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The effects of local application of retinoic acid on limb development and regeneration in tadpoles of *Xenopus laevis*

S. R. SCADDING
Department of Zoology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada
AND M. MADEN
National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, UK

SUMMARY

Vitamin A can have different effects on developing and regenerating limbs depending on the mode of administration. Previous work has demonstrated the differential effect of retinol palmitate on limb development and regeneration in *Xenopus laevis*. The purpose of the present investigation was to determine the effects of vitamin A on limb development and regeneration in *Xenopus* when administered by a local implantation method. *Xenopus* tadpoles had both hindlimbs implanted with either a block of silastin carrying retinoic acid or an anion exchange resin bead carrying retinoic acid and then the right hindlimb was amputated and the effect of the retinoic acid on limb development and regeneration was studied. The results showed that in developing hindlimbs the effects of silastin implants carrying retinoic acid was to cause skeletal reductions or deletions similar to those induced by immersion of the tadpole in retinol palmitate. On the other hand, in regenerating hindlimbs, the silastin implants caused a range of skeletal reductions and deletions as well as occasional accessory structures but notably induced no proximodistal (PD) duplications, unlike the effect of immersion in retinol palmitate where PD duplications were a common response. Implantation of anion exchange resin beads carrying retinoic acid had no significant effect on either development or regeneration beyond stage 50, presumably because the dose of the retinoic acid was so low. Thus the results suggest that the mode of administration of vitamin A has a very significant influence on its effects. The significance of this observation for vitamin A experiments on limbs is discussed.

INTRODUCTION

Vitamin A has impressive effects on both limb development and regeneration. In regenerating amphibian limbs, vitamin A can induce both proximodistal (PD) and anteroposterior (AP) duplications in the regenerate (Maden, 1982, 1983*a*,*b*). It has been known for some time that the effect of maternal injection of vitamin A on developing mammalian limbs is to cause skeletal reduction or deletions (Kochhar, 1977). Recently, we have shown that in developing amphibian limbs as well, vitamin A causes skeletal reduction and deletions, but never duplications

Key words: vitamin A, retinoic acid, limb development, regeneration, *Xenopus laevis*.

(Scadding & Maden, 1986*a*,*b*). Chick limb buds are unlike all other developing limbs, in that vitamin A causes AP mirror-image duplications (Tickle, Alberts, Wolpert & Lee, 1982; Summerbell, 1983). However, in both regenerating axolotl limbs and in developing chick limbs, the mode of administration of vitamin A modifies its effects. In the axolotl, administration of retinoic acid by implantation in silastin blocks, induces accessory limbs (Maden, Keeble & Cox, 1985). This effect was not seen when the retinoic was administered by immersing the axolotl in a retinoic acid solution. Similarly, in the developing chick, systemic administration of vitamin A has no effect on limb development at doses permitting survival (Summerbell & Harvey, 1983), while local administration on a paper carrier (Summerbell, 1983) or on ion exchange beads (Tickle, Lee & Eichele, 1985) induces AP duplication. Therefore, it is apparent that the mode of administration of vitamin A can have a profound effect on the results.

Thus, the primary objective of this investigation was to examine the response of both developing and regenerating *Xenopus laevis* hindlimbs to vitamin A administered by local implantation in both silastin blocks and in ion exchange beads. This would then allow us to evaluate these alternative modes of administration and to see if they gave different results compared to the administration of vitamin A by immersion (Scadding & Maden, 1986*b*). In particular, we wished to see if administration of the retinoid by implantation into developing limbs caused duplications as it does in the developing chick limbs.

As mentioned above, among developing limbs, chick limb buds are anomalous in that they can respond to vitamin A by producing AP duplications, but only when vitamin A is administered by local implantation. Thus, what is common to chick limbs and regenerating amphibian limbs is a very substantial wound in every case where limb duplication is observed. This leads to the hypothesis that wounding is essential for the duplication response, perhaps by initiating the release of mitogenic factors which then counteract the cell cycle inhibition caused by vitamin A. This might then allow pattern regulation processes to occur which otherwise might be masked, since if the cell cycle is inhibited by vitamin A, then clearly no morphogenetic effects can be observed. Thus, the secondary objective of this investigation was to determine if duplications could be induced in developing *Xenopus* limb buds when retinoic acid was administered by implantation methods which cause a definite local wounding of the limb bud during implantation.

