Effects of radius–ulna removal on forelimb regeneration in *Xenopus laevis* froglets

By ROBERT G. KORNELUK AND RICHARD A. LIVERSAGE

Ramsay Wright Zoological Laboratories, University of Toronto,
25 Harbord Street, Toronto, Ontario, Canada M5S-1A1

SUMMARY

Regeneration of boneless amputated forearms of adult newts was found to progress at a rate and to a degree comparable to amputated control limbs in which stump bones were not removed. In contrast, regeneration of boneless amputated *Xenopus* froglet forearms was significantly delayed and did not occur until two to three weeks following amputation. This is in comparison with the initiation of distal cartilage formation observed one week postamputation in control forelimbs of *Xenopus* froglets. The regeneration of cartilage in boneless forearms of adult newts was found to occur distal to the amputation level. In contrast, distal as well as proximal (centripetal) regeneration of cartilage was observed in the amputated boneless forearms of *Xenopus*. In froglets and newts, unamputated forelimbs in which forearm bones were extirpated did not initiate cartilage regeneration. Our findings support the hypothesis that forelimb regeneration in *Xenopus* froglets is primarily a tissue response. In comparison, limb regeneration in the adult newt is predominantly an epimorphic response.

INTRODUCTION

Histological evidence from Korneluk (1982) and Korneluk, Anderson & Liversage (1982) suggests that tissue regeneration is the dominant response to amputation of *Xenopus* froglet forelimbs. One major source of cells for the regenerating cartilage outgrowth appears to be the connective tissue sheaths of bone (periosteum) or of cartilage (perichondrium). A direct test of this hypothesis can be made by examining the effect of radius–ulna removal on the progress of forelimb regeneration in *Xenopus* froglets.

Previous studies employing such an approach with *Xenopus* have not been documented. However, in a preliminary study, Goss (1953) reported that in newly metamorphosed bullfrogs, surgical extirpation of the radius–ulna followed by forelimb amputation, resulted in enhanced regeneration. An heteromorphic cartilaginous spike regenerated following these treatments, in contrast to stumping of the amputated control limbs. A frog limb in which the long bones have been removed will not regenerate cartilaginous elements unless the limb skin-sleeve itself is amputated. This suggests that removal of these bones may improve the capacity for regeneration in anurans. In a comparative study, Goss (1958) showed that regeneration of extirpated skeletal parts in non-amputated limbs of anuran tadpoles did not occur except in a few very young
animals. Larval urodeles, however, were found to replace removed skeletal elements even in non-amputated limbs (Goss, 1958), but forelimb amputation was necessary before regeneration of bones could take place in adult newts (see also Rieck, 1960).

The reconstitution of skeletal elements in amputated, boneless limbs of urodeles has been the subject of numerous investigations (review Goss, 1956). In the present work, experiments corroborate and extend previous conclusions. Goss' results (1956) emphasize that normal regeneration of an adult urodele limb, including skeletal elements, ensues distal to the amputation level, even in absence of stump bones. Very little proximal (centripetal) bone reconstitution is found except where the entire radius–ulna is removed. In contrast, centripetal regeneration of extirpated skeletal elements occurs more readily in the amputated limbs of larval urodeles (Thornton, 1938). Goss (1956, 1958) suggests that this is because skeletal elements of salamander larvae are cartilaginous, and presumably 'potential chondroblasts' (mesenchymal cells capable of chondrogenesis) are sufficiently dispersed throughout the limb. Extirpation of a larval radius–ulna does not remove all of these potential chondroblasts. Consistent with this interpretation is the observation that skeletal regeneration also occurs in non-amputated limbs of urodele larvae (Goss, 1958). In the adult newt limb, however, chondrogenic cells are presumably restricted to the periosteum where they are capable of cartilage callus formation in the event of a fracture or severance of a limb. In adult urodeles, extirpation of such bones (Goss, 1956, 1958) removes virtually all of these cells, and skeletal regeneration does not occur unless the limb is also amputated.

Urodele bone removal experiments have provided information regarding two major considerations of skeletal reconstitution; a) the source of cells contributing to the regenerating skeletal parts, and b) the nature of the morphogenetic influence of stump bones on regenerating skeletal patterns. Accordingly, the present study of the effect of bone removal on the progress of forearm regeneration in *Xenopus* is directed toward these two major aspects. Furthermore, the reconstitution of extirpated skeletal elements in amputated and non-amputated forelimbs of *Xenopus* froglets and the effect of bone removal on the epimorphic regeneration of adult newt forelimbs will be examined, in order to compare the nature of skeletal regeneration in these two amphibians.

