Interdigital regeneration in the amphibian limb and the rule of distal transformation

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SUMMARY

The applicability to the amphibian limb of the rule of distal transformation, which states that tissue from any level can only become more distal, has been tested during intercalary regeneration following various types of shift-level transplantation. Following the grafting of distal blastemas to proximal levels, such that part of the presumptive pattern is missing, complete limbs nevertheless formed (Series I). That the intercalated tissue arose entirely from the stump was shown by exchanging blastemas between black and white animals. When the proximal stump was irradiated and its contribution eliminated, intercalary deletions were produced (Series II). This was not due to the inability of irradiated and normal tissue to communicate since irritated distal blastemas grafted onto proximal stumps still stimulated intercalary regeneration (Series III). When proximal blastemas were grafted to distal levels intercalary regenerates were obtained in about 20% of the cases (Series IV) and under these circumstances the grafted blastema was the sole source of intercalated tissue. The precise structure of these intercalated elements was impossible to ascertain, but it is suggested that they might be of reversed polarity as found in insects. These results are discussed in relation to similar experiments on the insect limb.

INTRODUCTION

The concept known as the rule of distal transformation emerged from developmental studies of Tubularia in which the pattern of regeneration following various cutting and splicing operations was observed (Rose, 1957). It seemed that under these circumstances regeneration consisted of a sequence in which the tissue to be reorganized transformed to the most distal structure not already developing distal to it until the final form was attained. Although this distal to proximal inhibitory control of differentiation is not thought to be the particular modus operandi of amphibian limb regeneration, the rule of distal transformation has, nevertheless, been applied to this system (Rose, 1962) and to limb regeneration in insects.

On a superficial level the rule seems to be applicable since insect and amphibian limbs only replace distal structures following amputation. The classical experiment which demonstrates that both cut ends of the amphibian limb transform distally was performed by Butler (1955). He inserted the hand of a larval

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salamander into the muscles of the flank and allowed it to heal in. Then the limb was amputated through the humerus to create two stumps both of which could regenerate a new humerus, radius and ulna and hand. Thus the stump protruding from the flank regenerated a mirror-image of itself, a phenomenon which also occurs in the insect limb (Bohn, 1965).

However, contemporary theories of limb regeneration (Maden, 1977; Stocum, 1978) envisage the entire epidermis as the distal boundary of the limb so that whenever the epidermis covers any wound, distal transformation must inevitably take place. Therefore the striking behaviour of regeneration from both cut ends described above does not, in fact, provide a good test of the rule of distal transformation. This is because during regeneration from a cut end the epidermis (distal boundary) covers the wound and there is no proximal tissue present with which to induce proximal transformation in the stump tissues. The possibility of proximal transformation in insects and amphibians can only adequately be tested not during terminal regeneration, but during intercalary regeneration when proximal and distal tissue can be placed adjacent to each other. Thus when a cockroach foreleg amputated at the distal femur level was grafted to the hindleg proximal tibia level, the intercalary regenerate consisted mostly of proximal femoral structures, demonstrating that proximal transformation of the graft had occurred (Bohn, 1976). Indeed, Bohn's results implied that proximal transformation of the graft was the rule rather than the exception during this type of intercalary regeneration. In the converse situation (proximal to distal grafts) French (1976), using differently coloured mutants of Blatella, also revealed the occurrence of proximal transformation in the intercalary regenerate of reversed orientation resulting from a proximal tibia to distal tibia graft.

However, similar experiments on amphibian limbs (Iten & Bryant, 1975; Stocum, 1975; Stocum & Melton, 1977) seem to have revealed a clear difference in behaviour. In the case of distal to proximal grafts intercalation occurs and the resulting limb is complete, but it has been surmised from the use of tritiated-thymidine-labelled grafts that only the stump contributes to such an intercalary regenerate (Stocum, 1975). The distal graft does not provide tissue by proximal transformation as in the insect experiments described above. In similar contrast to insects, the discontinuity created by grafting newt blastemas from proximal to distal levels is not known to be intercalated.