MATERIALS AND METHODS

The *Xenopus laevis* tadpoles were raised in the laboratory from the induced breeding of adult frogs. Tadpoles were fed daily on a suspension of dried baby food and nettle powder. During the experiment, the tadpoles were maintained in small groups (usually seven) in plastic bins of constantly aerated tap water. Aeration seemed to be very important and this may be due more to the fact that it keeps the food particles in suspension, than to its role in oxygenation of the water. The tadpoles were anaesthetized in $0{\cdot}2\,g\,l^{-1}$ tricaine methane sulphonate neutralized with sodium bicarbonate, for implantation and amputation.

Silastin blocks, 200×200×200 μm, prepared with 200 mg ml^{-1} of retinoic acid (Sigma) as previously described and carrying an effective dose of about 1 μg (Maden *et al.* 1985), or control blocks without retinoic acid, were implanted into both hindlimbs buds in tadpoles at stages 52, 53, or 54 (Nieuwkoop & Faber, 1967). Implantation was carried out by puncturing a hole in the dorsolateral surface of the limb bud at about the prospective knee level with an electrolytically sharpened tungsten needle, enlarging the hole into a tunnel with one tine of fine watchmakers forceps, and then inserting the silastin block into the tunnel by spearing it on the end of a blunt tungsten needle. The right hindlimb was then amputated through the prospective zeugopodium. Control tadpoles with no implants at all were also amputated.

Anion exchange resin beads, AG1-X2 (BioRad), were loaded with retinoic acid by soaking them for one hour in a 1 mg ml^{-1} solution of retinoic acid in dimethyl sulphoxide (DMSO), rinsing them twice in distilled water, and allowing them to dry. This procedure was adapted from Eichele, Tickle & Alberts (1984) with the intent of maximizing the amount of retinoic acid on the bead. Control beads were prepared in an identical manner except that beads were soaked in DMSO without retinoic acid. Beads of about 125 μm in diameter were selected and implanted into the proximal half of both hindlimb buds of tadpoles at stages 50, 51, and 52. Implantation was achieved by puncturing a hole in the limb bud with a sharp tungsten needle, enlarging the hole with one tine of fine watchmakers forceps, and then pushing the bead into the tunnel using a glass micropipette with an outside diameter of about that of the bead. About one half of the right hindlimb bud was then amputated using fine scissors and tungsten needles.

The tadpoles were reanaesthetized and fixed after limb development or regeneration was complete at stage 59 or later. The entire tadpole was fixed in neutral buffered formalin, stained with Victoria Blue B, and cleared in methyl salicylate using a method similar to that of Bryant & Iten (1974). The blue-stained skeletal structures were then examined and analysed.

RESULTS

The normal limb skeleton of *Xenopus laevis* tadpoles has been described previously (Scadding & Maden, 1986*b*) and is the basis for comparison and evaluation of the differences noted in regenerating and retinoic-acid-treated limbs (Fig. 1A). Untreated right hindlimbs amputated at stage 50 to 52 regenerated skeletal patterns essentially the same as seen in intact limbs. However loss of one, two, or three phalanges was occasionally observed especially at stage 52. Hence, limbs were arbitrarily defined as 'normal' so long as not more than three phalanges were missing. Amputation at stage 53, when the foot plate was developing, resulted in regenerates which were always slightly hypomorphic, usually missing one complete digit as well as several phalanges. Amputation at stage 54, when digital indentations first became apparent in the foot plate, resulted in regenerates which had four digits at best, and often had defects of the leg as well as those in the foot (Table 1).

