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Morphogenesis of the amphibian limb blastema: The relationship between pattern and form

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SUMMARY

A relationship between pattern formation and field shape is established following the formation of rounded blastemas on lower arm limb stumps after treatment with vitamin A. Pattern formation is not affected by alteration in blastemal shape caused by removal of the dermis from the thigh region of the leg. We conclude, therefore, that blastemal shape does not play a causal role in establishing limb pattern. Data relating the number of cells present between the cardinal axial poles of blastemas and the size of blastemas is discussed in terms of short arc intercalation and short range cell–cell interactions during pattern regulation.

INTRODUCTION

Morphogenesis and pattern formation are central concepts for the study of tissue levels of control during development. Morphogenesis is the creation of form and shape and the transition from one form or shape to another during development of a particular structure. Pattern formation is discussed in terms of the spatial relationships of the component parts of a particular structure such as the bones in a limb. In turn, each bone in the limb will have a precise shape and form, as does the limb as a whole. These two aspects of development can be studied independently but must bear some intimate relationship to one another. One can ask why the limb has a precise shape when it is first formed, and what are the causal relationships between limb shape and pattern formation? Does the positional information responsible for establishing the pattern of limb parts also regulate changes in limb shape at precise limb levels?

In this paper, we examine the relationship between pattern formation and blastemal shape. In normal limbs amputation in upper limb positions leads to the regeneration of a single bone at this level, the humerus or femur. In contrast, amputation at a more distal limb level leads to the formation of two bones, the radius and ulna or tibia and fibula. The initial question examined here is whether the blastemal shape differences at these two limb levels are causally related to this alteration in patterning. Several models have been put forward which intimately link alterations in field shape with alterations in pattern. Prepattern

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models, for example, such as those based on reaction diffusion kinetics (see, for example, Geirer & Meinhardt, 1972; Geirer, 1981; Murray, 1982) are very sensitive to the shape and size of the field of cells in which the diffusion of morphogens occurs. Indeed, a specific model based on such a system relies particularly on the changing shape and size of the limb field in the chick embryo as limb development proceeds, in order to create the spatial pattern of bones in the different limb segments (Newman & Fritsch, 1979). Similar diffusion based models and similar arguments have been presented to explain patterning in other systems such as the egg and imaginal discs of Drosophila (Bunow, Kernevez, Joly & Thomas, 1980; Kaufman, 1981; Kaufman, Shymko & Trabert, 1978; Meinhardt, 1977). Short range cell contact frameworks such as the polar coordinate model (French, Bryant & Bryant, 1976; Bryant, French & Bryant, 1981) do not rely on field shape to establish a particular pattern in the same way as a prepattern diffusion system but may be sensitive to shape changes governing cell contacts and healing modes (Bryant, Holder & Tank, 1982; French, 1981; Holder, 1981, 1983). It is of particular interest that during normal regeneration the polar coordinate model does not predict that blastemal shape alterations will affect pattern but that alterations in blastemal shape may affect cell contacts and consequently affect pattern of limb parts in surgically constructed limbs of abnormal structures, such as symmetrical limb stumps (Holder, Tank & Bryant, 1980; Krasner & Bryant, 1980). In this light we also present data concerned with the possibility of direct contact between cells in different regions of blastemas which are formed at different limb levels.

We have demonstrated previously that the amphibian limb blastema is approximately round in cross-section if it forms in an upper limb position and elliptical in cross-section if formed in a lower limb position (Holder, 1981; Holder & Reynolds, 1983). This alteration in shape is largely an autonomous property of the blatema rather than a simple reflection of the shape of the stump upon which the blastema forms (Holder & Reynolds, 1983). This was demonstrated by removing specific tissues from the stump and examining the shape of blastemas that formed following amputation through the operated region.

Two basic experiments were performed in this study. One of these is based on the observation by Maden (1982, 1983) that vitamin A causes limbs amputated at distal levels to regenerate a complete proximodistal sequence of limb parts. The results presented below establish that distal amputation levels produce round blastemas when proximal structures are produced at the amputation plane and elliptical blastemas when normal forearm structures are formed following vitamin A treatment. The second experiment is based on the observation that upper leg stumps from which the dermis has been removed produce abnormally elliptical blastemas. We establish that such operated legs regenerate normal patterns despite the abnormal shape of the blastemas which initially form. We conclude from the results of these two experiments that blastemal shape is determined by level-specific positional information but that pattern formation during

normal regeneration is not sensitive to these blastemal shape alterations. The relationship betwen pattern formation and morphogenesis, at least during limb regeneration, is therefore more clearly defined.

MATERIALS AND METHODS

All the experiments were performed on axolotls between 35 mm and 80 mm long spawned either at the colony at King's College or at the University of California at Irvine. They were fed on liver or heart and kept in individual plastic containers in standing tapwater. All operations were performed with the animals anaesthetised in MS222 (Sigma).

Three separate procedures will be described.

1. *Vitamin A treatment*

Both forelimbs of ten animals were amputated in the forearm midway between the proximal carpals and the elbow. The protruding cartilage elements were trimmed away to leave a flat amputation surface. Animals were immediately immersed in tap water containing 0·1 mM retinoic acid (Sigma) for 10 days. This concentration produces the maximum degree of proximodistal duplication with the size of animals used (Maden, 1982, 1983). Animals were then transferred to standing tap water alone. The right forelimb of each animal was cut off and fixed in Bouin's fluid at the stage of medium bud. These limbs were imbedded in wax and serially sectioned transversely at 10 μm. The shape of these blastemas was then quantitavely assessed by measuring the distance separating the anterior and posterior and dorsal and ventral poles and producing a ratio of these measurements (see Holder, 1981; Holder & Reynolds, 1983). The left limbs were allowed to regenerate a complete pattern and were fixed 6 to 8 weeks after the initial amputation. These limbs were stained with Victoria blue B and cleared to reveal the skeleton.

