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Stage-dependent effects of retinoic acid on regenerating urodele limbs

Iqbal A. Niazi[1], **Maurice J. Pescitelli**[2] **and David L. Stocum**[2]

[1] Developmental Biology Laboratory, Department of Zoology, University of Rajasthan, Jaipur 302004, India
[2] University of Illinois, Department of Genetics and Development, 515 Morrill Hall, 505 South Goodwin Avenue Urbans, IL 61801, USA

Summary. Following amputation through the distal zeugopodium, regenerating limbs of larval *Ambystoma mexicanum* and pre and post-metamorphic *Pleurodeles waltlii* were treated with 150 μg of retinoic acid (RA) per gram of body weight, at the dedifferentiation, early bud, medium bud, late bud or early redifferentiation stages of regeneration. The effect of RA on regenerate morphogenesis differed as a function of the stage at which it was administered. When given during dedifferentiation or at early bud stages, RA evoked proximodistal duplications of stump segments in the regenerates. The maximum duplication index (DI) in *Abystoma* was achieved when RA was injected at 4 days post-amputation, which corresponds to the stage of dedifferentiation; and in *Pleurodeles* at 10 days post-amputation, which corresponds to a stage midway between early bud and medium bud. When RA was administered at later stages, the DI declined progressively to zero or nearly zero by the stage of early redifferentiation in both species. The decline in DI was due to a decreased frequency of duplication, not to a decrease in the magnitude of duplication in individual regenerates. At the same time, there was an increase in hypomorphism and aberrant morphogenesis of both duplicating and non-duplicating regenerates. These results indicate that regenerative cells are differentially sensitive to RA in a stage-dependent way.

Key words: Retinoid – Limb regeneration – Pattern effects

Introduction

Vitamin A is a lipid molecule essential for normal growth, maintenance of cell differentiation, reproduction and vision in vertebrates. Derivatives of vitamin A, collectively called retinoids, have a variety of interesting teratogenic effects on embryonic cells that suggest their value in probing the cellular and molecular basis of development. Among these effects are: (1) the conversion of keratinizing epidermis to mucus epidermis (Fell 1957); (2) the differentiation of teratocarcinoma cells into endodermal cells (Strickland and Mahdavi 1978); (3) the alteration of adhesive and growth properties (Lotan 1980; Jetten 1984); and (4) the modification of glysoaminoglycan and glycoprotein synthesis (Lotan 1980; Elias et al. 1983; Levin et al. 1983; Robinson et al. 1983).

Retinoids have profound effects on the morphogenesis of embryonic tissues. They modify the pattern of mouse vibrissae follicles to a mucus gland pattern (Hardy 1983), and the pattern of chick scale epidermis to feather epidermis (Dhouailly et al. 1980). In mouse limb buds, retinoids cause phocomelia and micromelia when given at the initial stages of chondrogenesis (Kochhar 1973, 1977). Likewise, they inhibit further development of the feet in anuran hindlimbs when administered after chondrogenesis of the foot has begun (Jangir and Niazi 1978; Niazi and Ratnasamy 1984).

Some of the most interesting effects of retinoids on developing systems are seen in regenerating amphibian limbs. Regenerating anuran hindlimbs exhibit stage-dependent responses to retinoids. If tadpole hindlimbs, amputated through the distal zeugopodium, are treated with retinoid during dedifferentiation or blastema formation, the blastema cells are proximalized and the regenerates form proximodistal duplications of stump segments (Niazi and Saxena 1978; Jangir and Niazi 1978; Niazi and Alam 1984; Niazi and Ratnasamy 1984). As in the mouse or anuran limb bud, retinoids inhibit morphogenesis when given to regenerates initiating chondrogenesis (Jangir and Niazi 1978).

Regenerating urodele fore or hindlimbs amputated through the zeugopodium also duplicate in the proximodistal axis when treated with retinoids during stages of dedifferentiation and blastema formation (Maden 1982; Thoms and Stocum 1984). There is some indication that the response to retinoids of urodele regeneration blastemas initiating morphogenesis and redifferentiation is similar to that of the anuran regeneration blastema. Maden (1983b) noted a decreased frequency of duplication and an increased frequency of hypomorphism in axolotl regenerates treated after 8 days postamputation. However, a systematic study of the effects of retinoids on different morphological stages of urodele regeneration has not been made. Such studies are important to determine whether these effects parallel those observed at similar stages of mouse and anuran limb buds, and to provide a data base from which to investigate their cellular and molecular aspects. Hence, the purpose of this report is to describe the morphogenetic effects of retinoids on several stages of regeneration in two urodele species, the axolotl (*Ambystoma mexicanum*), and the Iberian newt, (*Pleurodeles waltlii*).

Offprint requests to: D.L. Stocum at the above address

Methods and materials

Animals and maintenance. Larval axolotls were obtained from laboratory spawnings or from the Indiana University axolotl colony. *Pleurodeles* were raised from embryos provided by Mr. Warren Fox, University of California at Irvine, USA. All animals were raised individually at room temperature (21–23°) in wax-paper cups containing 20% Holtfreter solution, and fed freshly-hatched brine shrimp until old enough to feed on adult, frozen brine shrimp.

