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Regeneration of Symmetrical Forelimbs in the Axolotl, *Ambystoma mexicanum*

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Surgically constructed symmetrical double-anterior and double-posterior upper forelimbs of the axolotl were amputated immediately after surgery. Double-anterior limbs either failed to regenerate or formed single digits or spikes. Double-posterior limbs formed symmetrical double-posterior regenerates in 60% of the cases, thus extending the previous finding that the amount of distal transformation in surgically constructed double-half limbs is inversely proportional to the time between grafting and amputation (Tank and Holder, 1978). When these symmetrical regenerates were amputated through the forearm region, all but one formed a symmetrical secondary regenerate. The majority of the secondary regenerates had a larger number of digits than did their corresponding primary regenerates. Reamputation of the secondary regenerates resulted in symmetrical tertiary regenerates, and the majority of these also had a larger number of digits than did their corresponding primary regenerates. The results are compared to those of Slack and Savage (1978a, b) on embryonically derived double-posterior limbs and they are discussed in terms of a formal model for distal transformation (Bryant and Baca, 1978).

INTRODUCTION

Several recent studies have shown that regenerates produced by upper forelimbs of both newts and axolotls consisting of surgically constructed symmetrical halves always regenerate reduced skeletal patterns. If the symmetrical tissues are allowed to heal for 30 days or longer prior to amputation, little or no regeneration occurs (Bryant, 1976; Bryant and Baca, 1978; Tank, 1978). However, when amputation is carried out nearer to the time of grafting, the extent of distal transformation increases markedly (Tank and Holder, 1978). In the initial experiments on axolotls, healing of the grafts was allowed to occur, and the shortest healing period examined was 5 days. Several double-posterior upper arms in this category regenerated symmetrical double-posterior limbs. The original purpose of this study was to examine the regenerative ability of double-posterior and double-anterior upper arms which were not permitted to heal prior to amputation in the hope that a higher incidence of more complete symmetrical structures would result.

The production of symmetrical regenerates has enabled us to compare their regenerative ability with that of symmetrical double-posterior limbs produced following surgical manipulations in the embryo (Slack and Savage, 1978a, b). One of the main points on which these experiments focus is the applicability of the complete circle rule for distal transformation to amphibians. This is one of two rules explicit in the original formulation of the polar coordinate model of French et al. (1976). Although we cannot be sure of which circum-
ferential values are present in the embryonically produced symmetrical limbs, several other lines of evidence suggest that this rule as originally stated may be insufficient to explain some types of cellular behavior in regenerating amphibian limbs and in imaginal discs of Drosophila (Schubiger and Schubiger, 1978). A recent interpretation of the relationship between circumferential positional values and the generation of more distal levels of the limb pattern (Bryant and Baca, 1978; Bryant et al., submitted) may help to resolve these apparent discrepancies. The results presented here will be shown to be consistent with this formal model for distal transformation.

MATERIALS AND METHODS

The animals used in this study were male and female larval axolotls (Ambystoma mexicanum) that were kindly provided from the axolotl colony of Indiana University. Animals were 3 to 4 months of age at the time of surgery and ranged in length from 95 to 135 mm. Experimental animals were fed daily on beef liver or Tubifex. They were maintained in 50% Holtfreter solution in individual plastic containers at room temperature (18 ± 2°C) for the duration of the study.

All double-half symmetrical forelimbs were prepared by the technique already described (Tank, 1978; Tank and Holder, 1978). Briefly, animals were anesthetized in MS222 (ethyl m-aminobenzoate methanesulfonic acid, Aldrich) at a concentration of 1 g/liter in 50% Holtfreter solution. Anterior halves of left upper forelimbs were exchanged with posterior halves of right upper forelimbs with the dorsal-ventral and proximal-distal axes of the grafts and stumps in harmony. Grafts were sutured in place, and the limbs were amputated immediately through the midpoint of the humerus. After bone and soft tissues were trimmed to produce a flat surface, at least 2 mm of grafted tissue remained proximal to the level of amputation (Fig. 1a). Animals were returned to individual containers and placed in a refrigerator (~5°C) for 2 days to reduce their mobility while the grafts healed. They were maintained at room temperature for the duration of the study. The limbs were carefully observed every other day for the first week to check

