Regulation and limb regeneration: 
the effect of partial irradiation

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SUMMARY

The regulative ability of the regeneration blastema has been studied by the technique of partial irradiation. Longitudinal halves of fore- and hindlimbs were irradiated and the product of regeneration from the unirradiated halves characterized. Such half irradiated limbs could regulate only to a very limited extent. In the upper arms the anterior halves merely produced a few autopodial elements whereas complete limbs regenerated from the posterior halves. This striking regenerative inequality was less marked in the lower arm where the two halves each produced regenerates of two to three digits. In the hindlimb dorsal halves produced more than ventral halves and thus it was concluded that the posterior-dorsal quadrant has some special regenerative property. These results highlight the inadequacy of current models of limb regeneration and are discussed with reference to the arrangement of positional values in the limb and the question of the totipotency of blastemal cells.

INTRODUCTION

The development of the vertebrate limb is currently envisaged as being under the control of discrete organizing regions which establish long range diffusion gradients of morphogens (review Saunders, 1977). The limb field consequently shows extensive powers of regulation as revealed in the work of Harrison earlier this century.

At first sight therefore we might expect the limb to regenerate by re-establishing these organizers and gradients, but this is not, in fact, the direction of contemporary thought on the mechanisms of limb regeneration. Instead, localized interactions between cells whose responses depend upon an imprinted memory of their developmental history is currently favoured, not only because of the difficulties involved in communication by gradients across the relatively enormous distances in the adult regenerating limb, but also because of the highly deterministic behaviour displayed by regeneration blastemas and individual limb tissues. The latter property is in stark contrast to the highly regulative powers of the developing limb mentioned above.

For instance, longitudinal half regenerates grafted into irradiated limb stumps (Stinson, 1964) or onto an indifferent site in the tail (Stocum, 1968)
produce half limbs and skin segments only regenerate highly deficient limbs when grafted onto irradiated stumps (Lheureux, 1975). It is surprising therefore that Bryant & Iten (1974) concluded that the newt regeneration blastema possesses extensive regulative ability up to early digit stages. In this work, following the removal of either the anterior, posterior, dorsal or ventral half of the blastema a complete limb was invariably produced. However, this experimental procedure did not actually test the regulative ability of the blastema since no distinction was made between the regulation of the remaining blastemal tissue and the regeneration of the excised portion from the stump. One method of preventing the replacement of tissue from the stump and avoiding the debilitating effects of isolating blastemas is to employ the technique of partial irradiation. Thus Goss (1957) reported that when either the anterior or posterior half of the newt lower arm is irradiated and then the limb is amputated through the lower arm, an average of two digits regenerates from the unirradiated half. These results add further support to the general thesis that the blastema is a relatively mosaic system. In the work reported here both upper and lower forelimbs and hindlimbs have been partially irradiated. A striking anterior-posterior developmental inequality is revealed and either partial or complete limbs can be regenerated depending on the segment that is irradiated. It is concluded that the blastema has a very limited capacity to regulate and thus blastemal cells are not truly totipotent as far as axial organization is concerned. The results highlight the inadequacy of current models of limb regeneration and are discussed with reference to the arrangement of positional values in the limb and the question of the totipotency of blastemal cells.

MATERIALS AND METHODS

The experiments were performed on axolotls, Ambystoma mexicanum, 80–100 mm long, whose limbs were irradiated with 2000 R of filtered X-rays at 90 kV, 5-5 mA from a Newton and Victor Ltd. X-ray machine. The rest of the body was shielded with 3 mm thick lead plates. A dose of 2000 R is sufficient to inhibit the regeneration of any tissue to which it is applied.

Forelimbs. The left forelimbs had their posterior halves irradiated while the anterior halves were shielded with a strip of lead. The right limbs had their anterior halves irradiated, the posterior halves being similarly shielded. These shields were carefully placed with their edge along the centre of the limbs between the radius and ulna and along the middle of the humerus (Fig. 1).

Hindlimbs. The same shielding patterns were applied to the hindlimbs (Fig. 1). However, the orientation of the thigh is different from that of the upper arm. In anaesthetized animals, when the hindlimb is laid flat as in Fig. 1 the ventral part of the thigh is aligned with the posterior part of the shank. Thus by placing shields along the femur the areas irradiated were ventral thigh in the left and dorsal thigh in the right limbs.
The shielding pattern used in these experiments. The position of the specimen is shown in outline, omitting the gills and the shaded area represents lead plates.

