IS RETINOIC ACID ESSENTIAL FOR PATTERNING DURING AXOLOTL LIMB REGENERATION?

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ABSTRACT

IS RETINOIC ACID ESSENTIAL FOR PATTERNING
DURING AXOLOTL LIMB REGENERATION?

Sonia Victoria del Rincón
University of Guelph, 1998

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Retinoic acid (RA) has been detected in the regenerating limb, and exogenous RA can proximalize, posteriorize, and ventralize blastemal cells. Thus RA may be an endogenous regulatory factor during limb regeneration. Retinoic acid receptors (RAR) form heterodimers with retinoic X receptors (RXR) and transactivate RAR/RXR responsive genes. This thesis examined whether endogenous RA is essential for patterning during axolotl (*Ambystoma mexicanum*) limb regeneration, by using retinoid antagonists that bind to specific RAR or RXR subtypes. Retinoid antagonists: Ro41-5253, Ro61-8431, LE135, and LE540 were implanted into the regenerating limb using silastin blocks. The skeletal pattern of regenerated limbs treated with Ro41-5253 or Ro61-8431 differed only slightly from control limbs. LE135 inhibited limb regeneration, and LE540 revealed relatively normal regenerated limbs. Implanting LE135 and LE540 together, regeneration was not completely inhibited: a hand-like process regenerated. These results demonstrate a possible role of endogenous RA during patterning of the regenerating limb.

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Abbreviations used:
RA = retinoic acid, Ro41 = Ro41-5253, and Ro61 = Ro61-8431

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Abbreviations used:
RA = retinoic acid, Ro41 = Ro41-5253, and Ro61 = Ro61-8431

Introduction

Amphibian Limb Regeneration

Urodeles (newts and salamanders), such as the axolotl, *Ambystoma mexicanum* regenerate limbs epimorphically after amputation to replace only those limb segments damaged or amputated. "Epimorphic regeneration", a term coined by Morgan (1901) to indicate regeneration by addition to an existing structure, results in a steadfast copy of the original limb. This implies that cells at the amputation plane retain positional memory (limb tissue retention of information about their original position). How do the cells of the regenerating limb build a new limb structure with a very specific shape? Limb regeneration partially resembles initial development of the same structure, consequently many biologists study limb regeneration in hope of uncovering some fundamental developmental mechanism. Furthermore, the basic mechanism of limb development in all tetrapod vertebrates is identical, and so one objective in studying limb regeneration is the stimulation of limb regeneration in other vertebrates, primarily humans.

The mechanism of limb regeneration (Figure 1) basically follows that of limb development but, differences exist between the two. Whereas the cells used during limb development arise from undifferentiated embryonic cells, the cells for limb regeneration arise from already differentiated tissues of the limb stump. It has been observed so consistently that only the skeletal elements distal to the amputation plane regenerate, that Rose (1962) described this as the "law of distal transformation" of the blastema. The initial and critical phase in epimorphic regeneration is the quick migration of epidermal cells over the wound

Figure 1: Mechanism of limb regeneration

A) Healed stump: The amputation surface is covered by a distal migration of the epidermis.

B) Early blastema: Cells undergo dedifferentiation.

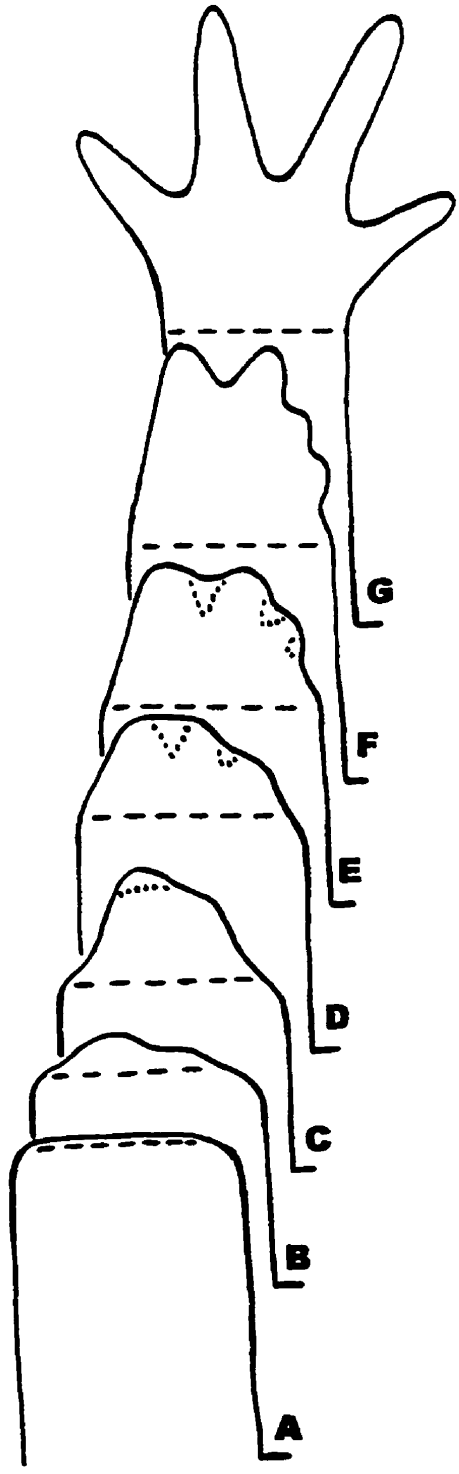
C) Cone stage: The blastema continues to elongate and grow as rapid cell division and redifferentiation occurs.

D) Palette stage: Redifferentiation and morphogenesis continues, and the blastema becomes flattened.

E) Notch stage: The first 2-3 digits are beginning to form.

F) Medium digit stage: The fourth digit is being formed.

G) Complete limb: Larval axolotls take approximately six weeks to fully regenerate a complete limb.


G
F
E
D
C
B
A

surface from the edge of the amputation site to cover the wound and create the wound epithelium. The development and maintenance of the wound epithelium is essential for limb regeneration: its removal results in cessation of limb regeneration. This distinct epithelium may provide the necessary signals for dedifferentiation to the underlying stump tissue and the signals for growth to the blastema cells (Tsonis, 1996). Subsequently, cells close to the amputation site lose their differentiated histological characteristics and become dedifferentiated, with extensive dedifferentiation seen by day 4 post-amputation. Dedifferentiated cells of the stump enter the cell cycle and undergo a dramatic increase in number, to produce a mass of blastema cells beneath the apical epidermal cap formed over the wound surface. This mass of cells then begins elongating to form a cone while cells are beginning to redifferentiate, with cartilage cells being the first to appear around the amputated end of the bone. As redifferentiation continues, the cartilaginous rudiments of all bones distal to the amputation plane are laid down. By six weeks post-amputation in larval axolotls, the exact limb pattern is complete.

Patterning Effects of Retinoic Acid

Vitamin A and other compunds similar in structure and effect are known collectively as retinoids, and greatly influence vertebrate development including the visual system, tissue morphogenesis, cell differentiation and embryonic development (Sporn et al., 1984). Patterning in developing and regenerating limbs is also affected by retinoids (Scadding and Maden 1986a, 1986b, 1986c), and there is evidence to support the hypothesis that retinoic acid is a morphogen that provides positional information in the regenerating limb system. Limb

regeneration is thus a fitting model for investigating the effects of retinoic acid on patterning.

Vitamin A (retinol) is reversibly converted to retinal via alcohol dehydrogenase, this in turn is irreversibly converted to all-trans retinoic acid (RA) via aldehyde dehydrogenase (Figure 2). Retinoids have the ability to modify positional information in the regenerating system along the three cardinal axes (Figure 3): proximodistal (PD), anteroposterior (AP), or dorsoventral (DV) axes.

Knowing pregnant rats exposed to vitamin A produce offspring with severe limb defects, Niazi and Saxena (1978) explored the effects of vitamin A on amphibian limb regeneration. They made the exciting discovery that frog tadpoles *Bufo andersonii*, treated with retinyl palmitate, regenerated structures already present proximal (towards the shoulder) to the amputation plane: this defect has since been called a proximodistal (PD) duplication. Developmental biologists began to investigate the effects of retinoids on regenerating limbs in greater depth. Maden (1982) and others (Scadding and Maden, 1986a; Thoms and Stocum, 1984) reported extra skeletal elements along the PD axis upon treatment with vitamin A during limb regeneration in the axolotl. It was also shown that retinoic acid-induced pattern modifications were not restricted to amphibians, limb regeneration, or to the PD axis. Tickle et al. (1982) and Summerbell (1983) showed retinoids disrupted patterning in the AP axis of the developing chick wing bud by mimicking the zone of polarizing activity (ZPA) located at the posterior edge of the wing bud. The ZPA regulates the AP axis, so that tissue grafted from the posterior wing bud margin to the anterior margin

Figure 2: Reaction pathway of endogenous retinoids

A) Retinol is reversibly converted to retinal via alcohol dehydrogenase.

B) Retinal is irreversibly converted to all-trans-retinoic acid (t-RA) via aldehyde dehydrogenase.

C) Interconversion of t-RA and 9-cis-RA (t-RA isomer) is thought to be cell-specific.