As a control, the limbs of animals of comparable size were amputated either in the mid-upper arm or mid-lower arm and allowed to regenerate to MB. These were then fixed, wax embedded and serially sectioned as above.

2. *The removal of dermis from the thigh region of the stump*

The first experiment was designed to establish whether removing the dermis altered the shape of blastemas in the thigh region. Whole cuffs of skin were removed between the knee and the flank and the animals were left for a week before amputation to allow epidermis to migrate over the wound. Limbs were amputated and allowed to regenerate to the stage of medium to late bud (stages according to Tank, Carlson & Connelly, 1976).

Control operations were performed where cuffs of skin were removed and replaced to heal for a week before amputation.

Having reached the required stage the hindlimbs were fixed in Bouin's fluid

and processed for wax sectioning at 10 μm. Serial transverse sections were cut, stained with Mallory's trichrome and the shape of blastemas assessed quantitatively.

Having established that dermis removal from the thigh region caused flattening of the blastema (see Fig. 3), cuffs of skin were removed in a larger series of animals and the limbs were amputated one week later. These limbs were allowed to regenerate for 6 weeks so that the limb pattern could be analysed. Animals in this part of the experiment were between 70 and 75 mm long. Some animals were operated on both legs whereas others had only one leg operated, the contralateral leg being amputated in the midthigh region and allowed to regenerate for the same period of time in order to assess normal regeneration over the defined time period. All limbs were then removed at the same time, fixed in Bouin's fluid, stained in Victoria blue and cleared in methyl salicylate.

In order to assess the normality of the pattern in these operated limbs the whole skeletal pattern was examined for gross anatomical features. The growth and form of the tibia and fibula and the metatarsal and first phalanx of digit 3 was assessed by measuring the proximal to distal length and anterior to posterior width of these elements from camera-lucida drawings of cleared specimens of both control and operated limbs.

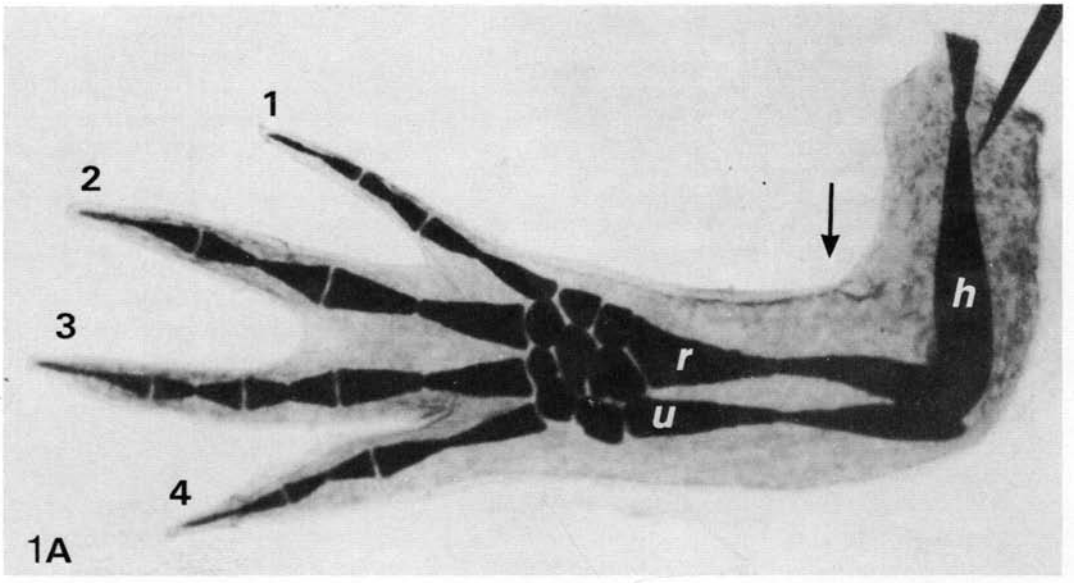
For this aspect of the study three blastema classes were analysed. Forelimbs were amputated in the midupper arm or midlower arm and allowed to regenrate to appropriate stages. The upper arm blastemas were fixed at either MB or palette and the lower arm blastemas were fixed at MB. Limbs were fixed in Bouin's fluid, wax embedded and serially sectioned as described above. In each of the three categories the number of cells separating the dorsal to ventral and anterior to posterior blastema poles were counted. In addition, the distance separating these poles was measured, using an eye-piece graticule, in the upper arm palette-staged blastemas.

RESULTS

1. *Vitamin A treatment*

Two curious features of vitamin A treatment are important in the analysis of the results. Firstly, the same concentration of vitamin A can produce different degrees of proximodistal duplication in different animals (Maden, 1982, 1983).

