A monoclonal antibody detects a difference in the cellular composition of developing and regenerating limbs of newts

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Summary

We have previously described a monoclonal antibody (called 22/18) that reacts with the early blastema cells of the regenerating limb of the newt (Notophthalmus viridescens). In embryos of two newt species the antibody reacts with the epidermis, glial cells in the neural tube, the lens and cells in a restricted region of the aorta. In the developing limb bud less than 1% of the mesenchymal cells were reactive with 22/18, although most cells stained brightly with an antibody to another cytoskeletal component. When limbs were amputated prior to the arrival of nerves (axons and Schwann cells) at the amputation plane there was no extra reactivity with 22/18 as compared to the contralateral unamputated control, even though the amputated buds regenerated satisfactorily. Limbs amputated after nerves are present at the plane of amputation respond by forming a 22/18-positive blastema. The appearance of the 22/18 response is a function of the stage of limb development as shown by amputation of forelimb and hindlimb buds at a larval stage where development of the forelimb is greatly advanced relative to the hindlimb. The distribution of the 22/18-positive cells in larval blastemas showed them to be closely associated with axons as detected by double staining with an antiserum to a neurofilament subunit. The clear antigenic difference between development and regeneration may be related to the relationship between embryonic regulation and epimorphic regeneration, and also to the acquisition of nerve-dependent proliferation of blastemal cells.

Key words: monoclonal antibody, limb regeneration, limb development, peripheral nerves, blastema, newt, Notophthalmus viridescens.

Introduction

Adult urodele amphibians such as newts and axolotls can regenerate their limbs after amputation. Several lines of evidence support the view that regeneration and development of the urodele limb proceed by similar mechanisms. During development a bud of undifferentiated mesenchymal cells appears on the flank of the embryo and progresses through a series of stages to form the mature limb (reviewed by Hinchcliffe & Johnson, 1980). The morphogenesis of a regenerating limb proceeds in a similar manner (reviewed by Wallace, 1981). After amputation the wound heals by formation of a specialized epithelium and a mass of undifferentiated progenitor cells, called a blastema, appears beneath it. The early blastema can be considered equivalent to the early bud in several respects. The blastemal cells differentiate and replace the missing parts of the limb by reiterating the developmental stages at the level of tissue organization and patterning of the limb. A two-dimensional electrophoretic analysis of the major proteins synthesized by mesenchymal cells of the developing bud and the regenerating blastema revealed only minor differences (Slack, 1982). Under certain experimental conditions designed to investigate the rules governing pattern formation, developing buds and regenerating blastemas behave similarly. Both respond to apposition of distal grafts to proximal stumps by intercalary regeneration of missing parts (Iten & Bryant, 1975; Stocum, 1975; Maden & Goodwin, 1980). Furthermore, both respond to inversions of the transverse axes by forming supernumerary limbs (Iten & Bryant, 1975; Tank, 1978; Wallace & Watson, 1979; Maden & Goodwin, 1980; Maden, 1980). In fact the regenerating blastema can substitute for the developing limb bud, and vice versa, in the generation of supernumerary limbs (Muneoka & Bryant, 1982, 1984).
On the other hand, there is evidence of differences between developing limb buds and regenerating limb blastemas. (1) Scadding & Maden (1986) showed in the same animal that morphogenesis of a regenerating forelimb blastema and a developing hindlimb bud is differentially affected by treatment with vitamin A (retinol palmitate). (2) The specific origin of the cells that form the regenerating blastema of urodeles has long been a matter of controversy, with much of the evidence favouring the view that the cells arise by dedifferentiation of mature tissues, in clear contrast to development (for example, Hay & Fischman, 1961; Namenwirth, 1974; reviewed by Hay, 1974; Wallace, 1981). (3) The role of the nervous system in growth control is clearly different during development and regeneration. Early limb outgrowth and development are initiated before the limb becomes innervated. In contrast, the dependence of limb regeneration on the nervous system has been recognized since the work of Todd (1823). In a comprehensive series of experiments, Singer demonstrated that limb regeneration in amphibians is dependent on the quantity of axons at the plane of amputation and that either motor or sensory axons can meet this requirement (reviewed by Singer, 1952, 1974). Denervation appears not to affect the initial appearance of blastemal cells but stops blastemal cell proliferation, thereby arresting regeneration (Singer, 1952; Kelly & Tassava, 1973; Tassava, Bennet & Zitnik, 1974; Mescher & Tassava, 1975; Maden, 1978).