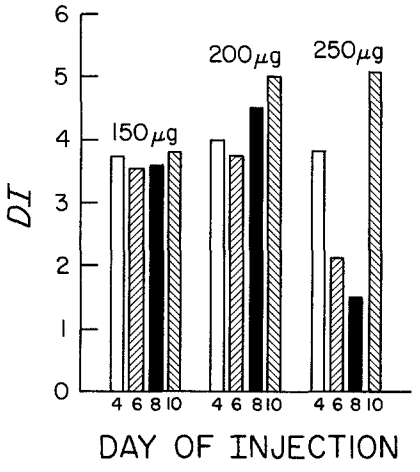


Fig. 1. Effects of administering 150, 200, and 250 μg RA/gm body wt. to metamorphosing *Pleurodeles* at 4, 6, 8, or 10 days postamputation through the distal zeugopodium. The maximum duplication index was obtained with the 250 μg dose at 10 days post-amputation. When animal survival was considered, however, the optimal dose for creating PD duplication was 150 μg

Table 1. Percent survival of *P. waltlii* amputated bilaterally through the distal zeugopodium of the forelimb and injected with various doses of RA at 4, 6, 8 or 10 days post-amputation. Numbers in parentheses indicate number of animals surviving/total number of animals

	Days post-amputation			
Dose	4	6	8	10
150	75 (9/12)	75 (12/16)	50 (6/12)	67 (8/12)
200	88 (6/7)	44 (8/18)	33 (4/12)	17 (2/12)
250	43 (3/7)	29 (5/17)	30 (3/10)	45 (5/11)

Amputation and rate of regeneration. Forelimbs were amputated bilaterally through the distal zeugopodium. At the time of amputation, the axolotl larvae were 40–50 mm in length. *Pleurodeles* were the same length, but were amputated shortly before metamorphosis, during metamorphosis, or shortly after metamorphosis. The different amputation times had no noticable effect on the time taken to

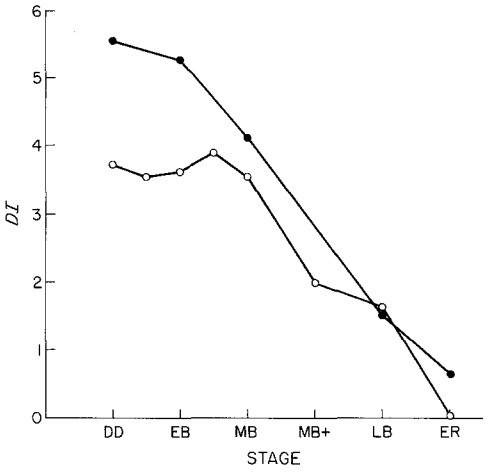


Fig. 2. Duplication index (DI) of axolotl (*closed circles*) and *Pleurodeles* (*open circles*) regenerates, resulting after injection of 150 μg RA/gm body wt. at the stages of *DD* (dedifferentiation), *EB* (early bud), *MB* (medium bud), *LB* (late bud), and *ER* (early redifferentiation). In axolotls, the DI is maximum at the DD and EB stages, and in *Pleurodeles* is maximum at 10 days post-amputation, between the *EB* and *MB* stages

Table 2. Frequency (f), mean degree of duplication (DD) and duplication index (DI) for limbs treated with 150 μg RA/gm body wt. at various stages of regeneration. DD = dedifferentiation; EB = early bud; MB = medium bud; LB = late bud; ER = early redifferentiation. N = number of regenerates

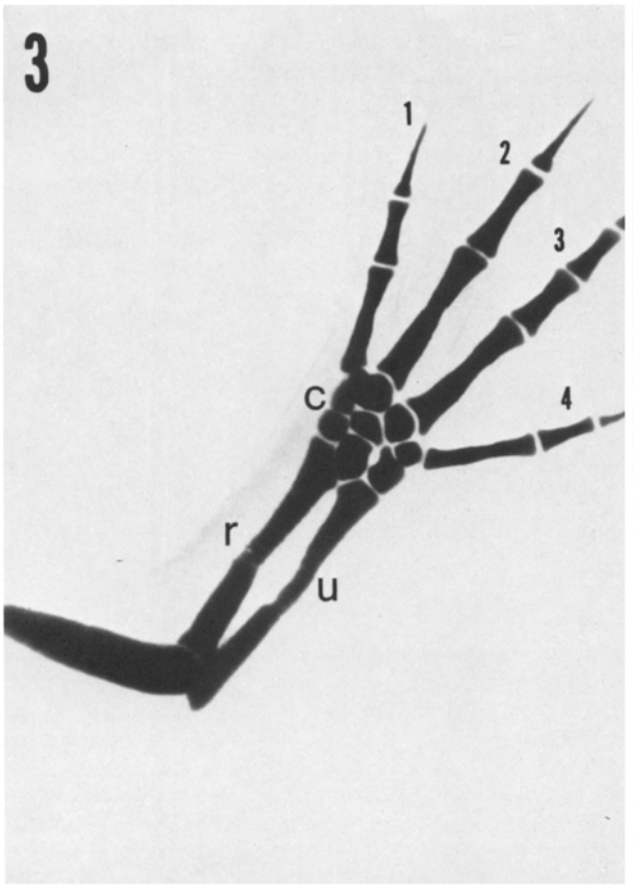
	Axolotls				Pleurodeles			
Stage	N	f	DD	DI	N	f	DD	DI
DD	17	1.00	5.52	5.52	28	0.85	3.57	3.03
EB	12	1.00	5.25	5.25	28	0.91	3.71	3.38
MB	10	0.70	5.85	4.10	7	0.85	4.17	3.54
MB+	–	–	–	–	12	0.58	3.42	1.98
LB	14	0.29	5.25	1.50	13	0.38	4.25	1.63
ER	14	0.14	4.50	0.64	21	0.00	0.00	0.00

Fig. 3. Control axolotl regenerate, left forelimb. r = radius, u = ulna, c = carpals, 1–4 = anteroposterior order of digits. Each digit is composed of a basal metacarpal, and phalanges. The first, second and fourth digits have two phalanges, and the third digit has three phalanges. × 60

Fig. 4. Control *Pleurodeles* right (*A, B, D*) and left (*C, E*) forelimb regenerates. r = radius, u = ulna, c = carpals, 1–4- = anteroposterior order of digits. Each digit is composed of a basal metacarpal and phalanges. Digits one, two and four each have two phalanges, and digit three has three phalanges. The normal number of carpals is seven, but incomplete segregation of carpals during regeneration usually reduces this number to five or six. × 30

