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The characteristics of local application of retinoic acid to the regenerating axolotl limb

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Summary. The work described here is concerned with the effects of local application of retinoic acid to the regenerating limb, administered in silastin blocks. Using 14 C RA we have shown that its rate of diffusion into medium was the same as into regenerating limbs and that the curve of percent loss shows a fast rise tailing off to a plateau. RA caused specific alterations in the proximodistal axis of the regenerate such that complete limbs could be produced from distal amputation planes. Increasing concentrations of RA caused regeneration to commence from increasingly more proximal levels. The effect of time of administration after amputation on proximodistal duplication has been investigated as well as position effects with silastin blocks placed either at the anterior, posterior or dorsal poles of the blastema. These data permitted an estimate of the absolute amount of RA needed to cause alterations in the proximodistal axis – 2 to 16 μg per limb or 1 ng per cell. Supernumerary limbs were also induced by these local implants and here there was a distinct position effect with the dorsal side causing the highest frequency. This reveals a possible effect of RA on the anteroposterior axis of regenerating limbs, but some of these supernumeraries were thought to arise from the irritant action of RA rather than a specific effect on pattern formation. The relevance of these results to those obtained on other systems is discussed.

Key words: Retinoic acid – Vitamin A – Limb regeneration – Pattern formation – Axolotls

Introduction

Vitamin A and its analogues have dramatic effects on limb development and regeneration. Local application of retinoic acid (RA) to the anterior side of the developing chick limb mimics the action of the polarizing region (Tickle et al. 1982; Summerbell 1983). When applied to the posterior side, however, either normal or deleted limbs appear, revealing a distinct asymmetry of action.

So far, vitamin A has only been applied systemically to the regenerating axolotl limb by immersing the animals in solutions of these compounds (Maden 1983a). In contrast to chick limbs, the primary effect in axolotls is to cause duplications in the proximodistal axis so that complete limbs can be regenerated from distal amputation planes. An analysis of the cellular effects of RA on axolotl limbs revealed that DNA synthesis and cell division were inhibited, cartilage matrix breakdown was stimulated, epidermal mucopolysaccharide production was stimulated and blastemal cells tended to clump together (Maden 1983a). It has been shown that effects on the epidermis are not involved in pattern changes (Maden 1984a) and thus we must concentrate on alterations in the mesodermal component of the blastema.

To analyse this phenomenon further, it was decided that local application of RA to the blastema would be a more fruitful approach than immersing animals in solutions. The work reported below describes the characteristics of a silastin delivery system for retinoic acid. The rate of release of 14 C labelled RA has been investigated along with time, concentration and position effects on pattern formation. From this data we can estimate the absolute amount of RA needed to cause specific pattern duplications. We show that there are no position effects on proximodistal duplication but that there are position effects on the induction of supernumerary limbs. Some of these supernumerary limbs can be interpreted as anteroposterior duplications, demonstrating that in this regard axolotls behave in the same fashion as chick (Tickle et al. 1982; Summerbell 1983) and *Rana* (Maden 1983b) limbs.

Materials and methods

The experiments were performed on young larval axolotls, *Ambystoma mexicanum*, 40–60 mm long. The larvae for each experimental series were within 5 mm in length to improve the consistency of the results. The animals had their limbs amputated through the radius and ulna and/or tibia and fibula under MS222 anaesthesia.

Silastin implants were prepared by mixing the required amount (0, 50, 100 or 200 mg) of retinoic acid (Type XX all-trans, Sigma) with 1 ml of silastin (Silastic 382 Medical Elastomer, Dow Corning, Michigan, USA) until an even mixture had been obtained. Two drops of catalyst were then added and the silastin allowed to set. $300 \times 300 \times 150$ μ (small) or $500 \times 500 \times 250$ μ (large) blocks of silastin were used for the rate of release studies and for implantation into limbs only large blocks were used. In all implantation experiments except the position effect series the blocks were placed under the skin on the dorsal surface of the limbs, immediately proximal to the blastema (Fig. 1). They were not pushed into the blastema for fear of any physical disturbance to regeneration caused by their presence.

14 C RA was prepared for incorporation into silastin by the following method. 25 μCi of carboxyl 14 C vitamin A acid (Amersham International) was allowed to evaporate to dryness in a dark

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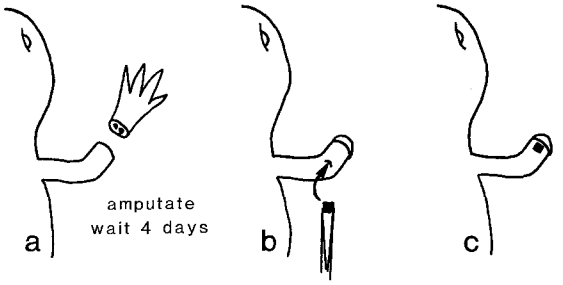

Fig. 1a–c. Diagram of the operation of local implantation. **a** The limbs of the animals are amputated through the radius and ulna (or tibia and fibula). Animals are left to regenerate for 4 days (except in the optimum time series). **b** A cut is made in the dorsal skin at the elbow and the block inserted through this cut. **c** The block is pushed down to the level of the blastema