One antibody, called 22/18, has provided new information about the cellular composition of the blastema, including the origin and nerve-dependent proliferation of a subset of blastemal cells. The antibody was raised by immunizing mice with a particulate fraction of blastemal cells from the adult newt, Notophthalmus viridescens (Kintner & Brockes, 1985). Screening of the resulting clones by immunofluorescence on sections of the early regenerating limb identified the 22/18 reagent by virtue of its reactivity with blastemal cells but not mature tissues. The 22/18 antigen is intracellular and filamentous (Fekete, Ferretti, Gordon & Brockes, 1987), appearing first in the processes of Schwann cells and interstitial cells of loose connective tissue at 36–48 h after amputation (Gordon & Brockes, 1987). By the second week after amputation, it is also expressed by muscle tissue and approximately 80% of the blastemal cells. The number of positive cells declines thereafter and reactivity with the antibody is no longer detectable some weeks before limb patterning and growth are complete (Kintner & Brockes, 1985). Most of the blastemal cells were expressing the 22/18 antigen during the time when the nerve was reported to exert its greatest influence on regeneration as assayed by the response of the regenerate to denervation (Schotté & Butler, 1944; Butler & Schotté, 1949). Interestingly, 22/18 was shown to be an excellent marker for a subset of blastemal cells whose division is dependent on the nerve (Kintner & Brockes, 1985; Brockes & Kintner, 1986).

With these facts in mind and, in particular, the utility of 22/18 as a marker of nerve-dependent cells in the early blastema, it was of obvious interest to determine if the 22/18 antigen is expressed during limb development. This paper presents evidence for a clear difference in the cellular composition of regenerating blastemas and developing limb buds as detected by immunostaining with 22/18. We suggest that this difference may be related to the role of the nerve and its accompanying Schwann cells, and discuss some future experiments to test this hypothesis.

Materials and methods

Care and feeding of animals

Pleurodeles walti embryos were supplied by Xenopus, Ltd within two days of spawning. N. viridescens embryos were bred in the laboratory from a colony of adult newts supplied by Xenopus, Ltd. Larvae were raised individually in aged tap water in plastic Petri dishes at room temperature and were fed three times per week. Bait consisted of live brine shrimp (Artemia) which were hatched in sea water and rinsed with tap water. Animals were staged according to Gallien & Durocher (1957).

Amputations

Animals were anaesthetized by immersion in 1:6000 tricaine (methylene sulphonate) in sterile distilled water. Prior to stage 40, the right limbs were amputated approximately midway along the proximal–distal axis using tungsten needles. At later stages, right limbs were amputated through the midhumerus using iridectomy scissors. Camera-lucida drawings of the limbs were made immediately before and after amputation and also just before fixation to assess the progress of regeneration. Animals were placed into sterile tap water following amputation and resumed swimming within a few minutes. The rate of limb regeneration tended to decrease as the animals aged and therefore survival times varied between 4–10 days. Amputation at the later stages often resulted in contraction of the soft tissues, leaving the humerus protruding from the cut surface. However, the epidermis healed over the exposed bone within 12 h. When such limbs were examined by histology at several days after amputation, the blastemas were observed to form around the circumference of the distalmost humerus (also described by Stocum, 1979). Animals were processed before the amputated limbs reached a late-bud stage (according to Stocum, 1979). Fewer than 10% of the animals were eliminated from the sample because of poorly formed blastemas.
Histology

It was necessary to remove the gelatinous capsules of embryos that were processed prior to staining by gentle teasing with fine forceps. All larvae were anaesthetized in tricaine, then immersed for 1 h in fixative containing 0.05% paraformaldehyde, 0.1 M-lysine, 0.01 M-sodium periodate diluted either in Hapes-buffered saline (120 mM-NaCl, 3 mM-KCl, 0.1 mM-CaCl₂, 5 mM-Hapes, pH 7.2) or an 80% solution of Gibco phosphate-buffered saline (PBS), pH 7.4. Animals were transferred through graded sucrose solutions and embedded in gelatin/sucrose at 4°C (Godsave, Wiley, Lane & Anderton, 1984). They were trimmed, oriented and frozen onto microtome chucks cooled with liquid N₂. Blocks were stored at -65°C until sectioned with a cryostat at 5–10 μm at -25°C. Slides were acid cleaned, dipped in 1:1 ethylene glycol:egg whites and air dried at 37°C.

Sections were collected sequentially onto five sets of slides so that adjacent sections could be reacted with different combinations of antibodies. In all but a few cases the larvae were sectioned transversely and all of the sections were saved from the rostral tip to the caudalmost extent of the limbs. Sections were stored in air-tight containers at -65°C for up to several months.

Sections were thawed at 37°C and in some cases they were fixed to the slide by dipping in periodate-lysine-paraformaldehyde fixative for 5 min. They were thoroughly rinsed before staining and between each antibody step with PB (0.1 M-phosphate buffer, 0.02% sodium azide, pH 7.4) with or without 0.05% Tween 20. All antibodies were diluted in 10% fetal or newborn calf serum in PB and incubated on the sections for 45 min to 1 h at room temperature.