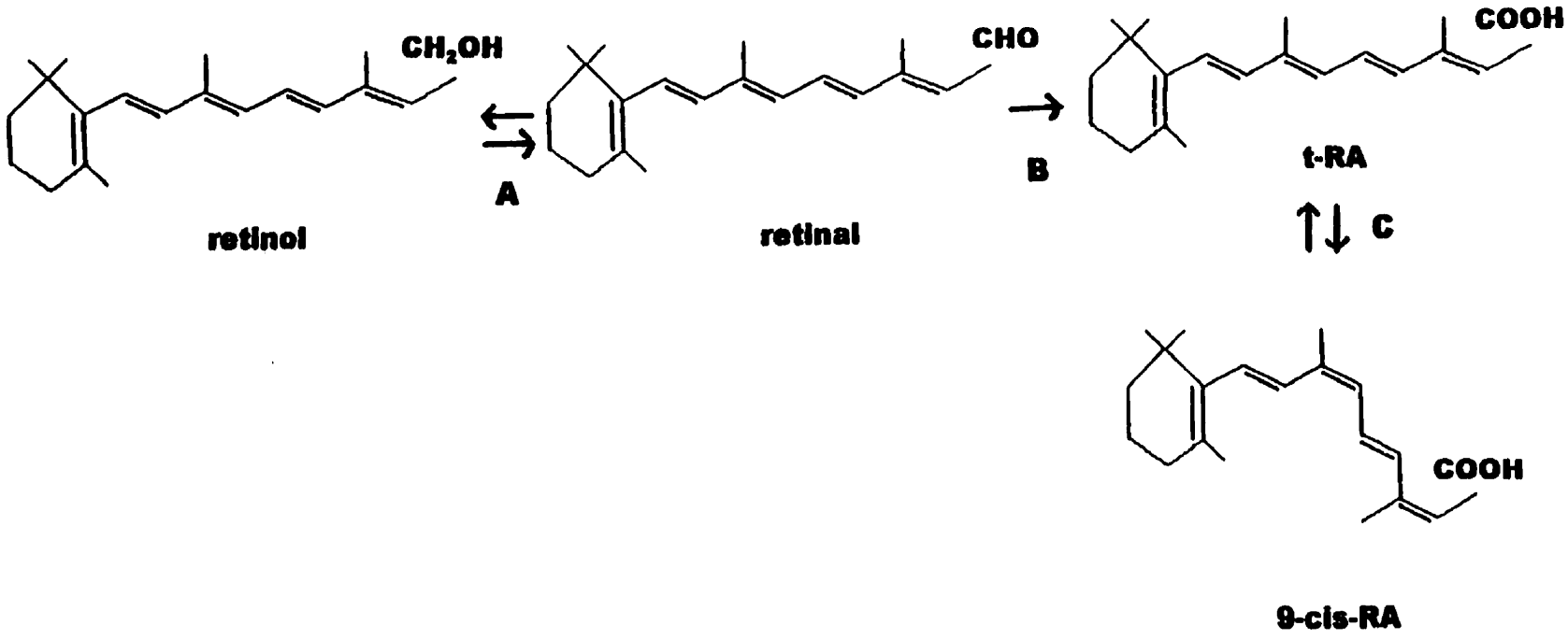
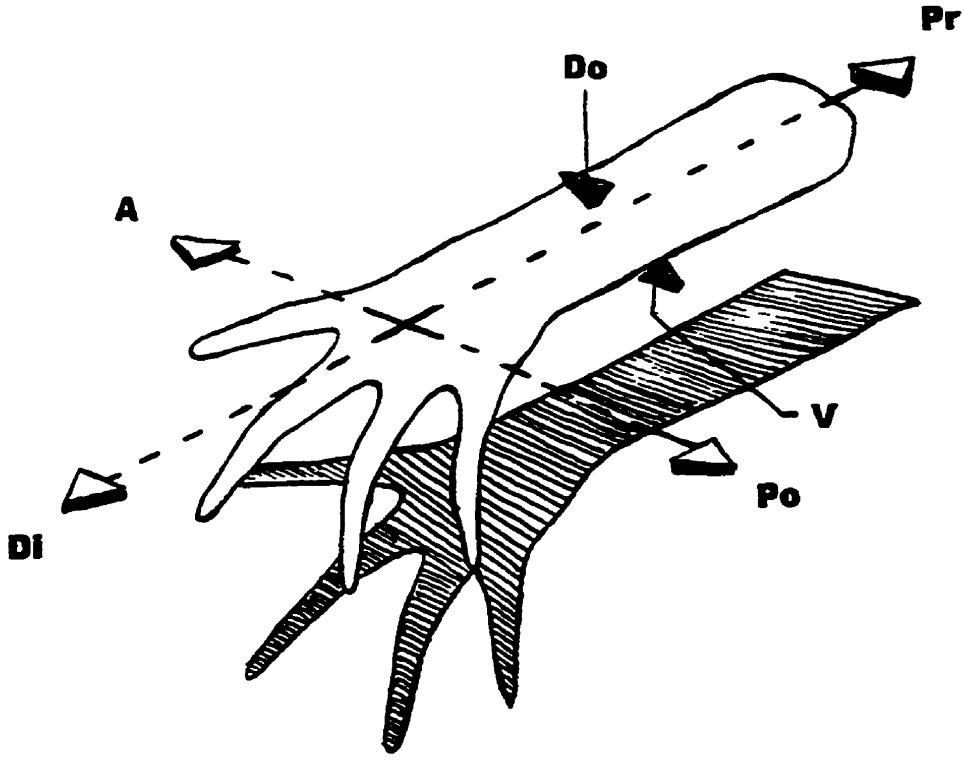

CH2OH
CHO
COOH
A
B
C
retinol
retinal
t-RA
COOH
9-cis-RA

Figure 3: The cardinal axes of the axolotl limb

1- Dorsal (Do) - Ventral (V) axis, also referred to as the dorsoventral axis.

2- Proximal (Pr) - Distal (Di) axis, also referred to as the proximodistal axis.

3- Anterior (A) - Posterior (Po) axis, also referred to as the anteroposterior axis.


Pr
Do
A
V
Po
Di

of a host chick wing bud causes mirror-symmetric digit duplications, that is, an AP duplication (Saunders and Gasseling, 1968; Tickle et al., 1975). Furthermore, when RA-loaded beads were implanted at the anterior edge of the chick wing bud, this same AP duplication was observed (Tickle et al. , 1982; Summerbell, 1983; Tickle et al., 1985).

RA also modifies regenerate pattern in the DV axis (Koussoulakos et al., 1986; Maden, 1997), thus RA appears to regulate patterning of all three axes of the regenerating limb. To show RA's ventralizing effect on the DV axis, Ludolph et al. (1990) cut the axolotl limb in half along the DV axis to produce half-ventral or half-dorsal limbs. Subsequently, on day 4 post-amputation, the axolotls were injected with RA. This study showed that half-ventral and half-dorsal limbs which were not treated with RA failed to regenerate the complementary half. However, RA-treated axolotls with half-ventral limbs failed to regenerate, while those with half-dorsal limbs regenerated intact limbs from the amputation surface. This then demonstrated the ability of RA to promote ventralization of limb structures.

The literature reviewed above shows RA's ability to modify the positional memory of blastemal cells along the three cardinal axes in only one direction. Positional memory is proximalized in the PD axis, posteriorized in the AP axis, and ventralized in the DV axis. It remains unclear whether RA is causing unidirectional modification of positional memory by being distributed nonuniformly or as a gradient across the limb of both regenerating and developing limbs.

Exogenous Retinoic Acid

Maden (1982, 1983) amputated axolotl limbs through the mid-radius-ulna, submersed them in varying concentrations of aqueous retinyl palmitate, and observed regenerates with extra carpals at very low doses, and extra part radius-ulna elements at higher doses (4 µg). At even higher doses an extra elbow joint appeared, and at the highest dose used (16 µg) a complete limb regenerated from the initial amputation site. It has also been shown that the method of administering retinoids does not have an effect on the concentration-dependant results. Silastin blocks can be used for local application of RA, and increasing the amount of RA present in a block causes regeneration to commence from a more proximal level; exactly what one would expect of a morphogenetic compound (Maden et al., 1985). Maden (1982) also reported that the effects are time- and stage-dependant: longer treatment times induce greater degrees of proximalization, and if treatment is delayed beyond a certain developmental stage, there is inhibition of limb regenerates. These properties (concentration-dependancy, and time and stage-dependancy) of retinoids serve as the foundation for the belief that positional memory can be provided by a compound existing as a gradient in the limb. Despite mechanism by which exogenous RA exerts its effects, is it employed as an endogenous signal in directing positional identity in the regenerating limb?

Endogenous Retinoic Acid

It is of great importance to try to determine whether endogenous RA is essential for patterning during limb regeneration. For many years researchers have been trying to elucidate whether RA is an endogenous morphogen acting

during limb regeneration. A morphogen is a gradient-forming molecule that dictates in a concentration-dependant manner the destiny of a group of cells and thus the specific fate of these cells (Tickle and Eichele, 1994). In 1969, Wolpert proposed the ZPA diffusible morphogen model, stating that the polarizing region releases an unknown morphogen which dictates patterning along the AP axis. Tickle et al. (1982) have shown that RA has the ability to mimic the effects of the ZPA (see above) and have thus speculated that RA may be the morphogen released by the polarizing zone. However, there exists the alternate possibility that RA may be acting indirectly on the limb by inducing the formation of a new ZPA at the anterior margin, which in turn releases the "real" signaling molecule, as suggested by Summerbell and Harvey (1983).

Definite support for the hypothesis that RA is the signaling molecule essential for limb patterning has been provided through the measurement of endogenous RA in the chick wing and amphibian limb. RA measurement is rendered difficult due to the minute amounts present; however, Thaller and Eichele (1987) and more recently Scott et al. (1994) were able to measure retinoid levels in the chick wing bud using high pressure liquid chromatography (HPLC). In accordance with the findings of Tickle et al. (1982) (see above), Thaller and Eichele (1987) found the posterior side of the wing bud contained approximately 2.5 times more RA than the anterior side, supporting the idea that RA is the gradient-forming molecule acting during limb development. Gradients of endogenous RA have also been reported in the regenerating limb system using HPLC (Scadding and Maden, 1994). A similar anteroposterior RA gradient to that of the chick was observed in the axolotl limb, with five times

more RA present in the posterior quarter than in the anterior quarter. Interestingly, the adult *Xenopus laevis* lacks an anteroposterior RA gradient, and lacks the ability for patterned limb regeneration (adults can regenerate only a spike-like outgrowth) (Scadding and Maden, 1994). Therefore, there is a correlation between the presence of endogenous RA gradients and the ability to pattern the regenerating limb.

In addition, Brockes (1992) devised a reporter construct containing a retinoic acid response element linked to a β-galactosidase gene, which he then transfected into blastemal cells and reported activation in the presence of endogenous RA. Using this technique, he observed a proximodistal RA gradient, with 3.5 times more RA present in the proximal blastemas as compared to distal blastemas. These observations support the hypothesis that endogenous RA is serving as a gradient-forming morphogen dictating positional information in the developing and regenerating limb system. However, to be classified as a classical morphogen, RA must also be able to establish the fate of blastemal cells in a concentration-dependant fashion. In this model, blastemal cells along the limb axis can then determine their position by interpreting the concentration of RA.

Evidence supporting endogenous RA as a putative morphogen or signaling molecule in vertebrate development and regeneration has been reviewed here, based on retinoid ability to respecify positional memory in a graded and dose-dependant manner. The presence of RA gradients within the limb should not be assumed to be the only factor when exploring the mechanism(s) behind patterned limb regeneration. However, it remains unclear

what other factors might be involved in designating positional identity of blastemal cells, and what role RA gradients play in relation to these factors.

Genes Involved in Patterning

Several Homeobox (Hox) genes of the developing limb bud are expressed primarily at the time when pattern is being specified. While Hox genes are clearly involved in limb patterning, the precise mechanism of their action is not clear. Based on their expression patterns in the limb, Hoxd genes are thought to regulate digit patterning (AP axis), while Hoxa genes regulate the formation of skeletal elements along the PD axis (Yokouchi et al., 1991). Dolle et al. (1993) were the first to introduce Hoxd-13 null mutations in mice, which resulted in an overall delay in limb development, and abnormal morphology of the digits and wrist bones. The most common observations were fused bones, and absent phalanges. The disruption of the Hoxa-11 gene in mice was performed by Small and Potter (1993), and they observed broadening of the radius and ulna, and fusion of two wrist bones (pisiform and triangular). These two studies have provided additional clues as to the roles Hox genes play in patterning.

Sonic hedgehog (Shh) is one of the key molecular components operating along the AP-axis of the limb, and was isolated by Riddle et al. (1993). Shh, a homolog of the *Drosophila* segment polarity gene *hedgehog*, is strongly expressed in many embryonic signaling tissues which can induce pattern duplications (Tickle and Eichele, 1994). Endogenous Shh is expressed at the posterior margin in the chick wing bud. Introducing ectopic Shh to the anterior margin can direct the formation of mirror-symmetric digit duplications in the same manner as grafting the ZPA or implanting a bead releasing RA to the anterior

margin of the wing bud can (Johnson et al., 1994). It was proposed that RA, which can induce Shh expression in 24 hours, first induces Shh, and Shh in turn activates Hoxd genes. However, knowing that RA induces Shh in 24 hours, and RA and Shh take about 24 and 20 hours respectively to induce Hoxd-11, is suggestive of two different pathways of Hoxd gene activation.

Retinoic Acid Receptor

The retinoic acid receptors (RAR), through which RA may be controlling gene expression, need to be thoroughly examined (Yoshimura et al., 1995). It is known that the biological effects exerted by RA are mediated by binding to and activating specific RARs, and this ligand-receptor complex then modulates gene transcription. It is not known however, what role these receptors may be playing in mediating the respecification of positional identity during the regenerative process.

