Effects of Amputation of Limbs and Digits of Lacertid Lizards

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ABSTRACT Although the majority of adult lizards are able to regenerate lost portions of tails, only rarely have instances of limb or digit regeneration been observed. The present work describes the histological features of the stumps of limbs and digits of *Lacerta vivipara* and *Lacerta dugesii* at various times after amputation, and compares these features with those which are seen after amputation or autotomy of the tail. The results show that the early stages of healing of the limbs and digits are similar to the early stages of tail regeneration. The epidermis which migrates over the wound becomes thickened, and cells released from dedifferentiating stump tissues accumulate beneath it. In one case, that of a digit, a papilla was seen extending from the wound epidermis into the blastema. During the later stages of healing differences between the events taking place in the limbs and digits, and the tail, become marked. The blastema fails to increase in size, and it appears that most of its cells are converted into a cartilage cap and sleeve investing the cut end of the bone. An investigation has also been made of the regenerative ability of the limbs of embryonic *Lacerta vivipara*. Limbs were amputated *in ovo* at various stages of embryonic life, but no cases of regeneration were observed.

Amputation of digits has often been practiced by herpetologists as a method of recognizing individuals in field and laboratory studies. While there is a general impression that such digits do not grow again, Marcucci ('30) described the appearance of a small outgrowth with a claw at its tip in one specimen of *Lacerta muralis*. It therefore seemed of interest to follow the histological changes which occur after amputation of the digits of lizards and to compare them with those seen after limb amputation, a procedure which is occasionally, though not normally, followed by regeneration (Marcucci, '30; Barber, '44). Attempts to determine the effects of limb amputation during the later stages of embryonic life have also been made.

MATERIALS AND METHODS

Amputations of the limbs of adult lizards were made through the middle of one thigh with a razor blade, under ether anaesthesia, in nine individuals of *Lacerta vivipara* and two of *Lacerta dugesii*, the Madeira wall lizard. Adult digits, usually the second, third or fourth, were clipped off with scissors through the second or third phalanx in five *L. vivipara* and ten *L. dugesii*. Serial sections of the stumps, fixed at various intervals after injury, were stained with Heidenhain's azan. The *Lacerta vivipara* were kept in the laboratory at a temperature of about 27°C. The *L. dugesii* were kept slightly warmer throughout, at 27–30°C.

Limb amputations were performed on 120 embryos of *L. vivipara* after the eggs had been removed from the mother and placed in Panigel culture (Moffat and Bellairs, '64). One hind limb was removed close to the body with a fine pair of scissors, after the shell membrane, chorio-allantois and amnio-allantois had been incised. In some cases incisions were made in the membranes, without the limb of the embryo being amputated. After operation, the embryos were allowed to develop for various periods at 28°C before being fixed in Bouin. Serial sections of the pelvic regions of the embryos were prepared and stained with Heidenhain's azan.

RESULTS

1. *Amputation of limbs in adults*. After the operation the limb usually bleeds for a few minutes and the lizards generally drink freely if they are placed partly in a

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dish of water when they have recovered from the anaesthetic. Removal of one hind limb has little effect on locomotion. The following observations refer to *Lacerta vivipara* except when stated.

In a lizard examined one week after operation the area of the wound was much the same size as immediately after injury, but was covered by a scab. Microscopic examination shows that new epidermis has grown out from the hinge regions of the scales bordering the wound and already extends some distance beyond its margins (fig. 1). This epidermis is greatly thickened, consisting in places of some 12 layers of cells, more than twice the number seen in normal epidermis. These are covered by several irregular layers of keratin, some of which have become detached in preparation. In places the severed muscles are undergoing extensive dedifferentiation and liberated cells are accumulating as a blastema beneath the new epidermis. The cut end of the femur projects slightly from the surface of the wound. A second lizard examined ten days after operation shows an almost healed limb stump. Here the shaft of the femur has been eroded by osteoclasts, and the epidermis has grown into the excavated bone. Presumably the distal portion of the bone would have been sloughed in time, and the advancing edges of the epidermis would have met in the center of the wound.

In a lizard examined after two weeks the area of the lesion is almost covered by new epidermis, but a large space filled with blood cells and covered by the remains of a scab is present in its central part which is still bare. The cut femur ends some distance beneath this, suggesting that a portion of the bone has either been cut off by epidermal penetration and sloughed, or has been absorbed. A dense accumulation of dedifferentiated blastema cells has formed around and distal to the end of the femur, and multinucleated giant cells are present at the surface of the bone. A specimen examined after three weeks is very similar except that a nodule of cartilage is forming near the cut end of the femur.