Table 1 shows the results of retinoic acid implantation in silastin blocks into developing and regenerating hindlimbs at three different stages (as well as the results of control experiments). The results are categorized as: normal (normal limb development or regeneration) (Fig. 1A), leg defects (absence or shortening of femur, tibia–fibula, or tibiale–fibulare) (Fig. 1B,D), foot defects (absence or irregularities of skeletal elements of the foot varying from loss of four phalanges to cases of extremely hypomorphic feet), both leg and foot defects (Fig. 1C,F,G), or complete absence of regeneration (Fig. 1G). Data are the number of hindlimbs observed in each category. Note that in each case the implantation of a silastin block alone has no effect when compared to limbs with no implant. The only

exceptions are two cases at stage 52 when the physical presence of the silastin block appears to have interfered with the development of one leg bone when it came to lie directly in the path of the developing bone. In developing left hindlimbs at all these stages, the retinoic acid implants clearly caused reductions or deletions of skeletal elements. Similarly, reductions were seen in regenerating right hindlimbs at stages 52 and 53, and occasionally complete inhibition of regeneration was also observed. At stage 54, the defects induced by retinoic acid are masked by the fact that even control limbs regenerate defectively at this stage. However, the defects observed did tend to be more severe in regenerating limbs with retinoic acid implants than in controls.

At each of stages 50, 51, and 52, nine to twelve *Xenopus* tadpoles were implanted with AG1-X2 beads into both hindlimbs, and then had the right hindlimb amputated. All 63 control implanted limbs developed or regenerated normally with the exception of one intact left hindlimb implanted at stage 52 which developed six digits. Of 29 intact left hindlimbs implanted with beads loaded with retinoic acid, all but one developed normally. This one specimen had a shortened femur in an otherwise normal limb. Of 22 amputated right hindlimbs implanted

Fig. 1. (A) Lateral view of a left hindlimb of a tadpole implanted with a control silastin block (arrowhead) at stage 52. Limb development was completely normal. The limb consisted of femur (*Fe*), fusing tibia (*T*) and fibula (*F*), tibiale (*t*) and fibulare (*f*), five tarsals (*ta*) (of which only three are visible here), five metatarsals (*mt*), and 14 phalanges arranged on digits I through V in the formula 2-2-3-4-3. Digit V (*V*) is dorsal (on right in photomicrograph). ×8. (B) Ventral view of the hindlimbs of a tadpole implanted with retinoic acid silastin blocks (arrowheads) at stage 52. In both the developing left (*L*) hindlimb and regenerating right (*R*) hindlimb, the femur and tibia–fibula were quite defective, and yet both feet were entirely normal. ×8. (C) Ventral view of the hindlimbs of a tadpole implanted with retinoic acid silastin blocks at stage 52. The left (*L*) developing hindlimb lacked distal femur, tibia–fibula, and proximal parts of tibiale and fibulare. Foot was moderately hypomorphic as well, with only three digits. The right (*R*) regenerating limb had a defective regenerate in which most of the leg was absent and yet the regenerated foot possessed 13 digits forming a ring as they emerge from an irregular set of tarsals. ×8. (D) Lateral view of a left hindlimb of a tadpole implanted with a retinoic acid silastin block at stage 53. The implant (arrowhead) came to lie in the region of the prospective fibula leading to the complete deletion of the element. The rest of the limb including the tibia (*T*) was apparently normal. Digit V (*V*) is dorsal. ×9. (E) Lateral view of a regenerated right hindlimb of a tadpole implanted with a retinoic acid silastin block (arrowhead) at stage 54. The leg was quite defective with a shortened femur, and completely irregular tibia–fibula and tibiale–fibulare. The four-digit hand was quite typical of regeneration subsequent to amputation at stage 54. Digit V (*V*) is dorsal. ×13. (F) Ventral view of the hindlimbs of a tadpole implanted with retinoic acid silastin blocks (arrowhead) at stage 52. In the developing left (*L*) hindlimb the femur, tibia–fibula, and tibiale–fibulare were largely fused into a continuous bent cartilage rod ending in a slightly hypomorphic four-digit foot. The right (*R*) hindlimb regenerate was completely hypomorphic and ended in a single digit. ×7. (G) Ventral view of the hindlimb of a tadpole implanted with retinoic acid silastin blocks at stage 53. The left (*L*) developing limb is greatly reduced with only two digits attached to the end of the femur. The right (*R*) regenerating limb has completely failed to regenerate. Only a V-shaped piece of cartilage is present beyond the femur and this probably represents a deformed stump tibia–fibula. ×10.

