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Expression of the Third Component of Complement, C3, in Regenerating Limb Blastema Cells of Urodeles[1,2]

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In this study we have shown that complement component C3 is expressed in the regenerating tissue during urodele limb regeneration. C3 was expressed in the dedifferentiated regeneration blastema and in the redifferentiated limb tissues in the axolotl, *Amblystoma mexicanum*, and in *Notophthalmus viridescens*. This expression was verified by immunofluorescent staining using an Ab against axolotl C3 and by in situ hybridization with an axolotl C3 cDNA probe. 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. We then demonstrated by hybridization experiments that a blastema cell line of myogenic origin expresses C3. All these findings implicate C3 in the dedifferentiation process and may indicate a new role for this molecule in muscle differentiation. ***The Journal of Immunology*, 1998, 161: 6819–6824.**

In several urodele (salamander) species, faithful regeneration of an amputated limb occurs within a few weeks of the injury. This remarkable regeneration process depends on the ability of the terminally differentiated cells of the limb (muscle, bone, and other connective tissues) to dedifferentiate and produce an embryonic-like cell mass, the blastema, at the site of injury. The blastema cells can then redifferentiate and reconstruct the lost part (1, 2). One of the major characteristics of dedifferentiation is the alteration and reorganization of the extracellular matrix. Previous studies have clearly demonstrated that several changes take place within the matrix to ensure that the appropriate interactions and communication occur among blastema cells; these changes involve factors that have been shown to play active roles in adhesion, cell differentiation, and tissue remodeling (3, 4).

It is our belief that components of the immune system may also be important in the regeneration process. Macrophages, for example, might be able to assist in the dedifferentiation processes by helping defend the organism against injury-related exposure to pathogens; such a role has been described for muscle macrophages in injury (5). If macrophages are indeed involved in the regeneration process, there must be signals to attract them to the site of injury. In investigating the possible factors that might be playing roles in such phenomena, we concentrated on the complement component C3, a multifaceted molecule (6, 7) that is expressed in all vertebrate (8) as well in invertebrate species (9). In addition to its interactions with several complement proteins, C3 is known to interact with several proteins that are involved in dedifferentiation, such as fibronectin and integrins (10–12). The intersection of the immune system with the developmental program needed for limb regeneration has been the subject of speculation in the past (1, 2), but no concrete cellular or molecular data are available to elucidate this possible interrelationship. Cells that are not part of the immune system, such as myoblasts, have been found to express complement proteins in vitro (13, 14), which suggests that molecules of the immune system might also be involved in nonimmunologic functions. Most likely, the presence of C3 in muscle provides protection in response to injury; likewise, it is conceivable that C3 should function in urodeles to ensure normal muscle dedifferentiation and regeneration rather than necrosis in response to amputation. Given the expression of C3 in muscle we thought it useful to determine whether C3 is expressed in urodele limb muscle, especially in response to injury that is associated with limb regeneration.

In the present study we used a specific Ab recognizing axolotl C3 as well as a partial axolotl C3 cDNA clone to establish that C3 is expressed in the blastema cells of the amputated limb as well as in those that differentiate to reconstitute the limb. The presence of the mRNA was also observed in cultured blastema cells of the myogenic lineage. These findings support the involvement of complement proteins in regenerative processes and also provide evidence suggesting a role for C3 in muscle differentiation.

Materials and Methods

Animals

Two urodeles, the axolotl *Amblystoma mexicanum* and the newt *Notophthalmus viridescens*, were used in this study. Axolotls were provided by the Indiana University Axolotl Colony (Bloomington, IN), and newts were purchased from Amphibia of North America (Nashville, TN). The limbs were amputated at the mid-ulna-radius level, and the regenerated portions were collected at various stages: early blastema (1 wk postamputation), mid-blastema (2 wk), or palette stage (3 wk). We also collected axolotl embryonic limb at the late bud stage. The collected limbs were either embedded in OCT (Miles, Elkhart, IN) and frozen at −70°C or embedded in paraffin.

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[2] The sequence described in this paper has been deposited in the GenBank database under accession number 3641563.

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Culture of limb cells

Blastema cells, especially those of myogenic lineage, can be cultured efficiently from muscle explants collected from an area immediately proximal to the amputation site. Dedifferentiated mononuclear cells grow out of these explants (15, 16). Such lines have previously been generated in our laboratory from the newt and were used in the present study. The cultures were kept in L-15 medium supplemented with 10% FCS and 2% CO_2.