Thus it seems that the amphibian limb does obey the rule of distal transformation whereas the insect limb does not. However, Bohn (1976) considers that in the light of the many features which insect and amphibian limbs share further experiments on the problem of proximal transformation will resolve the apparent differences in this regard. The work reported here was performed to this end. Proximal-to-distal and distal-to-proximal blastemal grafts have been performed using the natural colour difference between black and white axolotls to provide more effective marking of intercalary regenerates. Experiments are
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also reported in which either the graft or stump was irradiated in a direct attempt to induce proximal transformation.

MATERIALS AND METHODS

The experiments were performed on black (D/D or D/d) and white (d/d) larval axolotls, Ambystoma mexicanum. Animals that were used to provide either irradiated stumps or blastemas had their limbs and shoulders exposed to 2000 R of filtered X-rays delivered at 90 kV, 5-5 mA from a Newton Victor Ltd machine.

Distal blastemas were produced by amputation through the distal zeugopodium or autopodium and transplanted (and irradiated in the relevant series) at the late-bud/early-digit stage to the mid-stylopodium level. Proximal blastemas were produced by amputation through the mid-stylopodium and transplanted at similar stages either to the distal zeugopodium or autopodium level.

Blastemas were always transplanted to a fresh amputation site of the opposite coloration. Only the proximo-distal axis was altered, the circumferential axes kept in harmony. To minimize tissue loss due to transplantation trauma, blastemas were severed from donor limbs by cutting just proximal to the stump/blastema boundary such that about 0.5 mm of stump skin and underlying dedifferentiated tissues were included in the graft, but excluding any differentiated stump tissues. Following transplantation the animals were left for 2 h at 4 °C to facilitate healing of the graft.

Regeneration was allowed to proceed for between 2–6 months after which time they were sampled and stained with Victoria blue to examine the skeletal structure.

RESULTS

Series 1: Distal blastemas onto proximal stumps

Ten blastemas from amputation through the distal radius and ulna or carpals were each transplanted to the mid-humerus of an animal of opposite colour. All produced normal limbs with a full complement of skeletal elements (Fig. 1). An examination of the presence of melanophores in these limbs revealed that the intercalary regenerate in all cases was of the same colour as the stump. The distal blastema sometimes produced exactly what it would have done in situ, but in the majority of cases gave rise to somewhat less than would have been expected. This phenomenon is exemplified in Fig. 1, where a carpal level blastema from a black animal was grafted on to the mid-humerus of a white animal. Melanophores are present only around the carpals and in digits 2, 3 and 4, indicating that not only did the intercalary regenerate arise from the white stump but also digit 1 and the two anterior carpals. The same was true of white to black grafts which provides strong evidence to suggest that after a distal to proximal shift the intercalary regenerate arises entirely from the distal transformation of proximal stump tissue.
Fig. 1. The result of grafting a blastema from the carpal level of a black animal onto the mid-humerus level of a white animal. The graft has formed the central and posterior carpals and digits 2, 3 and 4 as indicated by the presence of melanophores in that area. The host stump has formed all the intercalary regenerate (1) as well as two anterior carpals and digit 1, as indicated by the absence of melanophores.

Fig. 2. The regenerate formed from grafting a tarsal-level blastema onto an irradiated mid-femur stump. When the stump contribution to the intercalary regenerate is prevented by irradiation the distal blastema cannot transform proximally to compensate and intercalary deletions result.

**Series II: Distal blastemas onto irradiated proximal stumps**

This series was an attempt to force distal grafted tissue to undergo proximal transformation by eliminating the stump contribution to the intercalary regenerate. Would the distal blastemas then provide the intercalary regenerate?

Twenty experiments of this type were performed, grafting white distal blastemas on to black irradiated proximal stumps. Seven limbs did not develop due to regression of the graft and in the remaining 13 cases all had severe deletions in the proximo-distal axis. In Fig. 2, for instance, the hind limb illustrated has the distal half of the stylopodium, the complete zeugopodium and several tarsals missing.