For each animal, one set of slides was double labelled with the monoclonal antibodies, 22/18 (IgM) and 12/101 (IgG; a newt muscle marker) according to Kintner & Brockes (1984). In brief, sections were incubated simultaneously with 22/18 ascites (1:75 or 1:150, depending on the batch) and 12/101 ascites (1:100), followed by rabbit anti-mouse-IgM antiserum (Miles; 1:200), and then simultaneously with rhodamine-conjugated goat anti-rabbit-Ig antiserum (Cappel; 1:250), fluorescein-conjugated goat anti-mouse-IgG (Cappel; 1:150) and 1-25 μg ml⁻¹ Hoechst dye 33258 (bisbenzimide). Sections were post-fixed in acid-alcohol (95% acetic acid in absolute ethanol), rinsed and coverslipped with DABCO (1,4-diazabicyclo[2.2.2]octane) in 90% glycerol, 10% PB, pH 8.6.

A second set of sections was double labelled with 22/18 and a rabbit antiserum (called NP70; gift of D. Bray) directed against the low molecular weight (70×10⁶ M₉) subunit of neurofilament protein (Jacob, Choo & Thomas, 1982). The neurofilament staining was improved by preculture treatment with acid-alcohol, but the 22/18 staining was drastically reduced on sections pretreated in this way. Therefore the following protocol was adopted: incubation with 22/18 ascites followed by fluorescein-conjugated goat anti-mouse-IgM (Miles; 1:100), then a 5 min treatment with acid-alcohol, then NP70 (1:100) followed by rhodamine-conjugated goat anti-rabbit Ig (Miles; 1:250). Sections were postfixed and coverslipped as described above.

We also tried a variety of other antibodies in double-labelling experiments with 22/18. If the other antibody was a mouse monoclonal of the IgG subclass, such as RT-97 (directed against the high molecular weight subunit of neurofilament protein, Wood & Anderton, 1981; gift of B. Anderton), then we used a protocol similar to that described above for 22/18 versus 12/101. If the other reagent was a rabbit antiserum such as anti-glial fibrillary acidic protein (anti-GFAP; gift of M. Noble) or anti-vimentin antiserum (Jacob et al. 1982; gift of D. Bray), then we used a protocol similar to that described for 22/18 versus NP70. In some cases, sections were stained with only one primary antibody, including 22/18, 22/31 (a mouse IgM monoclonal antibody; Kintner & Brockes, 1985), and Leu7 (gift of A. Mudge). Leu7 is a mouse IgM monoclonal antibody, also known as HNK-1, originally isolated as a marker for human natural killer cells (Abo & Barth, 1981) but later shown also to recognize the myelin-associated glycoprotein of Schwann cells and other neural glycoconjugates (Schuller-Petrovic, Gebhart, Lasman, Rumpold & Kraft, 1983; McGarry, Helfand, Quarles & Roder, 1983; Kruse, Mailhammer, Wernecke, Faissner, Sommer, Goridis & Schachner, 1984; Ilyas, Quarles & Brady, 1984; Inuzuka, Quarles, Noronha, Dobersen & Brady, 1984). In adult newts it serves as a good marker of Schwann cells in the limb (Gordon & Brockes, 1987). The primary antibodies were detected by the protocol described above using a rabbit anti-mouse IgM second layer followed by rhodamine-conjugated goat anti-rabbit antiserum.

Controls for 22/18 staining included elimination of 22/18 from the staining protocol or substitution of IgM antibodies of unrelated specificity in place of 22/18. In general, background fluorescence was present throughout the embryo but was clearly distinguishable from a positive signal. The pronephric tubules and the gut gave the highest background fluorescence. Autofluorescent cells were evident by virtue of their visibility under both filter systems and because their colour was different from either rhodamine or fluorescein. The tissue was viewed by epifluorescence or Nomarski optics and photographed as described by Kintner & Brockes (1985).

Tracings (×200 magnification) made using a drawing tube helped in comparing the staining patterns of different antibodies on adjacent sections. The distribution of both NP70-stained axons and 12/101-stained muscles (including myoblasts) was determined from camera-lucida tracings of every fifth section through 14 normal forelimbs at stages 35, 36, 38, 39, 40, 42 and 45. The more limited staining at earlier stages was assessed by direct observation of immunostained sections.

Results

The 22/18 antibody was initially isolated and characterized using blastemas of N. viridescens (Kintner & Brockes, 1985). As a species, N. viridescens is difficult to breed in captivity - our own colony unexpectedly started breeding for one season, yielding a small crop of embryos. For the present developmental study, we screened several different urodele species with the intention of finding an alternative to Notophthalmus. One or more adult newts of each species (including...
Fig. 1. Immunostaining of an early-bud-stage blastema of an adult *P. waltl*. The blastema was processed 8 days after amputation through the forelimb. (A) Most cells in the distal part of the blastema (*bl*) are stained by 22/18. Cells of the wound epidermis (*e*) and those in more proximal parts of the stump (not shown) are not stained by 22/18. (B) An adjacent section was processed exactly as that shown in A except that 22/18 was omitted from the protocol. The epidermal surface shows faint background fluorescence in both A and B. Bar, 100 μm.

