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Expression of Complement 3 and Complement 5 in Newt Limb and Lens Regeneration^{1,2}

Yuko Kimura,* Mayur Madhavan,[†] Mindy K. Call,[‡] William Santiago,[‡] Panagiotis A. Tsonis,[‡] John D. Lambris,^{3*} and Katia Del Rio-Tsonis^{3†}

Some urodele amphibians possess the capacity to regenerate their body parts, including the limbs and the lens of the eye. The molecular pathway(s) involved in urodele regeneration are largely unknown. We have previously suggested that complement may participate in limb regeneration in axolotls. To further define its role in the regenerative process, we have examined the pattern of distribution and spatiotemporal expression of two key components, C3 and C5, during limb and lens regeneration in the newt *Notophthalmus viridescens*. First, we have cloned newt cDNAs encoding C3 and C5 and have generated Abs specifically recognizing these molecules. Using these newt-specific probes, we have found by in situ hybridization and immunohistochemical analysis that these molecules are expressed during both limb and lens regeneration, but not in the normal limb and lens. The C3 and C5 proteins were expressed in a complementary fashion during limb regeneration, with C3 being expressed mainly in the blastema and C5 exclusively in the wound epithelium. Similarly, during the process of lens regeneration, C3 was detected in the iris and cornea, while C5 was present in the regenerating lens vesicle as well as the cornea. The distinct expression profile of complement proteins in regenerative tissues of the urodele lens and limb supports a nonimmunologic function of complement in tissue regeneration and constitutes the first systematic effort to dissect its involvement in regenerative processes of lower vertebrate species. *The Journal of Immunology*, 2003, 170: 2331–2339.

The complement system is comprised of several serum proteins, membrane-bound receptors, and regulatory proteins that constitute a phylogenetically ancient mechanism of innate immunity (1–2). The functions of the complement system in host defense and inflammation are mediated mainly through the sequential activation and proteolytic cleavage of serum proteins.

Complement activation occurs through three distinct pathways (classical, alternative, and lectin), all of which converge in the activation of C3, the third component of complement. C3 can interact with a wide spectrum of factors and therefore is able to mediate a wide variety of functions (3–5). In addition to complement proteins, C3 fragments interact with several proteins that are involved in differentiation, such as fibronectin and integrins (6, 7). Other complement factors share homologies with domains of extracellular matrix proteins, such as collagen binding domains, which might indicate that complement factors could be involved in such interactions in the extracellular matrix (8). Therefore, some of the functions of complement are apparently not immunologic. For

example, C3 is expressed in myoblasts (9, 10) and is also associated with proliferation and growth of B cells in vitro (11).

C5, the fifth component of complement, has also been found to have novel noninflammatory functions in various tissues. Studies in a human neuroblastoma cell line have suggested that C5a (a fragment of C5) participates in apoptotic signal transduction pathways through its binding to the neuronal C5aR (12, 13). It has also been shown that the terminal complex system C5b-9 (membrane attack complex), in sublytic doses, can induce DNA synthesis and cell proliferation in cultured mouse fibroblasts (14), human aortic smooth muscle cells (15), oligodendrocytes (16), and glomerular epithelial cells, in the absence of other growth factors (17). Also, sublytic concentrations can activate monocytes and induce cytokine release through activation of NF- κ B signaling pathways, which are critical for the cell cycle transition into DNA synthesis (18).

We have reported previously that C3 is expressed during limb regeneration in the axolotl (19) in the blastema, a cell population that gives rise to the various cell types that reconstitute the limb, through processes that involve dedifferentiation, transdifferentiation into different phenotypes, and extensive tissue remodeling. C3 was not expressed in the intact or developing limb, indicating its specificity for the regeneration process. Furthermore, we were able to show expression of C3 in dedifferentiated newt muscle cells in vitro. These findings were the first to indicate that complement might have a novel, possibly nonimmunologic, role in regenerative processes. This potential role for complement in tissue regeneration was supported by our recent observation that C5-deficient mice exhibit defective liver regeneration after acute toxic injury (20). In mice, the liver can regenerate by proliferation of the remaining hepatic cells. Mice lacking C5 are not able to repair their liver properly unless they are reconstituted with C5a (20).

To expand our understanding of this newly discovered role of the complement system in regenerative processes, we decided to use the newt *Notophthalmus viridescens* because of its extensive repertoire of regenerative capabilities. The newt can regenerate its

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² The sequences described in this paper have been deposited in GenBank database under accession numbers AY197698 for C3 and AY197699 for C5.

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limbs, tail, jaw, some of its internal organs, and its eye tissues, including the lens and the retina. In many cases, research in species other than humans is hampered by the lack of a wide range of tools to approach a specific problem. Abs represent one such tool that is a prerequisite for our studies addressing the function of newt C3 and C5. As a first step in our research, we decided to systematically generate the necessary tools (C3- and C5-specific probes and Abs) in the newt, so that they could be used to explore the role of complement in regeneration in this species. In this study, we describe the specific and robust expression of complement components C3 and C5 in regenerating tissues of the newt limb and lens, both at the mRNA and protein levels. In the effort to characterize the expression profile of these components, we isolated and characterized newt C3 and C5 cDNA and generated specific Abs against newt C3 and C5. The expression patterns of C3 and C5 suggest that both complement proteins are involved in the process of regeneration.

Materials and Methods

Animals

Newts (*N. viridescens*) were purchased from Mike Tolley Newt Farm (Nashville, TN). Newt limbs were amputated at the ulnar-radial plane, and the regenerated limbs were collected 7, 14, and 21 days after amputation. Lenses were removed from adult newts, and regenerating eyes were collected 5, 10, 15, and 20 days thereafter. The collected tissues were either immersed in OCT (Andwin Scientific, Warner Center, CA) and quick-frozen to be processed for immunohistochemical staining, or they were fixed in 4% paraformaldehyde overnight and then processed for paraffin embedding for subsequent *in situ* hybridization analysis. Blood was collected through a capillary tube after amputating the tail proximal to the body. Before these procedures, all animals were anesthetized with 3-aminobenzoic acid hydrochloride (MS222; Sigma-Aldrich, St. Louis, MO) and were handled in compliance with the regulations of the Institutional Animal Care and Use Committee.