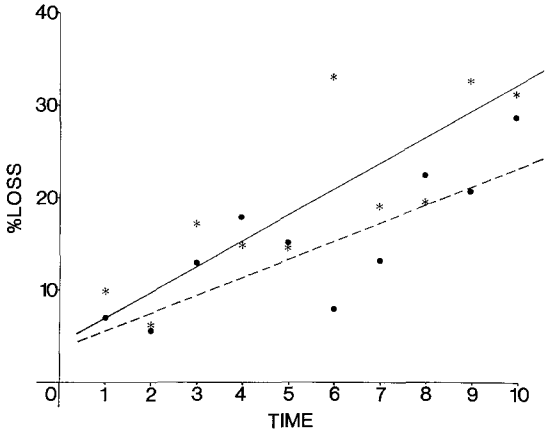

Fig. 2. Percent loss of 14 C RA from large (stars) and small (dots) blocks into 500 µl Niu-Twitty amphibian medium over a 10 h period. The regression lines (large blocks – solid line, small blocks – dashed line) are not significantly different from each other. Thus block size does not affect % loss, but since they contain different amounts of RA their absolute rates of loss are different (Table 1)

Table 1. Estimates of the absolute rates of release of 14 C RA from large and small blocks into medium, regenerating and non-regenerating limbs

Block size	Release into	Rate of initial release (µg/h)
Large	Non-regenerating forelimbs	0.23
Large	Regenerating forelimbs	0.26
Large	500 µl Niu-Twitty	0.20
Small	Regenerating forelimbs	0.073
Small	500 µl Niu-Twitty	0.025

cupboard. To this, 10 mg RA and 0.5 ml chloroform were added, mixed and again allowed to evaporate to dryness, forming crystals of labelled RA. The crystals were scraped from the tube, mixed with 0.1 ml silastin and set. Radioactive blocks to be inserted into limbs were counted on an Automatic TLC – Linear Analyser before and after implantation. Percent losses were calculated and put into a computer programme which fits non-linear mathematical models to experimental data. For analysis of the rates of diffusion into medium, blocks were placed in 500 µl of Niu-Twitty amphibian medium for periods of time up to 10 h and then removed. 9.5 ml of EP scintillant (Beckman) was added to each sample of medium and radioactivity counted on a Beckman LS 6800 scintillation counter. The blocks were then put back into the relevant scintillation tubes for 72 h to allow all the remaining radioactivity to

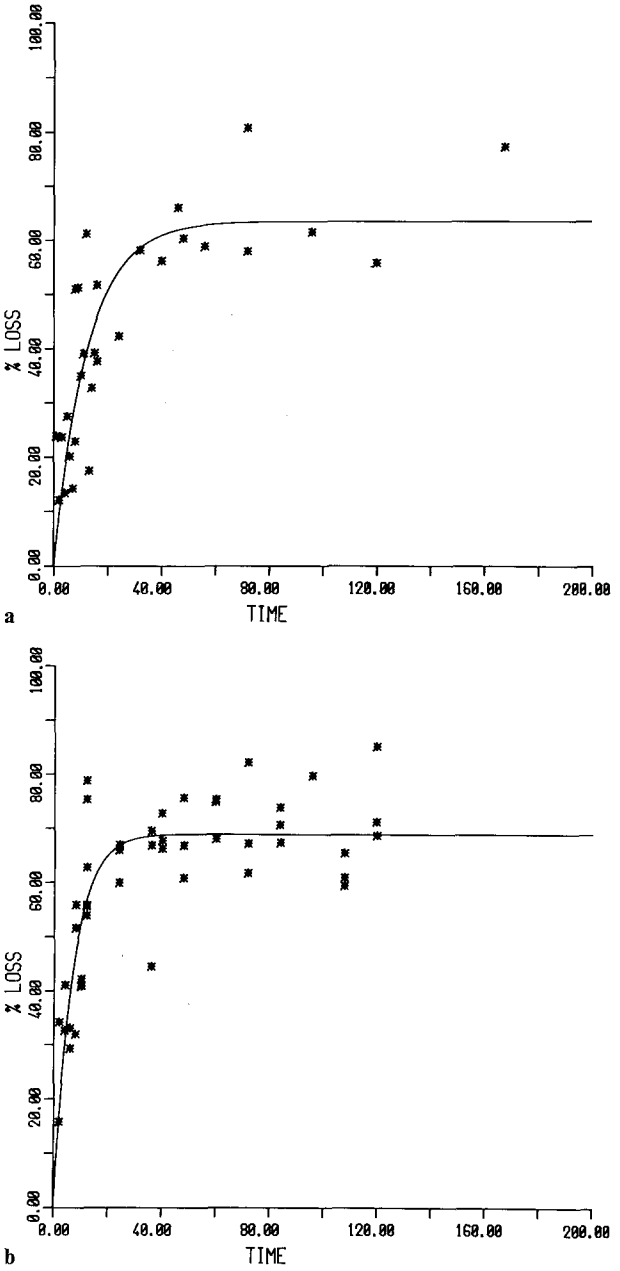

Fig. 3a, b. Percent loss of 14 C RA from blocks implanted into limbs measured on a TLC Linear Analyser over a 7 day period. The data was fed into a computer programme which fits non-linear mathematical models to experimental data and draws the curve. **a** Large blocks implanted into non-regenerating forelimbs. **b** Small blocks implanted into regenerating forelimbs. These curves confirm the data in Fig. 2 that large and small blocks show the same % loss. They also show that rates of loss are the same for regenerating and non-regenerating limbs

emerge, whereupon the samples were recounted. Preliminary experiments had revealed that after 72 h in scintillation fluid all the radioactivity is removed from a block.