Fig. 1. Victoria blue stained preparations of control and experimental forelimbs from experiment 1. The level of amputation in these limbs is shown by an arrow. A. A normal left forelimb regenerate. Magnification ×8. B. Left forelimb duplicated after vitamin A treatment. A complete limb has regenerated from the lower arm amputation level. Magnification ×9. C. Left forelimb duplicated after vitamin A treatment. The duplicated limb contains two forearm segments but no second humerus was formed. Magnification ×9. h, humerus; r, radius; u, ulna. Duplicated skeletal parts are marked with a superscript.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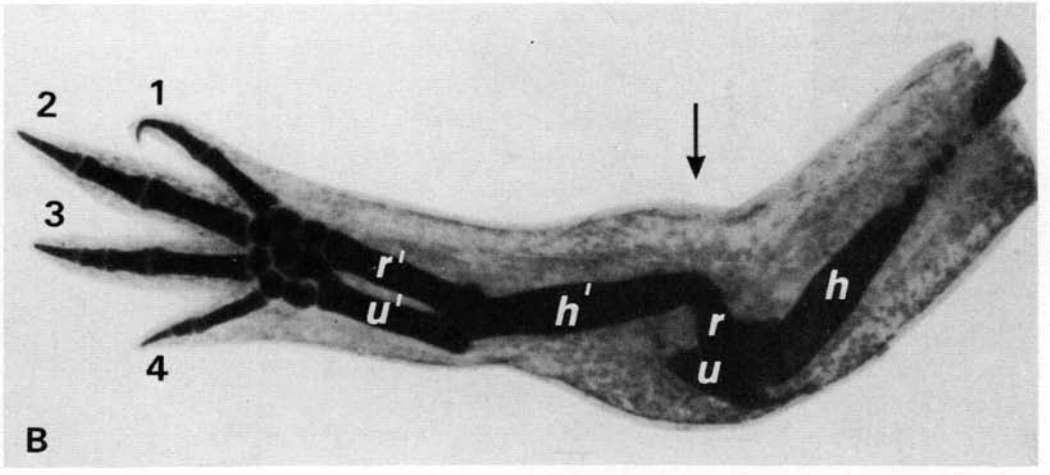
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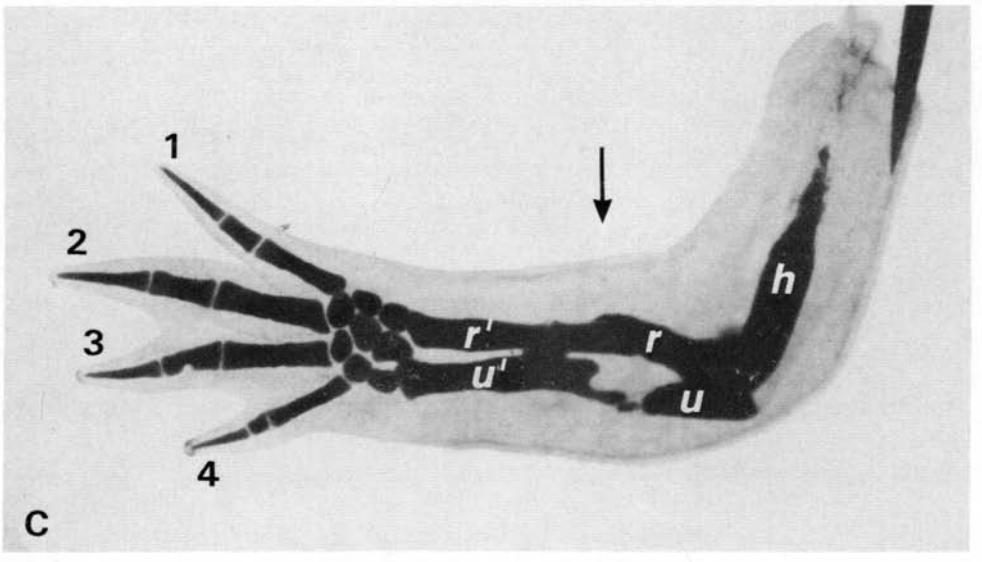
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With the concentration of vitamin A used in these experiments three types of limb were found. Limbs were either; 1, duplicated with a clear humerus present in the regenerate; 2, duplicated from the level of the forearm so that two forearm segments appeared or; 3, showed no regeneration. Secondly, the pattern of duplication seen in contralateral limbs within one animal is invariably the same (Maden, personal communication).

Skeletal analysis of fully regenerated limbs

A normal forelimb skeletal pattern is shown in Fig. 1A. Ten limbs were amputated in the midforearm and allowed to regenerate after exposure to retinoic acid. Of these, three showed complete duplication with a well formed complete limb regenerating from the midforearm level (Fig. 1B). Two of these limbs had a clear proximal articulation for the duplicated humerus with an abnormally shaped piece of cartilage that had the general form of the shoulder joint. This has been noted previously (Maden, 1982, 1983). In the first case the humerus appeared to be continuous with the radius in the stump. Five cases showed duplication of the forearm elements with no appearance of a second humerus. In two of these the forearm elements were not normal at the proximal part of the regenerate and fusion of the proximal regenerated forearm elements occurred. In the other two (Fig. 1C) the duplicated forearm elements were in the same plane as the respective elements in the stump but were separated from them by a small gap. In all five limbs the whole forearm region appeared relatively longer than in a normal limb, although this was not quantified (compare Fig. 1A with 1C). The remaining two limbs did not regenerate any clear structures, but MB blastemas were produced on the contralateral limbs in this group.

Morphometric analysis of contralateral limbs

Following morphometric analysis, the average r values for each individual MB blastema were derived. These are presented in Table 1, along with the level and type of duplication found in the appropriate contralateral limb.

Table 1. *The results of vitamin A treatment for the 10 pairs of limbs*

	Group 1 Mean r = 0·57 ± 0·01			Group 2 Mean r = 0·47 ± 0·01					Group 3	
Case number	1	2	3	4	5	6	7	8	9	10
Mean r value	0·55	0·55	0·60	0·41	0·47	0·49	0·43	0·52	0·54	0·54
Level of duplication										

Control amputations mean r values: lower arm = 0·38 ± 0·01
upper arm = 0·67 ± 0·02.

The limbs were divided into three categories: those in which the contralateral limb was duplicated at 1) the humerus, or 2) the forearm level, and 3) those which did not regenerate. The r values for each group were then averaged for each 100 μm proximal to distal blastemal level. Those values for the two categories where regeneration occurred are shown graphically in Fig. 2. It is clear that the first group average r value is higher than the second group ($0{\cdot}57 \pm 0{\cdot}01$ for humerus level duplication, $0{\cdot}47 \pm 0{\cdot}01$ for radius and ulna duplication – Table 1) yet neither are as round as normal upper arm controls (Fig. 2: $\bar{r} = 0{\cdot}67 \pm 0{\cdot}02$) and both are more round than normal lower arm controls (Fig. 2: $\bar{r} = 0{\cdot}38 \pm 0{\cdot}01$).