Fig. 5. Right forelimb regenerate of an axolotl injected with RA at the EB stage. The *arrow* indicates the regenerate-stump junction, beyond which a partial shoulder girdle (*g*), humerus (*h*), radius (*r*) and ulna (*u*) were duplicated. Note that the girdle is fused with the distal end of the stump radius, and the regenerate extends straight out from the stump. × 60

Fig. 6. Right (*R*) and left (*L*) forelimb regenerates of an axolotl injected with RA at the MB stage. The arrows indicate the regenerate-stump junction. The most proximal element duplicated in both limbs was a partial shoulder girdle (*g*), which is fused to the stump radius. The regenerates are oriented at a 90° angle to the stump. In the right limb, the stump radius and ulna regressed almost to the elbow joint. × 60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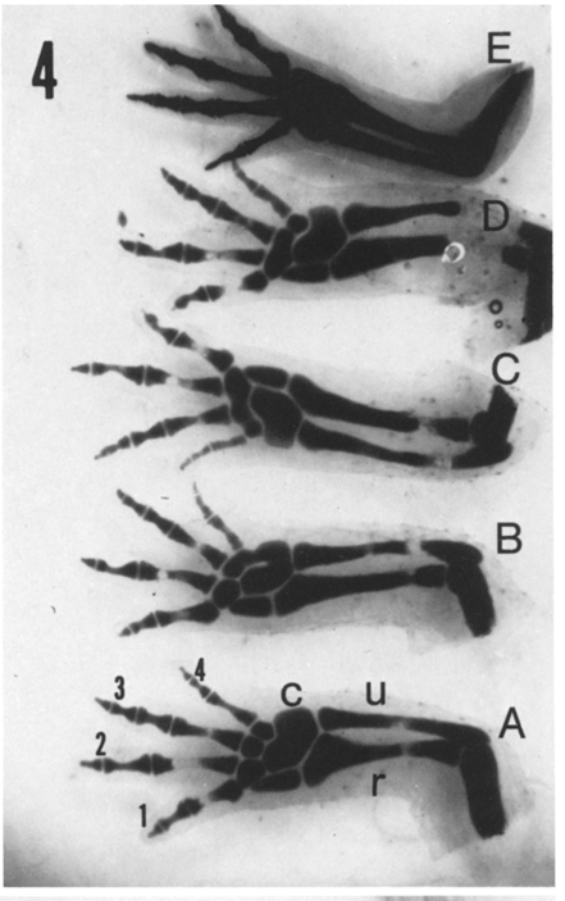
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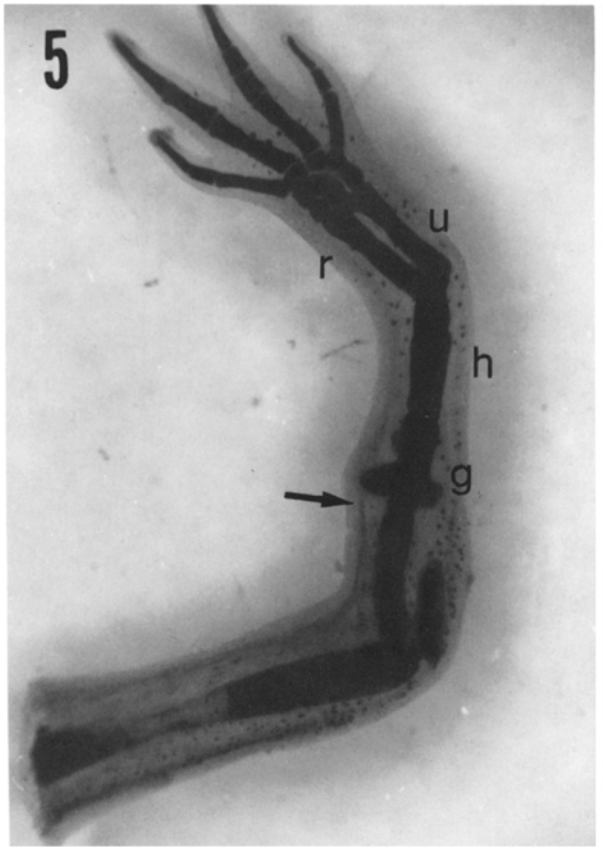
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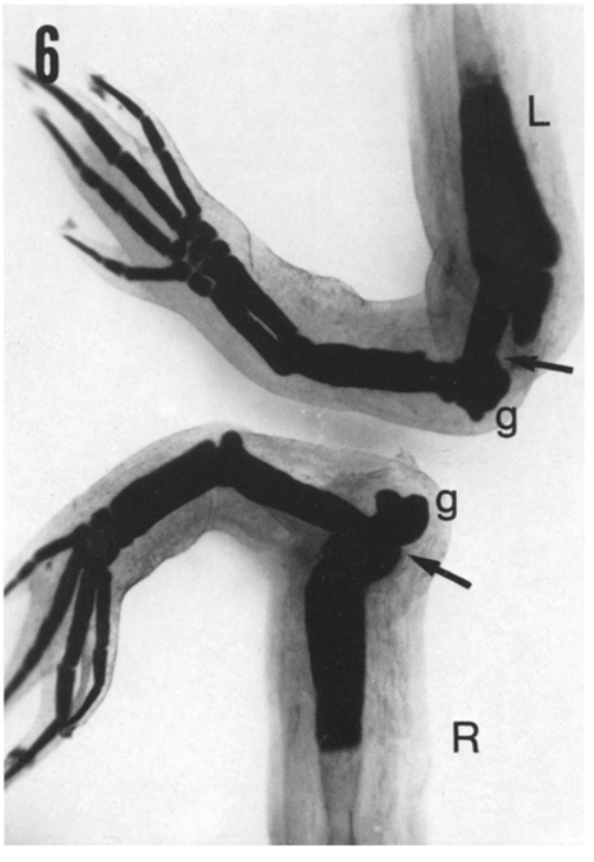
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reach a given stage of regeneration. However, the rates of regeneration were different in the two species. An early bud blastema formed on axolotl limbs by 5–6 days post-amputation, and by 8–9 days on *Pleurodeles* limbs (staging according to Stocum 1979). Later stages were also attained earlier in axolotls.