Purification of newt C3

Newt C3 was purified from newt plasma by a combination of polyethylene glycol (PEG)⁴ precipitation and anion exchange and gel filtration chromatography (Fig. 1A). The total recovery of purified C3 from 2 ml of plasma was 0.8 mg, which suggested that the concentration of C3 in newt plasma is similar to that found in other species. Purified C3 protein was analyzed by 7.5% SDS-PAGE under reducing and nonreducing conditions. To obtain partial protein sequence for C3 primer design, the purified protein was electrophoresed and electroblotted onto a polyvinylidene difluoride (PVDF) membrane, and the α -chain was subjected to Edman degradation.

Cloning of newt C3 and C5

First-strand cDNA was synthesized from newt liver total RNA using the Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions. A degenerate primer, 5'-AARGCNGGNGC NAARGC-3' (N = G, A, T, and C; R = A and G), was designed based on the sequence KAGAK which represents the C3 sequence KAGK with the insertion of A between residues G and K which is conserved in the C5 of various species; this primer was used to obtain both C3 and C5 cDNA. In addition, a sense primer, 5'-GNTGYGGNGARCARAAYATG-3' (Y = T and C), and an antisense primer, 5'-CATRTTYTGYTCNCCRCANC-3', were designed from a conserved sequence in the thioester region GCGEQNM as well as the primer 5'-ACRTANGCNGTNAARCA-3', which corresponds to the conserved sequence WLTAYV (21). Different combinations of primers were used to obtain the desired cDNA sequences. PCR were performed as follows: 30 cycles of denaturation at 94°C for 0.5 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min. The amplified PCR products were gel-purified and subcloned into the pGEM-T Easy vector (Promega) and sequenced.

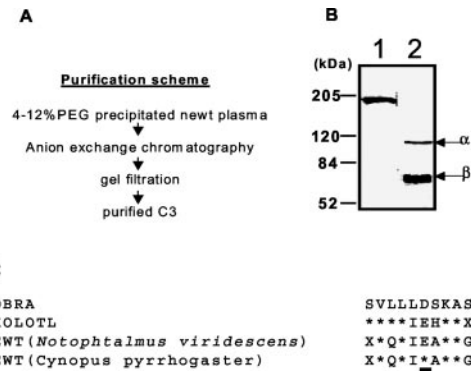


FIGURE 1. Purification and characterization of newt C3. *A*, Schematic of C3 purification. Newt plasma (4–12% PEG precipitated) was applied to a Mono Q HR 10/10 anion exchange chromatography column, and fractions were eluted with a 50–350 mM salt gradient. Based on SDS-PAGE analysis under reducing and nonreducing conditions, C3-containing fractions were concentrated and subjected to gel filtration on a Superose 12PG column. *B*, Coomassie-purified C3 was run on 7.5% SDS-PAGE under reducing (lane 2) and nonreducing (lane 1) conditions. *C*, Amino acid sequence comparison of the newt C3 α -chain N terminus to cobra and axolotl C3. An asterisk denotes identical residues. The amino acid sequence of newt C3 was obtained by N-terminal sequencing of purified C3.

Expression and characterization of newt C3a and C5a

The newt C3a fragment corresponding to residues 8–76 of the C3 sequence (Fig. 2A) was amplified by PCR from the cloned C3 fragment using the primers 5'-GGATCCAAAGCTGGCAAAGCT-3' and 5'-AAGCTTTTATCTTGACAAAAACAGTTG-3'. These primers added a *Bam*HI site at the beginning of the C3 sequence and a *Hind*III site at the end of the cDNA. For convenience, the fragment was cloned into pGEM-T Easy vector, and the sequence was verified. The fragment was excised with *Bam*HI and *Hind*III restriction enzymes and cloned into the expression vector pQE-30 (Qiagen, Stutigo, CA).

The C3a protein was expressed and purified from the *Escherichia coli* strain M15 (pREP4; Qiagen) according to the manufacturer's instructions. The *E. coli* was first treated with a lysogen solution (1 mg/ml), and then the pellet was resuspended with a solution of 0.1 M NaH₂PO₄ and 0.01 M Tris-HCl containing 10 mM 2-ME and 8 M urea (pH 8.0). The cleared lysate was mixed with Ni-NTA agarose (Qiagen) for 1–2 h and loaded onto a disposable column. The column was washed with 0.1 M NaH₂PO₄ and 0.01 M Tris-HCl containing 8 M urea (pH 8.0), followed by the same solution at pH 6.3 and then pH 5.9, and finally eluted at pH 4.5. The recombinant protein was refolded by dialysis overnight into 0.1 M Tris-HCl with 2 mM reduced glutathione, 0.2 mM glutathione, and 0.005% Tween 80 (22). Contaminating proteins were removed by a reversed-phase column using a RESORSE RPC 3-ml column (Amersham Pharmacia Biotech, Piscataway, NJ) as previously described (23). The purity and identity of recombinant newt C3a protein was assessed by SDS-PAGE and mass spectrometry. The newt C5a fragment (residues 9–79) (Fig. 2B) was prepared using the sense primer 5'-GGATCCATAGAGGCATTAACTCT-3' and the antisense primer 5'-AAGCTTTTATCGAGCCAGTCCAG-3' and was expressed and purified in the same fashion as recombinant newt C3a.

Preparation of anti-C3a- and anti-C5a-specific Abs

Rabbits were immunized by repeated s.c. injection at multiple sites with 30 μ g of purified C3a or C5a emulsified in CFA, followed by a booster injection at 3 wk. The IgG fraction of immune sera was prepared by a combination of caprylic acid and ammonium sulfate precipitation and was extensively dialyzed against PBS. The anti-C3a Ab was further purified by affinity chromatography using a C3a-Sepharose 4B column. The specificity of generated Abs was assessed by SDS-PAGE followed by Western blotting. Briefly, 0.25 μ l of normal newt serum treated with or without cobra venom factor were incubated at 37°C for 30 min and used for the detection of C3 and C5, respectively. Samples were subjected to SDS-PAGE on 7.5% gels under nonreducing conditions and electroblotted onto a PVDF membrane. The membrane was blocked with PBS containing 10% milk-0.05% Tween and incubated with the anti-C3a or anti-C5a IgG (5 μ g/ml) for 40 min. After washing with PBS-0.05% Tween, the membrane was incubated for 30 min with HRP-conjugated goat anti-rabbit IgG (1:1000).

