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A monoclonal antibody stains myogenic cells in regenerating newt muscle

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Summary

Monoclonal antibodies have been used to study minced muscle regeneration in the adult newt, *Notophthalmus viridescens*. The contralateral limb was amputated and the immunostaining patterns in the regenerating blastema were compared with the minced tissue in sectioned material. Staining with a myofibre-specific antibody, called 12/101 (Kintner & Brockes, 1984), showed that myofibre degeneration was complete by 8–10 days after mincing, with myogenesis commencing 2 days later. Another monoclonal antibody, called 22/18, previously shown to label a subset of cells in the regeneration blastema of the newt (Kintner & Brockes, 1984, 1985), was found also to recognize a population of cells in regenerating minced muscle. At 6 days after mincing, the number of 22/18-positive (22/18+) cells was low but by days 12–16, during the period of myogenesis, their number had increased to become a major population within the minced tissue. A small number of the 22/18+ cells could be double labelled with 12/101 at this time. Prior to this, there was a phase in which 12/101 staining had disappeared from the mince. Cells immunoreactive with both antibodies after this phase confirm that at least some of the 22/18+ cells are myogenic. The number of 22/18+ cells decreased as muscle repair and maturation progressed. These results show that 22/18 is not specifically associated with blastemal cells but is a more general marker for regenerating systems in the newt. They further suggest an alternative interpretation of the double-labelled cells used by Kintner & Brockes (1984) as evidence for myofibre dedifferentiation in limb regeneration. Instead, we propose that such cells represent new myogenesis occurring by tissue repair of locally damaged muscle fibres.

Key words: minced muscle, regeneration, pericyte, myogenesis, 22/18, dedifferentiation, newt, *Notophthalmus viridescens*.

Introduction

Adult urodele amphibians are able to regenerate their limbs after amputation by forming a localized growth zone, called a blastema, at the tip of the limb stump. It has been suggested that the cells of the blastema arise by local dedifferentiation of the mature tissues of the limb, including muscle, cartilage, connective tissue and Schwann cells. Dedifferentiation is the process whereby differentiated cells lose their cell-specific characteristics and take on those of undifferentiated precursor cells (Wallace, 1981). The process has been well described for Schwann cells of transected nerves (for example, see Politis, Sternberger, Ederle & Spencer, 1982) and for chondrocytes of amputated amphibian limbs during epimorphic regeneration (Hay, 1962; Steen, 1970). It has been suggested that dedifferentiation of myofibres also occurs after amputation (Thornton, 1938; Hay, 1959; Lentz, 1969), but this has been more difficult to prove conclusively since muscle contains a variety of cell types.

Among the vertebrates, limb regeneration is peculiar to the urodele amphibians, although other vertebrates are able to regenerate damaged muscle. The source of myogenic cells for this tissue mode of muscle regeneration, both *in vitro* (Bischoff, 1975; Konigsberg, Lipton & Konigsberg, 1975) and *in vivo*

(Snow, 1977, 1979), is the satellite cell, which normally lies within the basal lamina of the myofibre (Mauro, 1961). The contention that the muscle of adult newts lacks an observable satellite cell population has been used as evidence in favour of dedifferentiation, particularly during limb regeneration (Hay & Doyle, 1973; Hay, 1979). This view has been countered by others who point to the 'pericyte', or 'postsatellite cell' (which has its own basal lamina), as a possible source of myogenic cells in the newt (Popiela, 1976; Cherkasova, 1982; Cameron, Hilgers & Hinterberger, 1986).

A recent study by Kintner & Brockes (1984) attempted to address the issue of myofibre dedifferentiation by using monoclonal antibodies to identify cell types and to follow their progress into the blastema. The rationale was that, if one cell type is derived from another and specific markers for both cell types are available, then a small proportion of cells might show an intermediate phenotype and label with both markers. One monoclonal antibody, called 12/101, was isolated as a specific marker of skeletal muscle in newts (Kintner & Brockes, 1984) and also cross-reacts with other vertebrate muscle including frog (Gurdon, Fairman, Mohun & Brennan, 1985; Smith, 1987), chicken (Gordon, unpublished observations) and rabbit (D. M. Fekete and P. Bennett, unpublished observations). Comparisons of the 12/101 immunoreactivity in different skeletal muscle fibres of rabbit indicates that fast muscle fibres are more intensely labelled by 12/101 than slow fibres (Fekete, unpublished observations). In newts, the antigen appears very early in limb development (Fekete & Brockes, 1987) and is probably expressed prior to fusion of myocytes into myotubes. Kintner & Brockes described a second antibody, called 22/18, that recognized only a subset of cells in the blastema and had little or no staining in the normal limb of *Notophthalmus viridescens*. Although the biochemical nature of the 22/18 antigen has not been determined, it is an intracellular molecule whose immunostaining on cultured cells suggests that it is a component of the cytoskeleton (Fekete, Ferretti, Gordon & Brockes, 1987).