Administration of retinoic acid. All-trans retinoic acid (RA) (Sigma Chemical Co.) was dissolved under subdued light (to prevent photoxidation) in dimethyl sulfoxide (DMSO) at a concentration of 150 μg/μl, and aliquots of the solution stored frozen. RA was administered to animals via a single intraperitoneal injection, using a microliter syringe (Thoms and Stocum 1984). After injection, the RA precipitated in the body cavity, where it gradually dissolved, probably due to complexing with serum retinoid-binding proteins (see Lotan 1980).

Experiments

1. Dose-response relationships in Pleurodeles. Our observations have indicated that maximum duplication in regenerating axolotl forelimbs is achieved at a dose of 100–150 μg RA/gm body wt. (Kim and Stocum, unpublished observations). No reports are available concerning the effects of retinoids on regenerating *Pleurodeles* limbs. To determine the dose which evoked the maximum duplication in our *Pleurodeles,* groups of animals were amputated and injected with 150, 200 and 250 μg RA/gm body wt. on days 4, 6, 8 and 10 postamputation. Days 4–8 cover the dedifferentiation to early bud stages, and day 10 represents a stage between early and medium bud. Controls consisted of animals regenerating for the same periods injected with the maximum amount of DMSO used to deliver the RA.

2. Injection of a standard dose of RA at different stages of regeneration. The most effective dose producing proximodistal (PD) duplications in *Pleurodeles* was found to be 150 μg RA/gm body wt. (see Results). Hence, 150 μg RA/gm body wt. was injected into groups of both axolotls and *Pleurodeles* whose limbs had regenerated to the stage of dedifferentiation, early bud, medium bud, late bud, or early redifferentiation. Since DMSO had no discernable effect on regenerate morphology in the *Pleurodeles* controls of the dose-response experiment, nor does it have an effect on regenerates of axolotls or *Notophthalmus* (Thoms and Stocum 1984), the controls for this experiment consisted of untreated regenerates of each stage allowed to continue regeneration.

Morphological analysis of regenerates. All regenerates were allowed to develop for 40–45 days. They were then fixed in Bouin's or Gregg's fixative, stained in Victoria Blue B, and cleared in methyl salicylate to reveal the skeletal elements.

Individual limbs exhibiting duplication were scored for their *degree of duplication* (i.e., the most proximal element duplicated) as follows (Maden 1983b): 1 = duplicated carpals; 2 = duplication of a partial zeugopodium; 3 = duplication of a complete zeugopodium; 4 = duplication of a partial stylopodium; 5 = duplication of a complete stylopodium; 6 = duplication of a shoulder girdle. The mean degree of duplication (DD) for a given stage was then calculated by adding the scores and dividing by the number of duplicated limbs. Some limbs did not duplicate, so a second parameter measured was the *frequency* of duplication (f), calculated as the fraction of limbs of a given stage which exhibited duplication. Finally, the *duplication index* (DI) was defined as the product of DD × f.

Results

Dose-response relationships in Pleurodeles. Figure 1 shows that, in *Pleurodeles,* the DI varied little with dose when RA was given at 4 or 6 days post-amputation, except for a decrease at 6 days with the 250 μg dose. At 8 days post-amputation, DI increased considerably with increase in dose from 150 to 200 μg, but fell to a low value at 250 μg. This low value was due to the fact that only 3 of 10 animals survived at the 8 day/250 μg dose (see Table 1), and only 2 of the 6 forelimbs of the survivors duplicated. Only at 10 days was there a progressive increase in DI with increasing dose, this increase being very small between the 200 and 250 μg doses. Thus, the maximum DI was obtained at 10 days postamputation with a 250 μg dose. However, Table 1 shows that the percent survival of the animals on all days post-amputation tested was maximal (50–75%) at the 150 μg dose, decreasing sharply at 200 and 250 μg. Balancing DI with survival, the 150 μg dose seemed best to use for comparison of the effects of RA at different stages of regeneration.