How is positional identity encoded at the molecular level in relation to the compounds and cellular properties which may be needed for pattern formation? In an attempt to answer this question and further our understanding into the origins of complex regulatory systems such as limb regeneration, it is essential to analyse retinoic acid receptors (RAR). The RA-RAR complex may be a key player in determining commitment to specific cell lineages, as well as dictating positional information to blastemal cells. The diverse biological effects of RA are mediated through the RARs which belong to the nuclear receptor superfamily of ligand-inducible transcriptional regulators; comprising the steroid, retinoid, thyroid hormone and vitamin D_3 receptors. The discovery of RARs was made possible by the finger-swap experiment (Petkovich et al.,1987) which

demonstrated that conserved regions in receptors correspond to discrete functional domains. By exchanging the DNA-binding domain (DBD) of the RAR for the DBD of a glucocorticoid receptor (GR), a chimaeric receptor was constructed which could activate the GR-response element in response to RA. Thus, for the first time in a vertebrate system there was hope of investigating the mechanism of morphogenesis and patterning by identifying a set of developmentally controlled genes (Evans, 1988; Mangelsdorf et al., 1994).

All super-family receptors are composed of the following six functional domains (Figure 4):

A/B: The amino (N)-terminal region encodes the activation of transcription and contains the transcriptional regulating region AF-1. Transcriptional activation is cell-type specific; deleting the E-domain (see later) of an estrogen receptor (ER) results in a constitutively active ER in one cell type but an inactive ER in a different cell type. This suggests the existence of cell-type specific nuclear co-factors which interact this domain to mediate or inactivate its transcriptional activating function. This is the only domain differing among the RAR-isoforms (see below) and thus it may be involved in dictating the functional specificity of the receptors, that is, different isoforms may be mediating the distinct effects of RA by the activity of this domain (Gann et al., 1996).

C: This domain encodes the base-sequence-specific DNA binding function. It is responsible for specific response element recognition. This domain contains a high sequence homology among all nuclear receptors.

D: This is a short sequence which may be responsible for the intranuclear localization of the receptors.

Figure 4: Model for retinoid signalling and the functional domains of their nuclear receptors (modified from Hashimoto, 1991).

A) The retinoid molecule must first enter the nucleus.

B) The retinoid binds to the retinoid receptor (RAR):

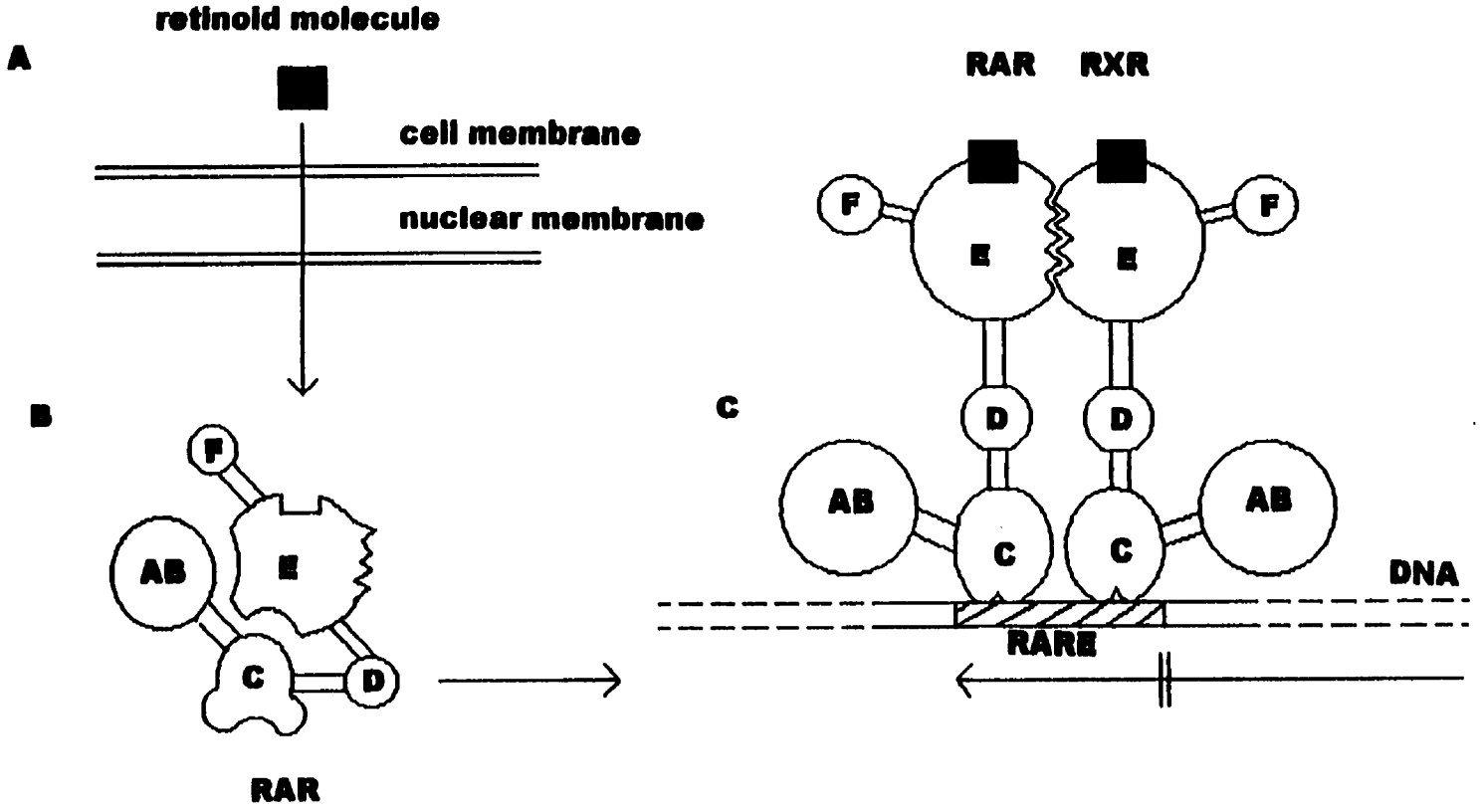
AB: Transcription activation domain.

C: DNA-binding domain.

D: Intranuclear localization of the receptors.

E: Ligand binding domain.

C) The Retinoid-RAR complex may form homodimers (with RAR) or heterodimers (with RXR) to bind to the retinoic acid response element (RARE) on the DNA to regulate gene transcription.


retinoid molecule
A
cell membrane
nuclear membrane
B
F
AB
E
C
D
RAR
C
RAR
RXR
F
E
E
F
D
D
AB
C
C
AB
DNA
RARE

E: Here lies the ligand binding function. It is also the region of ligand-dependant dimerization and the site of interaction with other nuclear factors.
The intrinsic transcriptional activating function of the A/B domain is hidden by the ligand free E-domain containing the transcriptional activating region called AF-2.

F: The function of this domain remains unknown.

The RAR gene family consists of three types: α, β, and γ. Each gene encodes a variable number of isoforms within each type (α1 and α2, β1 to β4, and γ1 and γ2), arising by differential splicing of primary RNA transcripts (Giguere et al., 1990). The existence of multiple isoforms may help explain the diverse biological effects (in teratogenesis, differentiation, vision, and patterning) of RA, and suggests that each isoform may have a precise function in mediating the pleiotropic effects of RA. As discussed, above these isoforms differ only in their amino terminal region which contain one of the transcriptional regulating regions. Thus, the isoforms may differ in their target genes, and consequently each may have distinct roles with respect to establishing the three distinct cardinal axes during regeneration. Moreover, RARs are differentially expressed spatially and temporally, they may then be regulating different sets of genes during embryonic and adult life. In tissues of adult animals, RARα is the most ubiquitously expressed, while β and γ display a more restricted pattern of distribution (Redfern, 1992).

Retinoid X Receptor

The complexity of retinoid signaling was further increased when Mangelsdorf et al. (1990) discovered another family of receptors for RA, the

retinoid X receptor (RXR), with three types α, β, and γ. Subsequently, a novel pathway for vitamin A was described, which used a stereo isomer of all-trans-RA as the ligand for RXR (Levin et al, 1992a; Tate et al., 1994). The RARs are activated by direct interaction with the major form of RA, all-trans-RA; although these receptors can also be bound by 9-cis-RA. Contrary to this, the RXR gene family can not bind the trans form of RA, instead, 9-cis-RA is their active ligand. That 9-cis-RA can bind to and transactivate not only RXRs but also RARs, suggests that it may serve as a bifunctional ligand. Thus, there exist two distinct receptor families and gene pathways with some overlap in the ligands binding the RAR (Levin et. al, 1992b).

Research of this decade led investigators of the nuclear receptor superfamily to consider the existence of nuclear accessory factors which are essential for high affinity binding of the vitamin D receptor (VDR), thyroid hormone receptor (THR), and RAR to their respective hormone response elements (HRE) (Liao et al., 1990; Yang et al., 1991). The common accessory factor was found to be RXR, which can form heterodimers *in vitro* with these receptors (Leid et al., 1993). Subsequently, the members of the nuclear receptor superfamily were categorized into four classes based on their dimerization and DNA-binding properties (Stunnenberg, 1993; Mangelsdorf et al., 1995). Class I receptors include the known steroid hormone receptors which function as ligand-induced homodimers (glucocorticoid, estrogen), class III receptors comprise orphan receptors which bind primarily as homodimers (RXR), and class IV receptors bind to the DNA as monomers . The class II receptors; VDR, THR, and RAR α, β, and γ; must heterodimerize with RXR for

high affinity binding of the receptor to its HRE, and enhanced receptor-dependant transactivation of HRE (Leid et al., 1992; Marks et al., 1992). Evidently, only one partner of the heterodimer complex needs to be occupied by its ligand to elicit gene transcription. Furthermore, it has been reported that ligand-induced transcription actions of RXR can be suppressed when heterodimerized with RAR (Kurokawa et al., 1994; Forman et al., 1995). The formation of the RXR/RAR heterodimer actually restricts 9-cis-RA from binding to the RXR partner, suggesting that 9-cis-RA responsiveness is not a compulsory consequence of heterodimerization with RXR, and that RXR is a silent partner.

That RXR is capable of heterodimerizing with receptors which bind different ligands, and assigns it a pivotal role in cross-talk between the various nuclear receptor signaling pathways. However, it remains unclear, in the retinoid signal pathway for example, what is the functional significance of forming RAR/RXR heterodimers. Leid et al. (1993) suggested two ways that RAR/RXR heterodimer interaction could increase diversity in the retinoid transduction pathway. Firstly, RAR/RXR heterodimers bind to the DNA binding domain with higher affinity than RAR- and RXR-homodimers. In addition, there is evidence that the liganded status of RAR in the RAR/RXR heterodimer can affect the activity of the RXR partner. Thus diversity can be generated at the level of the retinoid response element, where RAR/RXR heterodimers, and RAR- and RXR-homodimers may each transactivate different genes. Secondly, the various types and isoforms (with specific AF-1s, see Retinoic Acid Receptors) of RAR and RXR results in existence of various possible RAR/RXR heterodimeric combinations with different transcriptional outcomes (activation or repression).

Therefore, the multiplicity of RA receptors with specific AFs, and the formation of RAR/RXR heterodimers results in a large number of combinatorial possibilities which may account, at the molecular level, for the pleiotropic effects of the retinoid signal transduction pathway.