Fig. 1. Diagrammatic representation of the experimental procedures. (a) Double-posterior (right) and double-anterior (left) upper forelimbs were surgically constructed and amputated immediately through the grafted upper arm (1). Regeneration was allowed to continue for 50 days. (b) Symmetrical double-posterior regenerates were amputated a second time just distal to the elbow (2) and allowed to regenerate for 50 days. A third amputation was then performed at the same level of the forearm (3), and regeneration was allowed to continue for an additional 50 days.
that the graft was in place and once a week thereafter to record the condition of the graft and regenerate.

Limbs were allowed to regenerate for 50 days after the first amputation. Asymmetrical regenerates were removed at the shoulder and fixed in Bouin’s fluid. All animals with symmetrical regenerates with digits were reanesthetized, and their limbs were amputated a second time just distal to the elbow, i.e., through the symmetrical regenerate (Fig. 1b). The amputated structure was fixed in Bouin’s fluid, and the stump was trimmed to create a flat amputation surface distal to the elbow. These animals were then returned to their containers and allowed to regenerate for an additional 50 days. The symmetrical regenerates resulting from the second amputation were amputated for a third time at the same proximal forearm level (Fig. 1b). These limbs were in turn removed and fixed after 50 days, and all limbs were prepared as whole mounts for skeletal analysis by staining with Victoria blue B (Bryant and Iten, 1974).

Sham-operated controls of both right and left limbs were prepared by removing either posterior or anterior tissue and replacing it in situ. Amputation was performed immediately after grafting, and in each case a normal four-digit primary regenerate was formed. After the second amputation all formed basically normal regenerates, but in three cases, the regenerates had only three digits. A similar result was seen after the third amputation (six cases), where three limbs regenerated only three digits and three regenerate four digits (Table 1). A total of seven limbs was prepared as sham-operated double-anterior controls by removing the anterior half of the upper arm and replacing it in situ. Amputation was performed immediately after grafting, and in five cases normal regenerates were formed. One limb regenerated an extra digit and one limb was lacking a digit.

Experimental Limbs

The results of the first, second, and third amputations of double-posterior upper arms are given in Table 2. No second or third amputations of double-anterior limbs were carried out because the amount of tissue regenerated by these limbs was very small, and it was not possible to determine if the primary regenerates were symmetrical or not (Table 2).

Double-Posterior Limbs

Primary regenerates. In this group of 35 operated limbs, 21 of the regenerates were symmetrical double-posterior limbs which had from three to six digits (Figs. 2, 4, 5, and 6). Of these 21 regenerates, the majority (10) had five digits (Fig. 5). The three-digit symmetrical regenerates were comprised of digits IV, III, and IV (Fig. 2); the four-digit regenerates, IV, III, III, and IV

| Table 1
| Sham-Operated Control Regenerates |
|-----------------|------------------|--------|----------|
| 1st, 2nd, or 3rd regenerate | Limb type         | Total cases | Normal limbs | Asymmetrical 3-digit limbs |
| 1st Double-anterior | 7 | 5 | 2* |
| 1st Double-posterior | 8 | 8 | 0 |
| 2nd Double-posterior | 6 | 3 | 3 |
| 3rd Double-posterior | 6 | 3 | 3 |

* One of these regenerates was an asymmetrical 5-digit limb.
TABLE 2

| 1st, 2nd, 3rd | Limb type     | Total cases | Symmetrical limbs (# digits) | No regeneration | Spike$^a$ | One digit$^b$ | Asymmetrical two digit |
|---------------|---------------|-------------|-------------------------------|-----------------|-----------|-------------|-----------------------|
| 1st           | Double-anterior | 12          | 0 0 0 0 0 0 0                 | 4 5             | 2         | 1           |
| 1st           | Double-posterior| 35          | 5 4 10 2 0 0                 | 3 7             | 0         | 4           |
| 2nd           | Double-posterior| 19$^c$      | 0 0 7 6 5 0                  | 0 0             | 0         | 1           |
| 3rd           | Double-posterior| 13$^b$      | 0 3 2 2 6 0                  | 0 0             | 0         | 0           |

$^a$ Only regenerates with two or more digits could be clearly classified as symmetrical or asymmetrical.