Effects of standard dose on different stages of regeneration

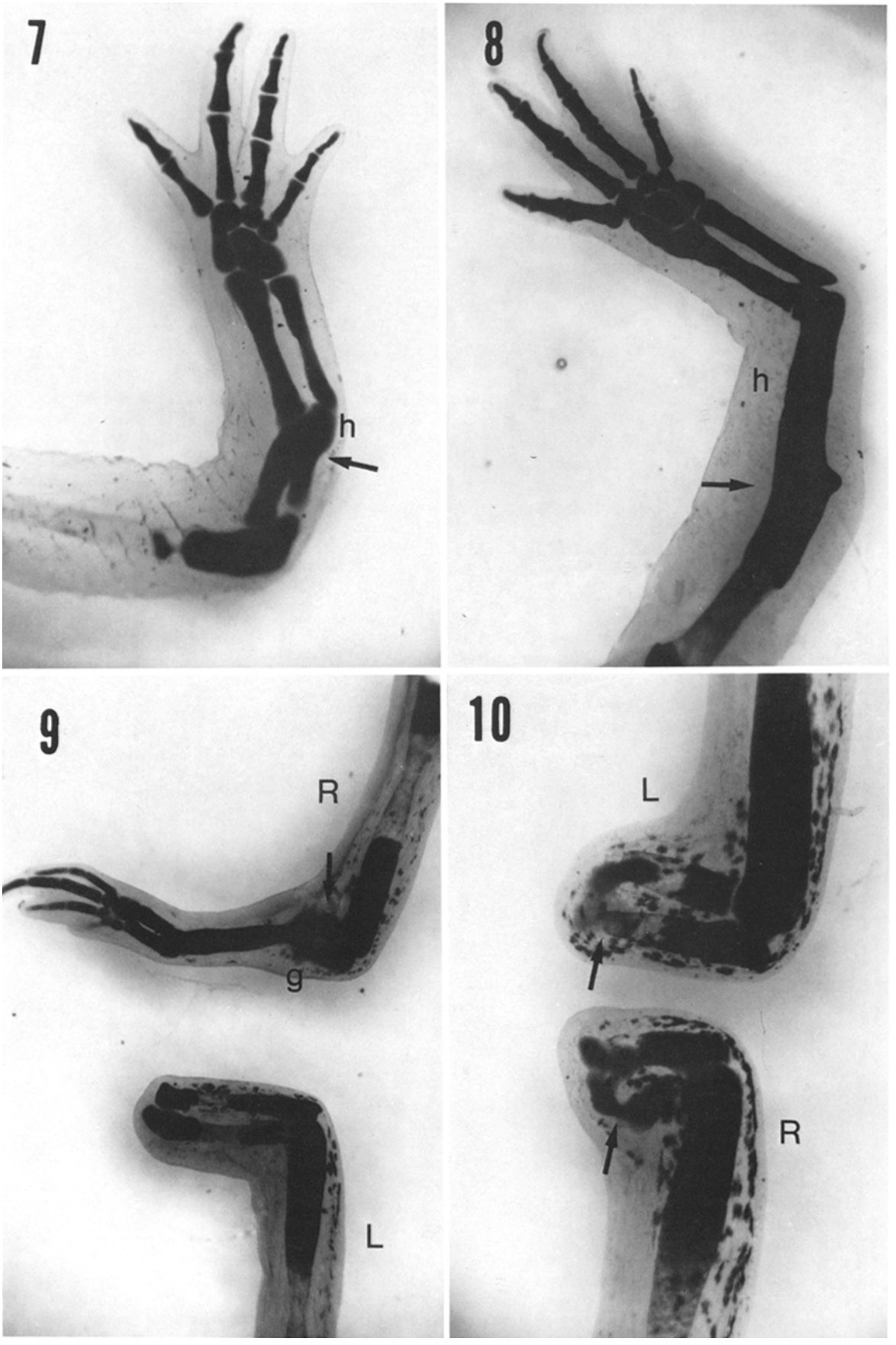
Figure 2 plots the DI for the different stages of regeneration after treatment with 150 μg RA/gm body wt. For both species, the maximum DI was elicited during stages of dedifferentiation and early blastema formation.

Fig. 7. *Pleurodeles* right forelimb regenerate after RA treatment at the EB stage. The *arrow* indicates the regenerate-stump junction. The most proximal element duplicated was the distal end of the humerus (*h*), which is fused to the stump ulna. Partial dedifferentiation without regression apparently took place in the stump radius and ulna, since they are completely cartilaginous. × 60

Fig. 8. Ventral view of a *Pleurodeles* left limb regenerate, treated with RA at the MB stage. The *arrow* indicates the regenerate-stump junction. The limb stump regressed up to the humerus, and the most proximal element duplicated by the regenerate was the humerus (*h*). × 60

Fig. 9. Right (*R*) and left (*L*) forelimb regenerates of an axolotl treated with RA at the MB stage. The *arrow* indicates the regenerate-stump junction in the right limb, in which a partial shoulder girdle (*g*) was the most proximal element duplicated. The stump radius and ulna regressed nearly to the elbow. Regeneration was inhibited in the left limb. × 60

Fig. 10. Right (*R*) and left (*L*) forelimb regenerates of an axolotl injected with RA at the ER stage. The *arrows* indicate the regenerate-stump junction. No duplication occurred, and the hand is represented only by 2–3 carpal-like elements. × 125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In axolotls, the peak DI was achieved when RA was injected at 4 days post-amputation (during dedifferentiation), or at the early bud stage. In *Pleurodeles,* the DI was highest from the dedifferentiation to medium bud stages, peaking at 10 days, between early and medium bud. Beyond early bud in axolotls, and medium bud in *Pleurodeles,* the DI declined steadily up through early redifferentiation, during blastemal growth and initiation of morphogenesis and redifferentiation. Control regenerates developed normally, forming the distal radius-ulna and hand.

Table 2 compares the frequency of duplication, degree of duplication and DI after RA treatment at different stages of regeneration. The data show that the decrease in DI resulting after treatment beyond the peak duplication stages is due to a decrease in the *frequency* of duplication, the degree of duplication varying little from stage to stage.

Figures 3 and 4 illustrate examples of the morphology of control regenerates and Figs. 5–14 illustrate examples of duplicated and non-duplicated regenerates produced after RA treatment. Shoulder girdles were duplicated only infrequently in *Pleurodeles* regenerates, but were duplicated with high frequency (50–65% of the cases at dedifferentiation to medium bud stages) in axolotl regenerates (Figs. 5 and 6). The girdles were always small cartilage nodules with a glenoid fossa.

In those cases in which the most proximal elements duplicated were the radius and ulna, these elements were a direct extension of the stump radius and ulna, or formed a joint with the stump elements. In cases where the most proximal element duplicated was a humerus or shoulder girdle, the regenerate was usually oriented at an angle to the stump (Fig. 6), but sometimes extended more or less straight from the stump (Figs. 5, 7 and 8). The girdles often had no connection to the stump cartilage elements, but when such a connection was present, it was the stump radius to which the duplicated girdle was fused (Figs. 5 and 6). Duplicated humeri also were usually fused with the stump radius (Fig. 8), but were occasionally fused with the ulna (Fig. 7). In some cases, the stump radius and ulna regressed prior to duplication, so that the most proximal duplicated element of the regenerate began from the humeral stump (Fig. 11).