Thus the distal blastemas produced only what it would have done *in situ* (or slightly less) and could not compensate for the inability of the stump to provide tissue for the intercalary regenerate. Consequently proximal transformation could not be induced in distal tissue.

**Series III: Irradiated distal blastemas onto proximal stumps**

This series was performed to ensure that the failure of proximal transformation in the previous series was not due to an inability of the irradiated stump to communicate level-specific information. Is proximo-distal positional signalling inhibited by X-irradiation of the tissue?

Twenty-nine irradiated white distal blastemas were transplanted onto black
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Fig. 3. An irradiated white distal blastema was grafted to a mid-humerus black stump. Despite being irradiated the graft stimulated an intercalary regenerate (I) which is black, since melanophores are present throughout. The graft itself only produced two fused carpals and one digit as indicated by the absence of melanophores in that area.

Fig. 4. A Y-shaped cartilage (arrow) resulting from grafting a proximal blastema onto a stump at the distal radius and ulna level.

proximal stumps. In all cases a normal intercalary regenerate consisting of the distal stylopodium and complete zeugopodium was produced from the unirradiated stump since these tissues were always black. Nine of these regenerates had a normal number of autopodial elements and the entire limb was composed of black tissue. In these cases it seemed that the irradiated graft was resorbed and the stump produced a normal regenerate.

In the remaining 20 limbs the presence of white tissue could be identified and these all exhibited varying degrees of distal deformities. The most severe cases (8 out of 20) had a reduced number of carpals/tarsals as well as missing digits or fewer phalanges (Fig. 3). The remainder had a normal number of carpals/tarsals but either missing digits or phalanges. That this phenomenon is due to a level-specific interaction between the two tissues and not simply a non-specific amputation response is demonstrated by the presence of a boundary between proximal, host-contributed tissue and distal, irradiated tissue at the appropriate proximo-distal level of pattern. That is, intercalary regeneration was stimulated and then "switched off" at the right time.

Thus the failure of proximal transformation in the previous series was not due to the inhibition of positional signalling in the proximo-distal axis.

Series IV: Proximal blastemas onto distal stumps

Of 36 proximal (mid-stylopodium) blastemas 28 were grafted onto the distal zeugopodium and the remainder onto the autopodium.

In the first group, 15 of the 28 cases behaved in a mosaic fashion and produced
simple tandem repeated limbs. In the remaining 13 cases an interaction occurred at the boundary of the graft and the stump. The most common result was the production of new cartilage in the form of a Y-shaped structure that fused the humerus and radius and ulna together (Fig. 4). This phenomenon might simply be attributed to disorganized growth resulting from fracture healing. Six cases, however, behaved differently and revealed quite extensive intercalary regenerates (Fig. 5). Unfortunately these growths were not of an instantly recognizable form (such as a zeugopodium and stylopodium of reversed polarity). Instead, they were single elements of excessive width which fused with the stylopodium at one end and forked to fuse with the zeugopodium of the stump at the other.

In the second group (stylopodium to autopodium) six of the eight grafts were mosaic and the humerus abutted directly on to the carpals. In the other two cases a significant interaction occurred resulting in similarly unidentifiable elements (Fig. 6).

From the use of grafts of opposite colour to the stump it was clear that in all these cases the intercalated elements came entirely from the graft. This is in striking contrast to intercalation after distal to proximal shifts where only the stump provides such tissue (Series I).

Although some animals were left up to 6 months to regenerate, increasing the time interval after about 2 months did not seem to affect the quality or quantity of intercalated elements.
DISCUSSION

When a blastema is grafted from a distal to a more proximal level a perfect
limb is, nevertheless, produced. It was deduced from the use of tritiated-
thymidine-labelled grafts that in such a situation the missing elements – the
intercalary regenerate – arise from the stump, in accordance with the rule of
distal transformation (Stocum, 1975). The work described here fully supports
this contention since the intercalary regenerate was more reliably identified
by grafting between black and white axolotls and it always corresponded to the
coloration of the stump, not the graft.