Regenerating limbs with silastin implants were allowed to develop for 4–6 weeks. They were then fixed in neutral formalin, stained in Victoria blue and cleared to reveal the cartilage structure. Abnormalities in the pattern of cartilage elements were scored according to the system previously described (Maden 1983a) i.e. normal regenerate −0 (Fig. 4a, b), extra carpals −1 (Fig. 4c), extra part radius and ulna −2 (Fig. 4d), extra full radius and ulna −3 (Fig. 4e), extra part humerus −4 (Fig. 4f), extra full humerus −5

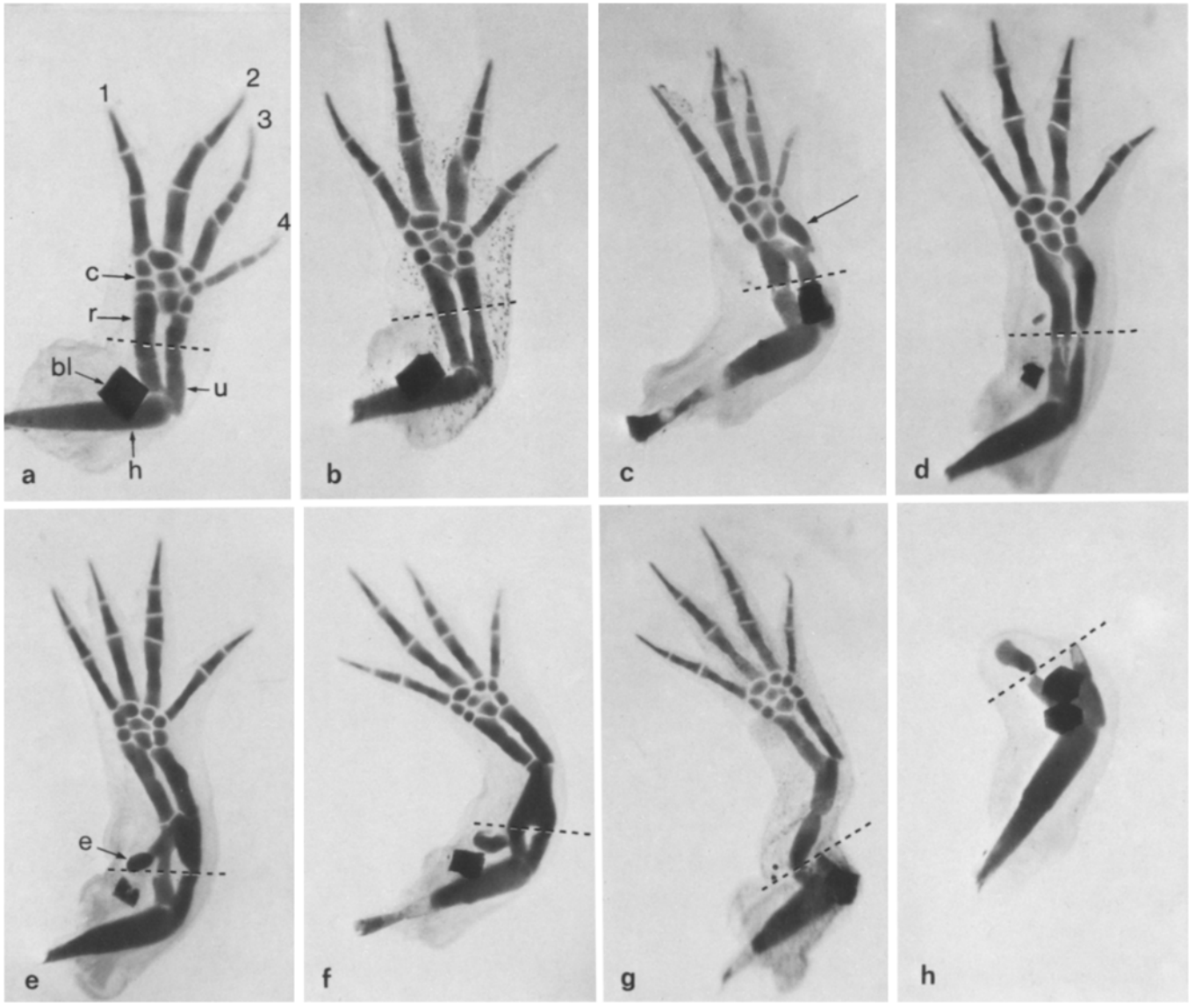

Fig. 4a–h. Increasing degrees of duplication caused by RA. Broken lines mark the amputation plane through the radius and ulna. The blocks (*bl*) in which the RA was administered can be seen in all these limbs just proximal to the amputation plane. Victoria blue staining. **a** Control limb (no RA in the block) which regenerated prefectly by replacing the ends of the radius and ulna, 8 carpals and 4 digits. *h*, humerus; *u*, ulna; *r*, radius; *c*, carpals; *1 2 3 4*, digits. Mag ×15. **b** Another control limb which had 1 extra phalange in digit 2 (3 instead of 2). Mag ×16. **c** An extra carpal-like piece of cartilage has regenerated (*arrow*). Mag ×15. **d** Here the radius and ulna are too long. Mag ×16. **e** An extra radius and ulna have regenerated in tandem. Also an ectopic piece of cartilage can be seen (*e*) differentiating next to the block, a common feature (see f). Mag ×15. **f** In this case the distal end of the humerus differentiated first, followed by the lower limb. Mag ×12. **g** Here a complete limb has regenerated from the amputation plane. Mag ×12. **h** An inhibited limb. No regeneration has occurred except for a small piece of cartilage. Mag ×16

(Fig. 4g), inhibited (no regeneration) −6 (Fig. 4h). The reason for including inhibited limbs in the analysis is because this represents the most potent effect of RA. If the concentration is higher or the animals treated for longer than needed to produce full duplications, then regeneration is inhibited (see also discussion of the strength of activity index in Summerbell 1983).