⁴ Abbreviations used in this paper: PEG, polyethylene glycol; PVDF, polyvinylidene difluoride; DAPI, 4',6'-diamidino-2-phenylindole; WE, wound epithelium; DIC, differential interference contrast.

A

5' primer
 5'AARGCNGGNCNAARGC 3'
 tggcaagctgcacaaagtacaaggcactgagagaaagtctgtgaagatggcatgaaagaaaaacaactggggcacagctgttcaaaagcagctcga
 K A G A K A G K A A K Y K G T E R K C C E D G M K E N N M G H S C S K R A R
 tataatcaggagaccttggagtgatgctgctgactgctgagcttctcagtaaaactcaggatggagaagagacgggagcaactgtttttgtcaagaagcgaagtgaagaa
 Y I R E T L E C V N A F M D C C E F I S K L R M E K R R E Q L F L S R S E V E E
 ggatacctatcggatgaagatatacctccaggaccgagttccagagagctggttctggaaagtggagcagctggggaaccaccggatgcaaatggaatctccagtaaaacattgcaa
 G Y L S D E D I T S R T E F P E S W F W K V E Q L V E P P D A N G I S S K T L Q
 atgttctgaaggattccatcaccactcgggaagttctgtgctcagcctctcaccogaaaggggatctgtgtagccgatccatataaatcactgtgttcaagaagtactttattgac
 M F L K D S I T T W E V L A V S L S P G K G I C V A D P Y E I T V F K K Y F I D
 ctgctctaccgtactctgtcgtcagaaatgaacagtggaatcogagctgtgcttttaactatgaggaagaccaagatgaaataaagggtcgcgctgagctcatgtacaatgaaaaa
 L R L P Y S V V R N E Q V E I R A V L F N Y E E D Q D E I K V R V E L M Y N E K
 gtctgcagcgtctccactcacaagaagaattcagtcagaatgtgatgatgacaggcaaatcttcaogtctgtgtccttattattgtgccctggagctgggtctgattgacattgaa
 V C S A S T S K K K F S Q N V M M T G K S S R A V S F I I V P L E A G L I D I E
 gtgaagcttattttcacaactacattggtagcggagtgaagaagaactaaaagtgtgctgaaggaatgaaatgagcaaacagtcacagtggtgacctggatccagcaactaaa
 V K A Y F P N Y I G D G V K K K L K V V P E G M K M S K T V T V V T L D P A T K
 ggaaaaagtggtgtccaggaagagaagattaaggctgctgattgaaggatattgttccaaaagtgaatctgagaccatggtagcgtccaaggaacccccatcacacaactaattgag
 G K D G V Q E E K I K A A D L K D I V P K S E S E T M V S V Q G T P I T Q L I E
 aatctatagatgggtcaaatcttgggcatcttattgtggttctctgcg
 K S I D G S N L G H L I V V P R G C G E Q N M

5' primer
 5'GNTGYGGNGARCARAAYATG 3'
 3' CNACRCCNCTYGTTRTAC 5'
 atgtcaatgactccggtagtcattgcaacatatttctggacaacaccggacagtgggagacctgggagtgaaaccgagagctgaagccatc
 G C G E Q N M M S M T P V V I A T Y F L D N T G Q W E T L G V N R R A E A I
 aagaacattaatctgggttacacacagcagttgacttaccgccaaggtgactcttcttactgctcctggataaacggacctggaagtacc
 K N I N L G Y T Q Q L T Y R Q G D S S Y A A W I N R P G S T W L T A Y V
 3'ACCRANTGNCGNATRCA 5'
 3' primer

B

5' primer
 AARGCNGGNCNAARGC
 agcaactgggtcaagataaacctcgcaccgatattgtgcgacaaaacgagatgttagtttcgatgataaaaggatagaggcattaaactcttttaaaaatatt
 K A G A K A A T G Q D K P C T D I V R P K R D V S F D D K R I E A L N S F K N I
 gttatgagaaagtgtgctggatggtatgaaggatataccccggaagagacactcogaagtgggctggctgatcaaatgagaaattccttccgcgctgcatgaaagccttcaag
 V M R K C C L D G M K G Y P T E E T C E V R A G R I K D R K F L P R C I E A F K
 agatgtgtgcttgggtggaagaggaagaggattacacaggcaaaaactggaactggctcgaatgogggcaaggacgctctttgatgtggattcaccacaaattcgcagctatttccct
 R C C R L V E E E E D Y T G K T L E L A R M R A R T L F D V D S P Q I R S Y F P
 gaaagctggatggtgaaacccacaaaatcacagacagatcgggttccagctcctgtcattcactctaccggactcttggactcgtgggaaattcaaggcattggattttctgacca
 E S W M W K T H K I T D R S G S Q S L S F T L P D S L T S W E I Q G I G I S D Q
 ggtctctgtggtgctcagtgagagtgaggtggttaaagatgtcttctcaacgcgcagctacttactcagttgtcgtggtgaaacagatcgagctgataggctctgtttataac
 G L C V A D P V R V E V V K D V F L N A H V P Y S V V R G E Q I E L I G S V Y N
 tacaagactccagagttagggtgtgtgcacaatggcagtcggagaggaatctgtttattcaaggcatgtccaccaagaaaaagggatccagaccaccactgacggcagctcatat
 Y K D S R V R V C V T M A V G E E I C L F K G M S T K E K G I Q T T T C S Q S Y
 ctccagcctcctccgtcacagtggtaaagttcaaatcctgcccctggaacttgggctgactccatcaacttcaactcctcaaatctgaatggaatgaaagtggtgaaaactttg
 L Q A S S V T V V K F K I L P L E L G L H S I N F T L K S E Y G H E K V V K T L
 agggctgctccggaaggcgtaaaaaggagaagatggttggattcactctggaccacaaggagttatgtgttactgaaaaggcgtcatgatattcgatacatggtgctcctaatgtg
 R V V P E G V K K E K M V G F T L D P Q G V Y G V L K R R H D I R Y M V P P N V
 gtcccaaaatccaagatcgacaggactctgagatataaaggccttattctgggtgacgtttatctctgctgtctgaaatcctgaaggcgtgaaactgctcaccatctacaaaagggtgac
 V P K S K I D R T L S I K G L I L G D V I S A V L N P E G V K L L T N L P K G S
 gccgagacagagctcatgagagtagtgcacaatattttatgtcttccattacttggaggcactggatgactggcaactcctcgtcctctgtccttaactcaaggacacaactgagacaa
 A E T E L M R V V P I F Y V F H Y L E A L D D W Q L L G P L S L T Q R T N M R Q
 cagatgaaagatggtattctaagcatcctgtcttttagaaaacgctgactctcataatagcatgtggaagggcogcagtgcaagcatg
 Q M K D G I L S I L S F R N A D F S Y S M W K G R E S S M W L T A Y V
 3'ACCRANTGNCGNATRCA 5'
 3' primer

FIGURE 2. Nucleotide and deduced amino acid sequences of C3 and C5. The translated amino acids of C3 (A) and C5 (B) are in single-letter code. Arrows indicate the sequence of primers used in the RT-PCR. The underlined sequences indicate sequences of probes used for the in situ hybridization.