$^b$ Only limbs developing from amputation of a symmetrical regenerate are included.

(Fig. 4); the five-digit regenerates, IV, III, II, III, and IV (Fig. 5) (6 cases) or IV, III, III, III, and IV (Fig. 9) (4 cases); and the six-digit regenerates, IV, III, II, II, III, and IV (Fig. 6). Of the remaining 14 regenerates, 4 produced asymmetrical two-digit outgrowths that were comprised of digits III and IV (Fig. 7), 7 produced short spikes of unknown symmetry, and 3 failed to regenerate any distal structures (Table 2).

Secondary regenerates. Nineteen of the symmetrical primary regenerates, one spike regenerate, and one asymmetrical, two-digit regenerate were amputated through the proximal forearm region just distal to the elbow (Fig. 1b). All but one of the secondary regenerates from symmetrical primary regenerates were double-posterior structures, and of these, 13 (68%) increased their number of digits compared to their primary regenerates. Digits were always added to the midline of the symmetrical limb pattern. Three of these limbs increased their digital count by one, seven increased the count by two, two increased the count by three, and one remarkable limb increased the count by four digits, from a three-digit primary regenerate (Fig. 2) to a seven-digit secondary regenerate (Fig. 3). Of the remaining six limbs from symmetrical primary regenerates, five were again symmetrical, and of these, three retained the same number of digits and two decreased the number by one digit. One symmetrical primary regenerate formed an asymmetrical two-digit secondary regenerate. The spike primary regenerate which was amputated gave rise to a four-digit symmetrical secondary regenerate; the two-digit asymmetrical primary regenerate formed a relatively normal asymmetrical four-digit hand.

Tertiary regenerates. Thirteen symmetrical secondary regenerates were amputated a third time through the proximal forearm region just distal to the elbow (Fig. 1b). All 13 formed double-posterior tertiary regenerates (Table 2). Of the 13, 7 limbs regenerated the same number of digits as their secondary regenerates, 3 regenerated an additional digit, 2 regenerated one less digit than the secondary regenerate, and 1 regenerated two less digits. Loss or addition of digits always took place along the line of symmetry of the regenerates. When compared to the primary symmetrical regenerates, the tertiary regenerates increased their number of digits in eight cases, had the same number in one case, and decreased the number of digits in four cases. One asymmetrical four-digit secondary regenerate was also amputated, and this regenerated an asymmetrical three-digit hand. The limb which formed a spike as a primary regenerate and a four-digit symmetrical secondary regenerate produced an asymmetrical half limb following the third amputation.
FIGS. 1–7.
**Double-Anterior Limbs**

In the 12 double-anterior limbs which were amputated immediately after grafting, the extent of distal transformation was markedly less than that seen in the double-posterior group (Table 2). Four limbs failed to regenerate any visible outgrowth, five produced short spikes of unknown symmetry and variable complexity, two limbs had a single digit (Fig. 8), and one limb had a single terminally branched digit.

**DISCUSSION**

Surgically constructed double-posterior upper arms amputated immediately after grafting produce symmetrical double-posterior regenerates in a high percentage of cases. These results expand on our initial observation that surgically constructed double-posterior upper arms produce symmetrical regenerates when the graft and host tissues are allowed to heal for 5 or 10 days (Tank and Holder, 1978). Furthermore, after immediate amputation 60% (21/35) of the double-posterior limbs produced symmetrical regenerates, compared to 40% (6/15) when the healing time of the graft was 5 days and 23% (3/13) when the healing time was 10 days (Tank and Holder, 1978). The present results are consistent with our previous finding that double-half upper arms display a gradual inability to distally transform as the healing time of graft and...
host tissues increases prior to amputation. A similar effect of graft healing time on the extent of distal transformation has also been found in double-half upper hindlimbs by Stocum (1978).