Results

Rate of release from silastin blocks

By using 14 C retinoic acid in silastin its rate of release from large and small blocks into medium, regenerating limbs and non-regenerating limbs could be investigated.

The release into 500 µl Niu-Twitty amphibian medium was determined at hourly intervals up to 10 h. Radioactivity in the medium and that remaining in the blocks was counted, thus allowing a percent loss graph to be drawn (Fig. 2). This shows that the rate of loss expressed as a percentage is not significantly different for different sized blocks. However, by estimating the amount of RA in a block the absolute rates of diffusion were found to be 25 ng/h for small blocks and 200 ng/h for large blocks (Table 1).

Release into regenerating and non-regenerating limbs could not be determined by this method. Instead we used a TLC linear analyser, which, rather than removing the radioactivity from the block to count it, measures the B-

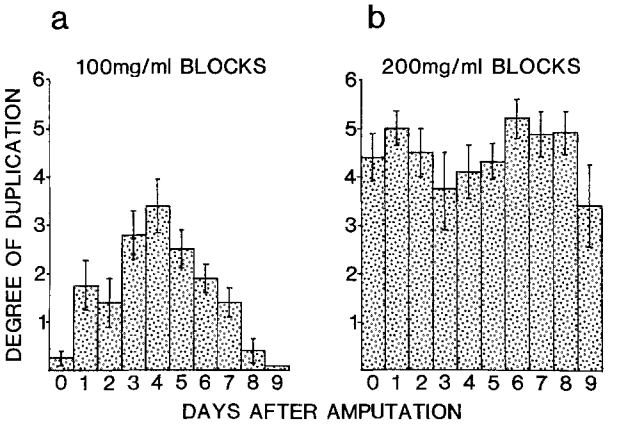

Fig. 5a, b. Results of the optimum time series (series 2) on degree of duplication. Either 100 mg/ml (**a**) or 200 mg/ml (**b**) blocks were inserted on days 0–9 after amputation. Bars mark standard errors

particles emitted in situ. Blocks were counted before and after insertion into limbs and percent loss curves drawn (Fig. 3a, b). These show three interesting features. Firstly, the rates of diffusion are the same in regenerating (Fig. 3b) and non-regenerating (Fig. 3a) limbs (Table 1). Secondly, the rate of loss is constant over the first 20 h until it begins to tail off, reaching a plateau of approximately 65–70% lost between 60–70 h after insertion. Thus only 65–70% of the RA in a block seems to be available to the limb (the remaining 30–35% is presumably in the centre and cannot diffuse out) and it is available only for 60–70 h after insertion. Thirdly, the rates of diffusion from big and small blocks into limbs are very similar to diffusion rates into medium measured above (Table 1). These conclusions suggest that diffusion alone is sufficient to account for the release of RA into the limb (but it does not exclude processes such as active absorption) and that the higher metabolic rate of regenerating limbs over normal limbs has no effect on this process.

Experimental effects of RA

When control silastin blocks were implanted into regenerating limbs normal regenerates were produced (see Series 1). But when silastin containing RA was implanted, instead of regenerating those elements removed by amputation (the distal half of the radius and ulna and hand) extra elements appeared. They can be seen to follow a graded series whereby the level from which regeneration commences gradually becomes more proximal. An example of each category of regenerate is shown in Fig. 4c–h. The most extreme example is a complete limb regenerating from a distal amputation plane (Fig. 4g). The same graded series was obtained in hindlimbs although not exemplified here. The scoring system is described in Material and Methods and for each of the subsequent series the total scores, averages and standard errors were calculated. The increasing proximalisation of the regenerates shown in Fig. 4 is scored as degree of duplication in subsequent figures.

Series 1 – Controls

A total of 28 limbs had silastin blocks implanted which contained no RA to examine any disturbances caused by its physical presence. All limbs regenerated normally except for an occasional alteration in the phalange number. A perfectly normal regenerate is shown in Fig. 4a and in Fig. 4b a typical abnormality – the presence of three phalanges instead of two in digit 2. Another typical alteration was the presence of two phalanges instead of three in digit 3. Therefore no significant deviations from normal were caused by the presence of silastin blocks in the regenerating limb and the degree of repetition for this control series was 0 (Fig. 7, first column).

Series 2 – Optimum time of implantation

Knowing that RA is only available for 60–70 h after an implant it was important to determine the time after amputation at which administration of RA would have the most profound effect on pattern formation.

In the first experiment on a total of 208 limbs, one 100 mg/ml implant was inserted into each limb on days 0–9 after amputation through the radius and ulna or tibia and fibula. After regeneration had been completed the results were plotted (Fig. 5a). It is clear that implantation immediately after amputation does not produce duplications (Fig. 6a), but the effectiveness increases over the first few days of regeneration up to a maximum at day 4 (Fig. 6b). It then tails off again to nothing by day 9. At these later days of administration, instead of proximodistal duplications appearing, regenerates were defective. They took the form of fused carpals/tarsals, missing digits (always posterior ones – Fig. 6c) or sometimes deleted zeugopodial elements (always anterior ones).