The membrane was washed and developed using ECL solution (Amersham, Arlington Heights, IL).

Immunohistochemistry

Frozen samples were sectioned and processed for immunohistochemistry as follows: frozen sections from the various stages of limb and lens regeneration, including intact limbs as well as intact lenses, were preincubated with 10% goat serum in 1× PBS for 1 h at room temperature, then with anti-C3 or anti-C5 Ab (1:100) in 1× PBS/10% goat serum/0.3% Triton X-100 (PBSGST) for 1 h at room temperature. After several washings in 1× PBS, the sections were incubated with a rhodamine-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 in PBSGST for 2 h at 37°C. The sections were then washed in PBSGST followed by 1× PBS and then counterstained with 4',6'-diamidino-2-phenylindole (DAPI; Roche, Indianapolis, IN), mounted with Vectashield (Vector Labs, Burlingame, CA), and examined under a fluorescence microscope.

In situ hybridization

In situ hybridizations were conducted essentially as previously described for newt tissues (19). Slides containing paraffin sections were deparaffinized in xylene and then hydrated through an ethanol series. The slides were rinsed in 1× PBS and then fixed in 4% paraformaldehyde for 15 min. After a rinse with 1× PBS, the slides were incubated with 250 µg/ml pepsin at 37°C for 11–15 min. They were again rinsed with 1× PBS and then treated with 0.1 M triethanolamine/0.25% acetic anhydride. The slides were then washed with 1× PBS and dehydrated through an ethanol series. After 1 h of air drying, the sections were hybridized at 58°C for 16 h with hybridization solution (10 mM Tris-HCl (pH 7.5) containing 50% formamide, 1 mM EDTA, 600 mM NaCl, 0.25% SDS, 10% PEG 6000, 1 Denhart's, 200 µg/ml tRNA, and 250 ng/ml digoxigenin-labeled probes). The next day, the slides were washed with 4× SSC, followed by treatment with 50 µg/ml RNase at 37°C for 1 h. The slides were incubated in 2× SSC at 63°C two times for 30 min each, then in 0.1× SSC at 63°C two times for 30 min each. For immunological detection, the slides were rinsed in buffer 1 (0.1 M Tris-HCl (pH 7.5) with 0.15 M NaCl) and then incubated in buffer 2 (buffer 1 with 1% blocking reagent (Roche, Indianapolis, IN)) for 1 h at room temperature. The sections were then incubated with alkaline

phosphatase-conjugated anti-digoxigenin Ab in buffer 2 at 1:2500 for 1 h at room temperature. After three washes with buffer 1 for 30 min each, the slides were incubated in buffer 3 (0.1 M Tris-HCl (pH 9.5) with 0.1 M NaCl and 50 mM MgCl₂) for 10 min, then in the same solution plus nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate for 16–24 h. The reaction was stopped with Tris-EDTA, and sections were mounted with Crystal Mount (Biomedica, Foster City, CA).

Results

Cloning of newt C3 and C5

To obtain partial amino acid sequence for newt C3, the protein was purified from newt serum. SDS-PAGE analysis of the purified C3 protein under nonreducing conditions showed a single 200-kDa band. Under reducing conditions, a 120-kDa α -chain and a 70-kDa β -chain were detected (Fig. 1B). To assess whether the purified protein was indeed C3, it was electrophoresed and electroblotted onto a PVDF membrane, and the α -chain was subjected to Edman degradation. The obtained sequence (Fig. 1C) showed high sequence similarity to C3 from other species. These data suggest that the isolated protein is indeed C3.

Partial cDNA sequences of newt C3 and C5 were obtained via RT-PCR as described in *Materials and Methods* (Fig. 2, A and B). The deduced amino acid sequence of newt C3 was 55% identical with that of human C3, 58% identical with that of *Xenopus* C3, and 62% identical with that of chicken C3. Likewise, the newt C5 amino acid sequence had 48% sequence identity with human C5, 46% with mouse C5, and 42% with trout C5.

Expression of recombinant C3a and C5a in *E. coli* and production of specific anti-C3 and anti-C5 Abs

Given the partial cDNA sequences of newt C3 and C5 that we had obtained, we decided to subclone and express putative C3a_{8–76} and C5a_{9–79} fragments in *E. coli* (22) (Fig. 3A). The observed mass of