Preparation of anti-C3 Ab, Western blotting, and immunofluorescence

A polyclonal Ab recognizing axolotl C3 was raised in rabbits using C3, which has been purified as previously described (17). The Ab was purified using either protein A or Sepharose-C3 affinity chromatography. This Ab was judged to be monospecific, since it reacted only with C3 in Western blot analysis of axolotl serum, blastema, liver, and kidney (Fig. 1). For Western blot analysis, tissues extracts were prepared as follows. First, tissues were dissected and frozen in liquid nitrogen immediately. They were then powdered while in N_2 and homogenized in lysis buffer (250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES (pH 7.0), 5 mM EDTA, 50 mM NaF, 0.1 mM sodium orthovanadate, 50 μg of PMSF/ml, 1 μg of leupeptin/ml, 1 μg of aprotinin/ml, and 1 mM DTT), passed through a 21-gauge needle 10 times, and incubated on ice for 30 min. The homogenates were spun at 10K for 3 min, and the supernatant was subjected to SDS-PAGE on a 7.5% SDS-polyacrylamide gel under reducing (2-ME) conditions followed by blotting onto polyvinylidene difluoride membranes. The membranes were blocked in 1% milk and probed with the affinity-purified anti-C3 Ab (2 μg/ml) followed by horseradish peroxidase-congugated anti-rabbit Ig and enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL).

For immunofluorescence, frozen sections from various blastema stages, from developing limb, intact limb, and liver, were reacted with the Ab (1/100) for 1 h at 25°C. After several washings, the sections were incubated with FITC-conjugated secondary goat anti-rabbit Ab at 1/200, washed, mounted, and examined through a fluorescent microscope. For the negative control, preimmune serum was used.

Cloning of axolotl C3

To isolate axolotl C3 cDNA, RT-PCR using total RNA isolated in this laboratory from axolotl liver was conducted essentially as described by Mavroidis et al. (18). Double-stranded cDNA was synthesized from 2 μg of total RNA and random hexanucleotide primers using the Super Script system cDNA synthesis kit (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. On the basis of *Xenopus* C3 amino acid sequences that are conserved in other species, we designed two degenerate oligonucleotides and used these as PCR primers: primer 1, 5′-(ctg)(cta)ctcca(tc)cca(ct)gtgtcctt-3′ (extends downstream); and primer 2, 5′-gtgga(tc)t(at)(tc)gt(gt)tacaa(ga)(ga)c-3′ (extends upstream).

The oligonucleotides were synthesized using an automated DNA synthesizer (Cyclone Plus, Millipore, Burlington, MA). The conditions for the PCR were denaturation at 95°C for 5 min, annealing at 45°C for 1 min, and polymerization at 72°C for 1 min. The reaction was initiated by adding 5 U of Taq DNA polymerase (Cetus, Northfolk, CT), after which 28 reaction cycles were conducted. The reaction products were separated by agarose gel electrophoresis, and the 266-bp PCR product was extracted from low melting point agarose and subcloned into a pCRII plasmid vector using the TA cloning kit (Invitrogen, San Diego, CA). Recombinant plasmid DNA was purified using the Qiagen kit (Qiagen, Stutio, CA) according to the procedure recommended by the supplier. DNA sequencing of both strands was performed according to Sanger et al. (19) using the Sequenase kit (U.S. Biochemical Corp., Cleveland, OH); each strand was sequenced twice. The PCR product was used in Northern blot analysis of samples from axolotl liver and blood RNA; we identified a 5.5-kb mRNA fragment that is similar in size those encoding C3 in other species.