A parallel histological study conducted on animals of the same size revealed the following stages. Day 2/3 – apical cap present, early dedifferentiation (Fig. 6d); day 4 – extensive dedifferentiation; day 5 – early blastema (Fig. 6e); day 7 – cone blastema; day 9 – late cone blastema, redifferentiation beginning (Fig. 6f). Thus the most effective time of administration for 100 mg/ml blocks is at the late dedifferentiation/early blastema stage.

This entire experiment was repeated with 200 mg/ml blocks on a total of 140 limbs. Here the results were quite different (Fig. 5b). There was no favoured time of administration, each day up to day 8 was a good as any other. By day 9 deletions in the regenerates were beginning to appear for the first time and so this probably represents the start of the decline in effectiveness. Thus for 200 mg/ml blocks any time of administration before the onset of redifferentiation in the blastema will produce maximal duplications.

Series 3 – Concentration effects

These were investigated in a series of 6 experiments involving a total of 180 limbs. Silastin blocks were prepared with RA present at concentrations of 50 mg/ml, 100 mg/ml and 200 mg/ml. In three experiments one block at each concentration was inserted into regenerating limbs and in another three experiments two blocks at each concentration were inserted. All implants were performed on day 4 of regeneration to ensure maximum effectiveness (see Series 2).

The results (Fig. 7) were interesting from several aspects. Firstly there was a concentration effect such that blocks with more RA produced regenerates which began from more proximal levels. Thus single block scores were 2.4 (50 mg/ml), 1.9 (100 mg/ml) and 3.75 (200 mg/ml). Double block scores were 3.75 (50 mg/ml), 4.2 (100 mg/ml) and 5 (200 mg/ml). Secondly the presence of 2 blocks at any one