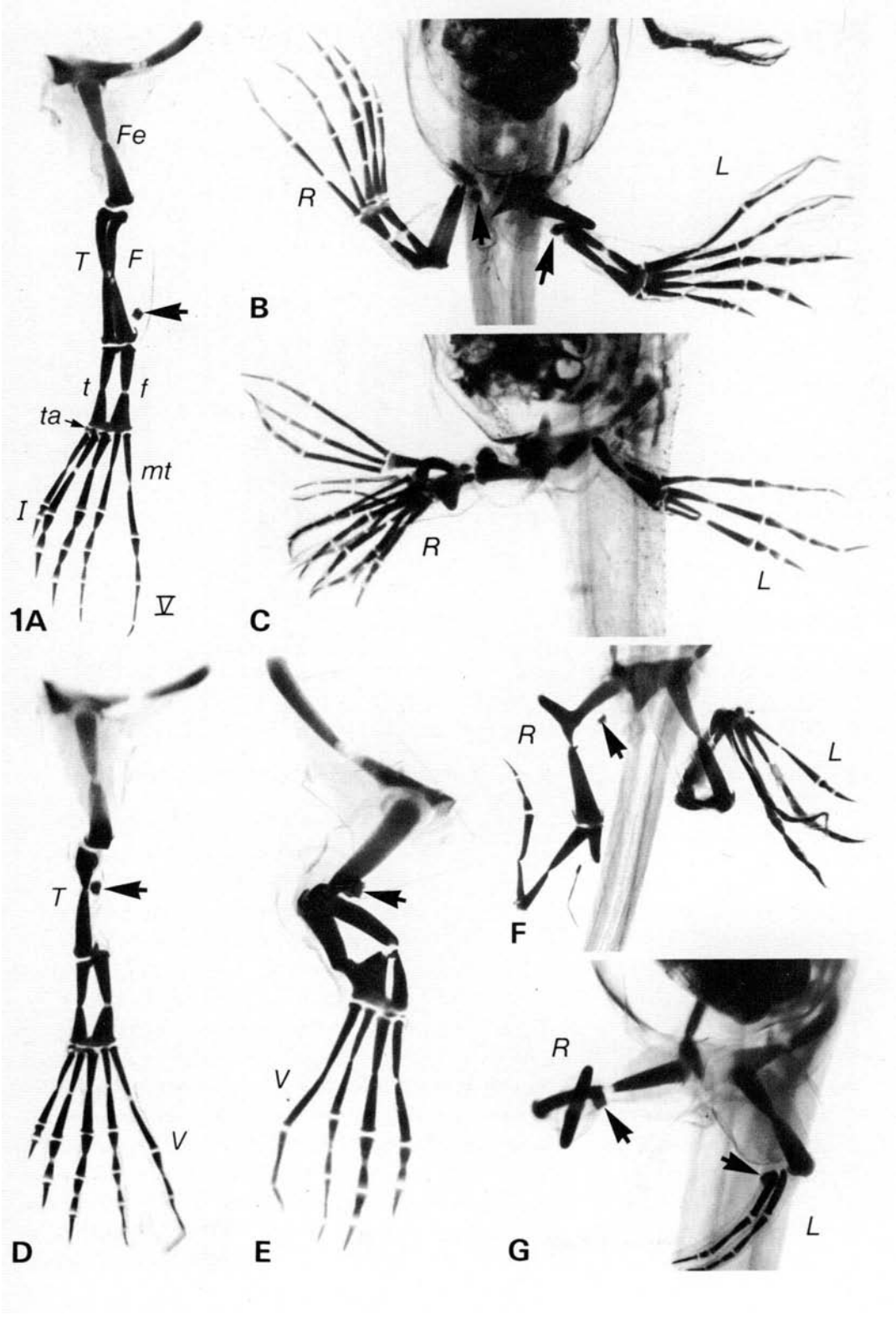
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Table 1. *Effects of silastin implants on developing left hindlimbs and regenerating right hindlimbs*