**MATERIALS AND METHODS**

The *Xenopus laevis* selected for this study were sibling froglets approximately two months postmetamorphosis with a snout–vent length of 2.5 to 3.0 cm. They were obtained by induced mating of a pair of mature, laboratory-bred *Xenopus* following injection of human chorionic gonadotropin (HCG, Sigma). The method of spawning and rearing *Xenopus* is described by Gurdon (1967).
Regeneration of boneless Xenopus forearms

Adult newts (*Notophthalmus viridescens*, 1.8–2.5 g body weight) were obtained from Charles Sullivan, Co., Tennessee. During the experiments, adult newts and *Xenopus* froglets were kept in dechlorinated tap water at 24(± 1)°C, maintained on a 12/12 h photocycle, and fed chopped beef heart or *Tubifex* worms twice weekly. A total of 74 newts and 146 *Xenopus* of both sexes were used in this study.

Unilateral or bilateral forelimb amputations were performed through the ossified diaphysis of the distal one-third of the radius–ulna in animals anaesthetized in MS 222 (0.10% w/v for newts, 0.04% w/v for frogs). Some forelimb amputations were immediately followed by removal of a 1 mm cuff of proximal whole skin (including dermis) from the distal portion of the stump in both froglets and newts.

In most experimental cases, removal of the radius–ulna was performed immediately following amputation. Beginning at the amputation surface, the adhering musculature was carefully teased away from the bones using fine forceps, and the elbow joint dissociated with iridectomy scissors. Bones were then removed distally through the amputated end of the stump, resulting in a boneless limb stump with an intact skin-sleeve, in which the only cut surface was the original amputation site. Sham removal of the radius–ulna was performed by separating the soft mesodermal tissue from the bones, but the bones were not removed nor the elbow joint cut. In *Xenopus* froglets, the radius and ulna are fused into one zeugopodial component, whereas these bones in the newt are separate.

For the sake of brevity, forelimbs from which the radius–ulna were removed are referred to as RUx limbs. In most RUx cases, amputation and bone removal were concomitant. However, in a few newts and froglets amputation of RUx forelimbs was delayed. In the latter cases, a longitudinal incision was made in the ventral forearm region of the intact limb, the radius and ulna were carefully separated from the surrounding tissues, the joints cut and the bones then removed (similar to methods of Weiss, 1925). Amputation of these RUx forearms and the corresponding control forearms was performed 10 days later.

The morphological progress of regeneration was observed and recorded under a dissecting microscope. At various stages, representative animals were anaesthetized and their forelimbs removed and fixed in G-Bouin’s fluid for histological sectioning (Liversage, 1967).

RESULTS

Experiment I

Left forelimb amputations were performed on 66 froglets; no further operation was performed upon the 18 control animals of this group. All other froglets in this experiment underwent either removal of the left forearm radius–ulna...
bones concomitant with amputation (i.e. 30 RUx cases) or sham radius–ulna extirpation concomitant with amputation (i.e. 18 sham RUx cases). The progress of the control froglet regenerates was scored periodically for up to 4 weeks postamputation. For a description of normal stages, see Korneluk et al. (1982).

Histologically, the sham RUx limbs differed from controls only slightly. One week following amputation, periosteal cartilage deposition was extensive, somewhat thicker than in comparable control limbs (Fig. 1). This may be related to the degree of stump tissue damage which occurred during the sham operation. Indeed, evidence of muscle bundle fragmentation along the entire length of the radius–ulna was visible one week following amputation (Fig. 1), but two weeks later stump muscle appeared to be repaired in the sham RUx limbs. Chondrogenesis distal to the level of amputation was advanced (Fig. 2). Furthermore, a sparse population of fibroblast-like cells is present in both sham RUx and control regenerates. We believe an appropriate term to describe this distal-most accumulation of cells is ‘fibroblast-blastema’ (foreshortened to ‘fibroblastema’, Fig. 2). Regeneration of the sham RUx and control limbs during subsequent weeks was identical (see Korneluk et al. 1982).