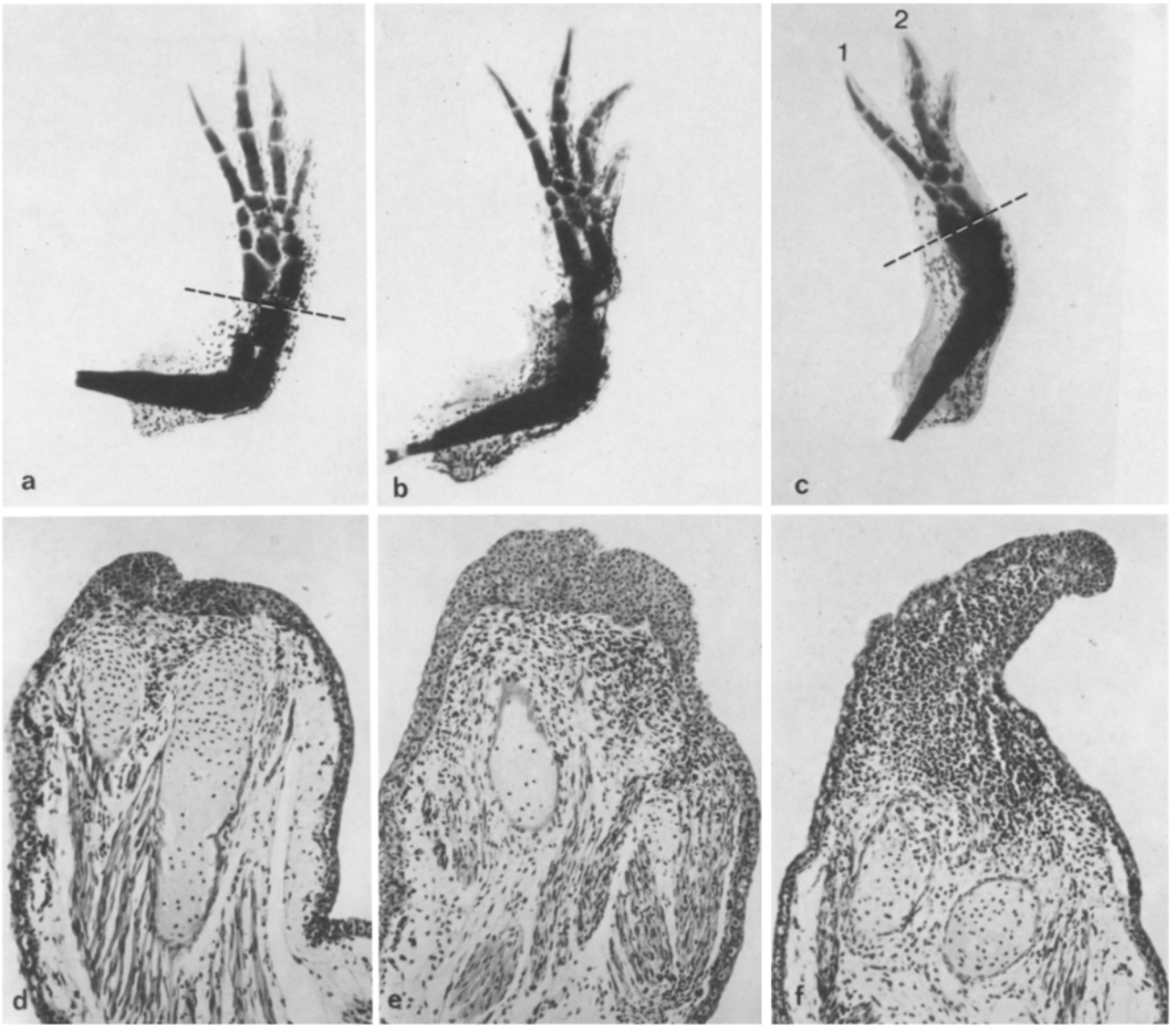

Fig. 6a–f. Some results from the 100 mg/ml optimum time series (Fig. 5a). **a** Normal limb produced after insertion of the block on day 0. Mag ×15. **b** Regenerate with a part humerus, radius and ulna and hand present after block insertion on day 4 after amputation. Mag ×15. **c** Defective regenerate after block insertion on day 9 after amputation. Digits 3 and 4 and posterior carpals are missing. Mag ×15. **d** Section through a day 2 regenerate at the early dedifferentiation stage. An apical cap is present. Haematoxylin and eosin staining. Mag ×60. **e** Section through a day 5 regenerate at the early blastema stage. Blastemal cells are beginning to accumulate beneath the apical cap. Mag ×60. **f** Section through a day 9 regenerate at the late cone stage. Redifferentiation is beginning and a central rod of cartilage condensation can be seen. Mag ×50

concentration is considerably better at inducing more proximal regenerates than one block at twice the concentration. Presumably this effect is due to the greater surface area of two blocks and thus greater availability of RA over a short period of time. Thirdly the frequency of inhibited limbs increased with increasing concentration of RA. Fourthly supernumerary limbs were produced at a frequency of 20–45% and this effect was not concentration dependent (Fig. 8). The characteristics of supernumerary limbs are discussed further in Series 6.

Since each concentration of RA produced a typical degree of duplication, we can use this to estimate the absolute amount of RA needed for such an effect. Knowing the size of the block, the amount of RA at each concentration can be calculated – 50 mg/ml blocks have approximately 3 μg RA in them, 100 mg/ml blocks 6 μg and 200 mg/ml 12 μg. Also knowing that only 65–70% seems to be released we can therefore estimate that 2–4 μg will produce an extra part radius and ulna, 4–8 μg an extra part humerus and an extra complete limb after 16 μg of RA has been administered. These figures are not intended to provide anything more than an idea of the order of magnitude.

Series 4 – Position effects

In all previous series the silastin blocks were implanted into the dorsal surface of the regenerating limb. To investigate any positional effects two experiments were performed with 200 mg/ml blocks on a total of 77 limbs. In half the cases blocks were grafted into the anterior side of the blastema

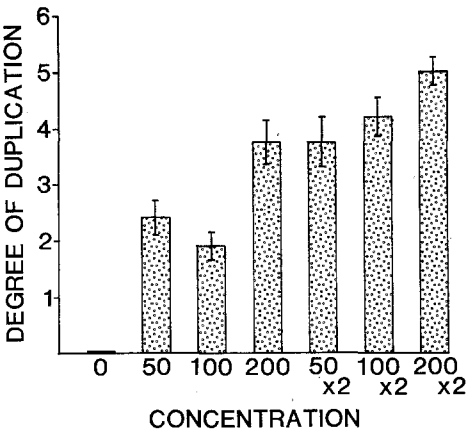

Fig. 7. The effect of concentration of RA in the blocks (in mg/ml) on degree of duplication (series 3). x 2 refers to 2 blocks. There is an effect of increasing concentration and of two blocks over one. Bars mark standard errors.

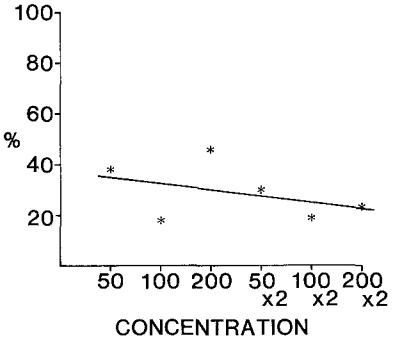

Fig. 8. Frequency of supernumerary limbs induced in each concentration series. There is no effect here of increasing concentration. Solid line marks the regression line

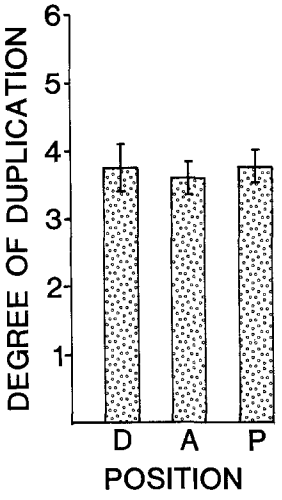

Fig. 9. The effect of position of block insertion (200 mg/ml) on degree of duplication. *D*, dorsal; *A*, anterior; *P*, posterior. Bars mark standard errors

and in half to the posterior side. For comparison with the data on 200 mg/ml blocks on the dorsal surface in Series 3, grafts were inserted on day 4 of regeneration.

The lack of effect of implant position on proximodistal duplication was quite clear (Fig. 9). The same score for degree of repetition was obtained in each experiment showing that position is not an important variable in this regard. However there was a difference in the induction of supernumerary limbs. From the dorsal implantation site (Series 3) 46% of limbs had supernumeraries, but from the posterior site only 7% and from the anterior site only 3% had supernumerary limbs. Therefore it seems that a dorsal implantation site results in a far higher frequency of supernumeraries.