The animals were divided into four groups to study the regeneration after amputating through either the forelimb stylopodium (upper arm), forelimb zeugopodium (forearm), hindlimb stylopodium (thigh) or hindlimb zeugopodium (shank). All animals were kept for 7 weeks after amputation, the resulting regenerates then being fixed in neutral formalin and stained with Victoria Blue to examine the skeletal structure.

RESULTS

The normal skeletal structure of axolotl limbs is: forelimb – humerus (stylopodium), radius and ulna (zeugopodium), eight carpals and four digits (autopodium); hindlimb – femur (stylopodium), tibia and fibula (zeugopodium), nine tarsals and five digits (autopodium).

**Series I: forelimb stylopodium.** The results for this group of eight individuals were strikingly consistent (Table 1). All posterior halves (anterior half irradiated) regenerated perfect limbs with a new distal humerus, radius and ulna, seven to nine carpals and four digits (Fig. 2b). In contrast the anterior halves (posterior half irradiated) produced severely deficient regenerates with an average of only 2-4 digits (Table 1) and 5 carpals. These autopodial elements were, as far as
Table 1  Numbers of digits regenerated by individual animals from two levels of the fore- and hindlimb after irradiation of longitudinal halves of the limb

Average numbers regenerated by each group are recorded at the bottom of the columns. Cases marked with * are proximo-distally complete limbs (see Fig. 2b); all the remaining limbs from stylopodial amputation were proximo-distally deleted (see Fig. 2a). The normal forelimb has four digits, the hindlimb five digits (see text).

| Forelimb |  |       |  |       |  |       |
|----------|  |       |  |       |  |       |
| Stylopodium | Zeugopodium | Stylopodium | Zeugopodium |  |       |
| Ant. half (left) | Post. half (right) | Ant. half (left) | Post. half (right) | Dorsal half (right) | Ventral half (right) | Ant. half (left) | Post. half (right) |
| 2 | 4* | 2 | 3 | 5* | 0 | 4 | 4 |
| 2 | 4* | 3 | 3 | 6* | 0 | 2 | 3 |
| 3 | 2 | 3 | 4 | 2 | 5 | 2 | 5 |
| 4 | 2 | 2 | 2 | 3 | 5 | 2 | 3 |
| 2 | 4* | 6* | 2 | 5* | 2 | 2 | 4 |
| 3 | 4* | 3* | 2 | 2 | 2 | 2 | 2 |
| 1 | 4* | 1 | 4 | 4 | 2-5 | 2-4 | 3-8 |
| Averages 2-4 | 4 | 2-4 | 2-8 | 4 | 2-5 | 2-4 | 3-8 |

could be judged, anterior ones. The only other structure present was a knob of cartilage presumed to be the distal head of the radius and in no case did anything resembling a humerus, ulna or proximal part of the radius regenerate (Fig. 2a).

An unequal regenerative potential between the anterior and posterior halves of the forelimb stylopodium is therefore very clear. The posterior half contains all the information for regenerating a complete arm, while the anterior half produces only distal, anterior structures. In the latter case it is peculiar that after a proximal amputation only distal structures regenerate, resulting in intercalary deletions; the significance of this observation is considered in the Discussion.

Since the two halves together produced more than the complement of cartilages in one normal limb (5 carpals and 2-4 digits more), some regulation had occurred. But it was limited for if both halves had been able to regulate fully two complete limbs would have been produced.

Series II: forelimb zeugopodium. The regenerative potential of zeugopodial halves was far more equal than in the stylopodium with the posterior half producing an average of 2-8 digits and anterior half, 2-4 digits (Table 1). Both halves seemed capable of a limited amount of regulation with perhaps a bias towards the former. That is, the posterior half could produce a digit 2 (and in one case a digit 1) in addition to the two posterior digits and the anterior half could produce a digit 3 (but not a digit 4) in addition to the two anterior digits (Fig. 3a, b). Only one complete limb regenerated, from a posterior half.
Fig. 2. The left (a) and right (b) forelimbs of one animal from Series I. (a) The posterior half of the stylopodium was irradiated and the limb amputated at the mid-humerus level. The arrow marks the amputation plane where the humerus was severed (it is not stained due to ossification). The regenerate has two anterior digits, five carpals and the other cartilaginous mass is possibly the distal end of the radius. This latter element abuts directly onto the end of the humerus. This figure shows the typical proximo-distal deletions that were characteristic of all the limbs so treated in this series. (b) The anterior half of the stylopodium was irradiated and the limb amputated at the mid-humerus level. As in all cases in this series a complete four-digit limb has regenerated.