Aside from those cases which duplicated with otherwise normal morphology (Figs. 5–8), RA-treated regenerates fell into four morphological categories: (1) blastemas that failed to redifferentiate (no regeneration); (2) normal regenerates that did not duplicate (as in controls. Figs. 3 and 4); (3) regenerates which failed to duplicate and were hypomorphic (Figs. 9–13); and (4) regenerates that duplicated, but were hypomorphic (Fig. 14). The distribution of cases among these categories is recorded for each stage in Table 3. The data show that, in both species, the incidence of hypomorphism increased rapidly from early-medium bud to early redifferentiation stages, the majority of the hypomorphic cases being found in limbs failing to duplicate. Varying degrees of hypomorphism were observed, from loss of a single digit accompanied by fused carpals (Fig. 14) to complete absence of the hand (Figs. 9, 10 and 13). Normal regenerates developed without duplication at nearly every stage in *Pleurodeles,* but no such regenerates were observed in axolotl limbs. In three cases, the blastema failed to redifferentiate, and these occurred only in axolotls.

Table 3. Percentages of the morphological classes of regenerates produced after RA treatment at various stages of regeneration. A = no regeneration; B = duplication and hypomorphic hand; C = no duplication and hypomorphic hand; D = normal regeneration; E = duplication and normal hand

	Axolotls					Pleurodeles				
Stage	A	B	C	D	E	A	B	C	D	E
DD	0	0	0	0	100	0	0	0	30	70
EB	0	17	0	0	83	0	0	0	20	80
MB	10	0	20	0	70	0	14	0	14	72
MB+	–	–	–	–	–	0	25	25	17	33
LB	14	0	57	0	29	0	30	60	0	10
ER	0	0	86	0	14	0	0	90	10	0

Overall, these data argue that the response of blastema cells to RA begins to change at the early to medium bud stage from one in which cells are proximalized and proximal-distal (PD) duplications with normal anteroposterior pattern are produced, to one in which PD duplication is inhibited and morphogenesis of normal regenerate hand pattern is hypomorphic.

Discussion

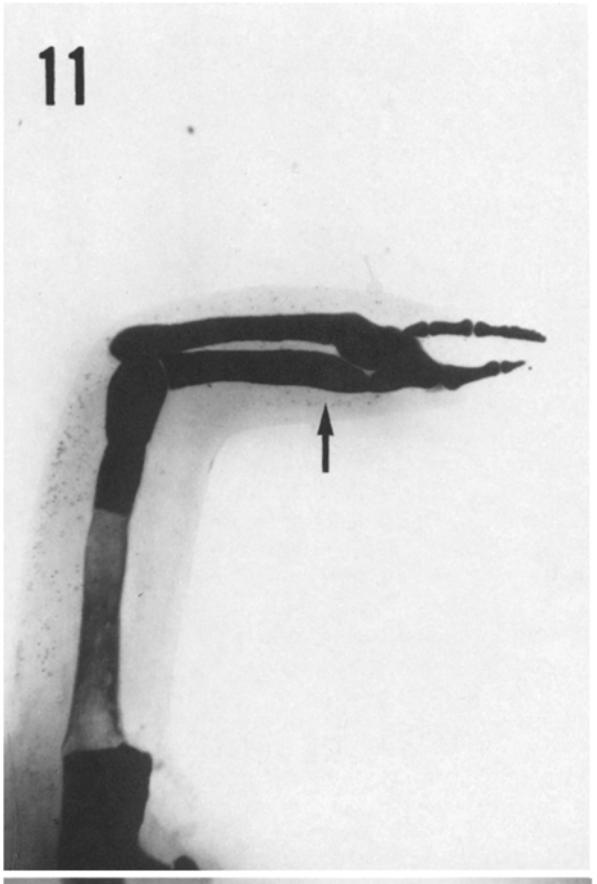
Proximodistal pattern-duplicating effects of retinoids on early limb regenerates have been previously reported in anurans (Niazi and Saxena 1978; Niazi and Alam 1984; Niazi and Ratnasamy 1984; Maden 1983a), and in urodele larvae

Fig. 11. Left forelimb regenerate of a *Pleurodeles* injected with RA at the ER stage. The *arrow* indicates the regenerate-stump junction. The regenerate did not duplicate, and the hand was hypomorphic, consisting of an unsegregated mass of carpal cartilage and two digits. × 60

Fig. 12. Hypomorphic right forelimb regenerate of a *Pleurodeles* injected with *RA* at the *ER* stage. The *arrow* indicates the regenerate-stump junction. There may have been an abortive attempt at duplication in the regenerate which resulted in two parallel masses of cartilage distal to the amputation plane. The hand is represented by a single digit. × 60

Fig. 13. Hypomorphic right (*R*) and left (*L*) forelimb regenerates of an axolotl injected with *RA* at the *ER* stage. The *arrows* indicate the regenerate-stump junction. Neither regenerate duplicated. The left regenerate formed a single mass of cartilage distal to the amputation plane. The right regenerate formed two distally fused masses of carpal cartilage, with a digit-like extension. × 125