*Ambystoma mexicanum, Triturus cristatus, Triturus helveticus* and *P. waltl* were amputated through the upper forelimb and allowed to regenerate to the early- to mid-bud stage (Iten & Bryant, 1973). The limbs were tested for 22/18 reactivity as previously described (Kintner & Brockes, 1985). Only *P. waltl* gave a similar staining pattern within the blastema (Fig. 1) to that reported previously for *T. viridescens*. In the axolotl, 22/18 cross reacted with the epidermis but failed to stain blastemal cells. Faint staining was observed in the severed nerves of the two species of *Triturus*, but no staining of blastemal cells or skin was evident. As a result of these observations, embryos and larvae of both *T. viridescens* and *P. waltl* were used in this study. There was essentially no difference in the results obtained for the two species either during normal development or following amputation.

This paper is particularly concerned with the expression during development and larval regeneration of 22/18, and its spatial and temporal relationship to nerve and muscle. Results obtained with other antibodies are presented as ancillary observations as these were generally not studied systematically throughout development.

Peripheral nerves were found to be labelled earlier in development by an antiserum directed against the low molecular weight neurofilament subunit (NP70) than by a monoclonal antibody against the high molecular weight neurofilament subunit (RT-97). The sequential expression during development of the neurofilament subunits (low and medium followed by a phosphorylated form of the high molecular weight subunit) has been reported previously for several vertebrate species (Shaw & Weber, 1982; Willard & Simon, 1983; Pachter & Liem, 1984; reviewed by Willard, Meiri & Glicksman, 1985). In the adult *T. viridescens* blastema, the distal tips of regenerating axons were more extensively stained using NP70 compared with RT-97 (data not shown). In the results that follow, all descriptions of limb innervation refer to observations made using the NP70 antiserum.

**Normal development**

Because a general description of limb innervation and its relationship to cytodifferentiation is lacking for the newt, this is presented first followed by a description of 22/18 reactivity during development. The urodele limb undergoes considerable growth before nerves can be detected among the mesenchymal cells. At the earliest stages (31–32) of outgrowth, a thin nerve bundle lies adjacent to the bud but does not penetrate into the limb mesenchyme. There is no evidence of overt cytodifferentiation of either cartilage or muscle. Cartilage prepatternning is not observed when the tissue is viewed with either Nomarski optics (Fig. 2A) or a nuclear dye stain. The muscle-specific
Comparisons of developing and regenerating limbs

antibody, 12/101, stains the differentiating myoblasts in the adjacent flank, but does not stain cells in the bud (Fig. 2B). From the outset, mesenchymal cells can be stained with an antiserum to vimentin and with a monoclonal antibody, 22/31 (Kintner & Brockes, 1985), which gives a staining pattern very similar to anti-vimentin (Fig. 2C).

By stage 35, the bud has elongated and a few axons are present in its proximal part. A ventrally directed nerve bundle enters the limb first, followed shortly afterwards by a smaller dorsal branch emerging from the brachial plexus. At that time, a few 12/101-positive myoblasts are found near the junction between the nerve bundles. Cartilage prepatternning is apparent as a swirling of mesenchymal cells in the central part of the bud.

Throughout limb morphogenesis the nerves were found in a more distal distribution than muscle. In contrast, weak cartilage prepatterns were evident in distal parts of the limb prior to the arrival of nerves. Because many fine nerve branches had sprouted by the notch-stage (38), it was not possible to reconstruct accurately the innervation pattern from semiserial sections. We focused instead on the relative amount of nerves at a given proximal-distal level and also on the distalmost extent of growing neurites at this and subsequent stages.

Cartilage prepatternning was present up to the base of the notch at stage 38–39. The muscles of the shoulder region were clearly differentiated as discrete entities. Beyond this, two main clusters of myoblasts, a dorsal and a ventral group, were present in the proximal half of the limb. Although the main nerve branches were also confined to the proximal half, several finer bundles extended about 150–200 μm beyond the 12/101-positive myoblasts.

By the two-digit stage (40–41) the limb is innervated along most of its length, although the axons in the distal 25 % are quite fine and do not form discrete bundles. Axons have barely penetrated into the proximal part of the forming digits. At later stages the number of axons continues to increase, with progressively more fibres entering the distal limb. Individual skeletal elements are well formed by stage 45.