Statistically, all four groups, that is control upper and lower amputations and the two vitamin A groups with duplications, are significantly different from each other at the 1 % level.

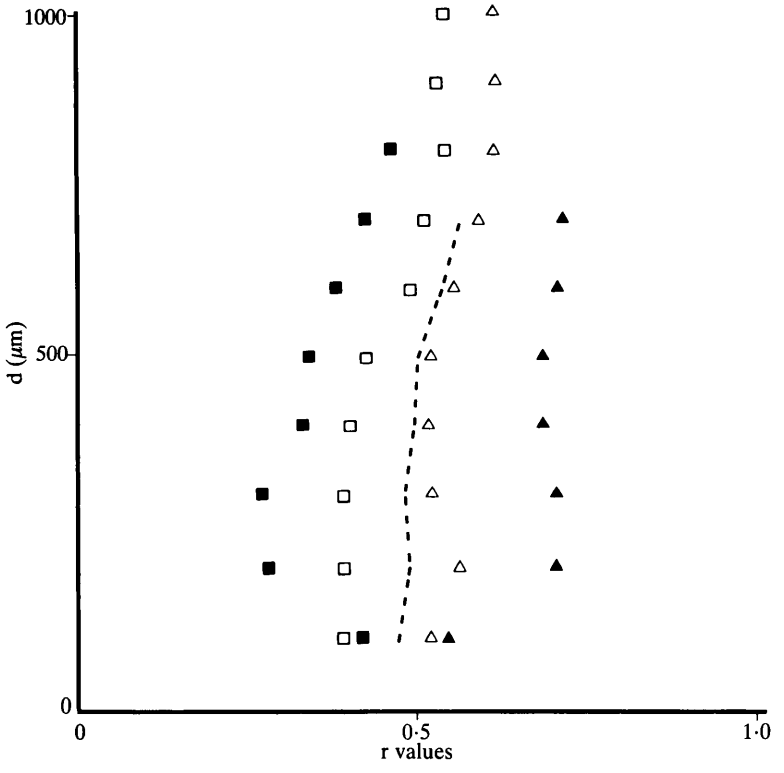


Fig. 2. Graphic representations of blastemal shapes from experiment 1. Control r values for MB upper arm (▲) and lower arm (■) blastemas are compared with MB blastemas from vitamin A duplicated limbs with either a duplicated humerus (△) or radius and ulna (□) as the most proximal extra structures. The dashed line represents the average of normal upper arm and lower arm r values (see text for further explanation). The 95 % confidence limits for these measurements are: upper arm control, $0{\cdot}67 + 0{\cdot}22$; lower arm control, $0{\cdot}38 + 0{\cdot}17$; duplicated humerus, $0{\cdot}57 + 0{\cdot}16$; duplicated radius and ulna, $0{\cdot}47 + 0{\cdot}17$. The distance (d) from distal to proximal extremes of the blastemas is measured in micrometres.

2. *Regeneration from stumps from which the dermis is removed*

i) *Blastemal shape*

Removal of dermis from the upper leg stump produced blastemas with an average r value of 0·43 ± 0·02 (Fig. 3A–B). This value is significantly more elliptical than the r value seen in control cases where the skin cuff is removed and replaced before amputation (r = 0·67 ± 0·03, Fig. 3A). For comparison, normal upper leg M/LB blastemas have an average r value of 0·57 ± 0·02 (see Fig. 3B) and lower leg M/LB blastemas an average r value of 0·24 ± 0·01 (Holder & Reynolds, 1983).

Close examination of the graph in Fig. 4B depicting the transition of r values at 100 μm steps for M/LB blastemas formed on stumps from which dermis was removed show them to have clearly abnormal shapes. Distally they appear to be

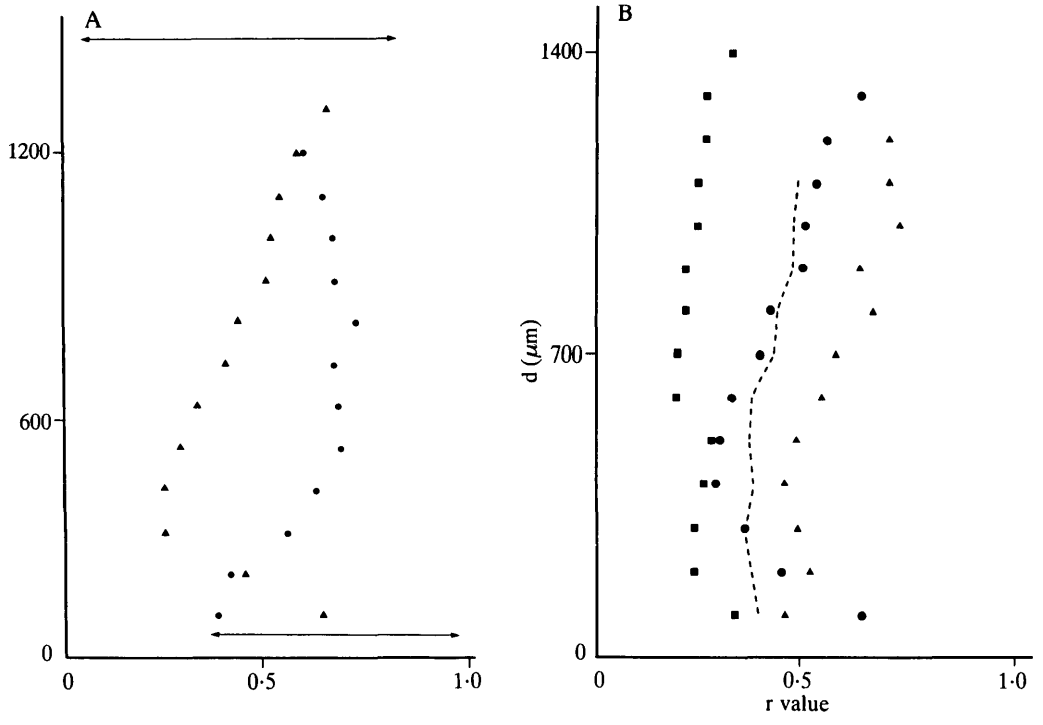


Fig. 3. The shape of blastemas derived from leg stumps from which the dermis was removed. A. Graphic representation of blastemal shape from dermis removals (▲) and sham operated controls (●). The arrows represent the 95 % confidence limits for the populations (lower arrow sham controls). B. The dermis removal blastemas (●) compared to normal, unoperated, M/LB upper leg (▲) blastemas and lower leg (■) blastemas. The normal leg blastemal shape data is from Holder & Reynolds (1983). The dashed line represents the intermediate values between normal blastemas at 100 μm levels. Note that the dermis removal cases transgress this line. Notation as for Fig. 2.