A subset of the 22/18+ cells in the regenerating limb could be double labelled with 12/101 (Kintner & Brockes, 1984). These double-labelled cells were observed at two time points after amputation of the limb. First, in the proximal area of the mid-bud blastema adjacent to the intact muscle of the limb stump at approximately 12–14 days after amputation. Second, in areas of myogenesis at the palette stage, approximately 25 days after amputation. If 22/18 specifically marks blastemal cells, then the double-labelled cells of the early and late phases of regeneration could be used as evidence for myofibre dedifferentiation and blastemal-derived myogenesis, respectively (Kintner & Brockes, 1984, 1985). If, however, the 22/18 antigen is expressed by cells other than blastemal cells, then other interpretations are possible. For example, if reserve cells express the 22/18 antigen during repair myogenesis, then the early phase of 22/18,12/101 double-labelled cells could reflect the tissue repair of myofibres directly injured by the amputation, as described by Carlson (1973, 1979). Kintner & Brockes (1984) dismissed this possibility because of the evidence against the existence of satellite cells in newts. In view of the renewed controversy concerning such cells (see Cameron *et al.* 1986), we felt that a direct comparison of 22/18 immunostaining in the tissue repair and the epimorphic regeneration of muscle was warranted. Minced muscle regeneration is known not to involve blastema formation but is a tissue repair phenomenon (Carlson, 1970*a*, 1979).

The present results show that large numbers of 22/18+ cells are present in regenerating minced muscle and at least some of these are myogenic. The initial appearance of cells double labelled with 22/18 and 12/101 is coincident in both minced muscles and early blastemas of the same animal. Since myofibre dedifferentiation is not normally a feature of minced muscle regeneration, we question the previous interpretation of such double-labelled cells as evidence for myofibre dedifferentiation during limb regeneration (Kintner & Brockes, 1984). We offer as an alternative the possibility that such cells are indicative of myogenesis by tissue repair during early limb regeneration among fibres injured by the amputation.

Materials and methods

Surgery

Notophthalmus viridescens were obtained from Lee's Newt Farm, Oak Ridge, Tennessee. For all operations, the newts were anaesthetized by immersion in 1:1000 MS-222 (Sigma). Two groups of animals were used. In one, the extensor muscle (anconeus) of the right forelimb was minced into small fragments and orthotopically replaced according to a method described earlier (Carlson, 1970*a*). The contralateral forelimb was amputated through the midstylopodium in order to obtain same-aged blastemal tissue resulting from epimorphic regeneration as an internal control. In another group of newts, a flexor muscle (puboischiotibialis) from the left hindlimb was minced and the right hindlimb amputated through the midstylopodium. Animals were killed at 6, 8, 10, 12, 14, 16, 18, 21, 25, 35 and 50 days after operation by ventricular perfusion with Hepes-buffered saline (HBS; 120 mM-NaCl, 3 mM-glucose, 2 mM-Hepes, 10 mM-$CaCl_2$, adjusted to pH 7·2) for 30 s, followed by periodate–lysine–paraformaldehyde fixative, containing 0·05 % paraformaldehyde (PLP; McLean &

Nakane, 1974) for 5 min. Limbs were removed by amputation at the shoulder or at the pelvis and postfixed in PLP for 30 min. Tissue was embedded in Tissue-Tek OCT (Miles Scientific), frozen onto notecards with liquid nitrogen, sealed in air-tight bags and stored at −65°C. Immediately before sectioning, the tissue was mounted onto metal chucks cooled with liquid nitrogen. Sections were cut at 10 μm on a cryostat at −14°C, collected on glass slides coated with Gatenby's solution and stored in air-tight containers at −65°C.

Immunocytochemistry

All primary antibodies were mouse monoclonal antibodies that have previously been characterized. These include: 22/18 (IgM) as a marker of newt blastemal cells; 12/101 (IgG) as a marker of newt myofibres (Kintner & Brockes, 1984, 1985); leu-7 (IgM; also known as HNK-1) isolated by Abo & Balch (1981), shown to cross-react with myelin-associated glycoprotein (McGarry, Helfand, Quarles & Roder, 1983) and recently used as a marker for Schwann cells in newt limbs (Gordon & Brockes, 1987); and RT-97 (IgG) as a neurofilament marker (Anderton *et al.* 1982). Mouse ascitic fluid was collected and diluted in 10% fetal calf serum in 0·1 M-phosphate buffer, 0·02% sodium azide as follows: 22/18 (1:150), 12/101 (1:200), leu-7 (1:150) and RT-97 (1:400). Sections were double labelled with one of the following pairs of antibodies: 22/18 and 12/101; leu-7 and RT-97, or 22/18 and leu-7. For differential staining of IgM and IgG, the two primary antibodies were added simultaneously, then rabbit anti-mouse IgM (1:200; Cappel, μ-chain specific), followed by rhodamine-conjugated goat anti-rabbit Ig (1:200; Miles) together with fluorescein-conjugated rabbit anti-mouse IgG (1:150; γ-chain specific, Cappel). Differential staining of two IgM antibodies was accomplished by direct conjugation of leu-7 to biotin (Kintner & Brockes, 1985). Sections were incubated with the following reagents sequentially: 22/18, rabbit anti-mouse IgM (*μ-chain specific*), 22/18, *biotinylated* leu-7 together with rhodamine-conjugated goat anti-rabbit Ig, and fluorescein-conjugated avidin. Nuclei were counterstained with Hoechst dye 33258. All incubations were for 45 min, except for the avidin incubation, which was for 10 min. Sections were washed in 0·1 M-phosphate buffer (PB) before and after each incubation. Postfixation was for 5 min in 5% glacial acetic acid in 95% alcohol at −20°C. After washing in 10 mM-PB at pH 8·6 to deacidify, sections were mounted in 90% glycerol, 10% PB containing 2·0% 1,4-diazabicyclo(2,2,2)octan at pH 8·6 and stored in the dark at +8°C. Control experiments in which primary antibodies were omitted gave low background staining. Autofluorescent cells were clearly distinguishable from positively stained cells as they fluoresced with a different colour to either rhodamine or fluorescein. Sections were viewed on a Zeiss photomicroscope II equipped with epifluorescence and appropriate filters, and photographed on Kodak Tri-X Pan film (ASA 400) using manually determined exposure times.