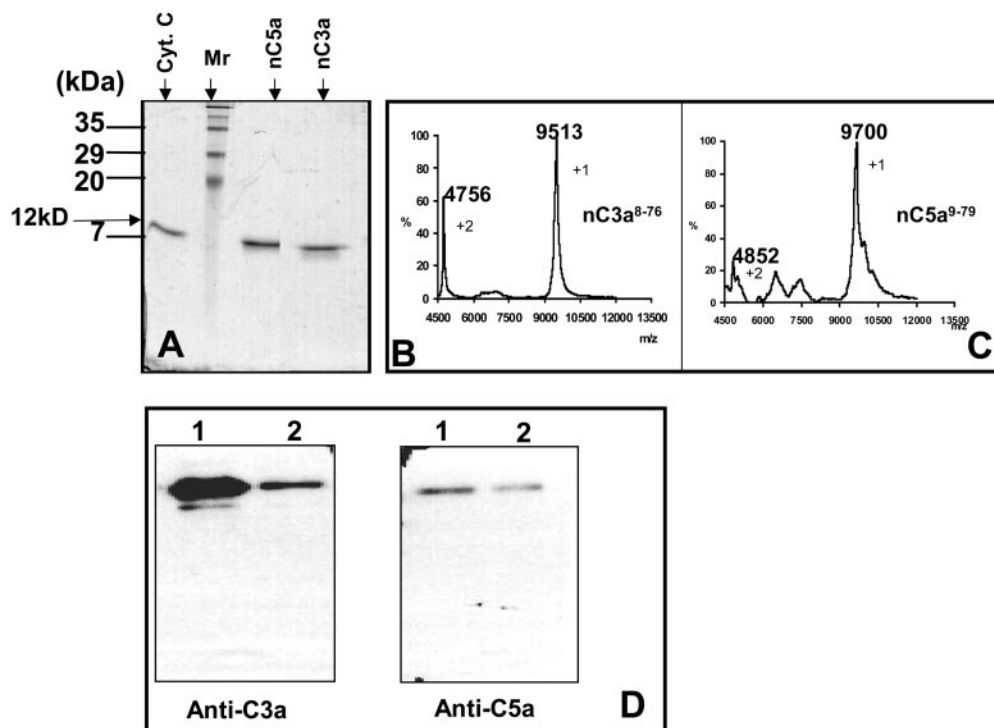


FIGURE 3. Characterization of expressed newt (n)C3a_{8–76} and nC5a_{9–79} and of Abs to these proteins. *A*, Coomassie-stained 16% SDS-PAGE gel. *Lanes* 1, 1 µg cytochrome *c*; 2, markers; 3, 1 µg nC5a_{9–79}; and 4, 1 µg nC3a_{8–76}. Mass spectrometric analysis of nC3a (*B*) and nC5a (*C*). *D*, Specificity of anti-C3 and anti-C5 Abs. Normal (*lane* 1) or cobra venom factor-treated (*lane* 2) serum was subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel under nonreducing conditions, blotted onto PVDF membrane, and then incubated with polyclonal anti-C3a (*left panel*) or anti-C5a Ab (*right panel*) followed by anti-rabbit IgG HRP-conjugated Ab and ECL solution.

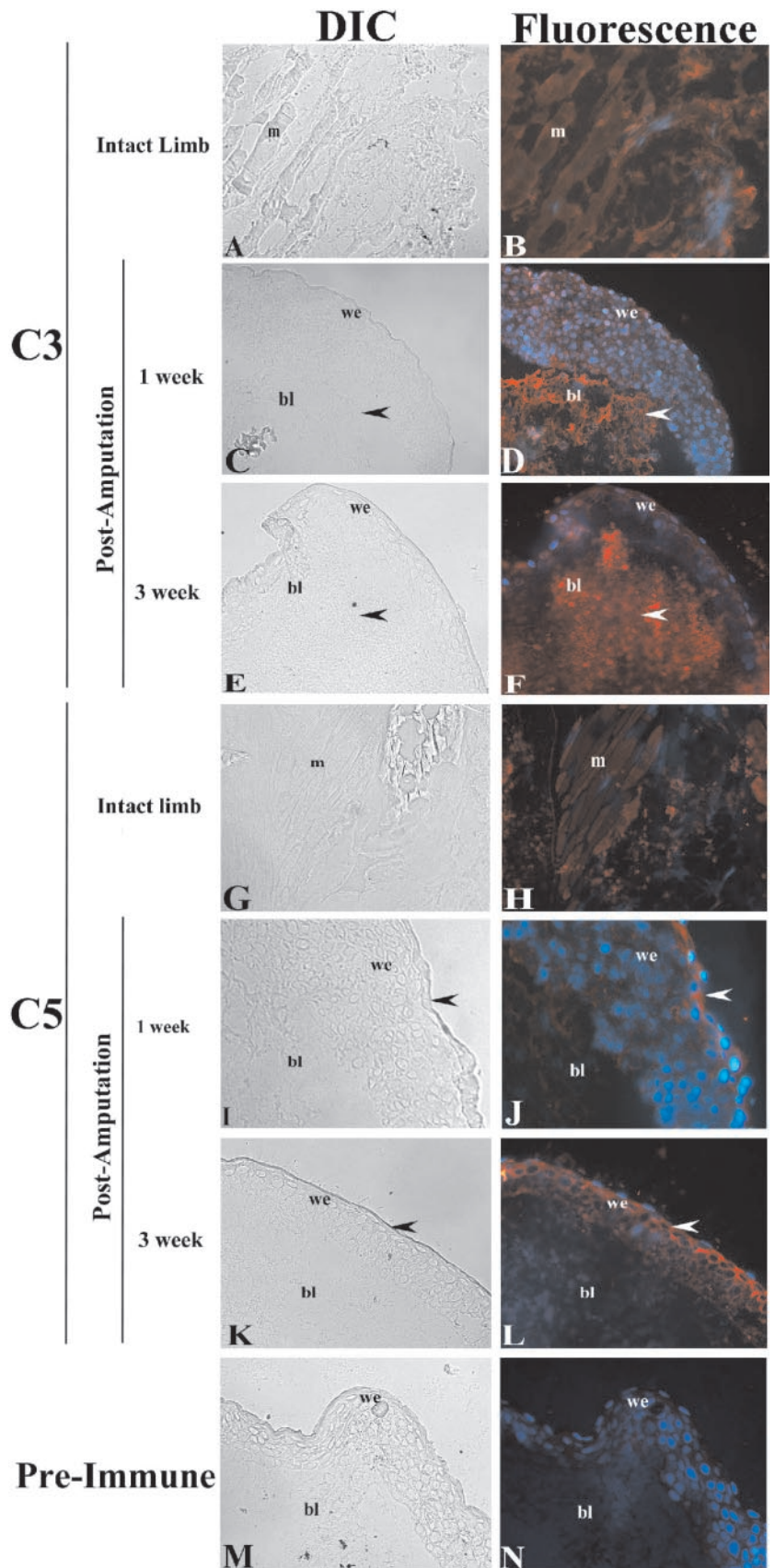


FIGURE 4. Expression of newt C3 and C5 proteins during limb regeneration. Expression of C3 (A–F) and C5 (G–N) proteins during limb regeneration. Column one contains differential interference contrast (DIC) images (magnification, $\times 20$); column two shows the corresponding fluorescence images. Intact muscle in the limb (A and B) shows no C3 expression. C3 (red) is predominantly expressed in the blastema as early as 1 wk (C and D) postamputation and is maintained even at 3 wk postamputation (E and F). C5 is not expressed in intact muscle (G and H) but is expressed in the WE of the regenerating limb at 1 wk (I and J) and 3 wk (K and L) postamputation. Using preimmune serum instead of anti-C3 or anti-C5 Ab yields no fluorescent signal (M and N). Sections have been counterstained with DAPI (blue). bl, Blastema; m, muscle; and we, WE. Arrowheads indicate areas of C3 or C5 protein expression.

expressed C3a protein is 9513 (Fig. 3B) and of newt C5a is 9700 (Fig. 3C), which are very close to the theoretical mass values of 9514 and 9704, respectively. Polyclonal Abs were then raised

against these recombinant proteins. These Abs specifically recognized newt C3 and C5, respectively, but only under nonreducing conditions (Fig. 3D).