The morphological progress of regeneration in the RUx forelimbs of *Xenopus* differed greatly compared with control and sham RUx regenerates. Following amputation and concomitant radius–ulna removal, the soft zeugopodial tissues of the limbs were observed to collapse and contract. By one week the elbow to distal tip length averaged 2.0 mm, about half the size of comparable control forearms. No external signs of regeneration were detected for two weeks after amputation. However, by the third week, some RUx limbs displayed small, narrow spike regenerates which developed slowly during subsequent weeks. By 4 weeks, the average size of the spike outgrowths was about 1.0 mm, compared with 3 to 4 mm in the control and sham RUx regenerates. All control and sham regenerates were normal. Nearly half of the RUx limbs (7/15) showed no external sign of regeneration 4 weeks postamputation, and displayed a pigmented dermis at the distal tip.

Epidermal wound healing of RUx limbs was normal as the distal tips were covered within 24 h. However, collapse and retraction of the mesodermal stump tissues in these limbs resulted in considerable lateral movement of the dermis from the edges of the wound by one week (Fig. 3). This limited the area of contact between the apical epidermal cap and the underlying mesodermal stump tissues. Furthermore, the fibroblastema normally located subjacent to the apical cap, was slight compared with control regenerates at this stage. Fibroblast-like cells, however, could be seen in the collapsed stump, interspersed amongst the disrupted mesodermal soft tissues (Fig. 3).

By two weeks, RUx limbs showed little external sign of regenerative activity. Histological sections revealed that the original differentiated tissue of the stump had not undergone extensive dedifferentiation nor histolysis (Fig. 4). As in the one week cases, disrupted but fully recognizable muscle and nerve bundles were
Regeneration of boneless Xenopus forearms

seen throughout the stump. Also, some proximal cartilage regeneration was evident, particularly where a remnant of the original radius-ulna had been inadvertently left in place (as in Fig. 4).

Histological sections of other Xenopus forelimbs two weeks following amputation and bone removal displayed a small, but distinct, fibroblastema subjacent to the apical epidermal cap (Fig. 5). The appearance of the fibroblastema was similar to that observed in control limbs during the first few weeks of regeneration. By 3 weeks, this distal population of fibroblast-like cells was differentiating into a small cartilage spike (Fig. 6).

RUx limbs 4 weeks after amputation showed either distinct regenerative activity, or stumping. Regenerating RUx limbs often formed cartilage in two separate areas of the stump (Fig. 7). The first region was the distal area; a continuation of cartilage spike formation initially observed between 2 and 3 weeks of regeneration. This cartilage developed in a distal as well as a centripetal (i.e. proximal) direction. Often a second or more proximal region of cartilage deposition was seen (Fig. 7). Cartilage formation in this latter instance was continuous with the cartilage cap around the epiphysis of the intact humerus (as in Fig. 5). About half (47%) of the RUx forelimbs at 4 weeks postamputation stumped and showed no morphological signs of regeneration. These limbs had distal tips completely covered by dermis (Fig. 8) and displayed only limited proximal cartilage formation.

Experiment II

Radius-ulna removal in froglets as well as newts was performed for comparative purposes. Bilateral, rather than unilateral, amputations were performed on both newts (50 cases) and froglets (50 cases); left limbs were amputated concomitantly with radius-ulna removal, while the right limbs served as amputated controls. Also, at the time both animal groups were amputated, stump dermis was cut back from the distal tip of the left and right forelimbs. Forelimbs were fixed at intervals up to 7 weeks postamputation.

As found in Experiment I, the Xenopus froglets of Experiment II displayed morphologically typical epidermal wound healing in the RUx and control forelimbs one day after amputation. However, due to the extent of dermis removal, the opaque area of wound epidermis remaining over the distal tip of the amputated limbs during the first two weeks was more extensive than that observed in froglets of Experiment I. Collapse and contraction of the mesodermal stump tissue in RUx limbs were evident by one week postamputation.

The delay of regenerative response of the RUx limbs was seen in the froglets of Experiments I and II. Regenerative activity was not externally visible in the Experiment II Xenopus froglets until the third week. Growth and development of the spike continued and by 7 weeks postamputation, these RUx limbs often attained a size comparable to contralateral control limbs. All RUx Xenopus limbs of Experiment II showed regenerative activity by 4 weeks postamputation,
Figs 1-8. Photomicrographs of longitudinal sections of forelimb regenerates of *Xenopus* froglets from Experiment I. The original level of amputation is indicated by arrows.