Results

Normal muscle

The 12/101 antibody stains newt skeletal muscle in a striated pattern by immunofluorescence (Fig. 1). It does not appear to label interstitial cells of muscle, including pericytes. There is a marked variation in the staining intensity of individual myofibres in normal, uninjured muscle, although the majority are very bright.

Regenerating limb blastemas

The 22/18 and 12/101 immunostaining patterns reported by Kintner & Brockes (1984, 1985) were repeated so that blastemas and minced muscles could be compared within the same animal. Our results are in general agreement with their reported findings, with the exception of a slightly later time course in the initial appearance of cells double labelled with 22/18 and 12/101. We first observed such cells in the proximal area of the blastema at 14–16 days after amputation, 2 days later than the previous descriptions. The present study extends the time course beyond that reported by Kintner and Brockes by including time points at 35 and 50 days after regeneration. At these later stages, discrete muscle groups

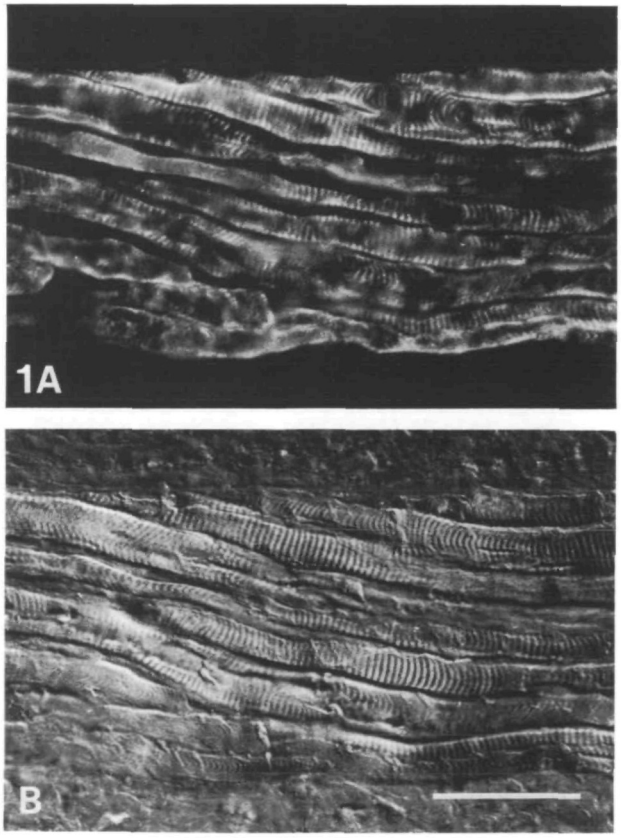


Fig. 1. 12/101 staining in normal myofibres, photographed under (A) fluorescein optics, and (B) Nomarski optics. Bar, 50 μm.

were forming in the regenerate and the small number of remaining 22/18+ cells were mostly, but not exclusively, confined to these regions. Muscle tissue located near the former plane of amputation also contained some 22/18+ cells. No 22/18,12/101 double-labelled cells were observed at late stages. By day 50, 22/18 staining was reduced in intensity and, in one case out of three, no filamentous 22/18+ cells were evident, either on the amputated side or on the minced side.

Minced muscle regeneration

22/18 and 12/101

The regeneration of the minced muscle may be conveniently divided into four periods based on changes in the pattern of 12/101 staining: a degenerative phase (day 6), a premyogenic phase (day 8 and 10), a myogenic phase (day 12, 14 and 16) and a maturational phase (day 18 and later).

Degenerative phase (day 6)

As expected from the mincing operation, the muscle tissue was highly disorganized (Fig. 2) compared with the uninjured muscle (Fig. 1). Some strong 12/101 staining was still evident, often with a dispersed granular appearance which was probably due to extensive sarcolysis and phagocytic degradation of myofibres (Fig. 2A). Pyknotic myonuclei were abundant. Staining with 22/18 was either totally absent or present only as occasional filamentous processes (Fig. 2B). When present, 22/18+ cells were more numerous in deep areas of the minced muscle than in the superficial tissue. No 22/18+ cells were labelled with 12/101.

Premyogenic phase (day 8 and 10)

At this stage, the morphology of the minced tissue was very poor when viewed with phase or Nomarksi optics, presumably because of the extensive degeneration of myofibres. The majority of the minced tissue was not stained with 12/101 at this stage. At day 8, staining with this antibody was confined to small fragments of myofibre not yet fully degenerated or to areas of noncellular granular staining (Fig. 3A). By day 10, immunoreactivity with 12/101 was essentially absent. The number of cells stained with 22/18 was greatly increased at this stage (Fig. 3B). Fewer 22/18+ cells were evident in areas that still contained some 12/101 staining. Longitudinal sections of the limb revealed that the 22/18+ cells were more abundant in the deeper areas of the regenerating muscle. Normal muscle adjacent to the minced tissue contained 22/18+ cells, bipolar in morphology, located in the interstitial tissue between myofibres (Fig. 3C). Muscle surrounding the minced tissue could become bruised during the mincing operation and such an injury is known to induce 22/18 expression in muscle tissue (Gordon & Brockes, 1987). This may account for the 22/18+ cells seen here, although it cannot be ruled out that these cells were responding to a diffusible signal from the minced tissue.