Reactivity of 22/18 in normal development
Initially the entire embryo was sectioned and stained in order to assess the overall distribution of 22/18. The staining of the larval epithelium was most striking and provided a convenient internal control with which to compare the staining of mesenchymal cells within the limb bud. The simple epithelium of the skin was difficult to preserve with such light fixation conditions so that many cells sloughed off during processing. It is for this reason that some of the photographs show only partial staining on the limb periphery, where the epithelial cells would be expected to lie. The staining of larval epithelium is in sharp contrast to results obtained from adult N. viridescens (Kintner & Brockes, 1985) and P. waltl (Fig. 1) where the stratified squamous epidermis is clearly not stained by 22/18.

Fig. 2. Immunostaining of a P. waltl larva at an early-bud stage of development (stage 33). (A) Nomarki optics shows that pigment cells are present in the early bud (p) but there is little evidence of other differentiation of the mesenchymal cells. (B) The same section has been stained with 12/101, a monoclonal antibody specific for newt muscle. Cells within the limb bud are negative while differentiating muscle (m) in the adjacent flank of the embryo is positive. (C) Nearly all of the limb mesenchymal cells can be stained by 22/31, a mouse monoclonal antibody that also stains cells in the adult stump and blastema (Kintner & Brockes, 1985). (D) Staining of a nearby section with 22/18 shows bright staining of the epidermal cells (e) and a few positive filaments among the mesenchyme (arrow). Bar, 50 μm.
In the developing brain and spinal cord, 22/18-positive cells that resembled radial glial cells were observed (Fig. 3A,B). Double labelling with anti-GFAP antiserum showed extensive overlap in the staining patterns of the two antibodies. There were regions where the staining with 22/18 was brighter than with anti-GFAP, and vice versa, suggesting that the two antibodies were not necessarily recognizing the same antigen. In the adult newt brain, the 22/18-positive cells were clearly a subset of the cells stained with anti-GFAP. In the larva a few cells weakly double labelled with the two antibodies were also observed in the trunk between the aorta and the pronephric tubules, and in the gut. These cells were most evident at the earliest stages (28–30).

Very strong staining with 22/18 was observed in the lens (Fig. 3C,D) at early stages and later along the wall of the aorta. Longitudinal sections through several late-staged larvae (stage 45 and beyond) revealed that positive cells stopped short of the heart and also extended only a short distance past the dorsal bifurcation of the aorta (Fig. 3E,F). No 22/18-positive cells were observed in the aorta of adult newts.

Prior to stage 36, examination of the limb buds relied primarily on unamputated animals. At later stages, the control limbs of animals whose right limbs had been amputated (see below) were also used. These data were supplemented with a few post-stage-36 larvae which were not subjected to amputation. A total of 67 unamputated limbs ranging from stage 30 through stage 52 were used.

At all of the stages examined, fewer than 1% of the limb mesenchymal cells were strongly stained with 22/18. A typical example of the staining pattern of 22/18 is shown in Fig. 2D. The epidermal cells of the limb bud are stained, as well as sparse filamentous staining in the limb mesenchyme. This pattern is in marked contrast to that obtained using another mouse monoclonal of the IgM subclass, 22/31, which stained all of the mesenchymal cells and nothing in the epidermis (compare Fig. 2C with 2D). In other cases there were essentially no 22/18-positive filaments detectable in the mesenchyme. A weak expression of the 22/18 antigen which is below the resolution of the immunofluorescence technique obviously cannot be ruled out, although brightly stained cells were observed in adult blastemas processed in parallel with the larval tissue and so the difference between them is unambiguous. When present, 22/18-positive staining appeared to be associated almost exclusively with cell processes, as such that shown in Fig. 2D, and was more prevalent in hindlimbs than in forelimbs. Only in rare cases was it possible to identify positive staining around the cell body of a limb mesenchymal cell. Usually it was still not possible to identify the stained cell as any differentiated type. They clearly did not represent pigment cells. In one case at stage 38; labelling with 22/18 closely overlapped with an area in which early myoblasts were differentiating, as evidenced by staining with 12/101. Several cells appeared to be double labelled by the two reagents. This result was obtained on both limbs of one N. viridescens larva, but could not be reproduced upon examination of 15 other limbs of similar stages from either N. viridescens or P. waltl.

After the limb was innervated, weak 22/18 staining was sometimes observed in association with areas through which the nerves were travelling. We suspect that these weakly positive cells could represent Schwann cells, but were unable to verify this because our marker of Schwann cells in adult limbs, Leu-7, was not specific in the larvae. The problem with Leu-7 was not an absence of staining (which might be interpreted as an absence of Schwann cells), but rather that more cells were labelled than would be expected for a Schwann cell-specific marker. Leu-7 labelled the spinal ganglia and peripheral nerves, but extended also to adjacent areas. An alternative possibility is that the 22/18-positive cells were associated with blood vessels which may be following a course similar to the nerves. It was not possible to test this due to the absence of an appropriate marker.