These recent findings have led to the updated model for retinoid signaling, taking into account current knowledge of retinoid ligands, their metabolism, and their receptors (see Figure 4) (Mangelsdorf et al., 1994; Mangelsdorf and Evans, 1995). When the appropriate ligand has been metabolically produced or transported to the target cell, it must then cross the nuclear envelope, via the nuclear pore complex, where the majority of unliganded RARs and RXRs lie. It has been previously suggested that cellular RA binding proteins (CRABP) could be acting as RA-transport shuttles between the cytoplasm and nucleus (Takase et al., 1986), however at present their function is still largely unknown (Mangelsdorf et al., 1994). Within the nucleus, all-trans-RAs interaction with RAR activates heterodimerization of RAR with RXR, and this RA-RAR/RXR complex then transcriptionally regulates its target gene by binding to the RARE. RXRs do not need to be bound by 9-cis-RA for heterodimer activation. However 9-cis-RA has been shown to act synergistically with RAR ligands, and in the presence of high levels of 9-cis-RA, RXR can bind not only as a RAR/RXR heterodimer but also as a RXR/RXR homodimer to activate RXR target genes. It has been observed that this function is repressed in the presence of low concentrations of 9-cis-RA or high concentrations of RAR when RAR/RXR heterodimer formation is favoured. Furthermore, the RAR/RXR heterodimer has a higher affinity for DNA than does

the RXR homodimer, and thus may win over the latter for binding to RXREs (Mangelsdorf et al., 1994). The presence of two distinct receptor systems with distinct response elements, implies the existence of target genes specifically responsive to each receptor type, and the possibility of controlling gene transcription via cross-talk between retinoid signalling pathways. If this model is correct, the pleiotropic effects exerted by RA may be due to the co-existence and interaction of RAR-RXR heterodimers, multiple RAR and RXR isoforms, and interconversion of RA isomers (trans/cis). Consequently examining these factors in relation to each other may help provide some insight into the molecular basis of retinoid action during limb regeneration.

In the past five years researchers have begun examining the mode of action of RARs and RXRs in mice by modifying or eliminating receptor function, using both null mutations in individual receptor genes (Li et al., 1993; Sucov et al., 1994; 1995; Luo et al., 1995), and double mutants of RARs (Lohnes et al., 1994). Null mutations of RARα, RARβ, or RARγ yielded mice without significant congenital defects or limb malformations. However the double mutants of RARα and RARγ did yield mice with many congenital defects: axial skeleton defects, and forelimb malformations of considerable variation such as; loss of the radius, carpal bone malformation, reduction or increase in digit number, and phalange abnormalities (Lohnes et al., 1994; Kastner et al., 1995). That severe congenital defects were reported only in RAR double mutants suggests that the multiple isoforms of both RARs and RXRs may be functionally redundant (Lohnes et al., 1994; Helms et al., 1996). An alternate way of investigating the actions of RA and its receptors would be to block the retinoid signaling pathway by creating a

"knockout" which would modify or eliminate receptor function and any downstream transcription events. As previously stated, RA mediates its actions through RARs, thus if RA is restricted from binding to its receptor via a retinoid antagonist, then retinoid and receptor action may be affected. Potentially, this "knockout" concept would help elucidate if endogenous RA is essential in mediating pattern formation during limb regeneration.

Retinoid Antagonists

An antagonist refers to a natural or synthetic compound, which resembles a ligand and competes with this ligand for the respective receptor, blocking the receptor and interfering with receptor action. Presently, there is little known about which RAR or RXR isoform(s) is/are responsible for mediating a specific response to RA, thus making behavior clarification of each RAR or RXR and their dimers one of the major current problems in the field of retinoid signaling (Eyrolles et al., 1994). However, receptor-selective retinoids or retinoid antagonists could serve as effective agents for the precise elucidation of the mechanisms of retinoid actions. Having said this, it is clearly important to investigate the consequence for patterned limb regeneration of blocking RA synthesis or inhibiting the activity of retinoid receptors by specific antagonists. The roles of retinol and RA in limb regeneration have been studied by using compounds such as citral and disulfiram which inhibit the enzymes acting to synthesize RA. Citral acts as a vitamin A antagonist by acting as a competitive inhibitor of alcohol dehydrogenase and aldehyde dehydrogenase, competing with retinol and retinal respectively for the active site of these enzymes, and inhibiting the formation of retinoic acid (Marsh-Armstrong et al., 1994; Tanaka et

al., 1996). Tanaka et al. (1996) have shown that endogenous RA plays a role in chick limb patterning by treating wing-buds with citral. Citral-treated wing buds induced malformed wings along the PD axis, with shorter radius/ulna bones and digits. Scadding treated axolotl limbs with citral and observed an inhibition of limb regeneration (unpublished data). Disulfiram or tetraethylthiuram disulfide is another retinoid antagonist, which at low levels is a specific inhibitor of cytosolic aldehyde dehydrogenase, inhibiting the conversion of retinal to RA, and causing developmental defects (McCaffery et al., 1992; Costardis et al., 1996). Maden (1996) and Maden (1997) reported that axolotl limb regeneration is inhibited in the presence of disulfiram, suggesting RA is essential during regeneration.

The problem with using metabolic inhibitors such as citral and disulfiram is that they are not specific. These compounds may be inhibiting systems other than the enzymatic machinery required to synthesize RA. Therefore, although citral and disulfiram have been shown to inhibit development and limb regeneration, we can not conclude that this is a result of blocking RA synthesis.

An alternate way of assessing retinoid signaling during limb development or regeneration is through the use of retinoid antagonists. Thaller and Eichele (1996) and Helms et al. (1996), looked at the consequence of blocking retinoid signaling on chick limb development by using beads soaked in the RAR and RXR antagonists: LG629 and LG754 (Lala et al., 1996). In general, treatment of the prospective wing region with these anti-retinoids resulted in a loss of limb structure. This result serves as evidence for RA possessing a role in patterning during early chick limb development. Therefore, it is possible to improve our understanding of retinoid involvement during patterning in limb development and

limb regeneration by using receptor antagonists. To this end, I worked with the following compounds:

1) Ro 41-5253 (Ro41), is a RA analogue and is believed to be an RAR α-selective antagonist (Figure 5a) (Apfel et al., 1992).

2) Ro 61-8431 (Ro61), is a RA analogue and is a RAR-specific antagonist (Figure 5b) (Yoshimura et al., 1995).

3) LE135, is a synthetic retinoid antagonist, and can bind selectively to RARα and RARβ, with higher affinity to RARβ (Figure 5c) (Umemiya et al., 1997).

4) LE540, is a benzolog of LE135 and can bind to all RARs (α,β,γ) and RXRs (α,β,γ)(Figure 5d) (Umemiya et al., 1997).

Ro41 causes a conformational change in the RAR which is not induced by RA, thereby impairing the receptors ability to interact with its transcriptional machinery (Keidel et al., 1994). There are a few concerns in using retinoid antagonists as a tool for studying RAs involvement during the regenerative process. First, it is not known which isoform the antagonists are specific. This could be significant in terms of functional redundancy, where isoforms could potentially be compensating/standing in for nonfunctional isoforms. Secondly, Ro41 can be critiqued for its α-selectivity, also stemming from the idea that the many isoforms of RARs and RXRs may be functionally redundant. It is thought that the multiple isoforms of RARs may overlap in function, as shown in comparing the outcome of mouse RARα or γ null mutations to RARαγ double mutants (see above). Thus the α-selectivity of Ro41 may limit this antagonists value in demonstrating the role of RARs in the regenerating system *in vivo* (Standeven et al., 1996). Therefore, an antagonist of RARα, β, and γ, and their

isoforms would prove to be a more effective compound than Ro41 for elucidating the role of RA and RARs *in vivo*. Lastly, is Ro41 really α-selective? There exists evidence that implicates Ro41 as being an inhibitor of both RARα and RARβ transactivation: Ro41 has been used to block retinoid mediated signaling during early *Xenopus* and chick embryogenesis (López et al., 1995). Using 0.75μM to 7.5μM Ro41, corresponding to 5- to 50-fold excess of antagonist over endogenous RA, they reported severe malformations of all three germ layers (central nervous system, heart, foregut derivatives) when *Xenopus* and chick embryos were treated with the highest dose (7.5μM) before or after gastrulation. Interestingly, the Ro41-affected heart and foregut structures seen in their study were also affected in RARαβ double mutant mouse embryos (Lohnes et al., 1994). Furthermore, limb malformations were not reported (even at 7.5μM), but were evident only in RARαγ double mutant mice. It is possible that the severe malformations seen only at 7.5μM of Ro41 were the result of RARα and RARβ having been blocked. If this is true, then Ro41 can not be labeled α-selective, and RARβ may be essential for heart and foregut formation, but not for limb development; since limb development was not affected by Ro41 (although limbs were abnormal in RARαγ double mutant mice).

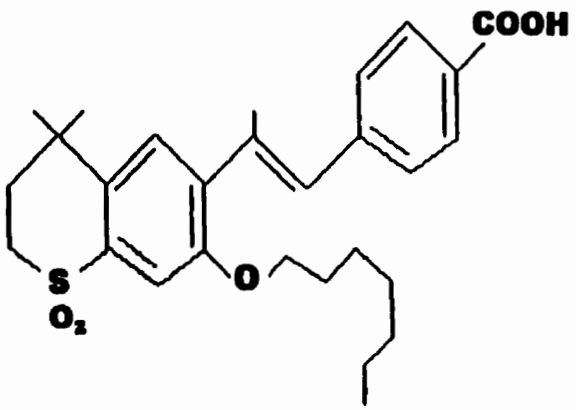
Figure 5: Structures of the retinoid antagonists used in this study.

A) Ro 41-5253: RARα-selective antagonist.

B) Ro 61-8431: RARα and RARβ antagonist.

C) LE135: RARα and RARβ antagonist, with higher affinity for RARβ.

D) LE540: Can bind to all RAR subtypes (α, β, γ) and all RXR subtypes (α, β, γ).



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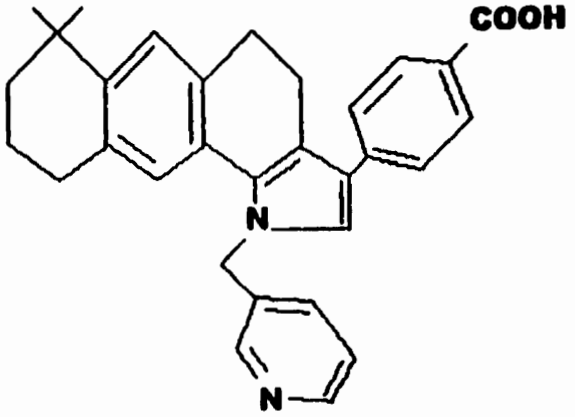

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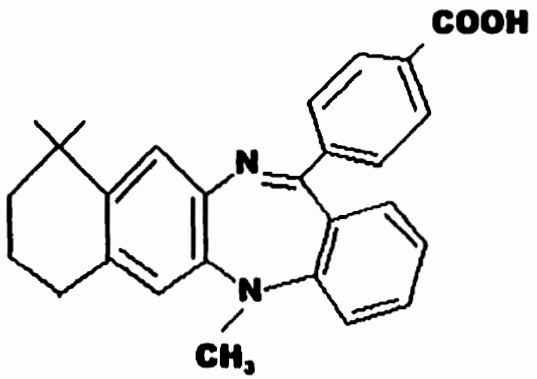

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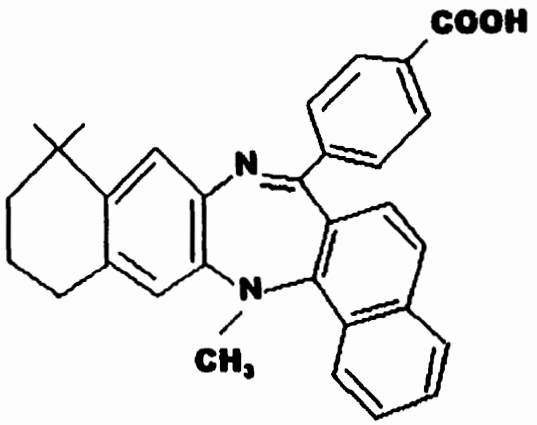


D

Hypothesis & Objectives

Retinoic acid is essential for patterning during amphibian limb regeneration. I hypothesize that the regenerating limbs of *Ambystoma mexicanum* treated with vitamin A antagonists; Ro 41-5253, Ro 61-8431, LE135, and LE540 will exhibit abnormal skeletal patterns, resulting from disruption to the retinoid signaling pathway.