Myogenic phase (day 12, 14 and 16)

Mononucleated 12/101+ cells were first observed in the minced muscle 12 days after regeneration (Fig. 4A), and in considerable numbers at 14 days. The 12/101+ cells were considered to be newly differentiated myocytes and they first appeared in small clusters. At 14 days, multinucleated 12/101+ myotubes appeared (Fig. 4C) and these were arranged as small bundles in random orientations to one another. Expression of the 22/18 antigen in the minced muscle was now maximal in terms of numbers of positive cells (Fig. 4B). The 22/18+ cells were

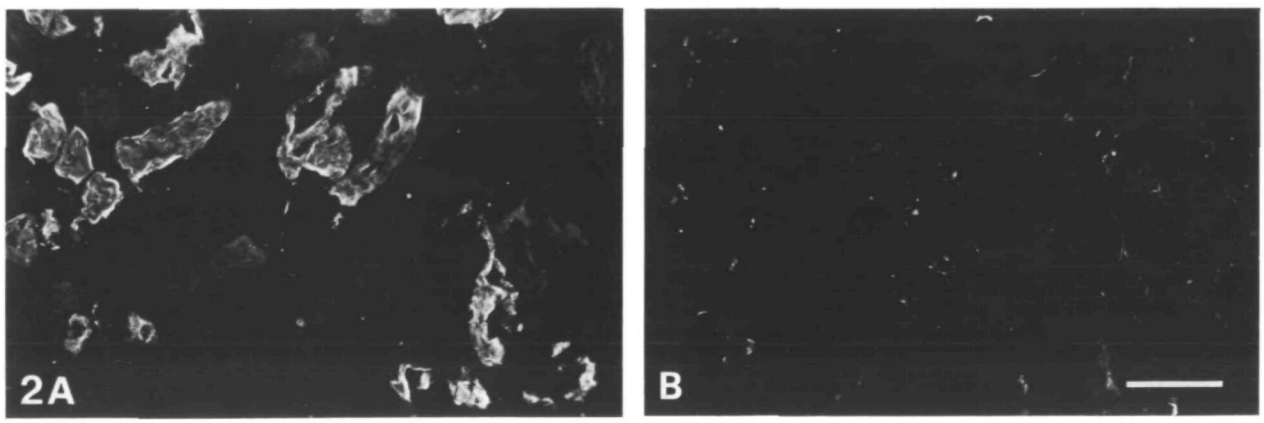


Fig. 2. 22/18 and myofibre staining in degenerating minced muscle (day 6). (A) An area from deep in the minced muscle stained with 12/101. Note that the muscle is highly disorganized and myofibre staining is patchy, though still strong in some cells. (B) The same field stained with 22/18. Bar, 100 μm.

observed much less frequently in areas which contained myocytes and multinucleated 12/101+ myotubes (compare Fig. 4A,B). Cells double labelled with 22/18 and 12/101 were observed frequently beginning at day 14 (Fig. 4E,F) when myocytes were most abundant (Fig. 4B,D).

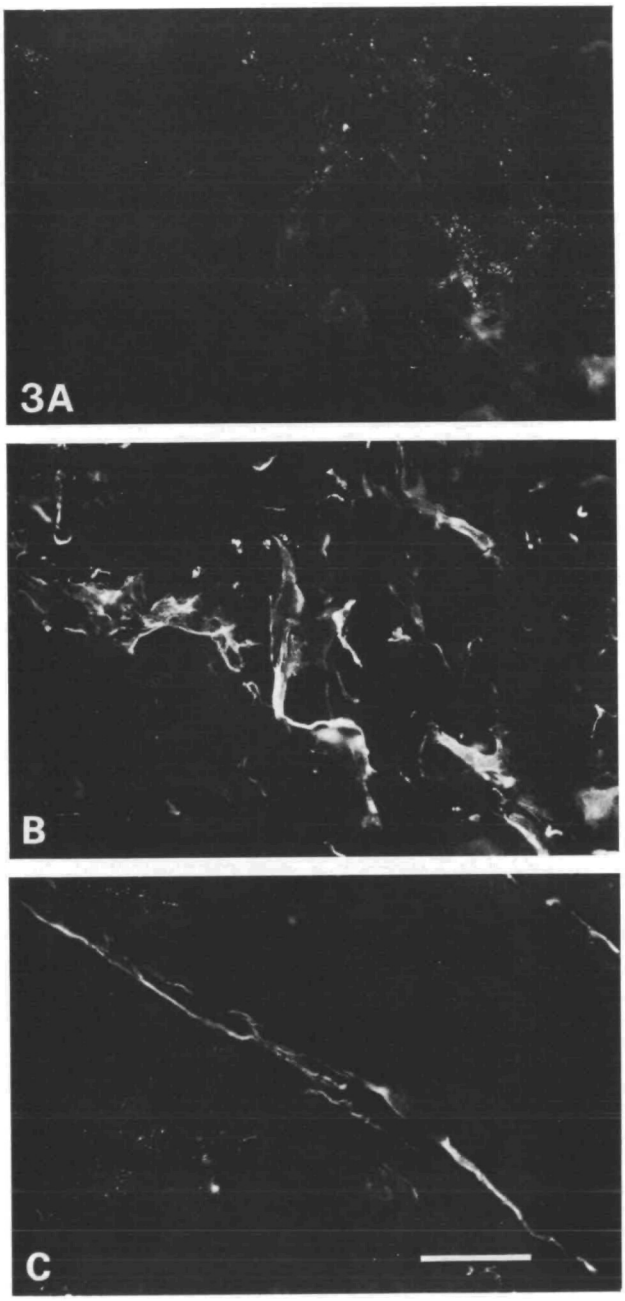


Fig. 3. 22/18 and myofibre staining in the premyogenic minced muscle (day 8 and 10). (A) Stained with 12/101. Note that muscle-specific staining has disappeared. The material causing the punctate staining is thought to be an artefact of sarcolysis. (B) The same field stained with 22/18. Expression of 22/18 is now intense in positive cells and their numbers are considerably higher than in day-6 regenerates. (C) 22/18 staining in a region of intact muscle adjacent to the minced muscle. Such 22/18+ bipolar cells do not double label with either of our markers for muscle or Schwann cell. Bar for A–C, 50 μm.