Expression of C3 and C5 during limb regeneration

After the newt limb is amputated, the first event that takes place is the closure of the wound by a specialized epithelium, the wound epithelium (WE). This epithelium provides the necessary signals to the underlying differentiated tissues, such as muscle and bone, to dedifferentiate. During dedifferentiation, these tissues lose the characteristics of their tissue of origin (i.e., muscle fibers become mononucleated), and by division, they produce a population of undifferentiated cells called the blastema. After ~2 wk, the blastema starts to differentiate into the various tissues that comprise the limb and to reconstitute the lost parts with exact replicas of the missing parts (24).

To analyze the expression patterns of C3 and C5 in this process, we examined sections taken from the blastema 1 or 3 wk after amputation. These stages represent an early and a late blastema. Immunohistochemical staining of regenerating limbs using the newt-specific anti-C3 and anti-C5 Abs indicated that C3 and C5 had nonoverlapping expression patterns. Even though C3 was present in the WE, most of the protein was found in the blastema, as was evident in the early regeneration stage, the 1-wk blastema (Fig. 4, C and D). This pattern continued in later stages of limb regeneration (Fig. 4, E and F). In contrast, C5 was present exclusively in the WE, with no expression in the blastema at any stage of regeneration (Fig. 4, I–L). Neither protein was found in the intact limb (Fig. 4, A and B (C3), and 4, G and H (C5)). In addition, in situ hybridization for C3 and C5 demonstrate that these molecules are being made locally, which suggests that systemic activation of complement is not a prerequisite for its involvement in limb regeneration. In early (1 wk; Fig. 5, A and D) and late (3 wk; Fig. 5, B and E) regenerates, both of these molecules are expressed in the WE and the blastema. This supports previously reported expression for C3 during axolotl limb regeneration (19).

Expression of C3 and C5 during the process of lens regeneration

After removal of the lens, the pigment epithelial cells of the dorsal iris dedifferentiate (lose their pigment) and form a small vesicle. At ~10 days after lensectomy, this vesicle begins to synthesize crys-

tallins. As the vesicle continues to grow, it differentiates into lens epithelial cells and lens fibers. This differentiation continues for ~2 wk, and at ~25–30 days, the lens has completely regenerated (25, 26).

C3 was not seen in the regenerating lens vesicle at any stage of lens regeneration (Fig. 6, D–I). However, it was present in the stroma and pigmented epithelium of the iris and in the cornea. In contrast, C5 was present in the regenerating vesicle at 10 (Fig. 6, M–O), 15 (not shown), and 20 days (Fig. 6, P–R) postlensectomy. C5 was also present in the cornea at all stages of regeneration (not shown). C3 and C5 were absent from the intact lens (Fig. 6, A–C (C3) and J–L (C5)); however, they were present in the corneal tissue (not shown). No reactivity was detected in the retina at any stage with either of the Abs (not shown).

To confirm that C3 and C5 were made locally in the eye tissues, we performed in situ hybridization studies using both C3- and C5-specific probes. Both C3 and C5 mRNA were found to be present in the regenerating eyes. C3 and C5 also showed similar expression patterns in the regenerating lens vesicle at all stages from 10–20 days postlensectomy (Fig. 7, B–E (C3), and G–J (C5)). C3 was also expressed in the retina (not shown), while C5 was exclusively expressed in the regenerating lens. No expression of either C3 or C5 was found in the intact lens (Fig. 7, A and F).

Discussion

We have previously reported that C3, a central component of the complement system, is expressed in the regenerating blastema of the amputated limb in axolotls. This finding suggested that complement may exert a novel, nonimmunologic role in complex developmental processes such as limb regeneration in urodeles. We have previously demonstrated by in situ hybridization that a newt blastema cell line expresses C3 mRNA, suggesting that these cells are actually synthesizing C3 locally (19). In the same report, we showed that C3 mRNA and protein were also expressed in the regenerating limb of another salamander species, the axolotl, which does not regenerate eye tissues. Extending this intriguing

