofactor activity in the presence of EDTA. These observations can most probably be explained by the fact that they had used human C3 instead of sand bass C3.

In summary, we have shown that the gilthead sea bream, a diploid fish [11], possesses five different forms of functionally active C3 that differ in their binding efficiencies to various complement-activating surfaces. These results indicate that the presence of multiple forms of C3 with different binding efficiencies is not restricted to trout, a quasi-tetraploid fish [10,19], species. The observed structural and functional diversity of C3 in fish might represent a way by which these animals increase their innate immune recognition capability to reinforce their immune response to foreign pathogens, because their specific immune response (based only on IgM) is delayed. This C3 diversity also suggests the generation of a C3-related gene family.

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