In situ hybridization

This was conducted essentially as previously described for newt tissues (20, 21). Slides containing paraffin sections were deparaffinized in xylene and subsequently 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 PBS, the slides were incubated with 250 μg/ml of pepsin at

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                V  D  F  V  Y  K  T  T  L  T
AXOLOTL C3  GTGGATTTTGTTTACAAAACTACTCTCACG -30
            |||||||||||.|||||..||||||||||||
XENOPUS C3  GTGGATTTTGTGTACAAGGCTACTCTCACG -30
                V  D  F  V  Y  K  A  T  L  T

                E  L  Q  P  S  D  N  F  D  N
AXOLOTL C3  GAGTTGCAGCCCAGCGACAACTTTGACAAC -60
            ||..|||||||||||.|||||||.|||||||
XENOPUS C3  GAAGTGCAGCCCAGTGACAACTATGACAAC -60
                E  V  Q  P  S  D  N  Y  D  N

                Y  V  M  T  I  K  K  V  I  K
AXOLOTL C3  TATGTTATGACAATTAAGAAGGTCATCAAG -90
            |||||||||||||||||||||||||||.|||
XENOPUS C3  TATGTTATGACAATTAAGAAGGTCATAAAG -90
                Y  V  M  T  I  K  K  V  I  K

                Q  G  T  D  E  D  P  E  D  K
AXOLOTL C3  CAAGGCACAGATGAGGATCCTGAGGACAAG -120
            ||||||||||||||||||||||||||||||
XENOPUS C3  CAAGGCACAGATGAGGATCCTGAGGACAAG -120
                Q  G  T  D  E  D  P  E  D  K

                T  R  N  F  I  S  H  I  K  C
AXOLOTL C3  ACACGTAATTTTATCAGCCATATCAAATGC -150
            ||||||||||||||||||||||||||||||
XENOPUS C3  ACACGTAATTTTATCAGCCATATCAAATGC -150
                T  R  N  F  I  S  H  I  K  C

                R  K  A  L  N  M  Q  L  N  R
AXOLOTL C3  CGAAAAGCTTTAAATATGCAGCTGAACCGA -180
            ||||||||||||||||||||||||||||||
XENOPUS C3  CGAAAAGCTTTAAATATGCAGCTGAACCGA -180
                R  K  A  L  N  M  Q  L  N  R

                D  Y  L  I  W  G  V  T  G  D
AXOLOTL C3  GATTATCTGATTTGGGGGGTAACTGGTGAC -210
            ||||||||||||||||||||||||||||||
XENOPUS C3  GATTATCTGATTTGGGGGGTAACTGGTGAC -210
                D  Y  L  I  W  G  V  T  G  D

                L  W  R  H  D  G  Y  S  Y  I
AXOLOTL C3  CTCTGGCGCCACGATGGATATTCCTACATC -240
            ||||||||..|..||||||||||||||||||
XENOPUS C3  CTCTGGCAGCCAGATGGATATTCCTACATC -240
                L  W  Q  P  D  G  Y  S  Y  I

                I  G  E  D  T  W  M  E
AXOLOTL C3  ATTGGAGAGGACACATGGATGGAGGG -266
            |||||..|||||||||||||||||.|
XENOPUS C3  ATTGGGAAGGACACATGGATGGAGTG -266
                I  G  K  D  T  W  M  E
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FIGURE 1. Alignment of axolotl and *Xenopus* C3 sequences. The bar indicates nucleotide identity, and dots indicate nonidentity.

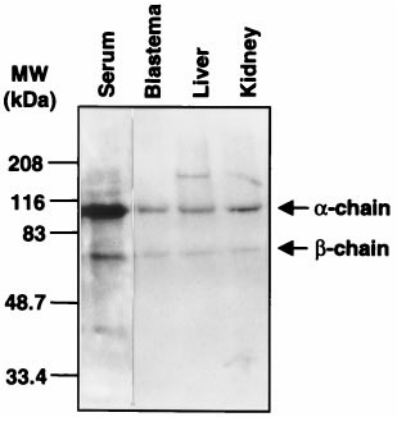

FIGURE 2. Reactivity of anti-axolotl C3 Ab with various axolotl tissues. Axolotl serum (0.25 μl) and tissues extract (20 μg) proteins were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel under reducing (2-ME) conditions, blotted onto polyvinylidene difluoride membranes, and then probed with the affinity-purified anti-C3 Ab followed by horseradish peroxidase-congugated anti-rabbit Ig and enhanced chemiluminescence (ECL, Amersham).