In one four-week specimen, as in that of ten days previously described, the epidermis is growing into the eroded shaft of the femur (fig. 2), while in another of the same age the wound is entirely covered by thickened epidermis which has not yet developed new scales; it is impossible to tell whether any part of the bone has sloughed. In both specimens large masses of cartilage have formed along the sides of the femur on the proximal aspect of the new epidermis. Blastema cells are less evident than in the previous specimens.

In two further lizards examined after six and eight weeks respectively the stumps of the limbs have become smooth and rounded. The new epidermis is thicker than normal and is not elevated to form scales. No dermal pigment cells are present beneath it; the band of dermal pigment ends abruptly at the margins of the original wound site in such a way as to suggest that no substantial contraction of the tissues has taken place. A massive investment of cartilage encases the cut end of the femur and continues for some distance up the shaft like a sleeve. Connective tissue intervenes between this cartilage and the epidermis.

In one specimen of *Lacerta vivipara* examined after 12 weeks (fig. 3) and in two *L. dugesii* after 13 weeks further differentiation of the limb tissues has taken place. New scales are beginning to form in the terminal epidermis and the cartilage which surrounds the femur has become calcified.

Portions of cartilage tube containing ependyma were taken from the mature tail regenerates of the two *Lacerta dugesii* and implanted into their limb stumps immediately after amputation. These implants did not have the effect of inducing regenerative changes in the limbs similar to those described by Simpson ('64) in the tail of the skink *Lygosoma*. In one *L. dugesii* the piece of cartilage tube remained readily identifiable and still retained a portion of ependymal tube, the structure regenerated in lieu of normal spinal cord (see Hughes and New, '59). In other respects the appearances were very similar to those in the 12-week limb stump of *Lacerta vivipara*. It does not seem possible that any of these specimens would have regenerated their limbs if they had been allowed to survive for longer, since the tissues at the tip of the stump are well differentiated.

2. *Amputation of digits in adults.* These observations were made on specimens of *L. vivipara* and *L. dugesii.*

Three specimens which were examined one to two weeks after injury, show the wound area covered by a thick scab which contains a separate fragment of the amputated phalanx; presumably this had been cut off by epithelial penetration combined with osteoclastic activity, like the ends of some of the femora after limb amputation.

In two specimens examined three weeks after amputation, the scab has disappeared and the wound is covered completely with new epidermis. In the specimen illustrated in figure 4, this epidermis is thicker than that over the rest of the digit, and has several layers of irregular keratin. Osteoclasts are numerous around the cut end of the phalanx which appears to be in the process of absorption. Many cells derived from the various tissues at the site of the wound have accumulated beneath the new epidermis; around the shaft of the bone a sleeve of cartilage has been formed. The appearance of this specimen is in particular very reminiscent of that of an early tail regenerate with blastema and an apical cap of thickened epidermis. The papilla growing proximally from the epidermis into the blastema is remarkably like the structure which has been described by Hughes and New ('59) in early tail regenerates of geckos.

In four specimens examined four to six weeks after injury the epidermis over the wound is still much thicker than normal, but no further growth of regenerative type beyond the rounding off of the cut end of the digit has taken place (fig. 5). Blastema cells are less evident than at three weeks, and the phalanx is now encased by cartilage and connective tissue which extends proximally almost as far as the next interphalangeal joint.

Five specimens examined after 9 and 15 weeks show that the new skin over the wound has become very similar to that of the adjacent sides of the digit. The blastema has now disappeared, and the bone is completely invested with cartilage. Many osteoclasts are present, and it is possible that resorption of the whole remaining part of the phalangeal shaft would eventually have taken place. None of these specimens had regenerated even a small part of the tissue which was amputated.

3. *Amputation of limbs in embryos.* Attempts were made to determine the regenerative capacity of the hind limb of *Lacerta vivipara* by amputating the appendage at various later stages in embryonic life. As reported by Marcucci ('15) in the oviparous *Lacerta muralis,* no regeneration was observed. The epidermis closes over the wound surface within two to three days, and if the specimen is allowed to survive for long enough, scales much like those elsewhere form on the limb tip. As in adult limbs and digits after amputation, cartilage is formed around the femur of embryos operated on during the later stages of embryonic life. Unfortunately, operations on lizard embryos are often followed by the development of constriction bands which may cause auto-amputation of an appendage such as the tail or limb. These may be extremely difficult to detect and the final result may appear similar to that of the experimental injury. Control experiments in which the amnion was incised but the embryo left intact were followed in a few cases by such auto-amputation. The effect of this self injury may be to inhibit regeneration, since amputation is brought about by constriction.