	Stage 52						Stage 53						Stage 54					
	Left devel.			Right regen.			Left devel.			Right regen.			Left devel.			Right regen.		
Classes of results	Control – no implant	Control – silastin only implant	Retinoic acid implant	Control – no implant	Control – silastin only implant	Retinoic acid implant	Control – no implant	Control – silastin only implant	Retinoic acid implant	Control – no implant	Control – silastin only implant	Retinoic acid implant	Control – no implant	Control – silastin only implant	Retinoic acid implant	Control – no implant	Control – silastin only implant	Retinoic acid implant
Normal	11	10	—	11	10	—	14	6	—	2	3	2	14	17	8	—	—	—
Leg defects only	—	1	5*	—	1	7†	—	—	11‡	—	—	3	—	—	11	1	—	1
Leg and foot defects	—	—	5	—	—	1	—	—	5	—	—	6	—	—	—	2	4	7
Foot defects only	—	—	—	—	—	1	—	—	—	11	2	—	—	—	—	8	13	7
Regeneration absent	—	—	—	—	—	2	—	—	—	—	—	6	—	—	—	2	—	4
Totals	11	11	10	11	11	11	14	6	16	13	5	17	14	17	19	13	17	19

* One of these cases had a small accessory outgrowth consisting of two cartilages.
† One of these in addition to leg defects exhibited 13 digits (Fig. 1C).
‡ One of these specimens had in addition an accessory limb, with phocomelic leg and seven digits, emerging from the mid-ventral pelvic girdle.

with retinoic-acid-loaded beads at stage 51 or 52, all but one regenerated normally. This one exception had a slightly hypomorphic hand with only four digits. The only group in which the retinoic-acid-loaded beads seemed to have any significant effect was that in which the right hindlimbs were amputated and implanted at stage 50. Of eight specimens in this group, four regenerated normally, one had an extremely hypomorphic hand consisting of four irregular digits, two exhibited complete inhibition of regeneration, and one was normal, but an accessory limb with a reduced femur and seven digits developed from an accessory pelvic girdle (Fig. 2).

The stage-50 hindlimb buds averaged about 0·4 mm wide by 0·5 mm long. By stage 51, they were about 0·5 mm wide by 0·8 mm long, and by stage 52 were 0·6 mm wide by 1·2 mm long. The silastin implants were 0·2×0·2×0·2 mm, consequently attempts to implant them into limbs at stages 50 or 51 resulted in extensive destruction of the limb bud. Our results suggest that the AG1-X2 beads

at 0·125 mm diameter, carried too little retinoic acid to have any significant effect later than stage 50.

DISCUSSION

The results demonstrated that the mode of administration of vitamin A can have a profound influence on its effects. Retinoids administered by immersing *Xenopus* tadpoles in a solution, frequently caused PD duplications (Scadding & Maden, 1986*b*), while silastin implant administration caused no PD duplications and only rarely caused AP duplications. Vitamin A administered by silastin implants tended to cause more highly localized defects than when administered by immersion. This is likely due to a gradient of the retinoid, set up in the vicinity of the implant with an extremely high concentration of retinoid immediately adjacent to the implant with rapidly decreasing levels at greater distances from it. The results point out the necessity for very detailed and extensive studies of vitamin A effects, since effects vary qualitatively with variation in concentrations or mode of administration. For example, if we had only the present results, without our previous observations on tadpoles immersed in retinol palmitate, we might have concluded that retinoids did not cause duplication in *Xenopus*, since similar retinoic acid silastin implants do cause duplications in regenerating axolotl limbs. The mode of administration of vitamin A is clearly critical. The great difference in responses between individual