37°C for 15 min, then rinsed again with PBS and treated with 0.1 M triethanolamine/0.25% acetic anhydride for 12 min. After a final wash with PBS, the slides were dehydrated through ethanol series, air dried for 1 h, and hybridized at 50°C for 16 h with hybridization solution (50% formamide, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5), 600 mM NaCl, 0.25% SDS, 10% polyethylene glycol 6000, 1× Denhart's, 200 μg/ml transfer RNA, and 250 ng/ml of digoxigenin-labeled probe); antisense and sense probes were labeled using the digoxigenin RNA labeling kit from Boehringer Mannheim (Indianapolis, IN). The next day the slides were washed with 4× SSC, then treated with 50 μg/ml RNase at 37°C for 1 h. Subsequently, the slides were incubated twice in 2× SSC at 50°C for 30 min each time, then twice in 0.1× SSC at 50°C for 30 min each time. For immunological detection, the slides were rinsed in buffer 1 (0.1 M Tris-HCl (pH 7.5), and 0.15 M NaCl) and then incubated in buffer 2 (buffer 1 with 1% blocking reagent (Boehringer Mannheim)) for 1 h at room temperature. The sections were then incubated with alkaline phosphatase conjugate-anti-digoxigenin Ab in buffer 2 at a 1/2500 dilution for 1 h at room temperature. After three washes with buffer 1 for 30 min each time, the slides were incubated in buffer 3 (0.1 M Tris-HCl (pH 9.5) with 0.1 M NaCl and 50 mM $MgCl_2$) for 10 min and later incubated in the same solution with nitro blue tetrazolium/5′-bromo-4-chloro-3-indolylphosphite for 16–24 h. The reaction was stopped with Tris-EDTA, pH 7.5, and the sections were mounted with Crystal Mount (Biomeda, Foster City, CA). Pictures were produced with a Sony video printer (Sony, Tokyo, Japan).

In situ hybridization was also used to examine C3 expression in cell cultures. For this purpose we used the radioactive method, which produces (in our experience) clearer results in cultured cells. The cells were also hybridized with a muscle lineage-specific probe, myf-5, that has been isolated from the newt (22). Cells attached to glass coverslips were fixed in 4% paraformaldehyde for 10 min, then incubated in 70% ethanol followed by 50% ethanol. The cells were incubated in 1× PBS with 0.5% Triton X-100 and 5 mM $MgCl_2$ for 10 min. The cells were then immersed in triethanolamine buffer for 2.5 min and in triethanolamine buffer plus 0.25% acetic anhydride for another 10 min, after which they were rinsed in 2× SSC and gradually dehydrated with ethanol. [^{35}S]UTP-labeled probes at 1×10^7 cpm/ml were used to hybridize the cells overnight at 50–55°C; labeling of the probes (antisense and sense) was performed using either T7 or SP6RNA polymerase and [α-^{35}S]thiol-UTP. The next day the cells were rinsed once in 2× SSC, then twice in solution 1 (50% formamide with 1× SSC, and 0.1% 2-ME) for 12 min each time at 50–55°C. They were then treated with 20 μg/ml of RNase A for 30 min at 37°C and washed twice in solution 1 at 50–55°C. Two more final washes were performed in solution 2 (1× SSC with 0.1% 2-ME) for 12 min each. The cells were dehydrated through a graded series of ethanol and allowed to air-dry before being