Maturational phase (day 18 onwards)

By day 18, myotubes were present in large numbers and their differentiation into myofibres was indicated by the presence of transverse striations, though myonuclei remained large and centrally located. Cells reactive with 22/18 were strongly positive, but the overall number of such cells was markedly reduced. The exclusion of 22/18 staining from areas containing myotubes was quite pronounced at these late stages (compare Fig. 5A,B). Perhaps the differentiation of myocytes from 22/18+ cells brings about a local depletion of 22/18+ cells. Alternatively, this exclusion may indicate a disparity in the rate of regeneration between different areas of the same muscle or perhaps it reflects differentiation of additional tissues, such as tendon, from 22/18+ cells. In other cases, intense 22/18 staining in 'excluded' areas seemed to be associated with fibrous scarring of the mince. By day 35, the level of 22/18 staining was still moderate, with 22/18+ cells interspersed among maturing myocytes (Fig. 5C,D). By day 50, the number and intensity of 22/18+ cells were significantly diminished in two cases (Fig. 5E,F), and absent from the third. At late stages, cells double labelled with 22/18 and 12/101 were rare; they were observed only in the day-21 regenerate and in the 50-day regenerate (Fig. 5G,H). The presence of these cells indicates that a low level of myogenesis is still occurring in some limbs even 50 days after injury.

Schwann cells and the nerve

Schwann cells, identified by immunostaining with leu-7, were shown to be present in the minced muscle at day 6, the earliest time point examined. Some 5–10 % of these cells could be labelled with 22/18 from day 6 through day 21. Schwann cells associated with some nerve bundles adjacent to the minced muscle also labelled with 22/18 (Fig. 6A,B). The 22/18+ nerves had probably supplied the muscle prior to mincing, or else they had been bruised by the mincing operation, as denervated or damaged Schwann cells express 22/18 (Gordon & Brockes, 1987). Individual axons were identified in the deeper areas of the minced tissue from day 6 onwards (superficial sections were not examined). In all cases, these axons were associated with Schwann cells, though a small number of Schwann cells were not associated with axons, as identified by RT-97 staining (data not shown).

Discussion

Tissue repair in the blastema

A considerable body of evidence suggests that there are two modes of muscle regeneration (Carlson, 1970*b*, 1979). In the tissue mode, which occurs in all classes of vertebrates, damaged muscle fibres repair themselves in the absence of a regeneration blastema and the attending morphogenetic controls that operate at the level of the entire limb. The tissue repair of