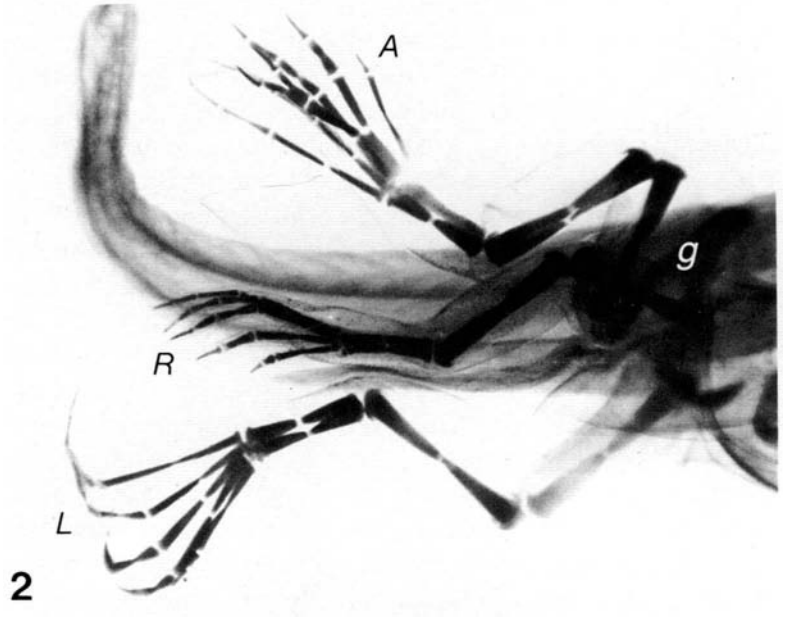

Fig. 2. Ventral view of the hindlimb region of a tadpole implanted with retinoic acid ion exchange beads at stage 50. The left developing hindlimb (*L*) is essentially normal as is the regenerated right hindlimb (*R*). The accessory limb (*A*) arises from an accessory pelvic girdle (*g*). In the accessory limb, the zeugopodium consists of three fused rods (tibia–fibula–tibia?) and the foot has seven somewhat irregular digits. ×9.

animals receiving identical retinoid treatments, noted both here and in previous studies (Scadding, 1983; Scadding & Maden, 1986*b*), suggests that genetic or physiological factors may significantly modify the response of an animal to vitamin A.

Since the available retinoic acid in either a silastin implant or ion exchange bead is largely gone within 3 days of implantation (Maden *et al.* 1985; Eichelle *et al.* 1984), it may be that the treatment is simply not long enough to induce PD duplications. In *Xenopus*, no duplications were induced after a 1-day immersion in retinol palmitate and very few after 3 days. 14 days was the optimal immersion treatment period for induction of PD duplications (Scadding & Maden, 1986*b*).

The fact that silastin and bead implant techniques have induced duplications in regenerating axolotl limbs or developing chick limbs and yet not in *Xenopus* tadpoles, may be related to the time course of development or regeneration. Chick limb buds develop over a period of two weeks, axolotl limbs regenerate over a period of about four to six weeks, while *Xenopus* limbs typically take about two months to develop or regenerate (although this period is extremely variable). It may be that cellular processes are much slower in *Xenopus* and that cells require a much longer exposure to vitamin A for it to have any effect.

The total amount of retinoic acid delivered to the limb bud via the silastin implants was about 1 μg. The much lower dose provided by the beads, was probably in the nanogram range (Eichele *et al.* 1984), evidently too low to have any effect except in the smallest limb buds at stage 50. However, the effects observed at stage 50 confirm that retinoic acid was indeed present in the ion exchange bead implants. It seems likely that *Xenopus* cells are much less sensitive to retinoic acid than chick embryo cells. In the chick embryo, ion exchange bead implants in the limb can cause beak defects at a considerable distance from the implant site (Tamarin, Crawley, Lee & Tickle, 1984).

It was impossible to prepare silastin blocks containing retinoic acid at sizes smaller than the 200 μm cubes used here, since the 'holes' in the silastin which contained the retinoic acid, were then relatively large compared to the size of the block. Thus, silastin blocks cannot be used to administer retinoic acid prior to stage 52 since the block is too large to implant into the limb bud without extensive damage to it.

We had previously suggested a hypothesis which suggested that wounding was a factor in the initiation of duplications since in all cases where duplications are produced wounding was a prior condition, i.e. wounding the chick wing bud by implant administration of retinoids or wounding the amphibian limb by amputation (Scadding & Maden, 1986*b*). We had intended this investigation to be a test of this hypothesis, since the developing *Xenopus* limbs would receive a significant wound by the implant procedure. However, no duplications were induced in developing limbs. This could be interpreted as evidence against the hypothesis, but the implant administration procedure also failed to induce PD duplications in regenerating limbs. Hence, further testing of this hypothesis is necessary.

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