A second major finding of this study is that when a symmetrical regenerate is amputated, a symmetrical limb usually regenerates. This result is similar to that of Slack and Savage (1978a, b), who showed that symmetrical limbs produced in the embryo regenerate symmetrical limbs upon amputation. To facilitate comparisons between these two studies, it is necessary to point out that the first amputation in the study of Slack and Savage is formally equivalent to our second amputation. This is because their symmetrical limbs were produced following operations on embryos rather than following amputation of surgically constructed upper arms (i.e., as a result of our first amputation). Despite the basic similarity between the results of the two studies, there is one important difference which concerns the fidelity of the patterns reproduced during regeneration of the symmetrical limbs. In our study, 68% (13/19) of the regenerates produced after secondary amputations showed an increase in the complexity of the limb pattern. This is in contrast to the results of Slack and Savage, where 77% (20/26) of the regenerates produced following the equivalent amputation (their first amputation) decreased in complexity. However, reamputations of these symmetrical regenerates in both studies (third amputations in our study, second and third amputations in their study) resulted in a more equal distribution of limbs that either increased or decreased their number of skeletal elements when compared to the preceding regenerate. However, when the later regenerates are compared to the original symmetrical limb, in our study 9/13 (69%) either increased the number of digits or had the same number, whereas in Slack and Savage's (1978a, b) study, 24/33 (73%; combining their second and third regenerates) formed fewer elements. These similarities and differences are crucial to the development of general models concerned with limb development and regeneration.

One model which has been used to explain the regeneration of symmetrical limbs from symmetrical stumps involves the production of a diffusible morphogen which would have a U-shaped distribution in double-posterior limbs (Slack and Savage, 1978b). In terms of this model, the increase or decrease of skeletal elements observed after the amputation of symmetrical stumps is seen as a consequence of the anterior-posterior width of the amputation plane. To us it seems that this idea would predict random increases or decreases within a given experiment in the number of limbs displaying increases or decreases in skeletal patterns, depending, for example, on slight variations in amputation techniques. However, features of both our results and their results demonstrate distinctly nonrandom behavior. Further evidence against a hypothesis which involves a gradient of a diffusible morphogen comes from experiments on the production of supernumerary limbs following blastemal transplantation when one of the partners in the combination has been X-irradiated (Holder, Bryant and Tank, 1979). In addition, it is clear from this paper and others (Tank and Holder, 1978; Bryant and Baca, 1978) that double-anterior as well as double-posterior upper arms are capable of some degree of distal transformation. We propose an alternate explanation for the results obtained.

It has been proposed previously that distal transformation occurs by a process of intercalation between cells at the same proximal-distal level of the limb. Interaction between cells of different circumferential positional values across short arcs leads to the generation of the next most distal complete circle of positional values. Thus, intercalation is seen as an integral part of the normal process of distal transformation (Bryant, 1978; Bryant and Baca, 1978). This model accounts for the results
obtained when surgically constructed symmetrical upper arms are amputated 30 days after grafting. In these cases, limbs either fail to regenerate or form short, distally incomplete spikes (Bryant, 1976; Bryant and Baca, 1978; Tank, 1978). In such limbs, intercalation across short arcs of the circle will result in progressive loss of midline positional values. Eventually, a point will be reached at which no further positional values will be generated and distal transformation will cease. The extent to which midline positional values will be lost will depend upon the degree of cellular interaction across the plane of symmetry (Fig. 10).