Reactivity of 22/18 in regenerating larval limbs

Because 22/18 reactivity was so limited during early limb outgrowth, we were able to assess the ontogeny of 22/18 expression in response to amputation of developing buds. The earliest stage at which we were able to amputate the limb bud and still retain a visible stump was approximately stage 34 for forelimbs, and stage 45 for hindlimbs. In each case, the right, amputated limb was compared with the left, control limb to assess both the intensity of 22/18 staining and the relative number of positive cells or cell processes. At all stages, the amputated limbs appeared to regenerate; a few animals were left to survive amputation and these regenerated normal limbs within 3–4 weeks.

Amputation of either fore- or hindlimbs at early stages does not provoke a 22/18 response when compared with the contralateral control (Fig. 4A,B). In these cases the nerve fibres have entered the limb, but most have not yet reached the plane of amputation. Forelimbs that were amputated between the notch stage (38) and the two-digit stage (40/41) and examined 3–6 days later show a few 22/18-positive cells at the plane of amputation. In several cases, we were able to verify that a few nerve fibres were also present near the amputation plane. Examination of adjacent or double labelled sections stained for 22/18...
Fig. 3. Immunostaining of 22/18 in nonlimb regions of developing larvae. For each pair of micrographs the left side shows nuclei stained by Hoechst dye and the right side shows the same field viewed by rhodamine fluorescence to locate 22/18-positive cells. (A,B) Thoracic spinal cord of a stage 38 N. viridescens larva. The radial processes of cells corresponding to GFAP-positive glial cells were stained in a filamentous pattern while mitotic figures in the centre of the field had a more punctate pattern. Bar, 50 µm. (C,D) The eye of a stage 38 P. waltl larva. The lens was strongly stained by 22/18. (E,F) The dorsal aorta of a stage 48 P. waltl larva shown just beyond the bifurcation point. Cells lining the lumen were 22/18-positive while those within the lumen were negative. Bar, C–F 100 µm.
Fig. 4. A 22/18-positive response to amputation occurs in late but not early developing limb buds. The drawings on the left show the approximate plane of section (arrows) for the immunofluorescence photographs of the control and amputated limbs shown on the right. The drawing in the upper left represents a P. walti larva whose right limb was amputated 5 days earlier at stage 37. (A) The left control limb developed to stage 38 and was 22/18-negative. (B) A 22/18-negative blastema formed on the right side. The drawing in the lower left represents a P. walti larva whose right forelimb was amputated at stage 49 and allowed to regenerate for 6 days. (C) At stage 52, the left control limb was 22/18-negative except for some staining of the epidermis. (D) On the right side, a blastema formed around the periphery of the severed humerus (the cell-sparse zone in the centre of the limb) and contained many 22/18-positive cells. Bar, 50 μm.

revealed a close spatial relationship between the nerve and the 22/18-positive cells. After the forelimbs had reached a three-digit stage (44), amputation through the midhumerus resulted in a robust 22/18 response (Fig. 4C,D). Many blastemal cells were brightly labelled by 22/18 and were often located close to nerve fibres (Fig. 5). The distalmost 100 μm of the blastema tends to have fewer positive cells than the region closer to the plane of amputation where nerve bundles are more prevalent. It was not possible to determine if the 22/18-positive cells originated from Schwann cells because of the lack of a Schwann cell marker for these stages (see above). The results of these experiments are summarized in Fig. 6.

The difference between the 22/18-response of early- and late-stage limb buds is most graphically demonstrated within a single animal. For example, by stage 46 a fourth digit is beginning to form on the forelimb whereas the hindlimb has only reached a midbud stage of development. Unilateral limb amputation at this stage results in a 22/18 response in the amputated forelimb compared with the contralateral control. In contrast, there is no difference between the amputated and control hindlimbs, with both showing a small amount of 22/18 reactivity that does not differ from unamputated animals.

Discussion

Normal development

The staining with 22/18 has revealed a clear difference in the cellular composition of developing and regenerating limbs of newts. Fewer than 1% of the limb mesenchymal cells were brightly labelled with the antibody during development. This contrasts sharply with limb regeneration in adults where as many as 80% of the blastemal cells are 22/18-positive at the early- to mid-bud stages. It is not clear what would be a comparable developmental stage, perhaps the pre-limb-bud stage, when the mesenchymal cells are just beginning to condense on the flank of the embryo. Examination of such limb rudiments did not
Fig. 5. The 22/18-positive cells in the blastema of a stage 46 *P. waltl* larva are spatially associated with nerves. (A) A slightly oblique section through the blastema sectioned and stained for nuclei 6 days after amputation. Closely packed blastemal cells surround the centrally positioned humerus in the distal part of the amputated limb (lower left). (B) The 12/101-positive muscles are located in the stump (upper right). (C) The same section as in A and B has been double labelled for 22/18. Numerous 22/18-positive cells are present in the blastema. (D) By staining an adjacent section with NP70 to detect nerve fibres, a close spatial association of nerves with 22/18-positive cells is seen in the distal blastema but not in the proximal stump. Bar, 100 μm.