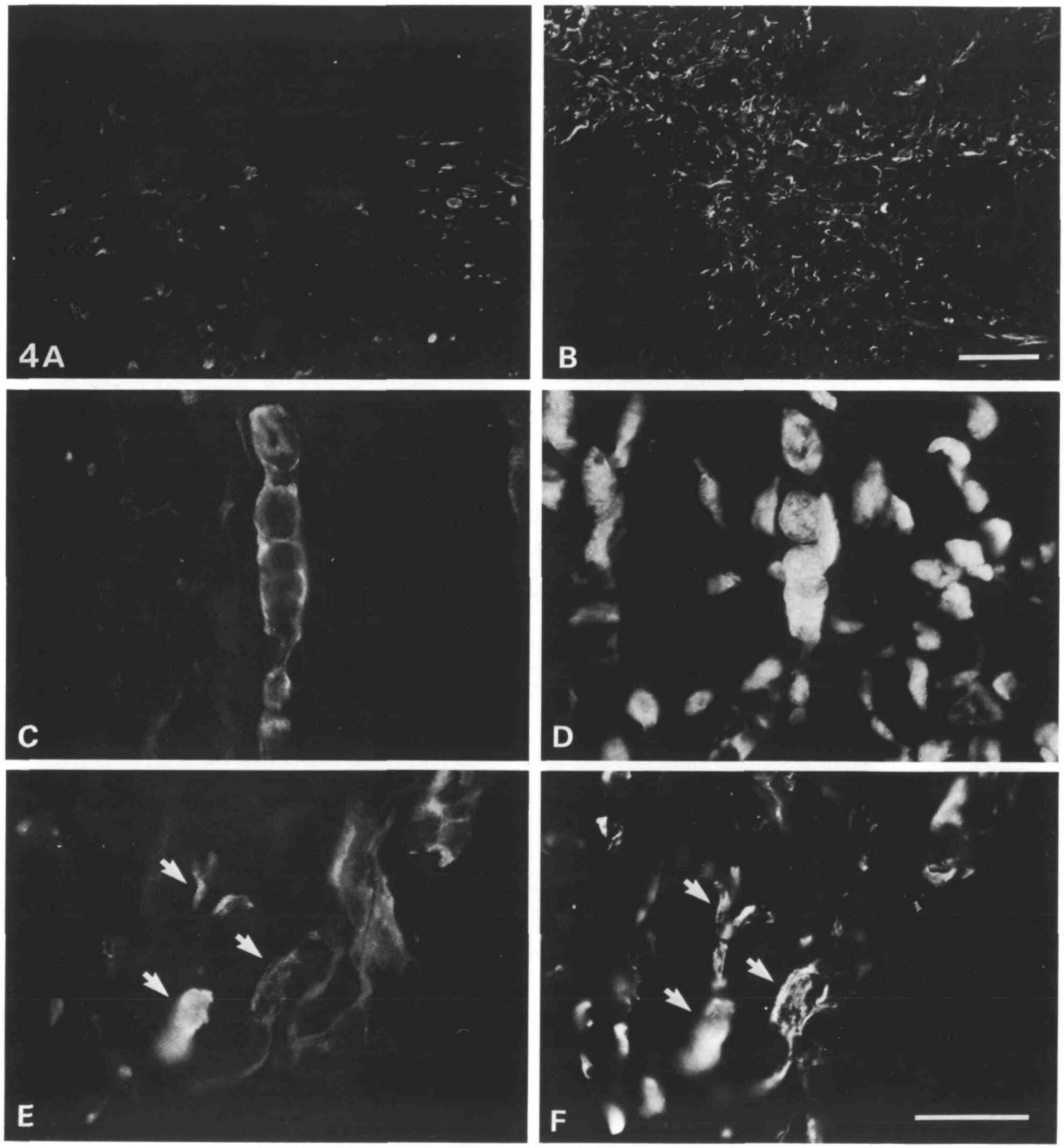


Fig. 4. 22/18 and 12/101 staining in the myogenic minced muscle. (A) A day-12 regenerate stained with 12/101 and (B) the same field stained with 22/18. Myocytes and myotubes are now evident. The number of 22/18+ cells is maximal, though they are obviously excluded from areas in which newly differentiated myocytes have accumulated. (Bar, 100 μm). (C) A multinucleated 12/101+ myotube from a day-14 regenerate, and (D) the same field stained with Hoechst dye to detect nuclei. (E) A day-14 regenerate stained with 12/101 and (F) the same field stained with 22/18. Arrows point to cells that stain with both markers. Cells double labelled in this way are most abundant in regenerates of this time group. Bar for C–F, 50 μm.

muscle is rapid and, in urodele amphibians, myotubes and cross-striated muscle fibres appear in minced muscle regenerates by the time a medium bud blastema has formed on an amputated limb (Carlson, 1970*a*). The morphology of muscle regenerated by the tissue mode is imperfect and is largely dictated by mechanical forces.

Epimorphic regeneration of muscle occurs within the regeneration blastema that forms at the end of an amputated limb. However, the tissue repair of muscle also occurs in amputated limbs. In limbs that are either naturally incapable of regenerating (e.g. the mouse) or that have been prevented from regenerating (e.g. urodele limbs after denervation or after being covered by a skin flap), a zone of tissue repair occurs at the ends of the muscles transected at amputation, with limited outgrowth of regenerating muscle fibres following a rapid time course and with imperfect morphology. In axolotl limbs from which most of the musculature has been removed (Carlson, 1972*a*) or in which limb amputation was carried out through a freshly minced muscle (Carlson, 1978), the influence of the epimorphic field affecting muscle regeneration was seen to extend a fraction of a millimetre proximal to the amputation surface. Limb regenerates in amphibians commonly have a thin zone between the intact stump muscle and the base of the essentially normal muscle of the regenerated limb where muscle fibres are not perfectly aligned. This is viewed as a zone where the most proximal stump muscle that was broken down during the phase of dedifferentiation of stump tissues has been regenerated by tissue repair outside of the influence of the epimorphic field and has subsequently made connections with muscle fibres that have regenerated from the blastema.

Using the 22/18 antibody and a muscle marker, this study has verified that myocytes of newt minced muscle first appear *in vivo* at 12 days after injury and has established that at least some of these are 22/18+. The present results show that cells double labelled with 22/18 and 12/101 appear simultaneously in the minced muscle and the blastema, in contralateral limbs of the same animal. In minced muscle, the double-labelled cells seen at day 14 are preceded by a 12/101-negative phase at day 10. Therefore, the double-labelled cells must represent differentiation of new myocytes from 22/18+ precursors and not the dedifferentiation of existing myofibres. The similar time course of immunoreactivity in both minced muscles and regenerating blastemas is consistent with the hypothesis that muscle repair by the tissue mode is occurring during early limb regeneration in muscle injured by the amputation. Thus the early and late phases of 22/18,12/101 double-labelled cells following limb amputation (Kintner & Brockes, 1984) would correspond to tissue repair and epimorphic regeneration, respectively.

Origin of myoblasts in tissue repair

Although repair myogenesis does not involve dedifferentiation of myofibres in other vertebrates species, we cannot as yet rule it out in the newt. This is because the origin of the 22/18+ cells cannot be traced in these experiments. If myofibres do give rise to 22/18+ cells, they must do so by first losing expression of the muscle antigen, 12/101. The nuclei of myofibres all appeared pyknotic and nonviable at day 6, in agreement with previous descriptions (Carlson, 1968, 1970*a*; Snow, 1977, 1979; Cameron, 1983). Thus it appears that few, if any, myofibre nuclei survive the mincing operation.