Fig. 14. Ventral view of a left forelimb regenerate of a *Pleurodeles* injected with *RA* at the *LB* stage. The *arrow* indicates the regenerate-stump junction. The stump regressed nearly up to the mid-humerus, and the most proximal element duplicated in the regenerate was a humerus (*h*). The hand was mildly hypomorphic, lacking the first digit. The third digit has only two phalanges, instead of the normal three. × 60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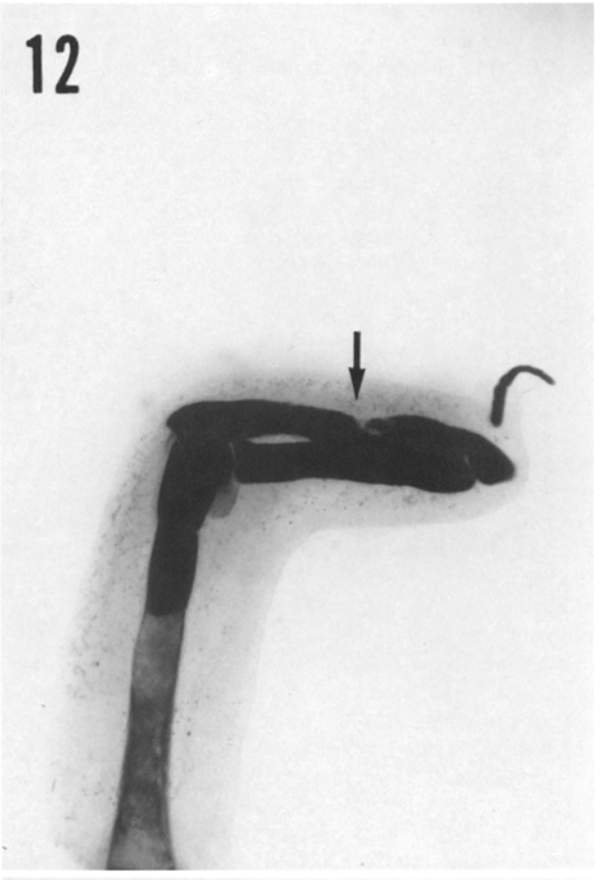
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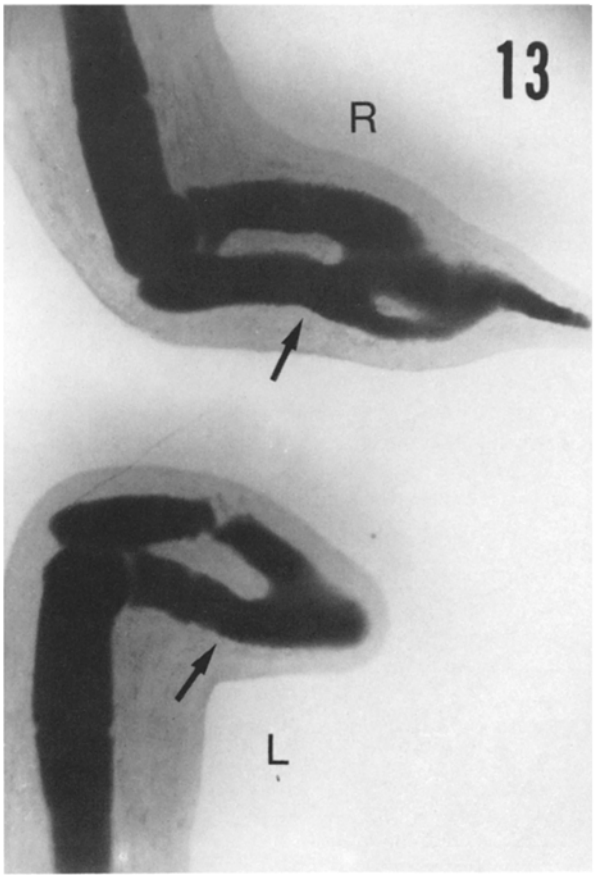
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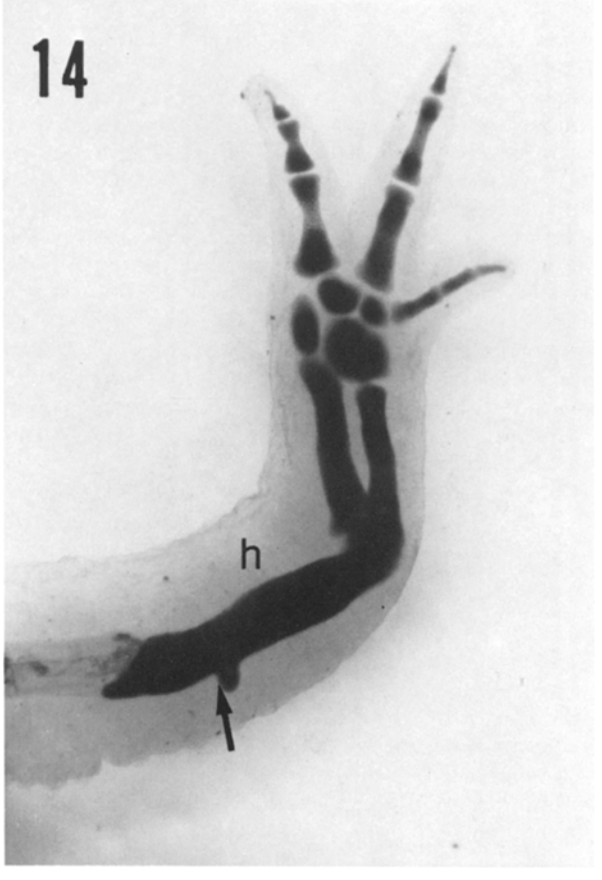
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(*Ambystoma mexicanum*; Maden 1982, 1983b; Thoms and Stocum 1984) and adults (*Notophthalmus viridescens*; Thoms and Stocum 1984). These effects can be interpreted as a resetting of positional value of blastema cells to a more proximal level (Maden 1982; Thoms and Stocum 1984). Here we have shown, for the first time, similar duplication effects of RA on early limb regenerates of a third urodele, *Pleurodeles,* treated prior to, during and just after metamorphosis. Hence, PD pattern duplication appears to be a general rule when the regenerating limbs of amphibians are treated with retinoid during stages of dedifferentiation or blastema formation.