rounded but immediately proximal to the blastema tip, within 100 to 300 μm, they become clearly elliptical. More proximally, 700 to 1300 μm from the distal tip, they return to being more rounded.

ii) *Limb pattern*

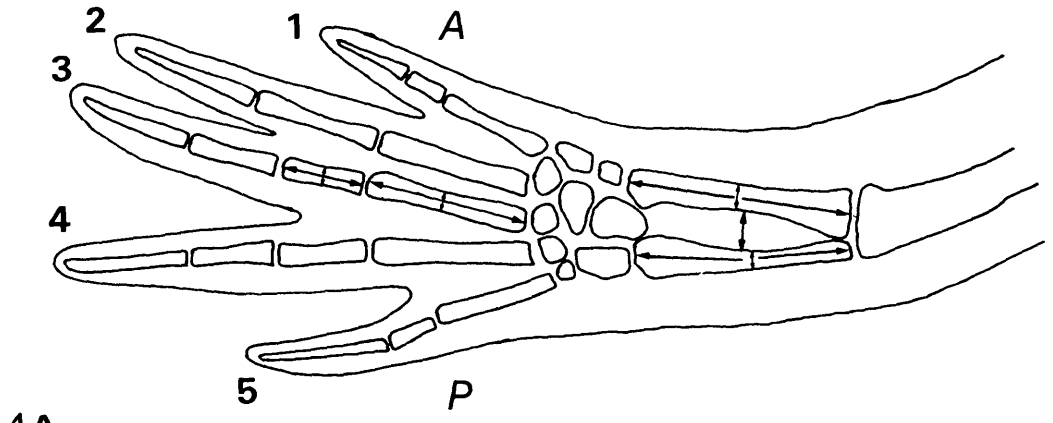
Prior to presenting the results of the quantitative assessment of pattern normality we will outline the basis for assuming that pattern alterations should be seen if blastemal shape is a determining factor in pattern specification.

The abnormality of the dermis removal M/LB blastemas is clearly indicated if the r values from the experimental cases are compared with those obtained from normal amputations performed at upper and lower leg levels and skin cuff removal controls (Fig. 3). The normal M/LB leg blastemal shape data is reproduced from a previous paper (Holder & Reynolds, 1983). If average values are calculated for each 100 μm level for each r value of normal upper and lower leg blastemas, an artificial transition line can be plotted (dashed line in Fig. 3B). This line is merely a guide to the possible transition between upper and lower leg blastemal shapes and upper and lower leg patterns. If shape determines pattern in some way then such a transition point must exist. The exact position of the transition point cannot be determined from these experiments, but the vitamin A results suggest that the average between upper and lower arm control values for each 100 μm blastemal level examined may be a close approximation, for the following reason. From Fig. 1 and Table 1 we can deduce that upper arm patterns are coincident with more elliptical blastemas. It so happens that the average between upper and lower arm control amputation r values for each 100 μm level falls directly between the two plots for the vitamin A groups (dashed line in Fig. 2). It must be the case, if pattern and shape are causally related, that the transition point must lie between the two vitamin A plots and therefore the average control artificial plot is a means of estimating this position. When the comparable artificial plot is compared with the blastemal shape of dermis removal operated upper legs it can be seen that the latter crosses the region of the estimated transition line between the 300 and 800 μm levels (Fig. 3B). It may therefore be expected that such an operation may lead to considerable distortion of the pattern regulation mechanisms if the shape of the blastema influences the pattern mechanism.

The skeletal patterns of the limbs regenerated following dermis removal were analysed in qualitative and quantitative terms in order to decide how normal the regenerated patterns were.

1) *General anatomical features:* A total of 13 limbs regenerated following dermis removal in the upper leg region. The general features were analysed with reference to the form of four parts of the skeleton: the knee joint, the tibia and fibula, the tarsals and the digits. The normal axolotl skeleton is shown in Fig. 4A.

Of the 13 operated limbs, all cases appeared to have normal knee joints and



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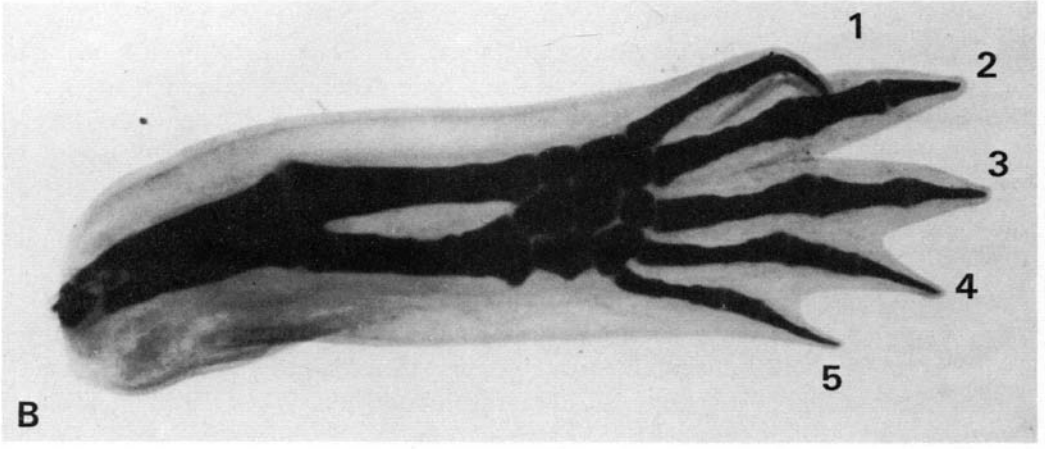