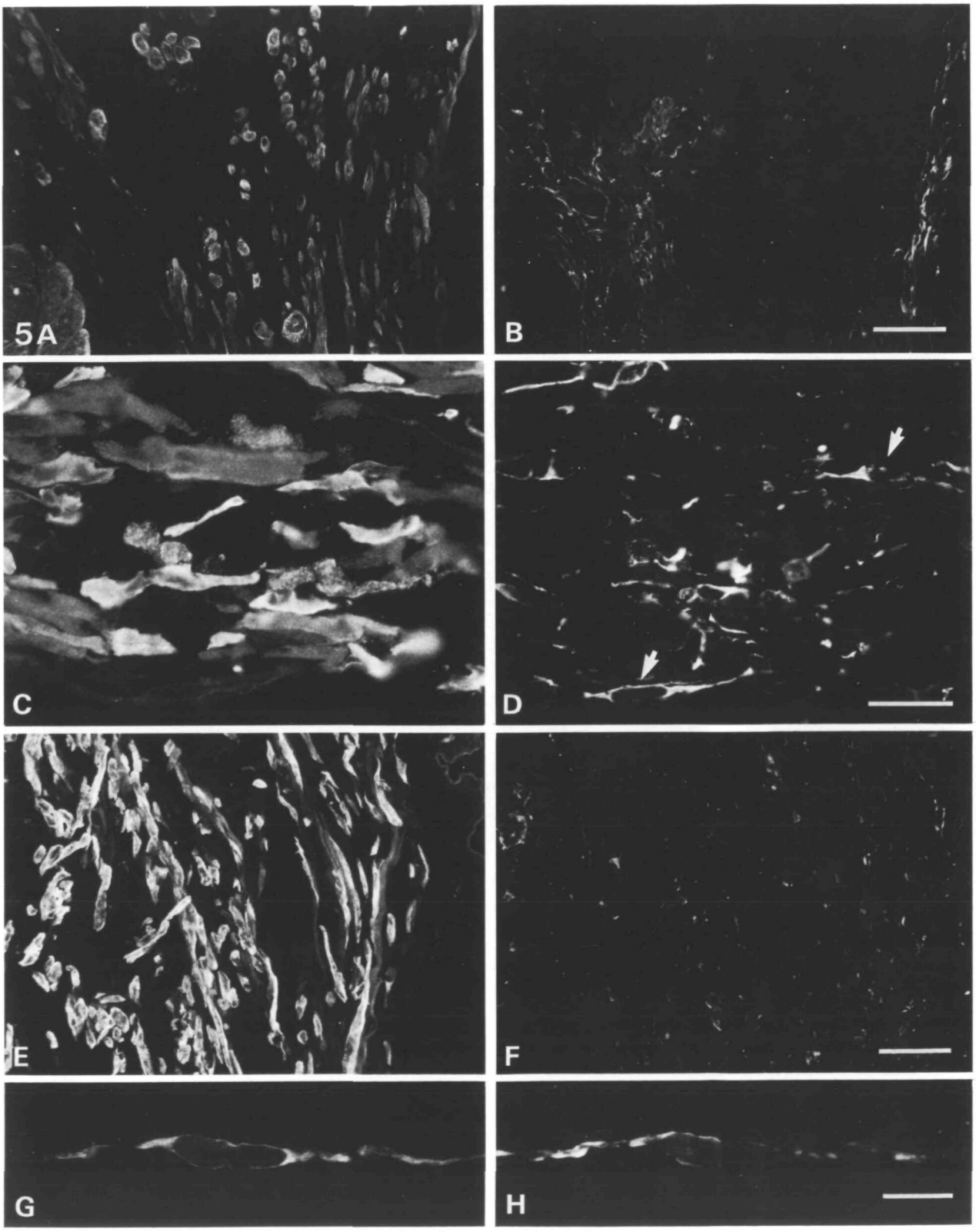
Other possible sources of 22/18+ cells include pericytes (Cameron *et al.* 1986), Schwann cells (Kintner & Brockes, 1985), and interstitial cells such as fibroblasts (Gordon & Brockes, 1987). In addition to myocytes, 22/18+ cells are probably contributing to other tissue types in the minced muscle regenerate, such as tendons, Schwann cells, fibroblasts and possibly endothelial cells. Nonetheless, we are concerned here only with the origin of those 22/18+ cells that give rise to myocytes. Cameron *et al.* (1986) describe a technique for selectively labelling about 30% of the pericyte population (also called 'postsatellite cells') using [^{3}H]thymidine. Explanted muscle labelled in this way yielded [^{3}H]thymidine-labelled myonuclei on regeneration *in vitro*, suggesting that the pericyte was the source of myogenic cells. Although the myotubes and some mononucleated cells were stained with 22/18, they did not explicitly show that identified pericytes were 22/18+. Nonetheless, the most parsimonious interpretation of the available data is that the pericyte/satellite cell is the myogenic cell for the tissue mode of muscle regeneration *in vivo*. The derivation of a specific marker for pericytes will be essential to prove this rigorously in newts.

Further characterization of 22/18

The 22/18 antigen was originally described within the context of the epimorphic regeneration of newt limbs where 22/18 was thought to be a specific marker for a subset of blastemal cells (Kintner & Brockes, 1984). Recent results have shown that in addition to amputation, the 22/18 antigen is expressed in response to other kinds of tissue damage, including nerve transection or chronic bruising (Gordon & Brockes, 1987). The present results provide another example of the induction of 22/18 expression during tissue repair following injury. In each case, 22/18 is expressed by Schwann cells and a population of interstitial cells. In regenerating muscle, 22/18 is also expressed by myocytes. The 22/18 antigen is rarely detected in

developing limb buds of newts and, with the exception of a few cells in a single animal, it is not expressed by the myogenic cells during development. It is turned on in the developing limb in response to amputation in the presence of nerves (Fekete & Brockes, 1987).