reveal 22/18-positive staining; positive cells were restricted to the nonlimb tissues described above, including the skin, lens, aorta and glia of the central and enteric nervous systems. At slightly later stages, a few 22/18-positive cells were sometimes observed within the limb bud mesenchyme, particularly in the hindlimb. The present analysis cannot distinguish between an absence of the 22/18 antigen during limb development and a situation where the antigen is present but requires post-translational modification (e.g. phosphorylation) before it can be recognized by the antibody.

This distinction between the cellular composition of buds and blastemas can be considered in relation to the results of Muneoka & Bryant (1982, 1984) which led them to conclude that the rules governing pattern formation are the same during development and regeneration of axolotl limbs. Our results on newts show that at stages similar to those used by Muneoka & Bryant in their grafting experiments, buds and blastemas are clearly different with respect to 22/18 expression. Although species differences pose an obvious difficulty when comparing such studies, we suspect instead that the disparate results emphasize the difference between the processes involved in blastema formation and growth (as detected by 22/18), and those that control subsequent pattern formation.

The staining of tissues other than limb does not lend itself to simple explanation. At the moment, we know little about the function of the 22/18 antigen, its molecular characteristics or the epitope recognized by the antibody. The intracellular distribution of the antigen *in situ* and in cultured blastemal cells is indicative of an intermediate filament protein, or perhaps an intermediate filament-associated protein (Fekete *et al.* 1987). Very little is known about the functional role of this large and varied group of proteins which share at least one antigenic determinant (Pruss, Mirsky, Raff, Thorpe, Dowding & Anderton, 1981). Other antibodies tend to be more specific, usually recognizing one or more proteins within a class, but not cross reacting with those of a different class (e.g. Wang, Fischman, Liem & Sun, 1985). If 22/18 is directed against intermediate filament protein(s), it has the unusual property of recognizing some but certainly not all cells which are known to contain intermediate filaments of very different classes, including GFAP, vimentin and keratins specific to simple epithelia. It is also possible that the antibody may be cross reacting with unrelated antigens, in which case the staining of different tissues may have no functional relevance. In any case, it may be noteworthy that the ventral wall of the aorta has been identified as a source of haematopoietic stem cells in developing chicks (Dieterlen-Lievre & Martin, 1981). Thus the cells of this structure may have some unique and unusual properties that would be lost at later stages of development. In this light, it is interesting that 22/18 stains the wall of the aorta in larval but not adult newts. The presence of GFAP-containing glia in the gut has been observed in the rat (Jessen & Mirsky, 1980, 1983). It is unclear why 22/18 should stain a subset of these cells or a subset of GFAP-positive glial cells in brain and spinal cord of newt.

**Response to amputation**

Amputation of the limb at early stages of development yields a 22/18-negative blastema which is capable of regenerating a normal limb. At later stages, after the limb is innervated, amputation yields a 22/18-positive blastema with similar regenerative capacity. When an early-staged hindlimb and a late-staged forelimb were both amputated in a single
Fig. 6. Summary of the experiments in which limbs were amputated at different stages of development and stained with 22/18. A schematic drawing of each stage according to Gallien & Durocher (1957) is shown across the top. The fine lines within each limb indicate the proximodistal extent of innervation as determined by staining with an antiserum directed against neurofilament protein. These drawings are not intended to portray individual nerve branches accurately, particularly after stage 37. Each arrow represents an individual animal and begins on the left at the stage of limb development when amputated. The arrowhead marks the stage to which the contralateral unamputated limb had developed during the course of the experiment. In all cases, the amputated limb formed a small blastema at the time of fixation. The symbol at the tip of the arrow indicates the degree of induction of 22/18 in response to amputation, determined by comparing immunostained sections of the amputated blastema with the contralateral control limb. A negative sign denotes no induction of 22/18. Increasing numbers of 22/18-positive cells within the regenerating blastema are indicated by the series of plus signs. Beginning at about stage 38, amputation results in a weak 22/18-response when examined several days later. Amputation at later stages results in a stronger 22/18-response.

animal, only the forelimb blastema developed reactivity to 22/18. This result suggests that the development of a 22/18 response to amputation cannot be explained by the appearance of a circulating factor.

At least two interpretations, somewhat related, could be advanced to explain the developmental transition of an amputated limb bud from a 22/18-negative to a 22/18-positive state. One possibility is that expression of the 22/18 antigen is related to a change whereby embryonic regulation gives way to epimorphic regeneration. A second possibility is that the nerve plays a critical role in the origin and subsequent proliferation of 22/18-positive blastemal cells. These two possibilities are schematically represented in Fig. 7 and will be discussed in turn.