Fig. 3. The left (a) and right (b) forelimbs of one animal from Series II. (a) The posterior half of the zeugopodium was irradiated and the limb amputated through the mid-radius and ulna. Two digits and five carpals regenerated in this case. It is not clear whether the element labelled e is the intermedium carpal or the regenerated end of the ulna. The latter ought not to have occurred since the ulna was irradiated. This limb reveals the difficulties in precise identification of digits - the carpal labelled bc is clearly basal carpal 1 + 2, yet the second digit has three phalanges and would normally be identified as digit 3 (see also b) (b) The anterior half of the zeugopodium was irradiated and the limb amputated through the mid-radius and ulna. Here three digits and seven carpals regenerated. As in (a) it seems that the distal end of the irradiated zeugopodial bone regenerated. Again problems with identification of digits are apparent.
Fig. 4. The left (a) and right (b) hindlimbs of one animal from Series III. (a) The ventral half of the stylopodium was irradiated and the limb amputated through the mid-femur. In this case a complete limb regenerated. Four out of eight limbs in this series were like this. (b) The dorsal half of the stylopodium was irradiated and the limb amputated through the mid-femur. In this case only two digits and two tarsals regenerated, abutting directly onto the cut end of the femur (marked by the arrow). Although the actual number of digits regenerated in this series was variable all limbs had these characteristic proximo-distal deletions.

Fig. 5. The left (a) and right (b) hindlimbs of one animal from Series IV. (a) The posterior half of the zeugopodium was irradiated and the limb amputated through the mid-tibia and fibula. Here two anterior digits, five tarsals, the distal end of the tibia and possibly the end of the fibula also regenerated. (b) The anterior half of the zeugopodium was irradiated and the limb amputated through the mid-tibia and fibula. Here digits 3, 4 and 5, three tarsals and the distal end of the fibula have regenerated.
Series III: hindlimb stylopodium. As explained in the Materials and Methods, due to the rotation of the hindlimb relative to the body axis, the dorsal and ventral halves were irradiated in this series.

The results were less consistent than in the forelimb stylopodium. From the dorsal halves (ventral half irradiated) four of the eight animals regenerated complete limbs (Fig. 4a) with a femur, tibia and fibula, nine or ten tarsals and five (or six) digits (Table 1). The remaining four regenerates had a reduced number of digits and tarsals (anterior ones), the distal end of the tibia and no femur – that is, proximo-distal deletions as observed in Series I. The average number of digits from the group as a whole was four. From the ventral halves (dorsal halves irradiated) no complete limbs were produced. Instead, all had proximo-distal deletions with only the distal end of the fibula and a variable number of posterior tarsals and digits regenerating (Fig. 4b). The average number of digits produced by this group was 2.5 (Table 1).

Thus, an unequal regenerative potential between the dorsal and ventral halves was also evident. The sum of the two halves was more than one complete limb (but less than two complete limbs), implying a limited amount of regulation. By combining this result with Series I we can locate the area of the stylopodium which has the greatest regenerative potential in the posterior-dorsal quadrant, the other segments only being competent to produce anterior, distal structures.

Series IV: hindlimb zeugopodium (Fig. 5a, b). Similar results were obtained in this series to those on the forelimb (Series II) although here the posterior bias was more apparent: posterior halves produced an average of 3.8 digits, anterior 2.4 digits (Table 1). It is to be remembered that the axolotl forelimb has four digits (each zeugopodial half could therefore yield an equal number of digits) whereas the hindlimb has five digits and we might expect one half to give three and the other two. Thus in terms of the number of digits the posterior half of the hindlimb zeugopodium has a greater regenerative potential than the anterior half. Only one complete limb regenerated in this group and that was, as in Series III, from a posterior half.

DISCUSSION

Three conclusions emerge from this study of the regenerative potential of partially irradiated limbs.

Firstly there is a distinct inequality of regenerative capacity between anterior and posterior forelimb stylopodial halves. The anterior half (posterior irradiated) is only capable of regenerating distal anterior structures (with proximo-distal deletions) whereas the posterior half (anterior irradiated) has the potential to form a complete limb. In the zeugopodium, however, this inequality was far less apparent since forearm halves each regenerated approximately half limbs. It is interesting to note that the posterior half of the forelimb zeugopodium produced an average of 2.8 digits (from a four-digit limb) and the same segment in the hindlimb an average of 3.8 digits (from a five-digit limb).
Thus it seems that the form of the urodele forelimb in only having four digits is due to the loss of a posterior digit rather than an anterior one from the basic pentadactyl limb.