It seems unlikely that the transition of cells to a 22/18+ condition is simply a transient response to injury, since the antigen continues to be expressed for weeks or months if tissue repair is blocked or if newt cells are maintained in culture (Fekete *et al.* 1987). Interestingly, cultured 22/18+ cells derived from



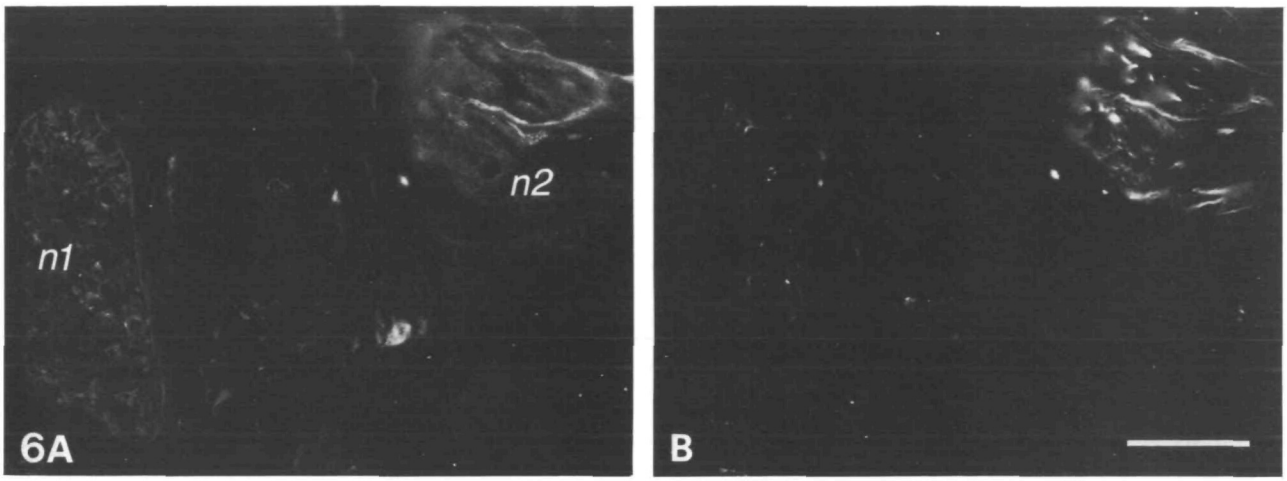


Fig. 6. 22/18 and Schwann cell staining in early minced muscle regeneration. (A) An 8-day regenerate photographed under fluorescein optics to detect leu-7 and (B) the same field photographed under rhodamine optics to detect 22/18. The area illustrated is very deep in the limb, close to the bone, and adjacent to the region of minced muscle. Two nerve bundles are evident (*n1* and *n2*), only one of which (*n2*) stains extensively with 22/18. Bar, 50 μm.

explanted muscle can differentiate into myotubes *in vitro* and this is accompanied by the loss of 22/18 expression (Ferretti & Brockes, 1987). These *in vitro* results parallel those obtained *in vivo* after muscle mincing, where an increase in myotube formation is accompanied by a marked decrease in 22/18 expression. This suggests that although the initial expression of 22/18 may occur in response to injury, perhaps accompanied by dedifferentiation of the responding cell, the antigen is not turned off until the cell is undergoing terminal differentiation.

Fig. 5. 22/18 and myofibre staining in the maturing minced muscle regenerate (day 18 and onwards). (A) A day-18 regenerate stained with 12/101 and (B) the same field stained with 22/18. The newly developed myofibres are now present in large numbers and are easily distinguishable from mature myofibres (bottom left) by their thickness in cross section. Note that the newly developed myofibres are in many orientations relative to one another. 22/18 is excluded from the areas containing large numbers of myofibres. (Bar, 100 μm). (C) Higher power view of a day-35 regenerate stained with 12/101. Note that muscle fibres differ noticeably in the intensity of staining with 12/101. (D) Same field stained with 22/18. Note the presence of 22/18+ bipolar cells (arrowheads) interspersed among the muscle fibres. (Bar, 50 μm). (E) A low-power view of a 50-day regenerate stained with 12/101. (F) The same field stained with 22/18 to show the diminished number of 22/18+ cells present in some of the regenerates at this time. (Bar, 100 μm). (G) High-power view of a double-labelled cell from a 50-day regenerate stained with 12/101 and (H) stained with 22/18. (Bar, 25 μm).

The nerve dependence of regeneration

At early stages of limb regeneration, the proliferation of blastemal cells is strictly dependent on one or more mitogenic growth factors provided by the nerve (Singer, 1952; Tassava & Mescher, 1975), and 22/18 is an excellent marker for cells that show this dependence (Kintner & Brockes, 1985). It is of interest to determine whether the 22/18+ cells that appear after muscle mincing show a similar nerve dependence. In other species, including frog, rat and axolotl (Carlson, 1968, 1970*a*), the regeneration of minced muscle follows the pattern of revascularization; and, in frog and rat, is not dependent on the nerve in the early stages of regeneration (Carlson, 1972*b*). To our knowledge no studies on the nerve dependence of muscle regeneration in urodeles have been carried out. It is tempting to speculate that minced muscle regeneration in the newt might prove to be nerve-dependent, due to the involvement of the 22/18+ cell type. If this hypothesis is correct then there should be a clear difference between the mitotic indices of 22/18+ cells of denervated and innervated minced muscles at early stages (i.e. 8–12 days). This would also indicate the extent to which 22/18+ cells of tissue repair and epimorphic regeneration are equivalent.

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