Control limbs should show no skeletal pattern malformations. The retinoid antagonists bind to different RARs (α, β, γ) or RXRs without activating them, resulting in inhibition of RA induced gene transcription, and loss of RA effects mediated by the RARs. My aim is to perform a receptor knockout experiment, by using the various retinoid antagonists in the axolotl regenerating limb system, alone or in combination. By inhibiting RAR-induced transactivation I will be able to assess whether endogenous RA is essential for patterning and which receptors may be acting in concert with RA.

Materials and Methods

Animal Husbandry

The axolotl larvae (*Ambystoma mexicanum*) used in this investigation were obtained from the Indiana University axolotl colony. Upon arrival they were housed in tap water in individual 150 ml plastic containers (D8 cups, Canada Cup Inc., Toronto, Ontario), preventing damage to native limbs resulting from the predations by other axolotls. They were fed a diet of brine shrimp, and when they were large enough they were fed salmon pellets. The axolotl larvae used in my experiments possessed fully developed forelimbs with complete digits and were on average 4.0 - 5.0 cm long (total length).

Treatments

All-trans-RA (RA)was obtained from Sigma. Retinoid antagonists, Ro 41-5253 (Ro41), and Ro 61-8431 (Ro61) were obtained from F. Hoffmann-La Roche Ltd.. Retinoid antagonists, LE135, and LE540, were obtained from the University of Tokyo, Graduate school of pharmaceutical sciences. The molecular weights of Ro41, Ro61, LE135, and LE540 are 484.65 g/mole, 490.65 g/mole, 438.54 g/mole, and 488.60 g/mole, respectively. Apfel et al. (1992), labeled Ro41 a RAR α-selective antagonist because it binds to RARβ and RARγ with 40-fold (IC_{50} = 2.4 × 10^{-6}M) and 55-fold (IC_{50} = 3.3 × 10^{-6}M) lower affinity, respectively, as compared to RARα, for which it binds with high affinity (IC_{50} = 6.0 × 10^{-8}M) (IC_{50} = retinoid concentration required to inhibit 50% of specific RA binding)(Apfel et al., 1992). It should be noted that RA has a higher binding affinity for RARα (IC_{50} = 1.4 × 10^{-8}M) than Ro41, therefore a 2- to 10-fold excess of Ro41 is needed for its antagonism to be effective. Furthermore, RARβ

transactivation was inhibited when a 50- to 100-fold excess of Ro41 was used (Apfel et al., 1992; and Moroni et al., 1993).

The second antagonist used was Ro61. In the assay system used by Yoshimura et al. (1995), it exhibited a higher binding affinity ($IC_{50} = 3.4 \times 10^{-10}M$) than RA ($IC_{50} = 6.3 \times 10^{-8}M$). This antagonist does not need to be used in excess of RA, because it is such a potent inhibitor of receptor function. No experiments to date have employed this particular antagonist for studying retinoid-receptor function.

The third antagonist employed was LE135; it does not bind to RARγ, but can bind selectively to RARα and RARβ, with highest affinity for RARβ. This binding affinity makes LE135 useful in creating a RARα/β knockout to determine if limb regeneration is at all affected.

The fourth antagonist used was LE540; which was shown to posses a 1 order of magnitude higher antagonistic potential ($IC_{50} = 3.6 \times 10^{-8}M$) than the parent molecule LE135 ($IC_{50} = 1.5 \times 10^{-7}M$). LE540 can bind to all RARs and RXRs, however, it binds to RARα and RARβ with the same affinity as the less potent LE135. The ability of LE540 to bind to RXRs may significantly contribute to its more potent antagonistic ability as compared to LE135. Being able to potentially knock out all retinoid receptors should theoretically yield some interesting results when LE540 is used to examine the role of endogenous RA in patterning during limb regeneration.

All retinoids were administered via a silastin block implanted into the treatment limb, a technique initially used for the administration of RA in regeneration studies (Maden et al., 1985). This method allows effective local

concentrations of the retinoids without high systemic doses. Ro41 silastin blocks were prepared by mixing 30 mg of this compound with 0.3 ml of silastin (Silastic MDX-4-4210 Medical Grade Elastomer, Dow Corning, Michigan, USA), and stirring until a uniformly mixed patty was obtained. Immediately, 30 µl of curing agent was added and mixed into the patty, which was then stored in the dark at room temperature, and cured for 48 hours. All other retinoids were similarly prepared, to ensure the concentration of the various retinoid silastin patties was approximately constant; e.g. for RA patty: mix 100mg RA with 1 ml silastin and 0.1 ml curing agent. Once cured, a firm patty is formed, making it possible to cut silastin blocks of the following sizes: 500 × 250 × 250 µm (small), 500 × 500 × 250 µm (medium) and 500 × 500 × 500 µm (large). These blocks were placed in aluminum foil covered petri dishes, and stored in the refrigerator. Small, medium, and large blocks contained approximately 3.12 µg, 6.25 µg or 12.5 µg, respectively, of the test drug mixed with the silastin.

Experimental Design

Amputation of both forelimbs through the distal radius-ulna was performed with a single edged razor blade under anaesthesia with 0.3 g/L tricaine methane sulphonate neutralized with sodium bicarbonate (Robinson and Scadding, 1983). On either day 2, 3, 4, 6, 7, or 10 post-amputation, experimental animals (6 per trial) were re-anaesthesised in preparation for the following implantation technique (identical for experiments 1 to 6, unless otherwise specified). Silastin blocks (one to six) of varying sizes, containing one of the test substances (experimental groups) or no drug (control group) were implanted into each forearm. To implant, fine forceps were used to pierce the epidermis and dermis

and make a tunnel in the forelimb. A tungsten needle was used to spear the block, insert it into the tunnel, and place it directly proximal to the blastema (Figure 6). To avoid disturbing the blastema, the block was inserted just proximal to the blastema. The animals were observed weekly to evaluate the regenerative progress. After 6 weeks post-amputation, the axolotls were again anaesthetized and the regenerated forelimbs were amputated at the shoulder level. The limbs were then fixed in 10% neutral buffered formalin, stained with Victoria Blue B, a cartilage specific stain, and then cleared in methyl salicylate (Bryant and Iten, 1974). Limbs were viewed under the dissecting scope to assess skeletal patterns.

Control Groups

Native limbs (limbs which have never regenerated) were amputated to assess their skeletal pattern

Regenerated limbs which had not been implanted with blocks were examined to assess their skeletal pattern.

Regenerated limbs which had been implanted with 4 large control blocks were examined to assess if the presence of silastin blocks resulted in skeletal variations not seen in native limbs or regenerated limbs without blocks.

Experiment 1 - Implantation Time (Table 1)

Maden et al. (1985) determined that RA is available for 60-70 hours after an implant, and deduced that day 4 post-amputation was the optimum time for RA to exert the most profound effect on pattern formation. Therefore, it was important to determine the time after amputation at which the retinoid antagonists (Ro41 and Ro61) would have the most profound effect on

Figure 6: Implantation technique

A) The limb has been amputated through the distal radius and ulna (dashed line). After four days post-amputation a well developed blastema (bl) is visible.

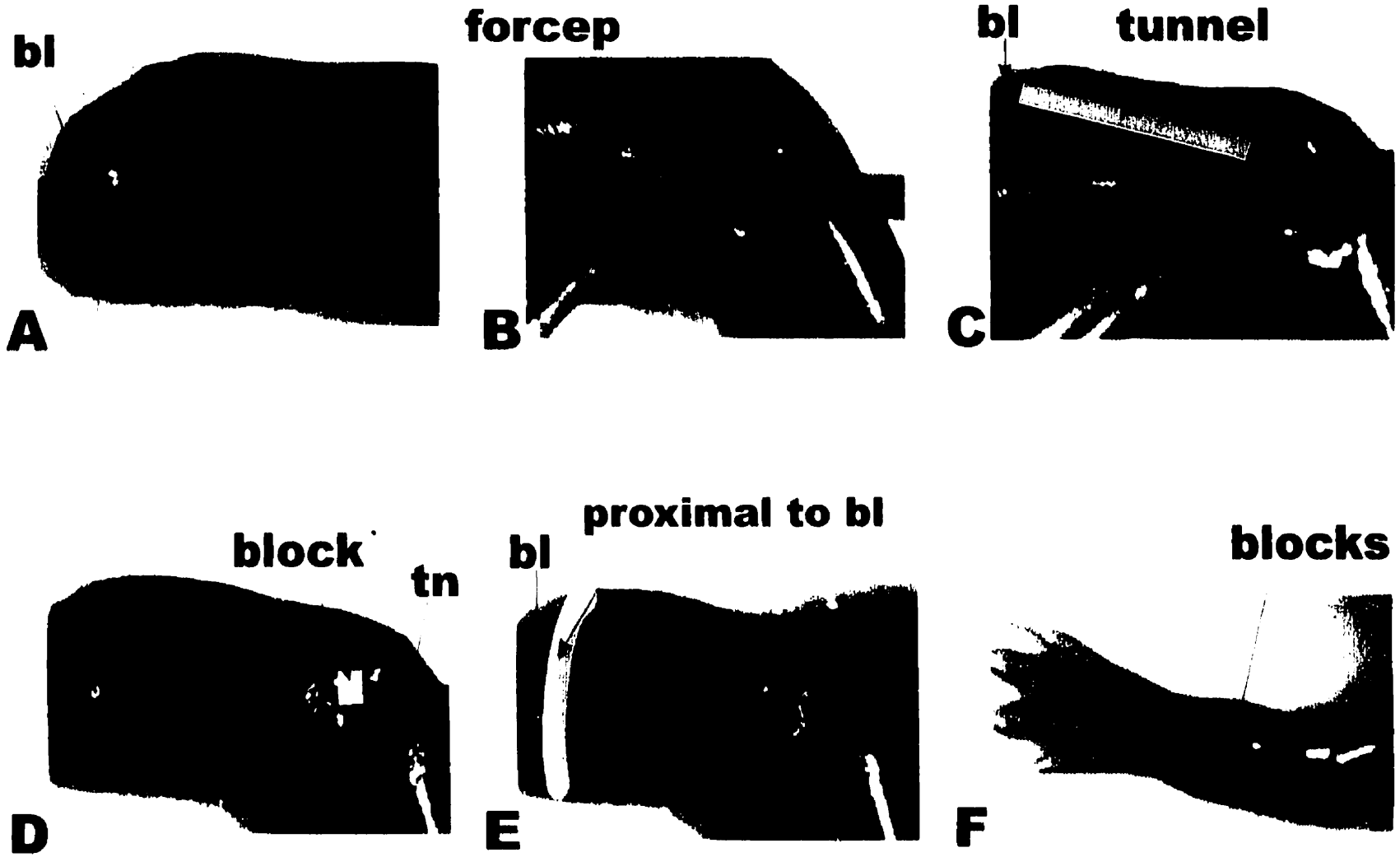
B) The epidermis must first be pierced (dashed circle) using fine forceps.