In contrast to the results obtained when symmetrical limbs are amputated 30 days after grafting, limbs amputated immedi-

Fig. 10. Diagram to illustrate proposed method of distal transformation from a symmetrical double-posterior limb in which the graft and host tissues are well healed. (a) Circumferential positional values at the level of amputation (A) interact with each other across short arcs of the circumference to produce new positional values of the next most distal limb level (B). (b) Interactions across the line of symmetry do not cause intercalation, hence, some positional values which are present at level A are missing in the newly generated level B. (c) Cells of the level B circumference now interact across short arcs to form level C, with a much reduced number of positional values. (d) Eventually, a point is reached, prior to complete restoration of all distal limb levels, where intercalation cannot proceed because no positional disparities remain. Intercalation shown by dashed lines; newly intercalated positional values are in boldface.
ately after grafting (or at 5 and 10 days) often produce symmetrical regenerates that have formed the most distal tip of the pattern. These symmetrical regenerates are also wider (i.e., contain more midline structures) than regenerates formed after amputation of double-half limbs at later healing times. We propose that the two sides of the double-half limbs behave independently of one another; that is, they do not interact across the midline of the circle unless they have healed for several days prior to amputation. This lack of interaction between cells across the midline may be seen as a reflection of lack of integration between graft and host at short healing times. As a result of this lack of interaction across the midline, the extent to which midline values are lost will be considerably reduced (Fig. 11). In short, when lack of interaction across the midline occurs, each half is behaving autonomously. This idea is

![Diagram](image)

**Fig. 11.** Diagram to illustrate the proposed method of distal transformation from a symmetrical double-posterior limb in which graft and host tissues are not well healed together and fail to interact across the line of symmetry. The process is similar to that explained in Fig. 10. In this diagram, interactions between different circumferential positional values will take place only within each half of the limb and not between halves of the limb. As can be seen from the diagram, some limited loss of positional values around the midline is expected with each round of intercalation, but this loss is minimal when compared to the loss of values predicted as a consequence of interactions across the midline as shown in Fig. 10. Distal transformation will proceed until distal parts of the pattern have been formed.
supported by the fact that we have obtained a number of half-limb regenerates from double-half stumps (Table 3, Fig. 7). It must be noted, however, that in cases when interaction across the midline is not possible, limited loss of values at the midline should still occur as shown in Fig. 11. The half-posterior regenerates which occasionally result from amputation through double-posterior stumps always form digits IV and III. Assuming that limited midline loss occurs in the distally transforming half of the limb, this implies that the posterior half of the upper arm is actually capable of producing more than two digits (i.e., digits IV and III, and part or all of digit II). As a consequence, the anterior half would be capable of forming a maximum of one to one and a half digits. As we have shown, half limbs resulting from amputation of double-anterior upper arms (Table 3; see Tank and Holder, 1978) produce regenerates with one digit in almost every case. We believe that these half-limb regenerates clearly demonstrate the autonomous behavior of one half of the double-half stump. It is interesting to note that these half-limb regenerates always form on the host side of the double-half limb. It seems reasonable to assume that in these cases the grafted tissue did not regenerate due to lack of adequate nerve or blood supplies. However, the physical presence of the grafted tissue prevents the formation of a complete circle of positional values on the lateral wound surface of the host half of the limb. Hence, complete regenerates from half limb stumps, as described elsewhere (Bryant and Baca, 1978), are not formed.

The majority of the amputated symmetrical posterior upper arms produced primary regenerates with five digits. Assuming, as discussed above, that the two half limbs behave autonomously after immediate amputation, it follows that each posterior half has sufficient numbers of positional values to produce the two to three most posterior digits. Hence, an anterior half limb only has sufficient positional values to produce only one or two digits. This idea is consistent with other evidence which indicates a clustering of positional values on the posterior side and fewer positional values on the anterior side of the upper arm of the axolotl (Tank, unpublished). This unequal spacing of positional values can also account for the difference in the amount of distal transformation shown by double-anterior and double-posterior upper arms after initial amputation (Table 2; Tank and Holder, 1978).

As discussed above, we argue that the posterior half of the upper arm will normally produce digits IV and III and part or all of digit II. Digit I will be formed by positional values found on the anterior edge of the limb. It is interesting to note that when double posterior symmetrical regenerates are reamputated just below the elbow, several limbs formed regenerates that consisted of digits IV, III, II, I, II, III, and IV. Such seven-digit limbs also resulted in some cases after the third amputation. The presence of digit I and possibly part of digit II.