Fig. 1. Sham RUx forelimb regenerate of *Xenopus* one week following amputation. The apical epidermal cap (ac) covers the distal accumulation of fibroblast-like cells (f). Dermis (d; indicated by dermal skin glands) does not extend into the regeneration area. Early chondrogenesis or procartilage (p) formation distal to the amputation level are visible. Sham RUx regenerates at this stage differ only slightly from
Regeneration of boneless Xenopus forearms

15

control forelimb regenerates. That is, periosteal cartilage (pc) deposition is more extensive in sham RUx limbs. Note muscle fragmentation (m) along bone complex (b). Stump reactions appear to be the result of injury which occurred during the sham operation (×40).

Fig. 2. Sham RUx forelimb of a froglet two weeks after amputation. Immediately proximal to the apical cap (ac), a sparse population of fibroblast-like cells or fibroblastema (f) can be seen. Dermis (d) is regenerating in a proximodistal direction, along the length of the cartilage spike (c). Periosteal cartilage (pc) formation
is continuous with distal cartilage regeneration. Stump muscle (m) observed to have been fragmented by the previous week (see Fig. 1) is less dissociated at this time (×40).

Fig. 3. RUx regenerate of a Xenopus froglet one week postamputation. Convolutions of the apical epidermal cap (ac) are due, presumably, to the collapse and contraction of the mesodermal stump tissues following bone removal. The integrity of the dermis (d) is less affected. Disruption of stump muscle (m) and nerve bundles (n) is apparent, but dissociation of these tissues is not extensive at this or any of the later stages of regeneration. A ring of fibroblast-like cells (rf) is evident distal to the epiphysis (ep) of the intact humerus (×40).

Fig. 4. Xenopus froglet forelimb regenerate two weeks following concomitant amputation and radius–ulna removal. Stump tissue contraction in conjunction with subsequent lateral movement of whole skin resulted in the dermis (d) covering the distal tip. Consequently, only a small area of wound epidermis (i.e. apical cap – ac) covers the distal portion of the amputated limb. Distal regeneration of cartilage is not seen in this preparation, as found in comparable sham control limbs (e.g. Fig. 2), but cartilage regeneration (re) has occurred in the proximal region of the stump, which extends from a cartilage remnant (r) of the extirpated radius–ulna. The remnant (r) stains darker, as found in the cartilaginous epiphysis (ep) of the intact humerus, compared with the less dense cell population and lighter staining of regenerated cartilage (rc). The original soft tissues of the stump, such as muscle (m), have not undergone histolysis (×40).

Fig. 5. RUx regenerate of an Experiment I froglet, two weeks postamputation. A small distal accumulation of fibroblast-like cells (f), covered by an apical cap (ac), is present. At the amputation level, early signs of procartilage formation (p) are visible and similar to those found in control regenerates from earlier stages (see Fig. 1). A regenerated cartilage cap (rc) is seen distal to the humeral epiphysis (ep); it is detectable by the difference in its staining properties compared with the epiphysis cartilage (ep). This cap appears to have regenerated from the ring of fibroblast cells (see Fig. 3) found at one week postamputation (×40).

Fig. 6. Xenopus RUx regenerate 3 weeks postamputation. A small cartilaginous spike (c) is seen in the distal area, regenerating in the region beneath the apical epidermal cap (ac). Note proximity of the dermis (d) to the spike. Except for the size difference, this spike is similar to that found in a normal Xenopus regenerate (see Fig. 2). Proximal regeneration of cartilage (rc) is also evident, and is distinct, on the basis of its staining intensity, from the cartilage remnant (r) of the original radius–ulna (×40).

Fig. 7. RUx forelimb regenerate of Xenopus 4 weeks following amputation. An apical epidermal cap (ac) covers the small accumulation of fibroblast-like cells (f). Distal cartilage formation (c) is a continuation of the distal chondrogenesis (as c in Fig. 6). This distal cartilage regeneration appears to have progressed in both a distal as well as a centripetal (i.e. proximal) direction relative to the amputation level. The regenerated cartilage (rc) found in the inner regions of the boneless stump is a continuation distally of proximal chondrogenesis seen in earlier stages (see Fig. 5). This cartilage appears to have regenerated directly from the cartilage remnant (r) of the original radius–ulna and/or from the ring of fibroblast-like cells described previously in Fig. 3 (×40).