C) Using one arm of the fine forcep a tunnel is made towards the blastema (bl).

D) Using a tungsten needle (tn), the silastin block is inserted under the epidermis.

E) Using the tungsten needle the block is pushed and placed just proximal to the blastema (bl).

F) The arm is allowed to regenerate for six weeks at which time the blocks may still be visible beneath the skin.


bl
A
forcep
B
bl
tunnel
C
block
tn
D
proximal to bl
bl
E
blocks
F

pattern formation. Two large blocks, each containing 25 µg Ro41 or Ro61, were implanted on various days post-amputation (day 2, 3, 4, 6, or 7).

Experiment 2 - Varying Concentration (Table 1)

Maden et. al (1985) showed a concentration effect of RA such that silastin blocks with more RA produced regenerates which began from more proximal levels. I wanted to determine if there was a concentration effect of Ro41 or Ro61 when implanted into the regenerating limb. The doses of Ro41 or Ro61 implanted on day 4 post-amputation were: 31.25 µg/limb , 37.5 µg/limb, 50 µg/limb, and 75 µg/limb.

Experiment 3 - Extending Treatment Time (Table 1)

The dose range of 25 µg/limb to 75 µg/limb yeilded very similar results. This prompted me to assess the effect of extending the treatment time of Ro41 or Ro61 using the highest possible drug dose (75 µg/limb). In experiment 3a and 3b, 3 large blocks were implanted on day 3 post-amputation and then an additional 3 were implanted on day 7. In experiment 3c and 3d, 2 large blocks were implanted on day 2, and an additional large block was inserted on day 4. On day 10, these three large blocks were removed, and 3 new large blocks were implanted. (See Table 1).

Experiment 4 - Effects of Exogenous RA (Table 1)

Ro41 and Ro61 were inferred to be retinoid antagonists because of their ability to counteract exogenous RA induced-effects on HL-60 cell differentiation *in vitro*. Therefore, I thought it would be useful to examine the ability of Ro41 and Ro61 to counteract exogenous RAs effects *in vivo*. This experiment examined the effects of implanting exogenous RA (9.37 µg /limb) into the

regenerating limb, and the effects of using various concentrations of Ro41 or Ro61 (6.25 µg /limb, 9.37 µg /limb, or 12.5 µg /limb) to antagonize exogenous RA (6.25 µg /limb).

Experiment 5 - Ro41 and Ro61 in Combination (Table 1)

This experiment briefly looked at the combined effect of implanting 18.75 µg /limb Ro41 and 18.75 µg /limb Ro61.

Experiment 6 - Preliminary Work using LE135 and LE540 (Table 1)

Having obtained the more recently synthesized retinoid antagonists LE135 and LE540, I looked at the effects of implanting LE135 or LE540 alone or in combination. It should be noted that the method of application of LE135 differed somewhat from that of Ro41 or Ro61. When LE135 was mixed with the silastin and curing agent, a solid patty did not form making it impossible to cut silastin blocks. Therefore, it was used as a thick paste and the amount of LE135 was estimated to be about the amount of Ro41 or Ro61 in one to two large silastin blocks (about 12.5µg/limb to 25µg/limb).

The following notes apply to Table 1:
*Block sizes used: lrg = large, med = medium, sm = small
**The drug dose administered was approximately the amount contained in 1 to 2 large blocks.

TABLE 1 - List of Treatments		
Expt #	Drug dose per arm (µg)	Implantation: Block size, #, and days post-amputation
1a	25 Ro41	2 lrg*, day 2
1b	25 Ro61	2 lrg, day 2
1c	25 Ro41	2 lrg, day 3
1d	25 Ro61	2 lrg, day 3
1e	25 Ro41	2 lrg, day 4
1f	25 Ro61	2 lrg, day 4
1g	25 Ro41	2 lrg, day 6
1h	25 Ro61	2 lrg, day 6
1i	25 Ro41	1 lrg, day 3 & 1 lrg day 7
1j	25 Ro61	1 lrg, day 3 & 1 lrg, day 7
2a	31.25 Ro41	2 lrg + 1 med*, day 4
2b	31.25 Ro61	2 lrg + 1 med, day 4
2c	37.5 Ro41	3 lrg, day 4
2d	37.5 Ro61	3 lrg, day 4
2e	50 Ro41	4 lrg, day 4
2f	50 Ro61	4 lrg, day 4
2g	75 Ro41	6 lrg, day 4
2h	75 Ro61	6 lrg, day 4

The following notes apply to Table 1 continued:
*Block sizes used: lrg = large, med = medium, sm = small
**The drug dose administered was approximately the amount contained in 1 to 2 large blocks.

TABLE 1 CONTINUED - List of Treatments		
Expt #	Drug dose per arm (µg)	Implantation: Block size, #, and days post-amputation
3a	75 Ro41	3 lrg, day 3 & 3 lrg, day 7
3b	75 Ro61	3 lrg, day 3 & 3 lrg, day 7
3c	75 Ro41	3 lrg, day 3 & 1 lrg, day 4; then Remove 3 blocks & implant 3 lrg, day 10
3d	75 Ro61	2 lrg, day 2 & 1 lrg, day 4; then Remove 3 blocks & implant 3 lrg, day 10
4a	9.37 RA	1 sm* RA + 1 med RA, day 4
4b	6.25 RA + 6.25 Ro41	1 med RA + 1 med Ro41, day 4
4c	6.25 RA + 6.25 Ro61	1 med RA + 1 med Ro61, day 4
4d	6.25 RA + 9.37 Ro41	1 med RA + 1 sm, 1med Ro41, day 4
4	6.25 RA + 9.37 Ro61	1 med RA + 1 sm, 1med Ro61, day 4
4f	6.25 RA + 12.5 Ro41	1 med RA + 1 large Ro41, day 4
4g	6.25 RA + 12.5 Ro41	1 med RA + 1 large Ro41, day 4
5	18.75 Ro41 + 18.75 Ro61	1 med,1 lrg Ro41 + 1med, 1 lrg Ro61, day 4
6a	**LE135	**, day 4
6b	**LE540	**, day 4
6c	LE135 + LE540	LE135 + LE540, day 4
	NATIVE LIMBS	limbs which never regenerated
	CONTROL-A	no blocks
	CONTROL-B	4 lrg, day 4

Results

Native Limbs (Table 2A)

A total of 28 limbs which have never regenerated (native) were amputated, and found to have no skeletal defects.

Controls - Without Blocks (Table 2B)

A total of 12 limbs were amputated, and allowed to regenerate without being implanted with any silastin blocks. There were variants involving carpal fusions, primarily fusion of distal carpal 1 (d1) and the radiale (r) (50%), thus reducing the carpal number to seven. Other carpal fusions involved: d3 with d4, and d1-r (17%), or fusion of the intermedium and centrale with d1-r (8%) thereby reducing the carpal number to six. All other limbs examined were complete (25%).

Controls - With Blocks (Table 2C)

A total of 21 limbs were amputated, and on day 4 postamputation, 4 large control silastin blocks were implanted. There were variants involving carpal fusions, primarily fusion of distal carpal 1 (d1) and the radiale (r), thus reducing the carpal number to seven. There was one case where the carpal number was reduced to six.

TABLE 2A

Native Limbs	
PATTERN	**No. of limbs = 28 (100%)**
complete	27 (96%)
7 carpals	1 (4%)

TABLE 2B

Control Regenerated Limbs - Without silastin blocks	
PATTERN	**No. of limbs = 12 (100%)**
complete	3 (25%)
7 carpals	6 (50%)
6 carpals	3 (25%)

TABLE 2C

Control Regenerated Limbs - With silastin blocks	
PATTERN	**No. of limbs = 21 (100%)**
complete	12 (57%)
7 carpals	8 (38%)
6 carpals	1 (5%)

Note: Blocks = control blocks without drug

For experiments 1 to 4 the results obtained using the antagonists Ro 41-5253 (Ro41) and Ro 61-8431 (Ro61) did not yield noticeably different effects on pattern formation from each other. Therefore, both sets of results have been presented together.

Experiment 1 - Implantation Time Effects (Table 3)

No observable differences in skeletal defects were reported between groups 1a to 1 j when implanting silastin blocks containing 25 µg/limb of either Ro41 or Ro61 on different days post-amputation. The main skeletal defects were primarily reductions in carpal number from eight to seven, or six, but this is the usual number for regenerated limbs. Figure 7B illustrates two of the most common carpal variants: fusion of distal carpal 1 (d1) with the radiale (r), and fusion of the centrale (c) with the intermedium (i). In addition to these carpal fusions, other carpal fusions appeared to a lesser extent: in Figure 7C and 7D, respectively; fusion of distal carpal 4 (d4) with the ulnare (u), and fusion of distal carpal 3 (d3) with distal carpal 4 (d4) were also seen. Therefore some limbs were noticeably different from control limbs (Figure 7A), exhibiting a reduction in carpals from eight to five, and there was 1 incidence of four carpals. Very few limbs exhibited phalange losses on digits 1,2, 3, and 4.

The following notes apply to Table 3:
-The double line found within the table separates the effects of the treatment from unaffected limbs.
* The numbers do not necessarily add up to 100%, since both reduction of carpals, and missing phalanges may have been observed in the same limbs.
** D4 = digit 4, and (-phalanges) = phalange loss.

TABLE 3

EXPERIMENT 1- DOSE = 25 µg/limb Ro41 or Ro61	
PATTERN	**No. of limbs = 97 (100%*)**
complete	33 (34%)
7 carpals	36 (37%)
6 carpals	18 (19%)
5 carpals	9 (9%)
4 carpals	1 (1%)
**D4 (-phalanges)	3 (3%)
D3 (-phalanges)	1 (1%)
D2 (-phalanges)	1 (1%)
D1 (-phalanges)	2 (2%)