Jangir and Niazi (1978) and Niazi and Alam (1984) showed that retinol palmitate inhibits anuran hindlimb regeneration when administered at 3 days postamputation just prior to, or during, redifferentiation, in contrast to its PD duplication effects when given prior to these times. Maden (1983b) observed a decreased frequency of duplication and an increased frequency of hypomorphism in axolotl regenerates treated with RA after 8 days postamputation. Here, we compared the effects of RA on different morphological stages of axolotl and *Pleurodeles* regenerates, since staging reduces variation in developmental state of the blastema that may be caused by variations in animal size (Connelly 1977). We found that after the EB stage in axolotls, and between the EB and MB stages in *Pleurodeles,* DI declined rapidly and the response to RA changed to one of increasing regenerate hypomorphism. By the time chondrogenesis is detectable, at the ER stage, the latter response occurred in nearly 100% of the cases. In up to 30% of the cases, at stages of regeneration where the DI was rapidly declining, RA caused duplication of stump structures while simultaneously causing hypomorphism of the hand. Although we presently do not know the reason for this mixed effect, one possible explanation might be related to the regression of the stump that was observed in some cases. Regression would lead to the production of freshly dedifferentiated cells whose positional value would be proximalized by the retinoid, resulting in duplication. At the same time, the cells of the original blastema, which would be in a more advanced developmental state, would respond to the retinoid by hypomorphic morphogenesis.

No normal regenerates were observed in RA-treated axolotl regenerates, but some normal regenerates were observed in *Pleurodeles* at nearly every stage of regeneration treated. Since DI was always lower in *Pleurodeles,* this difference might reflect a differential sensitivity of regenerate cells to retinoid in the two species, or different concentrations of retinoid circulating in the blood due to different concentrations of plasma retinoid binding proteins (see Lotan 1980). Since unamputated urodele limbs show no gross morphological effects of retinoid treatment (Thoms and Stocum 1984), we would expect that at some point between early redifferentiation and late digit stages, regenerate cells would lose their sensitivity to retinoids entirely, and normal limbs would regenerate in 100% of the cases.

Our results clearly show that urodele limb regenerates exhibit a differential sensitivity to RA that is stage-dependent, and emphasize the importance of the state of morphogenesis and differentiation of developmental systems in determining their response to a teratogen. This differential sensitivity to RA is both similar to and different from that observed in developing mammalian limbs. In the mouse, RA treatment *in utero* prior to 11 days gestation, when the forelimb is at the mesenchymal bud stage, has no morphogenetic effect on limb development, in contrast to the duplication induced in regenerating amphibian limbs. However, treatment at 12–13 days, just prior to or during the initiation of chondrogenesis, causes phocomelia and micromelia (Kochhar 1973, 1977; Kwasigroch and Kochhar 1980). After day 13, the mouse limb bud is again insensitive to RA. It is thus apparent that both embryonic mouse limb buds and regenerating amphibian limbs are most sensitive to the inhibitory effects of RA just prior to and during differentiation and redifferentiation.

The reasons for the differential sensitivity of predifferentiation and redifferentiation regenerate stages to RA are unknown, but several clues exist. Blastema cells of RA-treated regenerates which will duplicate form aggregates and strings, suggesting an increase in intercellular contacts over that of controls (Maden 1983b; Kim and Stocum unpublished results). RA prevents chondrogenesis of 10 day mouse limb bud mesenchyme in vitro, and prevents the normal decrease in cell contact and junctions associated with chondrogenesis. Furthermore, the normal loss of a 240 KD surface glycoprotein is prevented in these cells, and the normal appearance of an 84 KD surface glycoprotein is delayed (Lewis et al. 1978).

Inhibition of regeneration in anuran tadpole limbs is characterized by widespread mesenchymal necrosis (Jangir and Niazi 1978). The abnormal morphogenesis of RA-treated mouse limb buds initiating chondrogenesis is associated with cell death, depression of cell division, a very reduced Golgi, reduction of protein synthesis, and a paucity of collagen and proteoglycan in the extracellular matrix (Kochhar 1977). These effects are confined to the chondrogenic region (which is the first to differentiate), there being little or no effect on undifferentiated premyogenic or apical mesenchyme.

Based on these facts, it is plausible that the stage-dependent, differential effects of RA on limb regenerates are related to different biosynthetic activities of blastema cells prior to and during re-expression of the cartilage cell phenotype. Prior to this re-expression, these activities may not be as sensitive to the known toxic effects of retinoids, and blastema cell positional values would be modifiable. In contrast, once re-expression was initiated, RA would interfere with the biosynthetic acitivities leading to cartilage redifferentiation and normal matrix production (or degrade existing matrix). This interference would, in turn, lead to abnormal skeletal morphogenesis. We are currently investigating these possibilities via ultrastructural and immunocytochemical approaches.

The intracellular mechanism of retinoid action in producing normal and teratogenic effects on growth, morphogenesis and differentiation is unclear, but the bulk of the evidence suggests they act in a steroid-like fashion on patterns of gene expression (Liau et al. 1981; Sporn and Roberts 1983; Cope et al. 1984). For example, RA induces degradation of cultured rat limb cartilage, an effect which requires RNA and protein synthesis, since it is blocked by inhibitors of such synthesis (Gallandre and Kistler 1982). However, other studies suggest that in some cell types, retinoids may exert their effects through a calcium-activated, phospholipid-dependent protein kinase (Kraft and Anderson 1983; Kistler 1984). A molecular understanding of the stage-dependent differential effects of retinoids on limb re-

generates must await the elucidation of this intracellular mechanism of action.

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