Although it seems likely that the majority of our specimens show the true result of experimental injury, we cannot with certainty state that the limb of the embryo is unable to regenerate.

DISCUSSION

The process of healing after amputation is essentially similar in both the hind limbs and digits of adult lizards. A notable feature is the great thickening of the new epidermis; this was also observed in the embryos and seems to be a characteristic reptilian reaction to injury of the type inflicted. The extensive formation of cartilage which spreads far beyond the immediate neighborhood of the wound is also interesting. It was observed by Barber ('44) after amputation of the forelimbs of *Anolis,* and by Pritchard and Ruzicka ('50) in the callus formed after experimental fractures of the long bones of *Lacerta vivipara.* The latter authors commented on the predominance of cartilage in healing bones of cold-blooded vertebrates (frog and lizard) as compared with conditions in the rat.

The ultimate fate of this cartilage in our lizards was not determined, and it was still present as long as three months after injury. It is possible that it would eventually have become ossified and then remodeled so that the swollen shaft of the bone would have reverted to its original shape.

The early stages of healing of the limbs and digits show many features in common with those of early tail regenerates. In both cases a substantial blastema is accumulated beneath a thickened wound epidermis. Dedifferentiation of muscle in the limb, however, is very extensive whereas in the autotomized tail the integrity of the muscle is preserved by the fibrous autotomy septum so that this tissue contributes little if at all to the blastema. In both limbs and digits there is a tendency for a small portion of the cut end of the femur or phalanx to become cut off by osteoclastic activity and sloughed soon after injury. Similar sloughing of a part of the remaining fragment of the autotomized caudal vertebra has been described in geckos by Werner ('67), but we have found no definite evidence of this in *Lacerta vivipara*.

Despite the fact that the limbs of *Lacerta* occasionally regenerate in part, the stumps of amputated limbs show a less regenerative appearance than those of the digits which we have examined. This appearance is particularly striking in the digit shown in figure 4 which has many of the characters seen in some tail regenerates, including an epidermal papilla. It is of course possible that this digit would have regenerated if it had been left for a longer period, but conditions in other specimens suggest that the early promise of regenerative capacity is not fulfilled. As in the healing limbs the epidermis does not remain thickened and eventually reverts to its normal state. The blastema does not increase in size and most of its cells become converted into cartilage around the bone.

Barber ('44) compared the results of amputation through the forearm in *Anolis* with those of breaking off the tail. She attributed the failure of the limb to regenerate to the fact that its tissues are more loosely packed than those of the tail and so permit much greater shrinkage after injury. We have not observed such shrinkage in our experiments. Moreover, we have found little evidence for the contraction of wounds which is characteristic of healing of not only excised skin wounds (Billingham and Medawar, '55; James, '64), but also of amputated digits (Schotté and Smith, '59) in mammals. In the limb stumps of *Lacerta* which were sectioned 12 or 13 weeks after injury the greater part of the wound is covered by new epidermis rather than by the approximated edges of the original skin. This also appears to be the case in a few reptiles which we have examined which had old injuries sustained before capture.

Our observations suggest that shrinkage is not a critical factor in determining the regenerative capacity of an amputated appendage. The structure of the digits which have very little soft tissue between their rigid scaly covering and the bone of the phalanx would seem to preclude shrinking, and little if any was observed to occur. The balance between healing and regeneration is probably determined by other factors, for example, as Zika and Singer ('65) have demonstrated in *Anolis*, the quantity of nervous tissue present in the region of the wound.

The limbs of a considerable number of lizards were amputated during the later stages of embryonic life, and in no case case was regeneration observed. However the complicating effects of the embryonic membranes make it impossible to be certain that the limb of the embryo is devoid of regenerative capacity.

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Abbreviations

B, blastema; C, cartilage; DP, band of dermal pigment cells; E, epidermis; EP, epidermal papilla; F, femur; MU, muscle; PH, phalanx; UN, small undifferentiated cells.