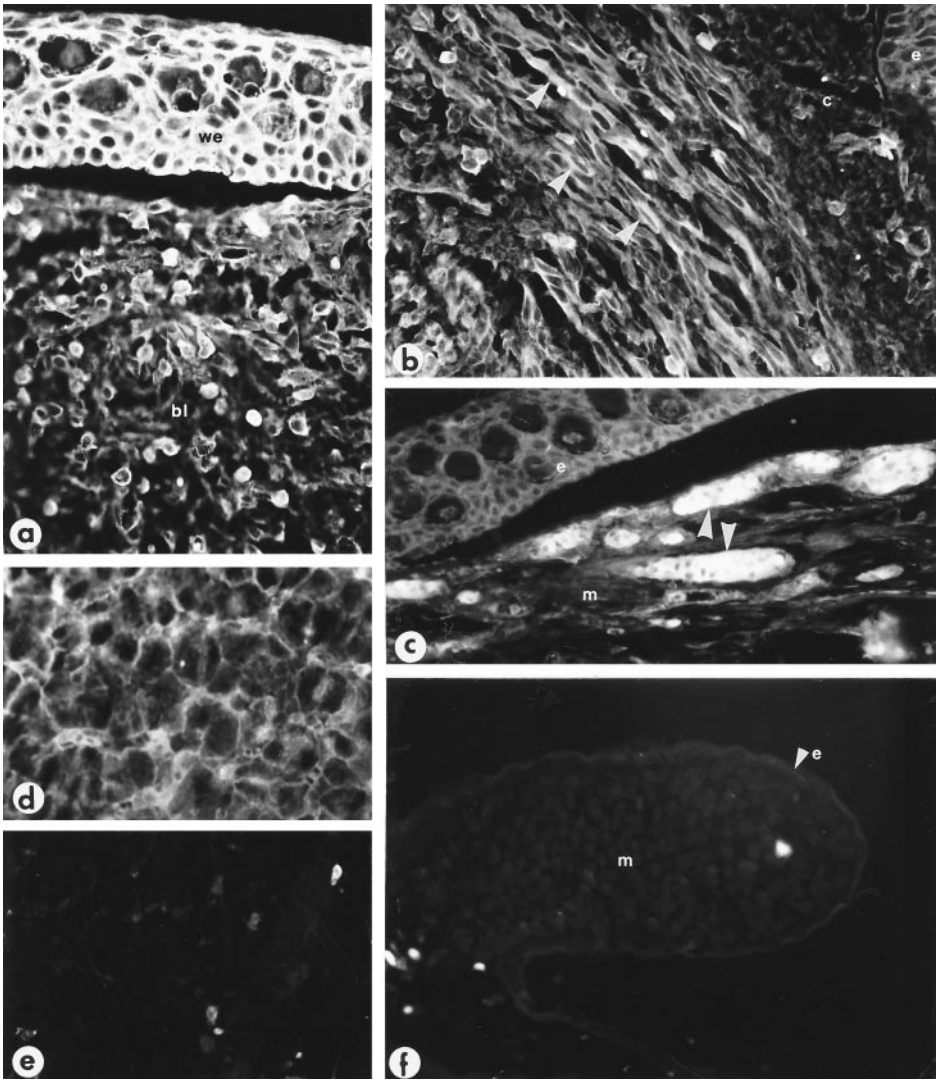

FIGURE 3. Presence of complement factor C3 in axolotl tissues as revealed by immunostaining. *a*, Frozen sections were reacted with rabbit anti-axolotl C3 and FITC-labeled goat anti-rabbit Ab as described in *Materials and Methods*. Sections through a 2-wk regenerating blastema showing positive cells in the wound epithelium (we) and the underlying blastema (bl). *b*, Section through a regenerating limb at the palette stage showing reactivity during the differentiation of the presumptive muscle (arrowheads), epidermis (e), and connective tissue (c). *c*, Section through a normal intact limb showing unstained epidermis (e) and muscle (m); only blood cells are positive (arrowheads). *d*, Section through the liver showing a positive reaction for C3. *e*, Preimmune serum for liver. *f*, Section through developing limb showing no reactivity with the Ab (m, mesenchymal cells; e, epidermis indicated by arrowheads).

exposed to NBT-2 emulsion (Kodak, Rochester, NY). Ten days later the samples were developed and observed under darkfield microscopy.

Results

Axolotl C3 sequences

As discussed in *Materials and Methods* we obtained via PCR a fragment of 266 nucleotides (Fig. 2). This fragment shows extensive sequence similarity (94.36%) to its *Xenopus* counterpart (23).

Expression of C3 during limb regeneration

Since we possessed both an Ab and a probe specific for axolotl (*A. mexicanum*) C3, our initial expression studies were performed using this urodele; protein A- and Sepharose-C3-purified Ab gave the same staining patterns. Once the pattern of expression of this C3 was established we also examined the expression of this molecule in the newt (*N. viridescens*). Both the Ab and the probe cross-reacted with newt C3, and the patterns of expression in the newt were identical with those in the axolotl. Therefore, we report here the expression data we obtained from the axolotl.

When an axolotl limb is amputated the wound epithelium covers the amputation site within a few days; this epithelium provides the underlying mesenchyme with signals to dedifferentiate. As the tissues of the mesenchyme (such as muscle and bone) dedifferentiate, they proliferate and form a blastema, from which the regenerated portion of the limb is derived. Muscle, for example, undergoes membrane lysis to give rise to embryonic-like mononucleated cells, which then begin active cell division. After blastema formation, these cells begin to redifferentiate to form the tissues that comprise the lost part, and an exact replica of the amputated portion of the limb is regenerated.