Supernumerary limbs

The characteristics of supernumerary limb induction were as follows. They appeared at a constant frequency unrelated to concentration of RA (Series 3, Fig. 8). In Series 2 with 100 mg/ml blocks there was a stage related induction – supernumeraries were induced for the first four days after amputation and only occasionally at later times. With 200 mg/ml blocks they were induced for the entire period of experimentation – up to 9 days after amputation. There was a distinct position effect – supernumeraries were virtually absent when blocks were inserted in anterior or posterior positions, but present with blocks inserted dorsally.

The structure of these supernumerary limbs varied. If they were induced near the insertion of the block, then they formed a completely seperate outgrowth (Fig. 10a, b, c). In the majority of these cases the supernumeraries were highly proximalised and usually commenced development with a complete humerus, at the very least half a humerus would be present. Occasionally the degree of duplication of the supernumerary and the original limb were the same (Fig. 10a), but more often than not they were different (Fig. 10b and c). For example, the supernumerary might have a complete humerus present, but the original limb be almost unaffected (Fig. 10b). As mentioned above, most of these seperate outgrowths originated on the dorsal surface, but occasionally one would develop side by side with the original limb, their anterior faces adjacent (Fig. 10c). These resembled double posterior limbs.

Another type of supernumerary structure was one which resulted only in extra digits and carpals (Fig. 10d). In these cases the degree of duplication of the supernumerary and the original limb were usually the same because they shared the same zeugopodial elements. These too resembled typical anteroposterior duplications with their anterior faces adjacent. Yet another type of structure was a minority of cases in which the supernumerary limb was itself duplicated in the anteroposterior axis (Fig. 10d), resulting in a triplicated limb. In these cases one of the supernumeraries formed a double posterior structure with the original limb, but the two supernumeraries between them formed a double anterior limb.

Discussion

The work reported here concerns the effects of local administration of retinoic acid to the regenerating axolotl limb. RA was applied in a silastin block and its rate of release from this vehicle was determined using the 14 C labelled compound. It was found that the rate of release into medium, non-regenerating or regenerating limbs was the same. Its diffusion from the block was rapid over the first 20 h and then tailed off, reaching a plateau of 65–70% lost in 60–70 h.