Fig. 8. Stumped RUx limb of a Xenopus froglet from Experiment I, 4 weeks postamputation. The dermis (d) covers the distal tip of the stump. A small, isolated nodule of cartilage (c) has developed in the central area. There is very limited proximal cartilage regeneration (rc), as well as a cartilage remnant (r) of the extirpated radius–ulna. A prominent nerve bundle (n) extends distally to the level of amputation (×40).
Regeneration of boneless Xenopus forearms compared with the recovery of regeneration in only 50% of the RUx limbs of Experiment I.

In contrast with Xenopus froglets, the majority of adult newt RUx regenerates progressed at the same rate and degree as their contralateral control forelimbs. Where a delay was observed, it was not significant and represented, at most, a lag of 2 to 4 days of regeneration.

The progress of RUx and control froglet regenerates of Experiment II was similar to that observed in the forelimbs of Experiment I. The main difference was seen in the formation of the wound epidermis. This was expected because of the extent of the dermis cut back. The apical cap of the RUx forelimbs one week after amputation covered a larger area of the distal tip and appeared thicker in the froglet forelimbs in Experiment II. However, the distal accumulation of fibroblast-like cells and the subsequent cartilage formation were delayed in a manner similar to that seen in the RUx limbs of the Experiment I froglets. Specifically, signs of distal chondrogenesis appeared 2 to 3 weeks following concomitant amputation and radius–ulna removal. Development of the distal cartilage spike progressed in both proximal and distal directions as evidenced by the density of cells and lighter staining of regenerated cartilage in numerous cases (e.g. Fig. 7). As in Experiment I, regeneration of proximal cartilage was observed, particularly where a proximal remnant of the original radius–ulna remained (Fig. 9).

Analysis of the newt regenerates of Experiment II indicated that the stages of regeneration were similar in RUx and contralateral control limbs. In the control forelimbs of bilaterally amputated newts, the stages of epidermal wound healing, dedifferentiation, blastema accumulation and growth, differentiation and morphogenesis were as previously described in unilaterally amputated newts (see review, Wallace, 1981).

RUx forelimbs of adult newts also showed epidermal wound healing within one day following amputation. By one week, the dedifferentiation of mesodermal soft tissue was observed to be particularly advanced (Fig. 10), in contrast to RUx regenerates of Xenopus froglets (compare Fig. 10 to Fig. 3). Newt RUx limbs by 10 days postamputation revealed large numbers of mesenchyme-like cells throughout the collapsed stump distal to the humerus. At this stage, dedifferentiation of the original mesodermal tissue was extensive (Fig. 11). The extent of dedifferentiation in a comparable Xenopus RUx limb was slight, if at all present (compare Figs 3, 4).

The differentiation of tissue in the distal region of older newt RUx regenerates was as observed in contralateral control limbs. That is, normal regeneration of distal skeletal elements and other differentiated tissues (e.g. muscle) was found in the control as well as in the RUx limbs of adult newts. However, the results revealed that regeneration of cartilage elements was virtually absent in the stump region proximal to the amputation level (Fig. 12), unlike that found in comparable RUx Xenopus limbs (compare Fig. 9).
Experiment III

Adult newts (24 cases) and juvenile *Xenopus* (30 cases) were used in this experiment. In this series, the left forelimb of the animal initially underwent radius-ulna removal and was allowed to recover from the surgery for 10 days when half of the animals were bilaterally amputated, and the remaining animals not subjected to further surgery. All animal limbs were scored periodically, and their forelimbs were examined histologically 4 weeks postamputation.
Regeneration of boneless Xenopus forearms

In the Xenopus froglets of Experiment III, amputated forelimbs regenerated in a manner similar to that described for the froglet limbs of the previous two experiments. Also, newt RUx regenerates of Experiment III progressed in a manner similar to that described previously. That is to say, the degree and rate of regeneration was similar in both RUx and control forelimbs of the bilaterally amputated adult newt.

RUx forelimbs of newts and froglets not amputated did not show any signs of cartilage regeneration in the region of the extirpated zeugopodium, even 5 weeks after bone removal.