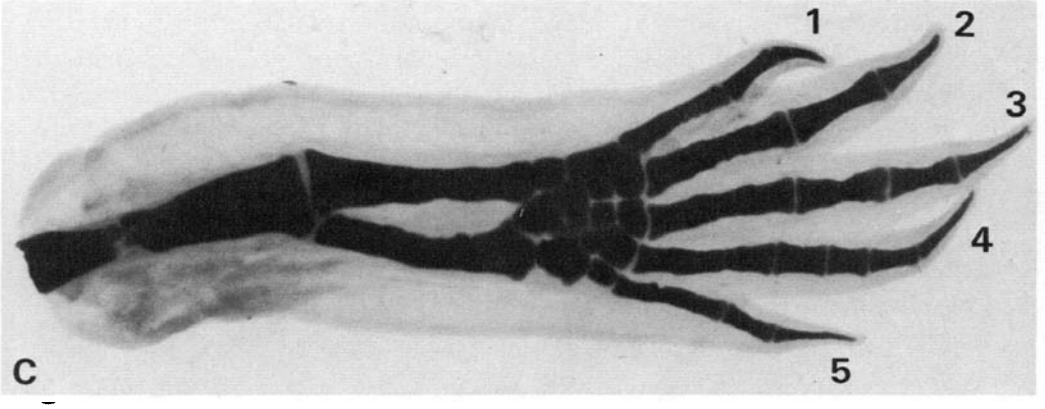

Fig. 4

the overall form and relative position of the tibia and fibula were also normal (Fig. 4B). The tarsal patterns did show some variations. Six cases had 8 tarsals, three cases 9 tarsals, three cases 10 tarsals and one case 12 tarsals (Fig. 4C). The overall pattern of tarsals was generally normal, but in five of the six cases in which 8 elements were present, the metatarsals of digits **4** and **5** articulated with 1 fused tarsal.

The digit patterns were also essentially normal, although minor variations occurred in some cases. Ten cases had 5 clear digits, eight of which had abnormal phalangeal formulae (**2, 2, 2, 3, 2** and **2, 2, 3, 4, 2**) (Fig. 4C). The remaining three cases had more severe abnormalities. One of these had only 4 digits (**2, 2, 3, 2**), one had 5 metatarsals but those of digits **4** and **5** were fused at the level of the first phalangeal joint and one had 4 metatarsals with digits **3** and **4** diverged from the first phalangeal joint to form 5 digit tips.

The overall impression from this qualitative appraisal is that the patterns are very close to normal. The knee joint and the form of the tibia and fibula are clearly normal in all cases, although minor pattern aberrations occur in the tarsals and digits in some cases.

2) *Quantitative analysis of pattern normality:* In order to make quantitative comparisons, three control regenerates were analysed to establish a norm. These cases were contralateral limbs from three operated animals which were allowed to regenerate for the same perod of time as the operated limbs to minimize growth differences. In order to quantitate pattern elements, nine measurements were made (Fig. 4A). These were the length of the tibia, fibula, metatarsal of digit **3** and the first phalanx of digit **3**, the width of these four elements at the mid-point of their proximodistal extent, and the distance separating the tibia and fibula at this mid-point position. The results of this analysis are presented in Table 2. The three measurements from the tibia/fibula region were taken from all 13 cases, but the measurements from digit **3** were taken only from the ten cases in which 5 clear digits regenerated. It is evident from Table 2 that the patterns regenerated from the operated limbs were quantitatively as well as qualitatively normal. This is clear even taking account of any growth variations which may be expected between animals over the six week period of regeneration.

Fig. 4. Limb patterns from cases where the dermis had been removed. A. A camera-lucida drawing of a control left leg regenerate. The normal leg has 9 tarsal elements and a phalangeal formula of **2, 2, 3, 3, 2** from anterior (A) to posterior (P). The arrows represent the measurements taken for the quantitative assessment of pattern normality. Magnification ×9. B. A right operated leg stained with Victoria blue. This limb shows a near normal pattern. Only 8 tarsals are present and the phalangeal formula is **2, 2, 2, 3, 2**. Magnification ×9. C. A right operated leg stained with Victoria blue. This limb has 12 tarsals and the phalangeal formula is **2, 2, 3, 4, 2**. Magnification ×9.

Table 2. *Quantitative assessment of the normality of legs regenerated following dermis removal in the thigh region*

	Tibia		Fibula		Width separating	Metatarsal d.3		1st phalanx d.3	
	Length	Width	Length	Width	Tibia and Fibula	Length	Width	Length	Width
Control	2·51	0·42	2·32	0·33	0·31	1·61	0·26	0·87	0·26
Range*	0·05	–	0·26	0·05	0·11	0·21	–	0·05	–
Operated	2·56	0·42	2·42	0·37	0·32	1·59	0·28	0·86	0·26
S.E.M. ±	0·03	0·01	0·04	0·01	0·01	0·06	0·01	0·02	0·01

* Range of the 3 measurements from regenerated controls is given as the difference between the minimum and maximum measurements.

3. *Cell counts and measurements along the dorsal to ventral and anterior to posterior axes of normal blastemas*

Cell counts were carried out in the upper and lower arm MB groups and in the upper arm palette blastemas. The cell counts at successive 100 μm distal to proximal blastemal levels are shown in Fig. 5A–C.