TABLE 3

| Limb type       | Total cases | Graft healing time in days | No. of digits | No. with identifiable digits | No. with unidentifiable digits |
|-----------------|-------------|----------------------------|---------------|-----------------------------|------------------------------|
|                 |             | 0  | 5  | 10 | 15 | 20 | 1 | 2 |                     |                             |
| Double-anterior | 19          | 3  | 8  | 6  | 2  | 0  | 17 | 2 | 7\*                   | 12                           |
| Double-posterior| 19          | 4  | 5  | 5  | 3  | 0  | 2  | 17| 9\#                   | 10                           |

*a Data from 5, 10, 15, and 20 days of graft healing from Tank and Holder (1978).

\# Identifiable as digits 3 and/or 4. These digits cannot be unequivocally identified.
II in these regenerates clearly indicates that structures normally produced by the anterior half of the limb have been formed from the posterior half. We suggest that anterior positional values corresponding to these anterior structures have been intercalated (see Fig. 12). Assuming that each posterior half of the limb contains more than half of the circumferential positional values, intercalation between extreme values will generate the missing anterior values through the middle of the limb. It is also possible to explain the apparently anomalous occurrence of limbs with a digital formula of IV, III, III, III, and IV as a result of intercalation. In these few limbs, if some loss of midline values occurred early in the process of distal transformation, the number of positional values in each half of the regenerate would be reduced to less than half of the total, and intercalation across the center of the limb would result in duplication of existing positional values and, hence, in duplication of existing structures at more distal levels. As can be seen in Fig. 9, some of these limbs show evidence of convergence of the elements of the pattern, followed at a more distal level by expansion and duplication. Another possibility, however, is that the apparently duplicated middle digit is abnormal, and hence a misidentified second digit.

Given that double-posterior limbs have the potential to intercalate all of the missing positional values, the question now becomes why double-posterior limbs do not always behave this way. For example, initial amputation through surgically constructed double-posterior upper arms never results in a greater number of digits than would be expected from the sum of the digits normally arising from each half, and usually results in the formation of less than that number, particularly when the graft is allowed time in which to heal. Furthermore, reamputation of symmetrical double-posterior regenerates at the level of the initial amputation plane results in regenerative failure (Tank and Holder, 1978). In the experiments of Slack and Savage (1978a, b), amputation of embryonically generated double-posterior limbs results in regenerates with reduced skeletal patterns. Similarly, when surgically constructed symmet-
rical forearms are amputated, loss of mid-line elements of the pattern occurs, that is, these limbs produce less than would be expected if each half behaved autonomously (Krasner and Bryant, 1980). Differences in the behavior of various symmetrical posterior limbs could be due to differences in the way that cells from various parts of the limb circumference come together during the early stages of regeneration under the dissimilar experimental conditions. For example, it is possible that the direction in which healing occurs in the forearm is different from that in the upper arm. The lack of any effect of healing time prior to amputation of double-posterior and double-anterior limbs in the forearm (Krasner and Bryant, 1980) suggests that cells may come together from the dorsal and ventral sides of the limb at this proximal-distal level. Factors such as the proximal-distal level of amputation and whether or not the amputation is performed through surgically constructed or regenerated symmetrical tissues may affect the ways in which cells are brought together, and thus the extent to which intercalation of new positional values or midline loss of existing positional values occurs. Following this line of argument we would predict that in those experiments in which expansion of the pattern is the major result after amputation of symmetrical limbs, the predominant interactions will favor intercalation of missing positional values. Conversely, it also follows that in those experiments in which reduction of the pattern is the major result, the predominant interactions will be across the line of symmetry and hence favor loss of positional values.

In summary, the spectrum of results discussed in this paper as well as a variety of other lines of evidence (Bryant et al., submitted) are consistent with the idea that distal transformation is brought about by interactions between circumferential positional values (Bryant and Baca, 1978). Hence, it is clear that a complete circle of positional values at the amputation plane is only necessary to produce a complete regenerate, and an incomplete circle will result in some distal transformation. In symmetrical limbs, the extent of distal transformation is related to the number of positional values present at the amputation plane, and it is modified by the extent to which intercalation of new values and midline loss of existing values occurs.

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