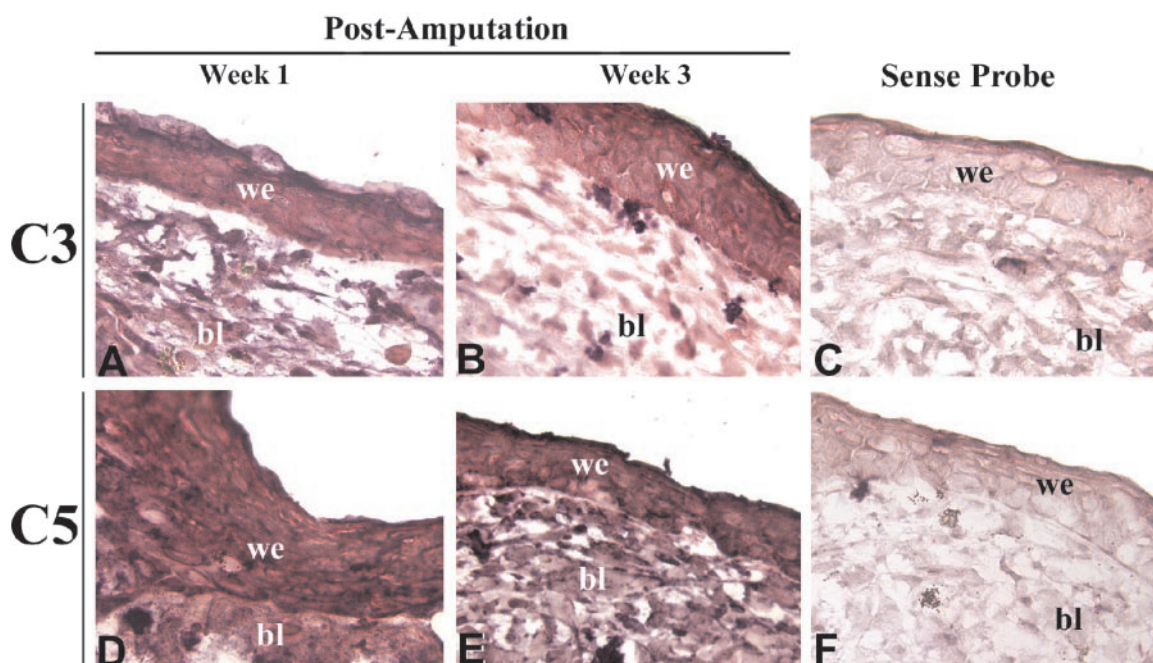


FIGURE 5. Expression of C3 and C5 mRNA during limb regeneration. In situ hybridization shows that C3 (A–C) and C5 (D–F) mRNA are expressed in both WE (we) and the blastema (bl). Images are $\times 40$.

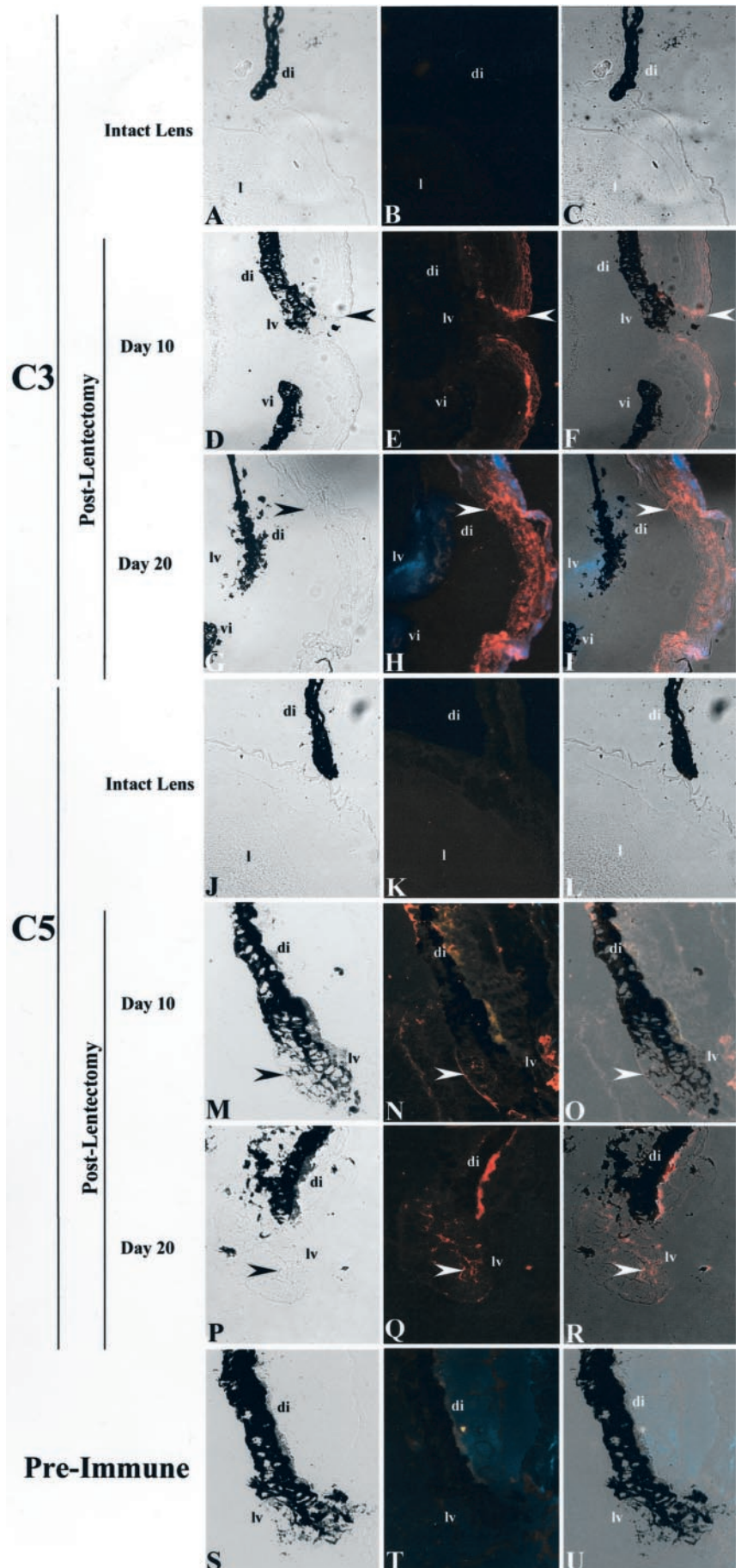


FIGURE 6. Protein expression of C3 and C5 during lens regeneration. The first column contains DIC images (magnification, $\times 20$ in A–L and $\times 40$ in M–U) of the sections in columns two and three. Column two contains fluorescence images, while the pictures in column three are overlays of the DIC and fluorescence images in columns one and two. The intact lens shows no expression of C3 protein (A–C). C3 (red) is expressed in the stroma and pigmented cells of dorsal iris at days 10 (D–F) and 20 (G–I) postlentectomy. Sections have been counterstained with DAPI (blue) to visualize nuclei. C5 protein expression (red) is shown in J–R. There is no C5 expression in the lens of an intact eye (J–K). C5 expression is seen in the dorsal iris and lens vesicle at days 10 (M–O) and 20 (P–R) postlentectomy. There was no fluorescent signal when preimmune serum was used instead of either anti-C3 or anti-C5 Ab (S–U). c, Cornea; di, dorsal iris; l, lens; lv, lens vesicle; and vi, ventral iris. Arrowheads indicate areas of C3 or C5 protein expression.