In our initial expression studies using immunofluorescent staining, C3 was detected in abundance during blastema formation. Positive cells were observed in the wound epithelium and in the undifferentiated blastema (Fig. 3*a*). In the later stages of blastema formation (such as the palette stage in which redifferentiation has begun) we observed a strong reaction in the presumptive future muscle, in areas where muscle fibers are differentiating, as well as in the growing cartilage (Fig. 3*b*). When we compared these staining patterns with those in the normal intact (unamputated) limb, the epidermal and muscle tissue showed weak staining; only blood cells were strongly positive (Fig. 3*c*). Similarly, C3 was not detected in developing axolotl limbs, in the ectoderm, or in the mesenchymal cells (Fig. 3*f*). Thus, the expression patterns we observed were strongly correlated with the process of limb regeneration.

Since C3 is secreted by blood cells (24, 25), the patterns we observed could have been attributed to C3 secreted from blood cells that had migrated into the blastema. To exclude this possibility we proceeded to detect the mRNA via in situ hybridization using our axolotl C3 probe. Again, the expression pattern was consistent with the Ab staining. Expression of the C3 mRNA was observed in the wound epithelium and the regeneration blastema (Fig. 4). One difference was that in the in situ hybridization experiments the mRNA in the epithelium was most prominent in the basal layer, as opposed to being seen in the whole epithelium, as in the case of the Ab staining (Fig. 4*b*). This overall staining could be explained by diffusion of C3 synthesized in the basal layer.

To further corroborate the finding that C3 is expressed in blastema cells of muscle origin, we employed in situ hybridization to examine a cell line generated from muscle explants in culture (16). The cells that grow out from muscle explants in culture are mononucleated, express blastema Ags, and have the ability to differentiate into myotubes in culture or into myotubes and cartilage when transplanted back into an amputated limb (16, 26). In other words, these are blastema cells derived from muscle and then express myogenic markers such as myf-5 (Fig. 5, *a–d*). Our in situ hybridizations showed that these cells also express C3 (Fig. 5, *e–h*).