DISCUSSION

Our results are discussed in terms of two major aspects of skeletal regeneration, the source of cells for the regenerating skeletal elements, and the nature of skeletal pattern formation. As skeletal reconstitution of amputated adult newt RUx forearms was found to progress at a rate and to a degree comparable to controls, the source of cells for the regenerating skeletal components in these limbs is the blastema (i.e. indicative of epimorphosis), and not the direct proliferation and differentiation of cells such as periosteal fibroblasts and osteoblasts (i.e. tissue regeneration). In support of our observations are the quantitative results of Chalkley (1954, 1959) which show that although periosteal cells contribute to the initial production of cartilage in the adult newt limb...
regenerate, their numbers are significantly augmented by the incorporation of dedifferentiated proliferative blastema cells from other sources.

In the present study, *Xenopus* RUx limbs show a significant delay in their regenerative response following amputation. As two major sources of cells for the regenerating cartilage spike of *Xenopus* are apparently cells of the periosteum (or perichondrium) and chondrocytes (Korneluk, 1982; Skowron & Komala, 1957), radius-ulna removal can be expected to result in a significant delay of distal cartilage formation in amputated froglet forearms. In contrast, a comparable, significant delay in the adult newt cases was not observed. Amputated RUx forelimbs of *Xenopus*, however, do eventually initiate distal cartilage formation. Apparently, a third potential source of cells forming the distal cartilage spike is the fibroblasts of the ubiquitous connective tissue sheaths of the stump (i.e. forming the fibroblastema). The delay in the regeneration of *Xenopus* RUx limbs indicates that the fibroblastema is not the initial nor sole source of cells for the regenerating distal cartilage spike as suggested by Goode (1967). Goode considered that most of the regenerating cartilage is derived from this source, and referred to this distal accumulation of fibroblast-like cells as a true 'blastema' (as does Dent, 1962). We have suggested that regeneration of amputated *Xenopus* limbs is also the result of the direct outgrowth of stump tissue, in agreement with Komala (1957) and Skowron & Komala (1957). They refer to the distal population of fibroblast-like cells as a 'pseudoblastema', and argue that it does not contribute at all to the regeneration of cartilage. However, the present study shows that amputated RUx limbs of *Xenopus* do ultimately initiate distal cartilage formation, and therefore, the role assigned to the fibroblastema must represent a compromise between both earlier interpretations. Although not an exclusive source of cells, the fibroblastema has the potential to differentiate into cartilage without the direct, inductive influence of stump bones. However, in absence of the dominating stump influences, morphogenesis is still limited to the differentiation of connective tissue elements, as in normal regeneration.

Following radius-ulna removal and amputation, a ring or collar of fibroblast-like cells formed just distal to the epiphysis of the intact humerus in both *Xenopus* froglets and adult newts. This subsequently differentiated into a cartilage cap. The degree of proximal cartilage regeneration was particularly extensive in *Xenopus*, especially when a remnant of the radius-ulna remained following the RUx operation. Cartilage regeneration from proximal bone remnants is, therefore, similar to the processes normally found during the regeneration of non-RUx froglet limbs. In the proximal regions of amputated RUx stumps and in the distal area of normal control regenerates of *Xenopus*, periosteal or perichondrial fibroblasts, or chondrocytes were found to contribute directly to cartilage regeneration.

The second major aspect of skeletal regeneration concerns the pattern of cartilage formation. In the amputated RUx limbs, cartilage formation was observed at two levels: at the amputation site, and more proximally, near the epiphysis
Regeneration of boneless Xenopus forearms of the intact humerus. In adult newt RUx limbs, most of the regeneration of skeletal elements was distal to the level of amputation; centripetal regeneration was virtually absent. Present and previous results (Goss, 1956) found in newt RUx regenerates conform to the 'rule of distal transformation', which states that only distal structures are regenerated outward from the plane of amputation (Rose, 1962). Although the information regarding blastema skeletal pattern formation appears to originate in the stump (Stocum, 1975; Maden, 1980), our results suggest that stump bones themselves are not necessarily responsible for this information, in agreement with Goss (1956) and Rieck (1960). Our findings are indicative of the 'dominance of epimorphosis' in regenerating urodele limbs, in which morphogenesis of the distal blastema region appears to suppress tissue regenerative responses in the proximal areas of the stump (Carlson, 1970, 1978, 1979).