PLATE 1

EXPLANATION OF FIGURES

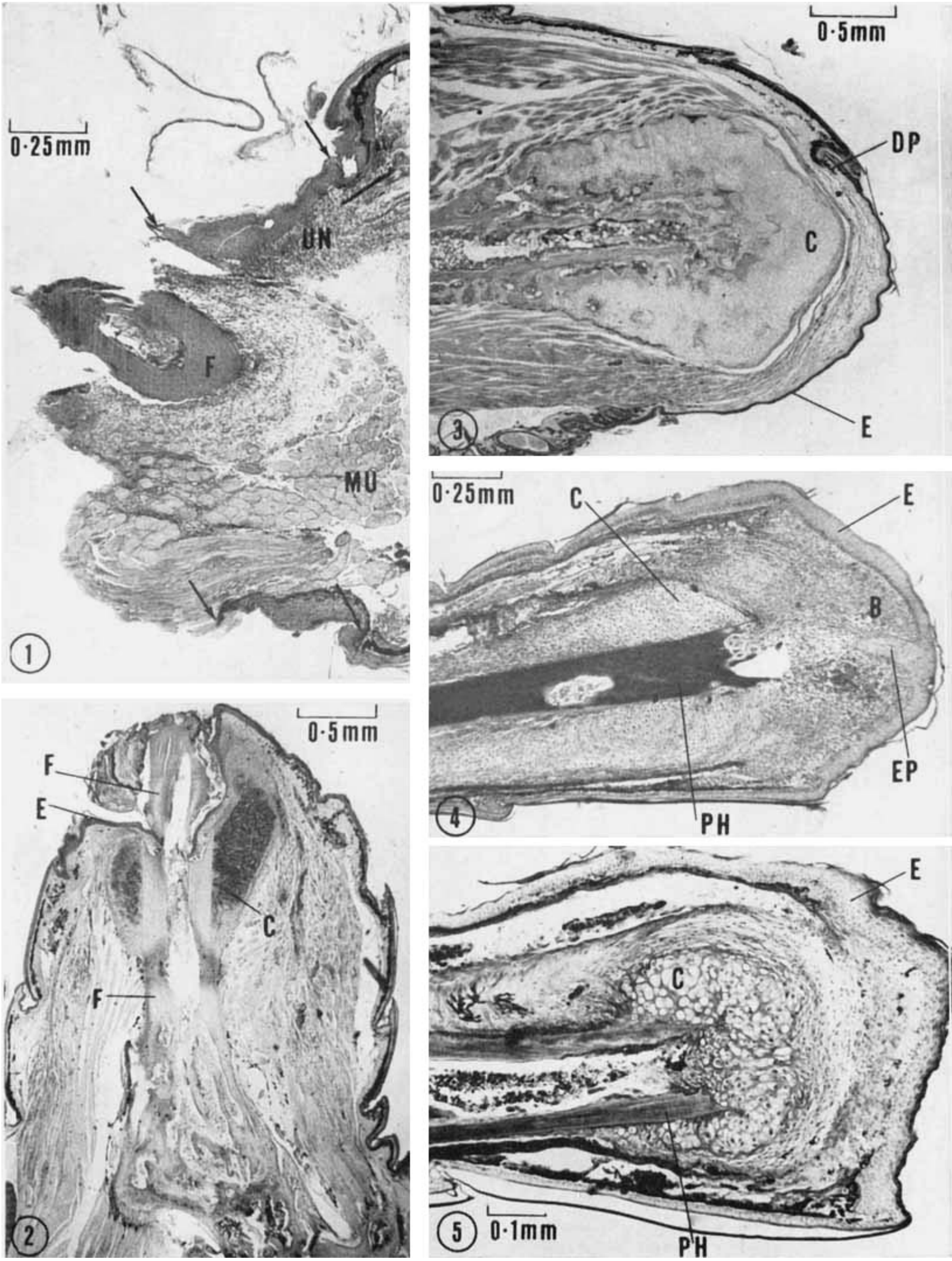
1 Limb stump of adult *Lacerta vivipara* one week after amputation. At each side of the wound, the epidermis between the arrows has migrated over the underlying tissues. The femur and some muscles project from the surface of the wound. Undifferentiated cells are accumulating beneath the epidermis.

2 Almost healed limb stump of adult *L. vivipara* four weeks after amputation, the femur projects beyond the level of the epidermis, its shaft is being eroded and penetrated by migrating epidermis and osteoclasts. Some undifferentiated cells are present beneath the new epidermis, but around the shaft of the femur, they have differentiated into cartilage.

3 Limb stump of *L. vivipara* 12 weeks after amputation. The new epidermis is still distinguishable from that elsewhere on the limb since scales are not fully differentiated and it does not possess an underlying layer of dermal pigment cells. A massive cartilage cap can be seen over the end of the femur.

4 Rounded stump of a digit of *L. dugesii* three weeks after amputation. Beneath the thickened epidermis which covers the tip of the digit a blastema of undifferentiated cells has accumulated. This is penetrated by a downgrowth of the epidermis, the epidermal papilla. Around the shaft of the phalanx, which appears to be undergoing resorption, a cartilage sleeve can be seen.

5 Stump of a digit of *L. vivipara* four weeks after amputation. A thickened layer of epidermis covers the tip of the stump. The cut end of the phalanx is sealed by cartilage, which also extends for some distance proximally along the shaft.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