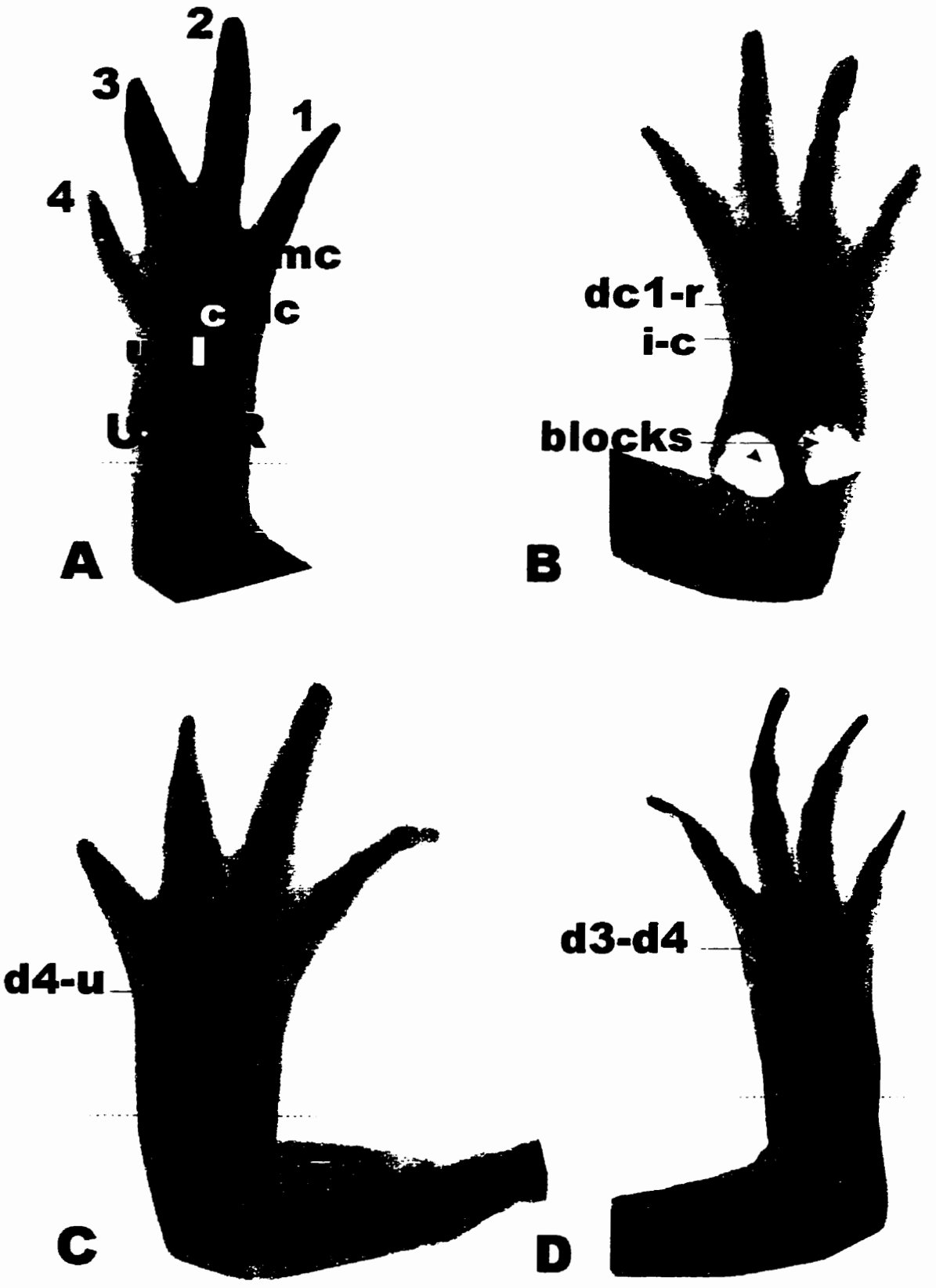
Figure 7: A) Control group, this is an untreated intact left forelimb showing normal skeletal elements: humerus (H), radius (R), ulna (U), radiale (r), ulnare (u), intermedium (I), centrale (c), four distal carpals (dc), 4 metacarpals (mc), and nine phalanges (ph) arranged in a 2-2-3-2 pattern on digits 1 to 4 respectively. ×10.

B) This right forelimb was treated with 25 µg/limb Ro 61-8431, shows two large silastin blocks. In addition, two common carpal variants can be seen in which distal carpal 1 and the radiale were fused (dc1-r), and the intermedium and the centrale have also fused into a single carpal (I-c). ×9.

C) This left forelimb was treated with 25 µg/limb Ro 61-8431, shows a reduction in carpal number from eight to five. In addition to the presence of dc1-r and I-c, the distal carpal 4 and the ulnare were also fused (d4-u). ×14.

D) This dorsal view of a right forelimb was treated with 25 µg/limb Ro 41-5253, shows another common carpal variant where distal carpal 3 and distal carpal 4 were fused into a single carpal (d3-d4). ×14.

NOTE: dashed lines represent the level of amputation through the distal radius-ulna.


2
3
1
4
mc
c
c
A
dc1-r
i-c
blocks
B
d4-u
C
d3-d4
D

Experiment 2 - Concentration Effects (Tables 4A to 4D)

Implanting silastin blocks containing 31.25 µg/limb of either Ro41 or Ro61 into regenerating limbs on day 4 post-amputation, resulted in an array of defects (Table 4A). In addition to the usual carpal number (seven or six) for regenerated limbs, some limbs possessed only five carpals. In addition to this reduction in carpals, this group also lacked one or two phalanges on digits 3 (6/23 limbs affected), and 4 (14/23 limbs affected) (Figures 8A, B). Other skeletal malformations observed were: loss of phalanges on digit 1 (4/23 limbs affected), fused or missing metacarpals (Figure 8C) , and abnormal radius and ulna (Figure 8D) (e.g., bent radius and ulna, radius fused with r).

Increasing the dose of either Ro41 or Ro61 by 6.25 µg/limb (37.5 µg/limb), did not yield noticeably different skeletal defects (Table 4B). Some regenerated limbs possessed a decrease in carpal number from eight to five. Digit 4 lacked one or two phalanges (8/20 limbs), and digit 3 (3/20 limbs) and digit 1 (4/20 limbs) continued to exhibit phalange losses.

Increasing the dose of either Ro41 or Ro61 to 50 µg/limb (day 4 post-amputation) revealed limbs which looked very similar to control limbs (Table 4C). The incidence of carpal fusions from eight to five was still apparent. Few limbs had phalange loss on digit 4 (3/22 limbs affected), and digits 1 and 3 were also observed to have few phalange losses.

The following notes apply to Tables 4A and 4B:
-The double line found within the table is used to separate the treatment effects from unaffected limbs.
• The numbers do not necessarily add up to 100%, since both reduction of carpals, and missing phalanges may have been observed in the same limbs.
** D4 = digit 4, and (-phalanges) = phalange loss.

TABLE 4A

EXPERIMENT 2- DOSE = 31.25 µg/limb Ro41 or Ro61	
PATTERN	**No. of limbs = 23 (100%)**
complete-	2 (9%)
7 carpals	7 (30%)
6 carpals	3 (13%)
5 carpals	9 (39%)
D4 (-phalanges)	14 (61%)
D3 (-phalanges)	6 (26%)
D2 (-phalanges)	2 (9%)
D1 (-phalanges)	4 (17%)

TABLE 4B

EXPERIMENT 2- DOSE = 37.5 µg/limb Ro41 or Ro61	
PATTERN	**No. of limbs = 20 (100%)**
complete	1 (5%)
7 carpals	11 (55%)
6 carpals	4 (20%)
5 carpals	2 (10%)
D4 (-phalanges)	8 (40%)
D3 (-phalanges)	3 (15%)
D2 (-phalanges)	1 (5%)
D1 (-phalanges)	4 (20%)

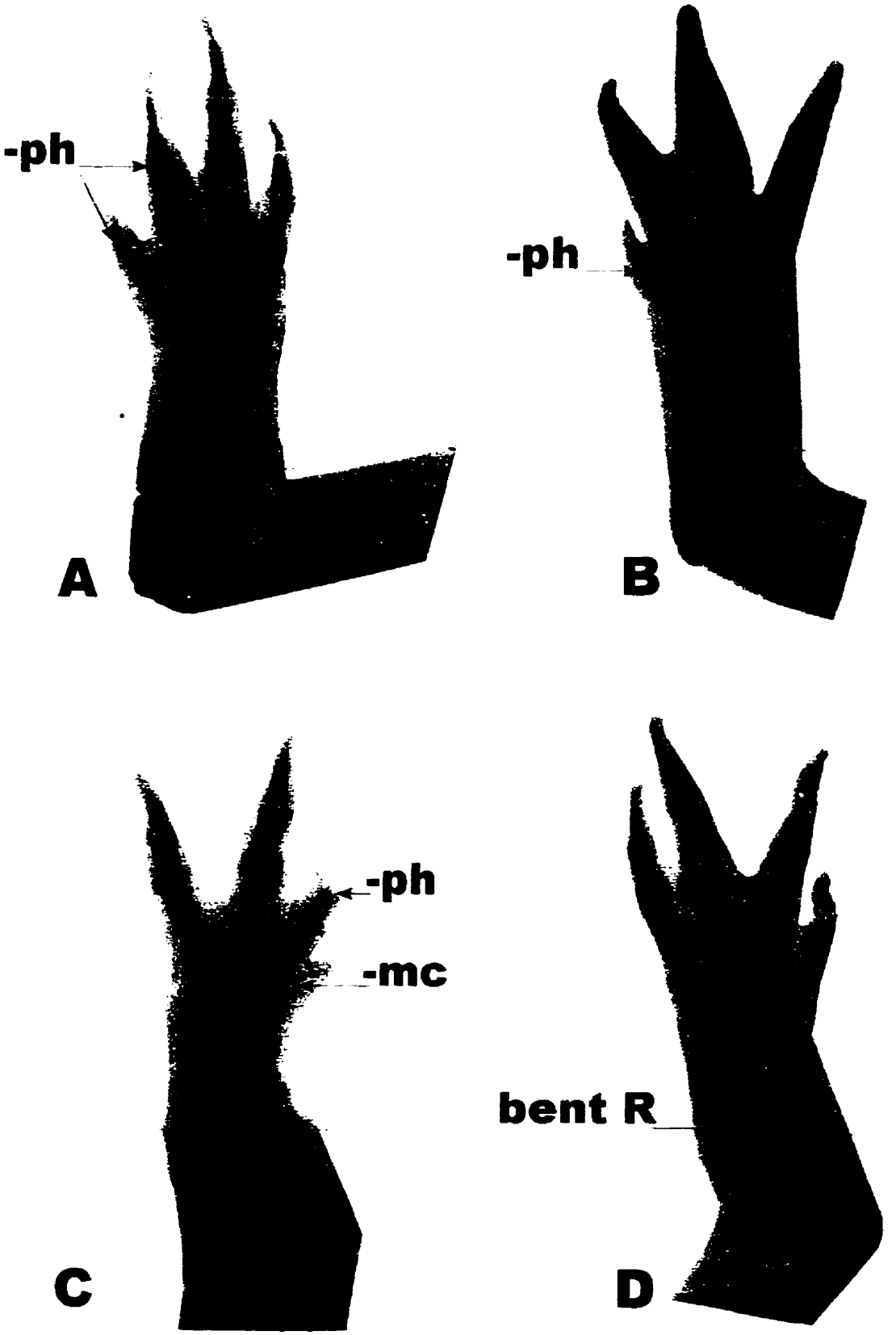
Figure 8: A) This left forelimb treated with 31.25 µg/limb Ro 61-8431, shows one phalange missing on digit 3 and another missing on digit 4 (-ph). ×15.

B) This left forelimb treated with 31.25 µg/limb Ro 61-8431, shows digit 4 lacking two phalanges (-ph), only metacarpal 4 is present. In addition only five carpals are shown. ×19.

C) This right forelimb treated with 31.25 µg/limb Ro 41-5253, shows digit 3 lacking almost all phalanges (-ph), only one phalange has been faintly stained. Digit 4 lacks all skeletal elements, even metacarpal 4 is absent (-mc). In addition only five carpals are shown. ×19.

D) This right forelimb treated with 31.25 µg/limb Ro 61-8431, shows digit 4 lacking a phalange (-ph), and there is a bend in the radius (R). ×19.

NOTE: dashed lines represent the level of amputation through the distal radius-ulna.


-ph
A
-ph
B
-ph
-mc
C
bent R
D

The following notes apply to Table 4C:
-The double line found within the table separates the treatment effects from unaffected limbs.
* The numbers do not necessarily add up to 100%, since both reduction of carpals, and missing phalanges may have been observed in the same limbs.
** D4 = digit 4, and (-phalanges) = phalange loss.