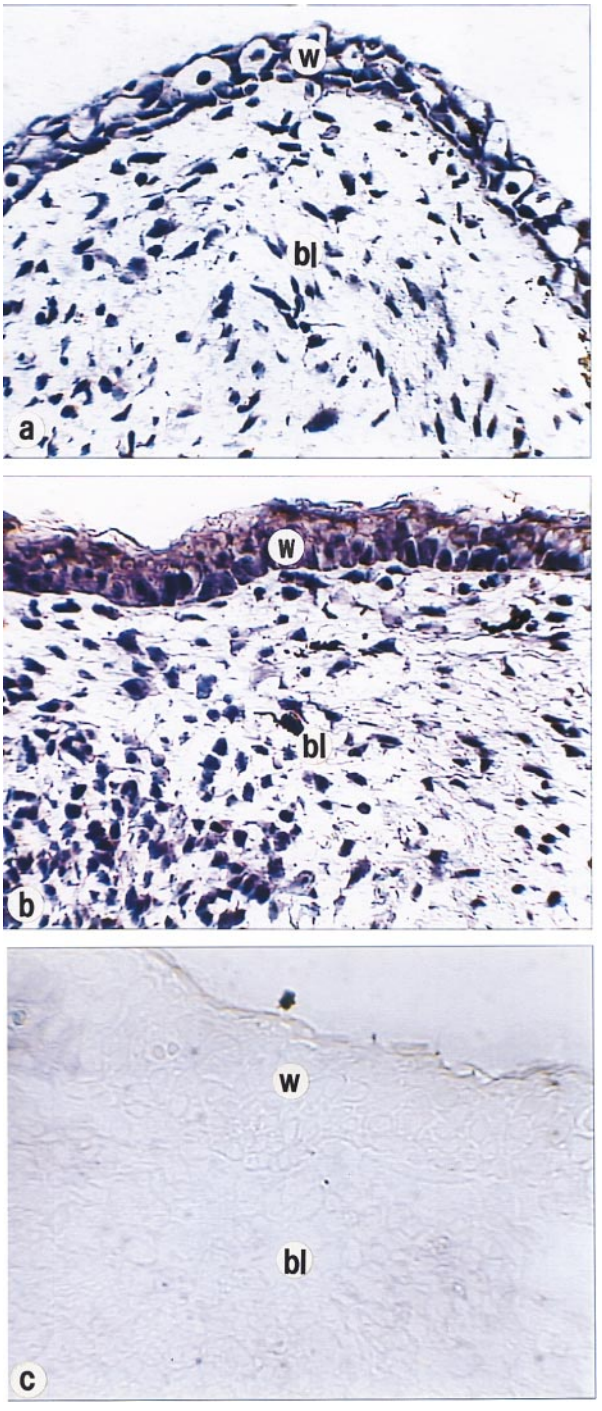

FIGURE 4. In situ hybridization of axolotl regenerating blastema with an axolotl C3 cDNA probe. In situ hybridization at 8 days (*a*) and 2 wk (*b*) after amputation, showing positive reactions (blue color) in the wound epithelium (w) and the blastema cells (bl). *c*, Negative control, hybridization with the sense probe. (Note in *b* that the basal layers (arrows) of the wound epithelium show a higher level of hybridization than do the surrounding tissues.)

Discussion

Our results clearly indicate that C3 is expressed in the blastema cells of the regenerating urodele limb. We have verified the expression of this molecule by demonstrating the presence of C3