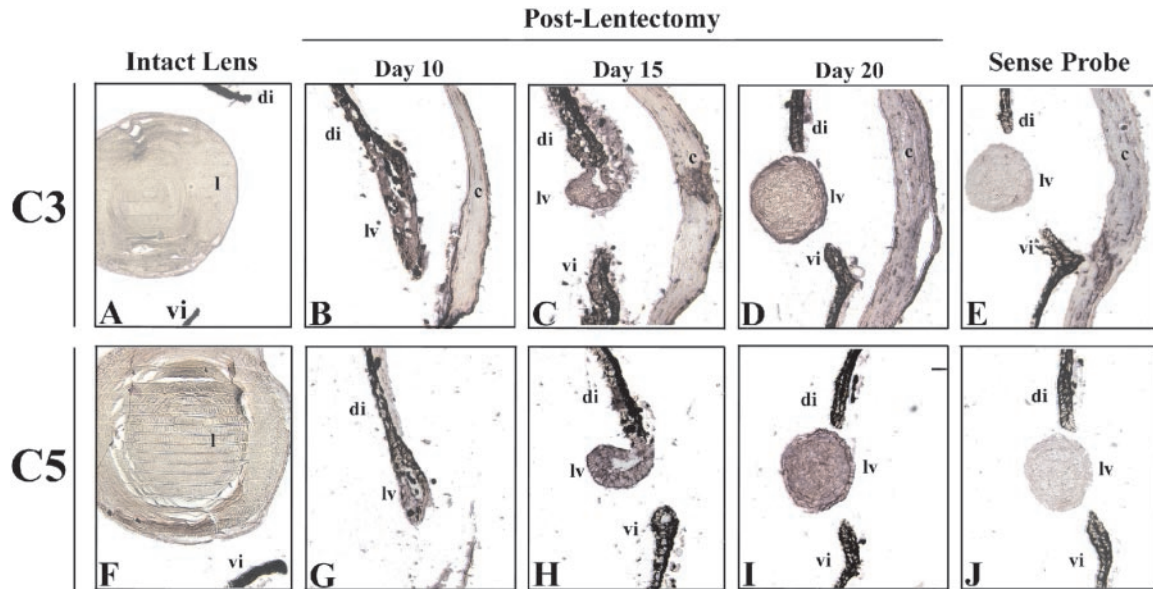


FIGURE 7. Expression of C3 and C5 mRNA during lens regeneration. In situ hybridization shows that C3 (A–D) and C5 (F–I) are expressed in the regenerating lens vesicle. C3 mRNA is not expressed in the intact lens (A), but C3 expression is seen in the lens vesicle at day 10 postlentectomy (B). A similar pattern is seen at days 15 (C) and 20 (D) postlentectomy. A day 20, regenerating lens vesicle probed with the sense probe gives no signal (E). Similarly, an intact lens does not show any C5 expression (F), but lens vesicles at days 10 (G), 15 (H), and 20 (I) postlentectomy express C5 mRNA. There is no staining when a C5 sense probe is used (J). c, Cornea; di, dorsal iris; l, lens; lv, lens vesicle; and vi, ventral iris.

observation to another urodele species that possesses the remarkable capacity to regenerate several of its body parts, we investigated the involvement of complement in urodele regeneration in a more rigorous and systematic manner. In that respect, we focused on dissecting the role of two key components of the complement activation cascade, C3 and C5, in two distinct regenerative processes, limb and lens regeneration.

We have now shown that C3 protein is present throughout the process of limb regeneration in the newt *N. viridescens* and is found mainly in the blastema, with some staining of the WE as well, matching the pattern that we have reported previously for the axolotl, but with a more distinct pattern in the blastema. In contrast, we observed that C5 (not previously reported) is strongly expressed in the WE at all stages of regeneration. This differential distribution of complement proteins is indicative of a specific role in the process of limb regeneration.

During lens regeneration in the newt, the distribution pattern was again complementary, even though both molecules were present in the cornea. C3 was found in the stroma and pigmented epithelial cells of the iris, while C5 was mostly found in the regenerating lens vesicle. No expression was observed in the intact lens. We also show in this study that complement components are locally being synthesized in the eye during the process of regeneration (Fig. 7). It is interesting to note that, even though both C3 and C5 mRNA were expressed in the regenerating lens vesicle, we detected only the C5 protein. There seems to be a regulatory mechanism in place that controls C3 translation, or a rapid degradation or processing of the protein product occurs. However, another possibility is that, in addition to the locally produced C3 and C5, circulating complement molecules move to these tissues during the process of regeneration.

It is possible that these complement molecules play a role in the proliferation and establishment of the blastema (limb) and lens vesicle (lens). It is known that complement components play a role in mitogenic events, as has been reported for C3 during the proliferation of a macrophage-monocyte progenitor lineage (20) and

during the proliferation and growth of B cells in vitro (9). Several studies have suggested that the terminal complexes (C5b-9) can exert a mitogenic effect on various cell types, including mouse fibroblasts (14), human aortic smooth muscle cells (15), and glomerular epithelial cells (17), when administered in sublytic doses (18). Moreover, the anaphylatoxic peptide C5a has been shown to be mitogenic for undifferentiated human neuroblastoma cells (27).

It is also possible that these molecules play a role in early differentiation processes, or even later processes such as differentiation. Reca et al. (28) have found expression of several complement receptors and complement components in normal human early stem/progenitor cells as well as in lineage-differentiated hematopoietic cells. Functional studies should further clarify the role/roles of these molecules in these regenerative processes. For now, it is important to stress that, in other systems, C5 has been shown essential for the process of liver regeneration, because C5 knockout mice exhibit defective liver regeneration after acute toxic injury (20).

Although our studies point to a possible role of the complement system in regenerative processes, its actual role in a pure developmental sense, such as pattern formation, remains unclear. In this respect, it is interesting to note that a molecule with homology to CD59, a protein that binds and inhibits membrane attack complex formation, has recently been labeled as an indicator of blastemal cell identity during limb regeneration (29), i.e., its expression depended on the position of the cells along the proximo-distal axis of the limb. Such discoveries, in association with our data, further support a new role for the complement system in regenerative processes.

Finally, it is interesting to speculate that complement components might be involved in tissue regeneration by binding to cell surface receptors and triggering signaling pathways that modulate cell-cell interactions and/or adhesion. All these could lead to proliferation, differentiation, or positional identity. Alternatively, cleavage of C3 and C5 could generate C3a and C5a which could stimulate the vascular and cellular elements of local inflammation.

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