Centripetal regeneration of cartilage in RUx limbs, from the amputation site and from more proximal stump regions near the intact humerus, occurred more readily and to a much greater degree in Xenopus than in newts. In froglets, the distal cartilage spike which initially formed at the amputation level in RUx limbs often appeared to regenerate in both a distal and a proximal direction. Therefore, the 'rule of distal transformation' does not strictly apply to the pattern of distal cartilage regeneration in the anuran. Furthermore, isolated distal and proximal cartilage elements were often formed in the same froglet limb. This shows that distal regenerative activity of an amputated Xenopus RUx forelimb does not appear to 'dominate' and suppress proximal cartilage formation.

Another consideration pertains to the correlation of dermis with the stumping of a froglet limb, as shown in the histological evidence of Experiment I. About half of the anuran RUx limbs failed to regenerate. These stumped limbs showed little, if any, sign of distal cartilage regeneration. The presence of dermis at the tip was probably due to the post-surgical collapse and retraction of whole skin over the boneless stump.

In the animals of Experiment II, the dermis was cut back from the amputation site. This resulted in most of these RUx limbs initiating distal cartilage spike formation. Regeneration of the spike appeared to occur only from mesodermal regions free of overlying dermis and covered by an apical epidermal cap. Although our correlative results do not provide direct evidence for the role of dermis in the stumping of Xenopus regenerates, such an interpretation is in agreement with studies in other amphibians including epimorphic limb regeneration in urodeles (review, Mescher, 1976). Results from other laboratories are consistent with the interpretation that the apical wound epidermis of urodeles may serve to establish the blastema (Singer & Salpeter, 1961; Thornton, 1968), and also to keep dedifferentiated cells in the cell cycle thereby preventing their immediate redifferentiation (Tassava & Mescher, 1975; Globus, Vethamany-Globus & Lee, 1980; Tassava & Olsen, 1982).

The formation of an apical epidermal cap is a normal regenerative response
of an amputated *Xenopus* forelimb. The extent of wound epidermis formation in RUx limbs, however, depended upon the degree of stump tissue collapse following amputation and subsequent dermal intervention. In *Xenopus* the relationship of epidermis to distal cartilage regeneration appears somewhat similar to that found in epimorphosis (see also Korneluk et al. 1982). However, the wound epidermis of *Xenopus* does not appear to prevent the immediate or precocious differentiation of fibroblast-like cells into cartilage, as is considered to be the case in the newt. The wound epidermis of a *Xenopus* regenerate does not attain the thickness found in newt regenerates (see also Dent, 1962; Goode, 1967). However, the difference in the prevention of immediate cartilage differentiation following amputation is more likely due to properties of the regeneration cells themselves, and not to the wound epidermis.

Another aspect concerning the importance of the wound epidermis in *Xenopus* and newt limb regeneration is a finding in non-amputated limbs from which the radius—ulna bone was extirpated. These limbs showed no signs of skeletal regeneration. Amputation must be performed and wound epidermis formation must occur in order for cartilage to regenerate in the limbs of adult newts (see also Goss, 1956, 1958; Rieck, 1960) and postmetamorphic *Xenopus*.

Lastly, general tissue injury including nerve damage (see Korneluk et al. 1982) due to bone removal, may have an effect on the progress of forelimb regeneration. The marked delay of regeneration observed in the amputated RUx limbs of *Xenopus* does not seem to be due to general tissue injury, but more likely was due to the removal of a major source of cells which normally contributes to cartilage regeneration. Amputated, sham RUx limbs regenerated at the normal rate. In the RUx forelimbs of *Xenopus* in which amputation was postponed 10 days (Experiment III), limbs underwent a similar delay in the regenerative response, even though considerable repair of stump tissue had taken place 10 days following bone removal. In contrast, the amputated RUx limbs of adult newts in Experiments II and III regenerated at control rates. It is unlikely that the regenerative response of newt forelimbs, compared with that of *Xenopus*, is significantly less vulnerable to the effects of general tissue injury.

The present results show that, in comparing limb regeneration of *Xenopus* froglets to that of adult newts, significant differences exist in the response of amputated forelimbs to stump bone removal. These differences add further support to the hypothesis that forelimb regeneration in *Xenopus* is predominantly a tissue response, in contrast to epimorphic regeneration in adult newt limbs.

We wish to express our appreciation to Mrs H. M. G. (Danielle S.) McLaughlin, Research Officer in this laboratory, for her expert assistance in editing the manuscript. The research was supported by grant A-1208 from The Natural Sciences and Engineering Research Council of Canada to R.A.L.
REFERENCES


(Accepted 16 March 1984)