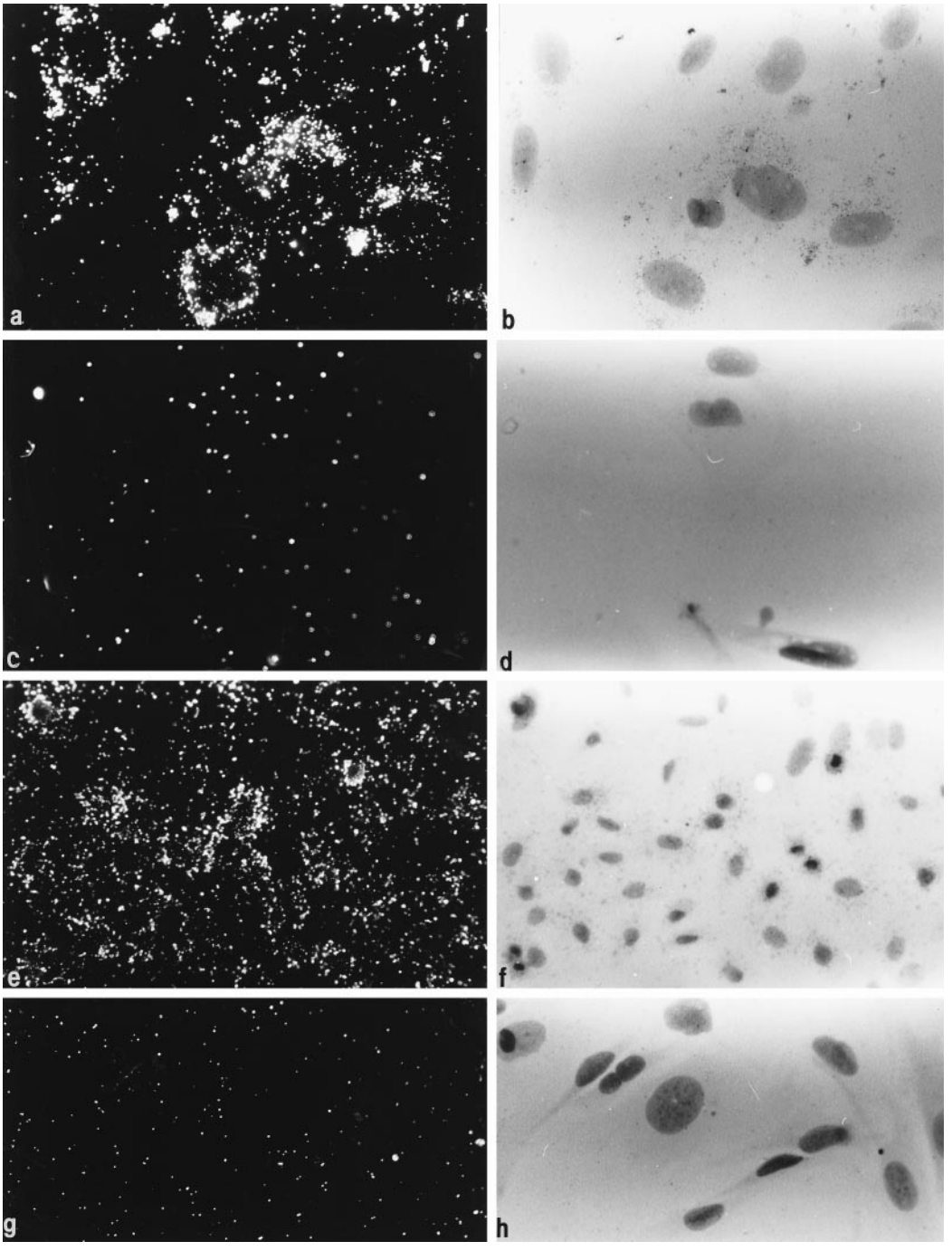

FIGURE 5. Expression of C3 in axolotl blastema cells in culture. *a*, Blastema cells were hybridized with the myogenic marker myf-5 as described in *Materials and Methods*. *c*, Negative control, hybridization with the sense probe. *b* and *d*, Brightfield images of *a* and *c*, indicating the counterstained nuclei. *e*, Hybridization of the C3 cDNA probe to blastema cells in culture. *g*, Negative control, hybridization with the sense probe. *f* and *h*, Brightfield images of *e* and *g*, indicating the counterstained nuclei.

mRNA as well as the protein, and we have shown that a blastema cell line of myogenic origin also expresses C3. In contrast, we did not detect any C3 expression in the developing axolotl limb. This difference between the developing and the regenerating limb suggests that C3 is involved in the process of dedifferentiation and regeneration. We believe that this expression of C3 is most likely associated with the process of dedifferentiation after injury of the muscle due to the amputation. The process of dedifferentiation that follows amputation is characterized by fragmentation of muscle cells and invasion of macrophages (27), the subsequent generation of mononucleated cells. Macrophages are necessary to provide protection of the dedifferentiated muscle from injury-related threats such as invasion by pathogens (13, 14, 27). In this sense, participation of complement proteins should be regarded as necessary for the appropriate initiation, maintenance, and completion of the dedifferentiation process that leads to faithful regeneration of the lost limb structures. Recent discoveries concerning the expression of complement factors in normal myoblasts in vitro (13, 14) support our conclusions.

Another interesting potential role for C3 is as a participant in cellular events such as tissue remodeling and cell adhesion. C3 is perhaps the most multifaceted molecule in the complement system

because it interacts with many proteins, including some that participate in or control cell adhesion and cell-to-cell communication. C3 has been found to interact with laminin and fibronectin and to bind to basement membranes in glomerulus and trophoblasts (12, 28). In addition, C3 binds to receptors that belong to the integrin family of proteins, which are involved in cell adhesion (29). It is possible that the interaction of C3 with proteins involved in cell adhesion is a part of the necessary remodeling that occurs during dedifferentiation. Several of these extracellular matrix proteins are regulated during dedifferentiation, including integrins and laminin (3, 4). Expression of laminin seems to be regulated during the synthesis of muscle membranes that occurs as part of the redifferentiation process (1), where C3 was particularly highly expressed as well.

Other complement factors show interesting sequence homologies to extracellular matrix proteins involved in cell adhesion. Complement factors B and C2 show homologies to cartilage matrix protein, von Willebrand factor, and the collagen binding domain of alkaline phosphatase (30, 31). The existence of these homologous domains strongly suggests that complement factors can, in fact, play a role in events that require cell adhesion and communication adjustments, such as differentiation and, in the present case, dedifferentiation. Such activities might also allow complement factors to act as growth factors. Indeed, this has been shown to be the case for C3 and C3 synthetic peptides, since they are known to support the growth of human CR-2-positive (EBV/C3d receptor-positive) lymphoblastoid B cells (32). Taken together, our results provide strong evidence for the involvement of C3 in the dedifferentiation and differentiation events that occur during limb regeneration.

Shedding light on the mechanisms of dedifferentiation would greatly enhance our understanding of the unique regenerative phenomena observed in some amphibia. In this sense the intersection of the immune system with regeneration could be particularly informative. At present, the role of the immune system in limb regeneration is largely obscure due to the lack of systematic studies and appropriate reagents. Our data provide the first concrete evidence for the specific expression of complement component C3 during the process of dedifferentiation and also suggest the involvement of this protein in muscle differentiation during limb regeneration.

Acknowledgments

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