In all the graphs the number of cells separating the anterior and posterior poles is greater than the number separating the dorsal and ventral poles. This difference is more clearly apparent at the distal tip of lower arm MB and upper arm palette blastemas (Fig. 5B–C), than at the tip of the upper arm MB blastema (Fig. 5A). The greatest difference in cell number along the axes occurs between 200 and 600 μm from the distal tip in all these blastema types. The overall difference between dorsal to ventral and anterior to posterior cell counts is considerably larger in the lower arm MB than the upper arm MB; this reflects the difference in shape (elliptical or round) at the two proximal–distal levels. In all three types of blastema examined between five and ten cells lie across the dorsal to ventral axis at the distal tip. Up to 500 μm from the blastemal tip in the distal palette blastema as few as 15 cells separate dorsal and ventral poles. The difference between dorsal to ventral and anterior to posterior cell numbers in upper and lower arm MB blastemas is evident at all proximal to distal levels (Fig. 5A–B). For example, at the 600 μm level in the upper arm MB 40 cells separate the A–P poles and 32 cells separate the D–V poles, a difference of only 8 cells;

Fig. 5. The number of cells separating the poles of the anterior to posterior and dorsal to ventral axes. The cell numbers were counted by assessing the number contacting a straight line drawn from dorsal to ventral and anterior to posterior at 100 μm levels from the distal tip (d). The bars represent standard errors for each mean value which were derived from three blastemas in each instance. A. Upper arm MB blastema. B. Lower arm MB blastema. C. Palette blastema derived from an upper arm amputation.

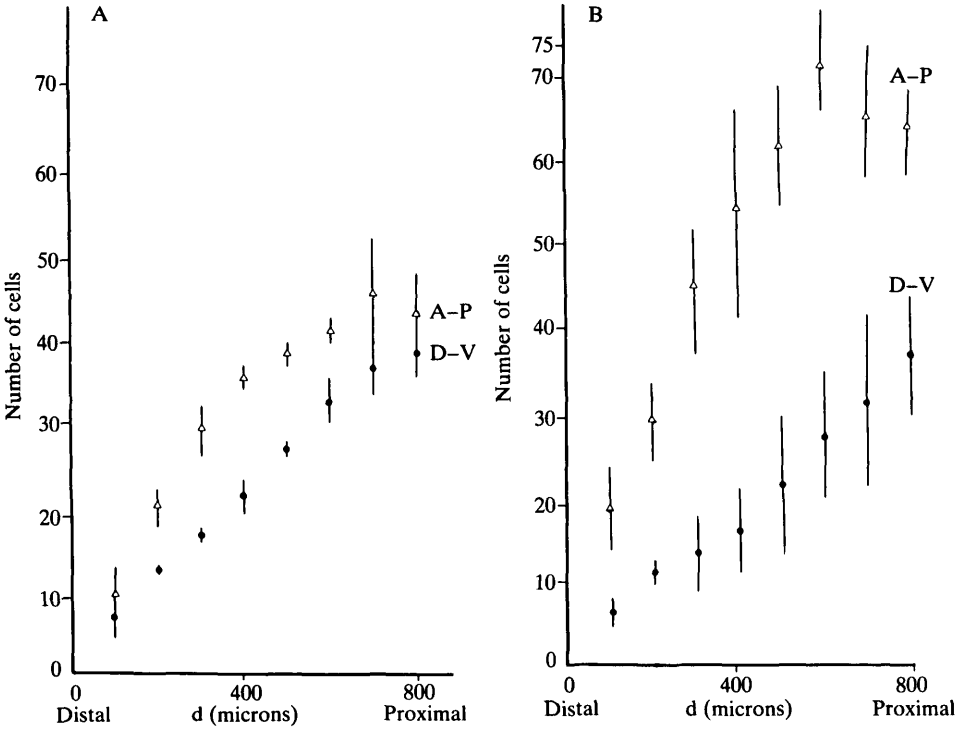
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Fig. 5

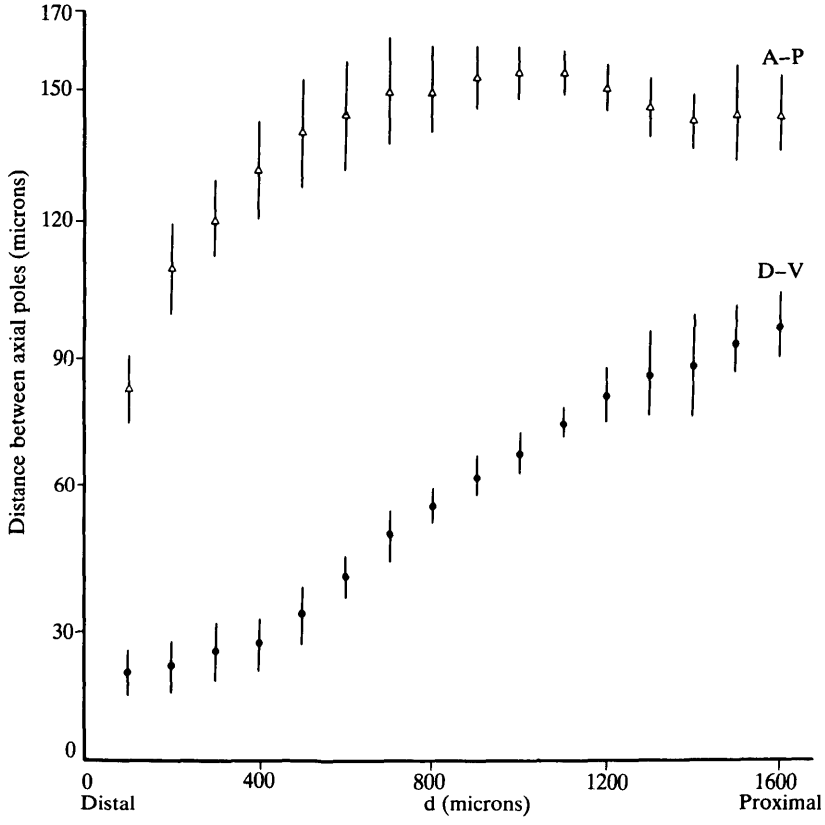

Fig. 6. The distance separating the anterior to posterior and dorsal to ventral axes of palette upper arm amputation blastemas. These distances were measured with an eye piece graticule at 100 μm levels from the distal tip of three blastemas. The bars represent standard errors.

whereas at the corresponding level in the lower arm MB 70 cells separate A–P poles and 26 cells separate D–V poles, a difference of 44 cells.