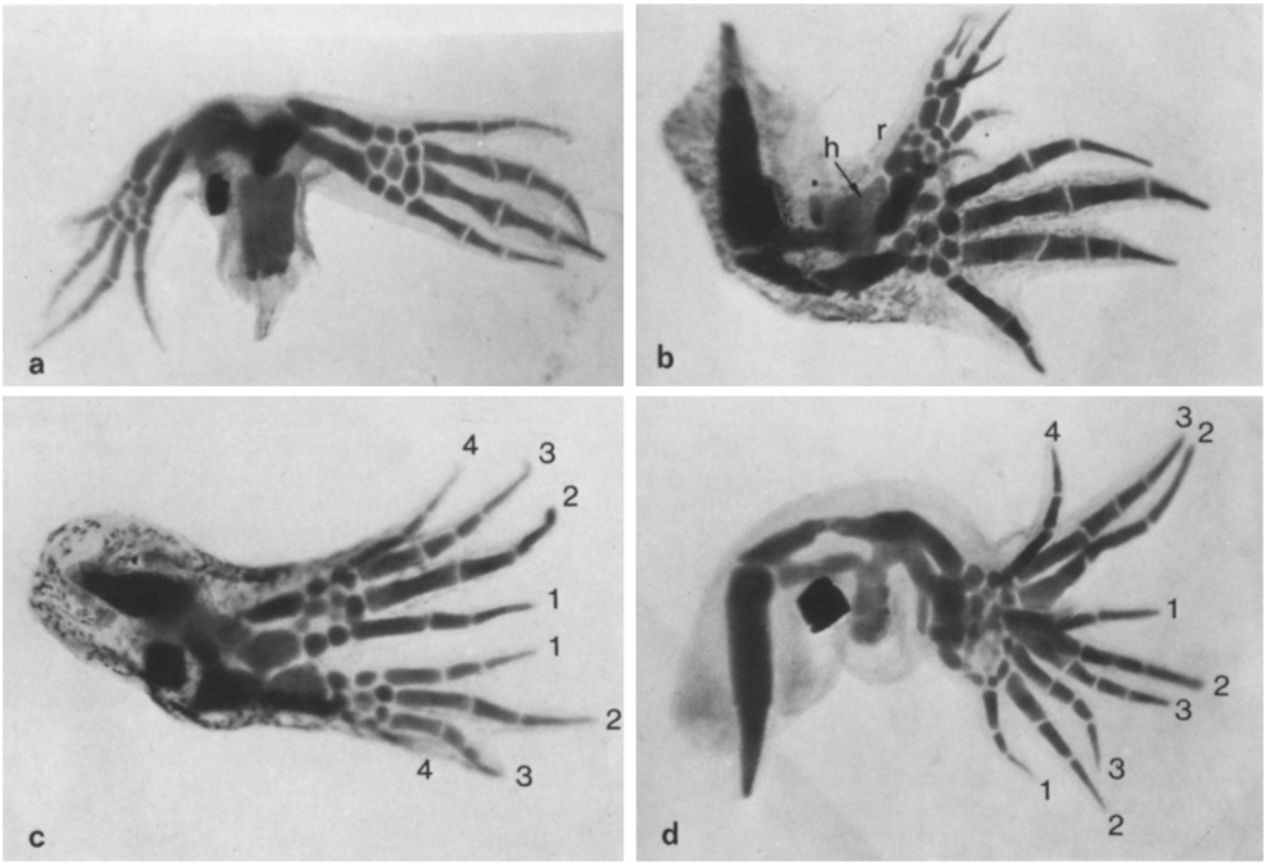

Fig. 10a–d. Examples of supernumeraries. **a** Here the supernumerary (left limb) and the original limb have the same degree of duplication, both commence at the humerus level. Mag ×15. **b** This supernumerary (upper limb) commences at the humerus (*h*) level whereas the original limb is almost normal. *r*, radius. Mag ×16. **c** A supernumerary (lower limb) and original limb which appeared in mirror-image relationship with their anterior faces adjacent. This resembles double posterior regenerates found in other organisms. Mag ×15. **d** A triplicated limb. The original limb consists of the upper 4 digits (*4 3 2 1*). One supernumerary consists of three digits (one shared) with its anterior face adjacent to the original thereby forming a double posterior limb (middle 3 digits). The other supernumerary (lower 3 digits) forms a double anterior structure with its partner. Mag ×15

Administration of RA by this method caused proximodistal duplications (Fig. 4) of the same type as systemic application (Maden 1983a). Thus after amputation through the forearm, regeneration does not replace those elements removed. Instead complete limbs can be regenerated from such distal amputation planes in the presence of RA. Other characteristics were also the same, such as the effect of concentration (Series 3) – more RA present in the block caused regeneration to commence from a more proximal level (greater degree of duplication). The optimum time of administration after amputation for 100 mg/ml blocks was at the late dedifferentiation/early blastema stage, but for a higher concentration (200 mg/ml) any day after amputation up to the onset of redifferentiation was as effective (Series 2). An estimate of the absolute amount of RA needed to produce duplications was 2–4 μg for an extra part radius and ulna to 16 μg for a complete new limb. Assuming there are about 10,000 cells in the blastema at the time of administration, this gives a value of ≈1 ng of RA per cell needed to cause maximal duplications.

There was no effect of position of implantation (either dorsal, anterior or posterior) on proximodistal duplication (Series 4), but there was an effect on the induction of supernumerary limbs. Surprisingly, implantation into anterior or posterior positions did not result in anteroposterior duplications as it does in the chick limb bud (Tickle et al. 1982; Summerbell 1983). Instead, a dorsal position produces a high frequency of supernumerary limbs. It is debatable whether all of these supernumeraries represent a real effect of RA on the anteroposterior axis of the regenerating limb. About half of them were completely seperate outgrowths from the axis of the main limb (Fig. 10a, b, c) and thus could equally represent an effect of RA on the dorsoventral axis. Apart from their proximodistal duplication caused specifically by RA, these structures resemble supernumerary limbs induced by local implantation of carcinogens (Breedis 1952), implantation of tissues such as liver and kidney (Ruben and Stevens 1963) or by nerve deviation (Bodemer 1958; Maden and Holder 1984). Limb induction by these methods is thought to arise as a result of the irritant or immunological reaction of the implant causing local tissue destruction and dedifferentiation of cells which divide to establish an accessory blastema. It is likely, therefore, that RA is an irritant or an immunogen (the control series showed that the silastin block itself is not an irritant or immunogen) as well as a proximalising agent.

On the other hand some of the supernumeraries were very like chick and *Rana* anteroposterior duplications with

their anterior faces adjacent, forming double posterior limbs (Fig. 10c). These are more likely to be formed as a result of RA acting on the anteroposterior axis of the limb by causing anterior cells to become posterior ones and thus establish a symmetrical axis. In favour of this interpretation is the data from series 2 where there was a stage related induction of supernumeraries which followed the stage related degree of duplication in the proximodistal axis.

Thus local application of RA affects the proximodistal axis and perhaps also the anteroposterior axis and/or the dorsoventral axis of the regenerating limb. Why anteroposterior duplications arise only after local application of RA and not systemic application (Maden 1983a) as they do in *Rana* (Maden 1983b) may have something to do with an additional stimulus provided by local injury. Stocum and Thoms (pers. comm.) have found that anteroposterior effects on the newt limb can be produced by systemic application of RA to regenerating double anterior limbs. These surgically created limbs obviously have been damaged and are in the process of regulating their anteroposterior axes. But this still does not explain why frogs produce anteroposterior duplications after systemic application, presumably this is a species difference. Further studies such as the effect of RA on double posterior limbs or half limbs will be needed to clarify these points.

The molecular explanation of these profound alterations in pattern formation is still a long way off, but the phenomenonology has been fitted into current models of pattern formation. The effects on the proximodistal axis have been interpreted as an alteration in the concentration gradient of morphogen which determines positional information along the proximodistal axis (Maden 1984b). Anteroposterior effects have been interpreted as RA mimicing the action of the morphogen produced by the zone of polarizing activity (Tickle et al. 1982) or that RA binds to the inhibitor in a Gierer-Meinhardt inhibitor – activator system, thereby causing a new peak of activator to be established (Summerbell 1983; Maden 1984b). Whether these models can withstand further more detailed experimentation remains to be seen, but we now have a system for answering such questions as the cellular site of action of RA in the regenerating limb.

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