These same differences in the behaviour of anterior and posterior segments and the stylopodium and zeugopodium emerged from a study of the regeneration of double half limbs (Stocum, 1978). Double anterior stylopodia only produced a cap of cartilage whereas double posterior stylopodia regenerated mirror image limbs with up to 8 digits. The inequality was present, but it was not as evident in double anterior and posterior zeugopodia.

Another experimental situation in which an anterior-posterior inequality has been revealed is during supernumery limb induction when, as here, one part of the system was irradiated (Maden, 1979). After rotating blastemas 180° onto irradiated stumps supernumerary limbs were produced, but they were never good four-digit limbs. The most deficient ones (one digit) were identified as anterior structures and the most complete ones (three digits) were composed of posterior elements.

The second conclusion to emerge from the above results is that the region of the stylopodium which has the greatest regenerative potential is located in the posterior-dorsal quadrant. This was deduced by combining the forelimb results (anterior v posterior stylopodial halves) with those from the hindlimb (dorsal v. ventral stylopodial halves). In his work on the regeneration of double half limbs Tank (1978) observed that most skeletal elements were produced by double posterior and double dorsal limbs and he too concluded that the posterior-dorsal quadrant has some special significance.

Exactly what is the unique property of this region is open to various interpretations. The simplest is to suggest that it is a growth centre: the greater proliferative potential of the posterior-dorsal quadrant could be responsible for the formation of a more complete pattern. Perhaps this is a manifestation of a developmental memory of the increased proliferation of the posterior part of the limb bud under the action of the zone of polarizing activity.

However, there are many indications that the organization of pattern is independent of growth and a more contemporary approach to the interpretation of this special posterior-dorsal quadrant is to attribute its properties to a clustering of positional values in that region. In the zeugopodium the positional values must be distributed more evenly though, implying a difference in organization along the proximo-distal axis. In the newt limb the arrangement of positional values deduced from the production of supernumerary limbs is exactly opposite to that in the axolotl, with a concentration of values in the anterior-ventral quadrant (Bryant & Iten, 1976). One would expect the properties of the limbs of such closely related animals to be the same and to eliminate this discrepancy the appearance of supernumeraries in the posterior-dorsal and anterior-ventral quadrants which was observed in the newt can equally well be explained by a clustering of positional values in the posterior-dorsal quadrant,
Regulation and limb regeneration

as in the axolotl limb. However, certain double half limb experiments performed on the newt (Bryant & Baca, 1978) cannot then be accounted for by this revised scheme.

It is pertinent here to consider the relevance of these results to the clockface model of regeneration (French, Bryant & Bryant, 1976). The development of partial limbs from zeugopodial halves clearly contravenes the complete circle rule of distal transformation as do many of the other experimental results discussed above. Furthermore, the production of proximo-distal deletions in Series I and II (autopodial elements on stylopodial stumps) has important implications in general for models of limb development and regeneration. It implies that the limb field can no longer be considered in terms of separate proximo-distal and circumferential axes(is), each having their own organizational properties. The whole limb field is thus a unit which cannot be meaningfully divided into discrete parts.

The third conclusion to emerge from these results is that the blastema has a very limited capacity to regulate, in agreement with other work considered in the Introduction. Clearly then, the dedifferentiated blastema is not truly totipotent nor capable of extensive reprogramming (Iten & Bryant, 1975). Certainly blastemal cells seem to be histologically totipotent in that cells from one tissue type can be transformed into another state of histological differentiation (Wallace & Wallace, 1973; Namenwirth, 1974; Maden & Wallace, 1975; Dunis & Namenwirth, 1977), but they do not seem to be axially totipotent. That is, anterior cells, for instance, cannot transform into posterior cells – they appear to remember their antero-posterior positions – a conclusion also apparent from the work of Lheureux (1975, 1977). It is thus becoming clear that the morphogenesis of the regenerating limb either in its normal form or a highly abnormal one (Slack & Savage, 1978) is a manifestation of a memorized signal which operates during limb development. Consequently the behaviour of adult limbs such as that reported here not only furthers our understanding of limb regeneration, but also provides insights into the phenomenology of limb development which might not otherwise be attainable.

REFERENCES


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