The term ‘regulation’ has been used to describe a variety of morphogenetic phenomena that occur following the removal of tissue from different developing systems. For the present discussion, we will use a definition suggested by Slack (1980): ‘regulation’ refers to a process whereby the fates of primordial cells within an embryonic field are altered by removal of a part of the field, so that the remaining cells give rise to a greater range of structures than normal. Preforelimb bud rudiments of early urodele embryos exhibit regulative behaviour when half of the tissue is removed (Harrison, 1918; Slack, 1980). At later
stages, amputated larval limbs are assumed to undergo the same process of epimorphic regeneration that occurs in adult limbs (e.g. Stocum, 1979). That is, the missing parts of an amputated limb are re-formed by the proliferation of undifferentiated blastemal cells that were derived from mature tissues. Between these two stages, the developing limb is at an intermediate point where some of the cells are still undetermined, while other cells are differentiating for the first time. Amputation at such stages is followed by the formation of a perfectly normal limb (Maden & Goodwin, 1980). The issue is whether this occurs by regulation or regeneration. Slack has proposed that the difference between regulation and regeneration corresponds to a difference between the response of undetermined rudiments and differentiated organs. He has emphasized the importance of dedifferentiation of mature tissue to yield a regenerating blastema, which then relies on the adjacent stump tissue to direct its subsequent morphogenesis.

When the developing limb bud regenerates after amputation, it must be doing so by a process which differs from that occurring in differentiated larval or adult limbs, since the 22/18 antigen is not expressed at the early stages. This observation may reflect the distinctions proposed by Slack in that the early 22/18-negative response to amputation would correspond to a situation in which there are still a sufficient number of pluripotent cells available that can alter their prospective fates. The later 22/18-positive response would correspond to a situation in which most of the cells are already determined so that now the missing parts must be reconstituted by cells that have to revert to a pluripotent condition. During this process, the cells, as in the adult limb, express the 22/18 antigen.

A second possible explanation for the ontogeny of the 22/18-response to amputation may be related to the arrival of nerves (axons plus Schwann cells) in the developing bud. Previous observations on adult limbs lend support to this suggestion. In the adult forelimb, the nerve has been shown to contribute 22/18-positive cells to the blastema and also to be required for the subsequent proliferation of these cells. In fact, these two roles of the nerve can be dissociated. The Schwann cells of the nerve are induced to express the 22/18 antigen after amputating the limb (Kintner & Brockes, 1985) or sectioning the nerve at the brachial plexus. If the limb is maintained in a denervated state, the Schwann cells continue to express the antigen for at least 6 weeks (Gordon & Brockes, 1987). If the limb is amputated and then denervated, 22/18 is expressed by Schwann cells and blastemal cells, but their division is severely reduced (Kintner & Brockes, 1985). Both a cellular contribution to the blastema by the nerve (Maden, 1977) and a mitogenic effect of the nerve (reviewed by Brockes, 1984) have been previously described. The additional information gained by using 22/18 was that the mitogenic effect was most pronounced for only a subset of blastemal cells and that some of these originate from Schwann cells.

The proximity of the nerve to the 22/18-positive cells in regenerating larval limbs suggests that as in the adult, some of these cells might be derived from Schwann cells. Unfortunately, there is little information about the temporal relationship between the arrival of axons and the arrival of Schwann cells at different proximodistal levels of a developing urodele limb. It is thought that peripheral axons are naked when they first leave the cord and that Schwann cells migrate down the axons some time later (Harrison,
dent on the nerve, since amputation of a denervated
Xenopus
and chick (Lamb, 1974; Hamburger, 1975; some point, the system becomes absolutely depen-
dent proliferation would also be manifest by an
A gradual shift from nerve-independent to nerve-
dependent proliferation would also be manifest by an
of development and would gradually acquire nerve
regeneration. If true, then regeneration (or regu-
lation) would be nerve independent at early stages
where amputated (Thornton & Thornton, 1970). We predict that, like the preinnervated
developing limb, the blastema of an aneurogenic
limb will not express the 22/18 antigen in response
to amputation (see also discussion by Brockes, 1984).
Likewise, the blastemal cells that arise will not be
dependent on the nerve-derived growth factor but
will respond to non-neural growth factors. We further
predict that after innervation of the aneurogenic
limb, 22/18-positive cells will be generated which can
only respond to the nerve-derived growth factor.
That is, the limb will have been restored to its normal
state of nerve dependence after amputation. Exper-
iments are currently under way to test the prediction
that an amputated, aneurogenic limb regenerates via
a 22/18-negative blastema. If it does, then the acqui-
sition of the 22/18 response to amputation does not
control the transition between regulation and regen-
eration, but rather represents the imposition of the
nerve-dependent system of growth control.

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