It could, however, be argued that the failure of the distal blastema to contri-
bute to the intercalary regenerate was due to a swamping effect of cells from
the stump. In order to overcome this objection the stump contribution was
eliminated by grafting distal blastemas on to irradiated stumps. In these circum-
stances the blastema could not compensate for the deficiency, even to a limited
extent, and severe intercalary deletions resulted.

To ensure that the failure of the blastema to intercalate proximally was not
due to the inability of the irradiated stump to communicate with normal
tissue, irradiated distal blastemas were grafted to normal proximal stumps.
Under these conditions intercalary regeneration commenced and terminated
at the correct level. This demonstrates that positional signalling in the proximo-
distal axis is not inhibited by X-rays and complements the same conclusion
concerning the circumferential axes (Maden, 1979; Holder, Bryant & Tank,
1979). Thus we can validly conclude that in the amphibian limb distal tissue
cannot undergo proximal transformation.

When proximal blastemas were grafted to distal levels the work reported
here showed for the first time that an interaction can take place between graft
and stump since it had previously been thought that no such interaction occurred
(Stocum & Melton, 1977). Unfortunately the precise structure of the intercalated
elements was not obvious and so we can only hypothesize about their true
identity. Three interpretations are apparent: firstly, they could have been
attempts to complete the proximal half of the stylopodium. If so, proximal
transformation would have occurred and we would have expected to have ob-
served the same phenomenon after distal to proximal shifts which was not the
case. Secondly, they could have been attempts to produce another stylopodium
in tandem. Why the limb should do this is obscure, but Carlson (1974) reported
tandem zeugopodial bones after 180° skin reversal experiments. Thirdly, and
perhaps most attractive, is the possibility that the intercalated elements are
stylopodial but of reversed polarity (e.g. Fig. 6). Certainly, in those cases where
their proximal ends were free they resembled the distal epiphysis at the elbow
or knee rather than the proximal epiphysis at the shoulder or hip. In two cases
(e.g. Fig. 5) it seemed that a partially fused zeugopodium had also been inter-
calated. Unfortunately, there are no polarity markers in the amphibian limb
such as the bristles on insect legs which can be employed to refute or confirm this hypothesis, so the true nature of these tissues must remain obscure for the present. However, if the intercalary regenerates are of reversed polarity then the rule of distal transformation remains inviolate since the colour marker demonstrated that such tissues were always of the same genotype as the proximal graft rather than the distal stump. This is in marked contrast to intercalation after distal to proximal shifts where the opposite was shown to be the case (Series I).

It seems therefore from the above analysis that distal transformation is indeed a fundamental rule governing the developmental behaviour of the amphibian limb. As considered in the Introduction, this is not the case in the insect limb, where proximal transformation routinely occurs during intercalary regeneration, and thus we are presumably led to the conclusion that insect and amphibian limbs are governed by different developmental laws. However, by a closer examination of the data it is possible to resolve these differences. The work of Bohn (1976) and French (1976) has only shown that distal tissue may form more proximal tissue of the same segment. Recent data have revealed that tissue from distal segments cannot form more proximal segments during intercalary regeneration either in leg (Strub, 1979) or wing (Haynie & Schubiger, 1979) imaginal discs. As these authors have emphasized, the proximal segment border cannot be crossed by distal tissue. Therefore the apparent differences between amphibian and insect limbs are due to the latter possessing repeated segmental gradients as well as a whole limb gradient and we have erroneously been comparing different gradient systems. It is the whole limb gradient in both organisms upon which the same developmental constraints, in the form of the distal transformation rule, are imposed. Furthermore, it may be that the amphibian limb too has segmental gradients (Stocum, pers. comm.). The latter suggestion requires detailed analysis to confirm or refute it, but it is already clear that one aspect of development in such widely divergent organisms seems to be remarkably constant.

REFERENCES


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