Measurements of distances: In the upper arm palette blastema group actual measurements of the distances in micrometres separating dorsal–ventral and anterior–posterior poles were measured using an eyepiece graticule. The results (shown graphically in Fig. 6) demonstrate the expected difference between the anterior–posterior and dorsal–ventral axes. In the distal 500 μm of blastema at least a threefold difference between means in width versus height of the blastema is evident. In the dorsal to ventral axis in the distal 500 μm as little as 20 to 32 μm separates the poles of the axis, compared with 84 to 142 μm in the anterior to posterior axis.

DISCUSSION

The results of three separate experiments are presented in this paper. In the first experiment vitamin A treatment is shown to affect blastemal shape as well

as the proximodistal limb pattern. This result clearly establishes a link between pattern regulation and morphogenesis because the formation of a single cartilage element, in this case the humerus, is coincident with the presence of a rounded blastema irrespective of the limb level at which the humerus is formed. This result does not distinguish cause and effect, however, and it is for this reason that the second experiment was performed. It was shown previously that removing the dermis from the upper limb segment caused significant flattening of the normally round blastema (Holder & Reynolds, 1983; and Fig. 3B). This observation allows the examination of the relationship between pattern regulation and blastemal shape and thus to distinguish cause and effect. It is clear from the analysis of skeletal patterns of legs regenerated from stumps in which the dermis had been removed that blastemal shape does not affect pattern regulation. Thus, the transition of blastemal shape in normal limbs from rounded in the upper limb to elliptical in the lower limb is a function of level-specific positional information. However, blastemal shape *per se* is not responsible for the alterations in limb pattern. It can be concluded that alterations in blastemal shape and dimensions cannot be used as basic mechanisms in models for pattern regulation in the normal amphibian limb. Therefore, the use of such mechanisms in other systems must also be treated with caution because the experiments reported here are the first to examine pattern formation following the experimental alteration of field shape and the subsequent examination of pattern.

It is important to emphasize that these conclusions relate to basic mechanisms for pattern regulation during normal limb regeneration. In other systems, such as the chick limb (Newman & Fritsch, 1979), and the insect embryo (Bunow *et al.* 1980; Kaufman, 1981; Kaufman *et al.* 1978; Meinhardt, 1977) and in reaction-diffusion kinetics in general (Murrary, 1982; Geirer, 1981), field shape is an intrincic feature of the basic patterning mechanism. If field shape alone is altered these models predict alterations in subsequent patterning. In contrast, the polar coordinate model does not rely on alterations in field shape as an intrinsic feature for pattern regulation. The essential features of this model are short-range cell–cell interations mediated by cell contact, intercalation and the two-dimensional polar array of positional values (Bryant *et al.* 1981). Thus, if field shape alone is altered, the polar coordinate model does not predict a subsequent alteration in pattern. However, if the arrangement of positional values in the limb stump is abnormal, such as the surgical creation of a symmetrical set of circumferential values (Holder et al. 1980; Bryant *et al.* 1982; Tank & Holder, 1978), field shape may have a secondary influence on pattern regulation. This is particularly clear if the short arc mechanism for cell contact during distal outgrowth is considered (Bryant, 1978; Bryant & baca, 1978). This hypothesis proposes that blastemal shape may be a moderator of cell contact which will affect outgrowth and patterning of symmetrical limbs but will not affect outgrowth and patterning of normal limbs. The third experiment described in this paper relates specifically to blastemal shape in terms of its role as a moderator

of healing mode and cell contact. The results of the third experiment demonstrate the possible effect of blastemal shape on cell contact between cells at different axial extremes of the blastema and are consistent with the assertion that blastemal shape can alter healing and thus affect patterning of abnormal limb stumps. However, the data presented here are from normal limbs and are, therefore, complementary in nature. Nonetheless, the results are entirely consistent with the notion that elliptical blastemas in the lower arm increase the likelihood of cells contacting from dorsal and ventral axial positions and decreases the likelihood of cells contacting from anterior and posterior axial poles. Indeed, given that blastema cells can contact other cells up to 100 μm away (Geraudie & Singer, 1981) cells from dorsal and ventral extremes of the lower arm blastema may contact directly (Fig. 6).

Finally, some comment is needed with regard to the demonstration that removal of the dermis in the upper leg causes flattening of the normally rounded upper leg blastema. Previous results have demonstrated that the removal of muscle and cartilage from the stump does not affect blastemal shape (Holder & Reynolds, 1983). The dermis seems, therefore, to have a unique property. Numerous other experiments have shown that the dermis is an important tissue for the control of pattern regulation (see, for example, Carlson, 1974, 1975; Tank, 1981). It is unclear, at present, how the dermis exerts its effect upon blastemal shape but other unpublished results from our laboratory suggest that the skin as a whole, and may be the dermis in particular, must be intact around the limb circumference for blastemal shape to be normal for a particular limb level. This is reflected by the control skin cuff removal and replacement experiment reported here (Fig. 4A–B), where the interruption of the skin by a cut in the proximodistal axis leads to an upper leg blastema more rounded than normal. The same effect is seen in the lower leg, where dermis removal leads to more elliptical blastemas whereas the controls are significantly more round (Holder and Reynolds, unpublished results). The possible mechanical and instructive roles of the dermis in determining blastemal shape are being examined further in the present time.

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