TABLE 4C

EXPERIMENT 2- DOSE = 50 µg/limb Ro41 or Ro61	
PATTERN	**No. of limbs = 22 (100%)**
complete	5 (23%)
7 carpals	4 (18%)
6 carpals	6 (27%)
9 carpals	1 (5%)
5 carpals	3 (14%)
D4 (-phalanges)	3 (14%)
D3 (-phalanges)	1 (5%)
D2 (-phalanges)	0 (0%)
D1 (-phalanges)	2 (9%)

The highest number of blocks implanted was 6 large blocks (Figure 9A), on day 4 post-amputation, of either Ro41 or Ro61 (dose =75 µg/limb)(Table 4D). At this dose, there was a large reduction in carpals from eight to five or four (11/22 limbs affected) (Figure 9B). Almost every limb had carpal fusions of d1 with r, and fusion of c with i. Other common carpal fusions were: d4 with u and, d3 with d4. Phalanges appeared normal, with the exception of 2/22 limbs which were both missing phalanges on digit 4.

Experiment 3 - Extending Treatment Time (Tables 5A and 5B)

Implanting 3 large blocks on day 3 post-amputation, and an additional 3 large blocks on day 7 post-amputation, of either Ro41 or Ro61 (dose =75 µg/limb), yielded arms with the following carpal fusions: i-c, r-d1, u-d4, and/ or d3-d4. Digit 1 (4/24 limbs), digit 3 (3/24 limbs), and digit 4 (7/24 limbs) were affected by loss of, or incomplete separation of phalanges (Table 5A).

Implanting 2 large blocks on day 2, 1 large block on day 4, and then removing these and implanting 3 new large blocks on day 10 of either Ro41 or Ro61, resulted in a reduction in carpal number from eight to five (Figure 9C). Digit 1 (5/20 limbs), digit 3 (4/20 limbs), and digit 4 (8/20 limbs) were abnormal due to loss of phalanges (Figure 9D).

The following notes apply to Table 4D:
-The double line found within the table separates the treatment effects from unaffected limbs.
* The numbers do not necessarily add up to 100%, since both reduction of carpals, and missing phalanges may have been observed in the same limbs.
** D4 = digit 4, and (-phalanges) = phalange loss.

TABLE 4D

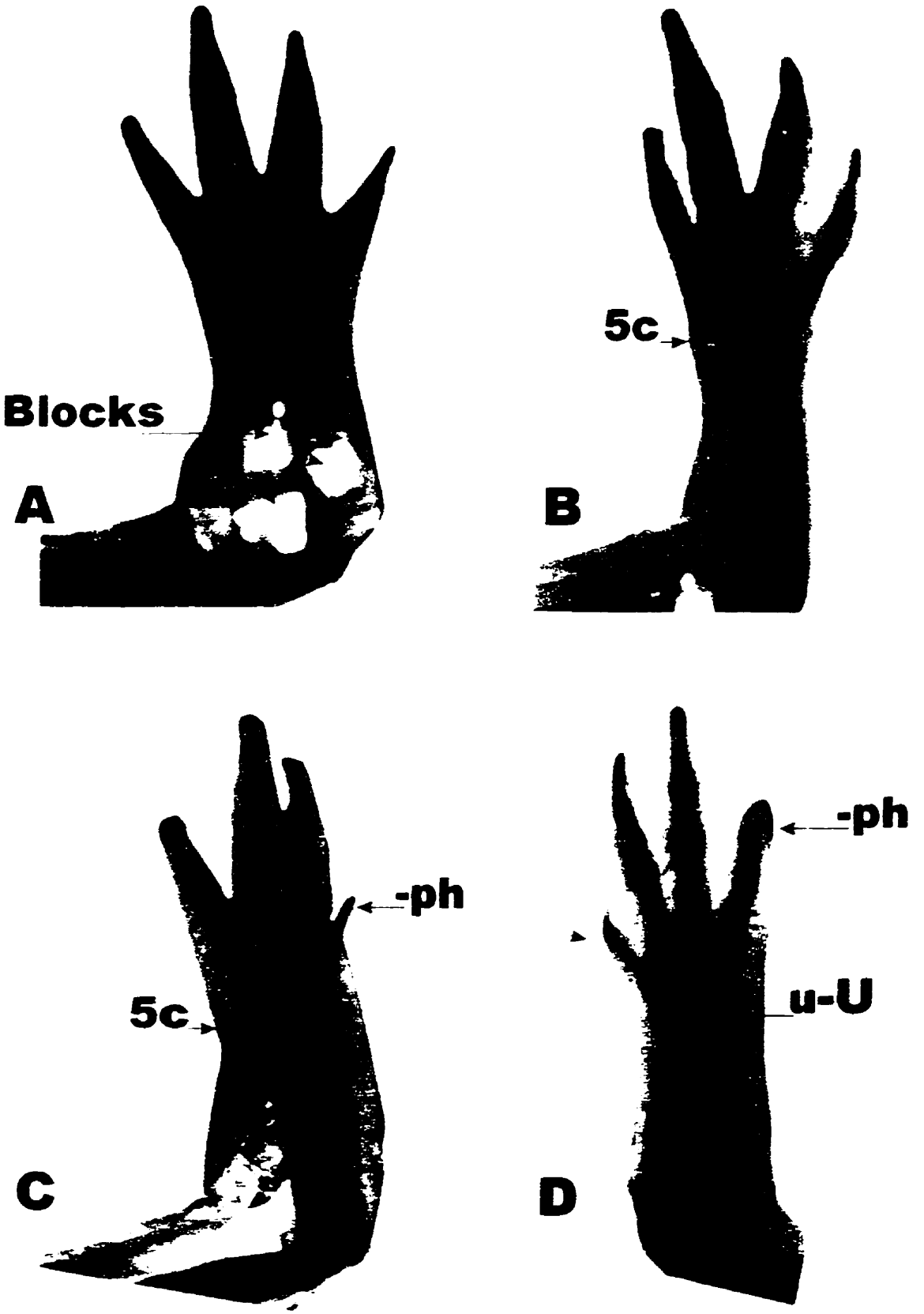
EXPERIMENT 2- DOSE = 75 µg/limb Ro41 or Ro61	
PATTERN	**No. of limbs = 22 (100%)**
complete	1 (5%)
7 carpals	4 (18%)
6 carpals	5 (23%)
5 carpals	8 (36%)
4 carpals	3 (14%)
D4 (-phalanges)	2 (9%)
D3 (-phalanges)	1 (5%)
D2 (-phalanges)	0 (0%)
D1 (-phalanges)	1 (5%)

Figure 9: A) This dorsal view of a right forelimb was treated with 75 µg/limb Ro 61-8431, shows six large silastin blocks used to locally deliver the drug to the limb. ×12.

B) This dorsal view of a right forelimb was treated with 75 µg/limb Ro 61-8431, shows a reduction in carpal number from eight to five (dashed circle), due to the fusion of distal carpal 1 with the radiale (dc1-r), fusion of the intermedium with the centrale (I-c), and distal carpal 4 fusing with the ulnare into a single carpal (d4-u). ×12.

C) This dorsal view of a right forelimb was treated with 75 µg/limb Ro 41-5253, shows a reduction in carpal number from eight to five (dashed circle), due to the fusion of dc1-r, I-c, and distal carpal 4 fusing with distal carpal 3. In addition, phalanges appear to be missing on digit 4 (-ph). ×12.

D) This dorsal view of a left forelimb was treated with 75 µg/limb Ro 61-8431, shows the incomplete separation between the ulna and ulnare (u-U). In addition, phalanges appear to be missing on digit 1 and digit 4 (-ph). ×12.


Blocks
A
5c
B
-ph
5c
C
-ph
u-U
D

The following notes apply to Table 5A:
-The double line found within the table separates the treatment effects from unaffected limbs.
* The numbers do not necessarily add up to 100%, since both reduction of carpals, and missing phalanges may have been observed in the same limbs.
** D4 = digit 4, and (-phalanges) = phalange loss.

TABLE 5A

EXPERIMENT 3- DOSE = 75 µg/limb Ro41 or Ro61, implantation on day 3 and day 7	
PATTERN	**No. of limbs = 24 (100%)**
complete	3 (13%)
7 carpals	8 (33%)
6 carpals	9 (38%)
5 carpals	3 (13%)
4 carpals	1 (4%)
D4 (-phalanges)	7 (29%)
D3 (-phalanges)	3 (13%)
D2 (-phalanges)	0 (0%)
D1 (-phalanges)	4 (17%)

The following notes apply to Table 5B:
-The double line found within the table separates the treatment effects from unaffected limbs.
* The numbers do not necessarily add up to 100%, since both reduction of carpals, and missing phalanges may have been observed in the same limbs.
** D4 = digit 4, and (-phalanges) = phalange loss.

TABLE 5B

EXPERIMENT 3- DOSE = 75 µg/limb Ro41 or Ro61, implantation on day 2, day 4, and day 10	
PATTERN	**No. of limbs = 20 (100%)**
complete	5 (25%)
7 carpals	1 (5%)
6 carpals	8 (40%)
9 carpals	1 (5%)
5 carpals	5 (25%)
D4 (-phalanges)	8 (40%)
D3 (-phalanges)	4 (20%)
D2 (-phalanges)	0 (0%)
D1 (-phalanges)	5 (25%)

Experiment 4 - Effects of Exogenous RA (Tables 6A and 6B)

Implanting 1 small and 1 medium silastin block of RA on day 4 post-amputation (9.37 µg/limb), revealed limbs containing extra long elements, and inhibited growth (8/18 limbs affected) (Table 6A). Figure 10A shows an example of an extra long radius-ulna, while Figure 10B shows an extra part radius-ulna, and 14c shows a complete inhibition of regeneration. There were also 2 cases supernumerary digits and one case of a supernumerary limb (Figures 10C,D).

To determine if Ro41 and Ro61 could antagonize the effects of exogenous RA, I implanted 1 medium block of RA with 1 medium block of either Ro41 or Ro61. There were no noticeable differences between the arms implanted with RA and Ro41 or RA and Ro61, thus I have grouped the results. All 22 limbs showed missing or incomplete development of phalanges (Figure 11A), and there was one incident of inhibited regeneration. The radius (R) and ulna (U) bones appeared frequently abnormal (15/22 limbs); e.g., R-U exhibited an abnormal curvature (Figure 11B), R-like element, extra long R-U. Four of the 22 regenerated limbs had eight carpals, the other limbs had a reduction in carpals from eight to seven (2/22), six (4/22), or five or fewer (8/22). Furthermore, two limbs had an extra carpal element, and there were no occurrences of supernumerary limbs.

Increasing the concentration of Ro41, 1 small and 1 medium block implanted, while keeping the concentration of exogenous RA constant (1 medium block implanted as above), the nature of limb defects showed considerable variation. The radius and ulna were abnormally arranged, as were the carpals. Figures 11C to 11F show the most severe defects. In figure 11C,

The following notes apply to Table 6A:
• The numbers do not necessarily add up to 100%, since both reduction of carpals, and missing phalanges may have been observed in the same limbs.
** D4 = digit 4, and (-phalanges) = phalange loss.

TABLE 6A

EXPERIMENT 4- RA DOSE = 9.37 µg/limb	
PATTERN	**No. of limbs =18 (100%)**
complete	0 (0%)
inhibited	8 (44%)
extra cartilaginous element	1 (6%)
D4 (-phalanges)	6 (33%)
D3 (-phalanges)	3 (17%)
D2 (-phalanges)	2 (11%)
D1 (-phalanges)	1 (6%)
extra humerus	3 (17%)
extra long radius-ulna	2 (11%)
extra part radius-ulna	2 (11%)
supernumerary limb	3 (17%)

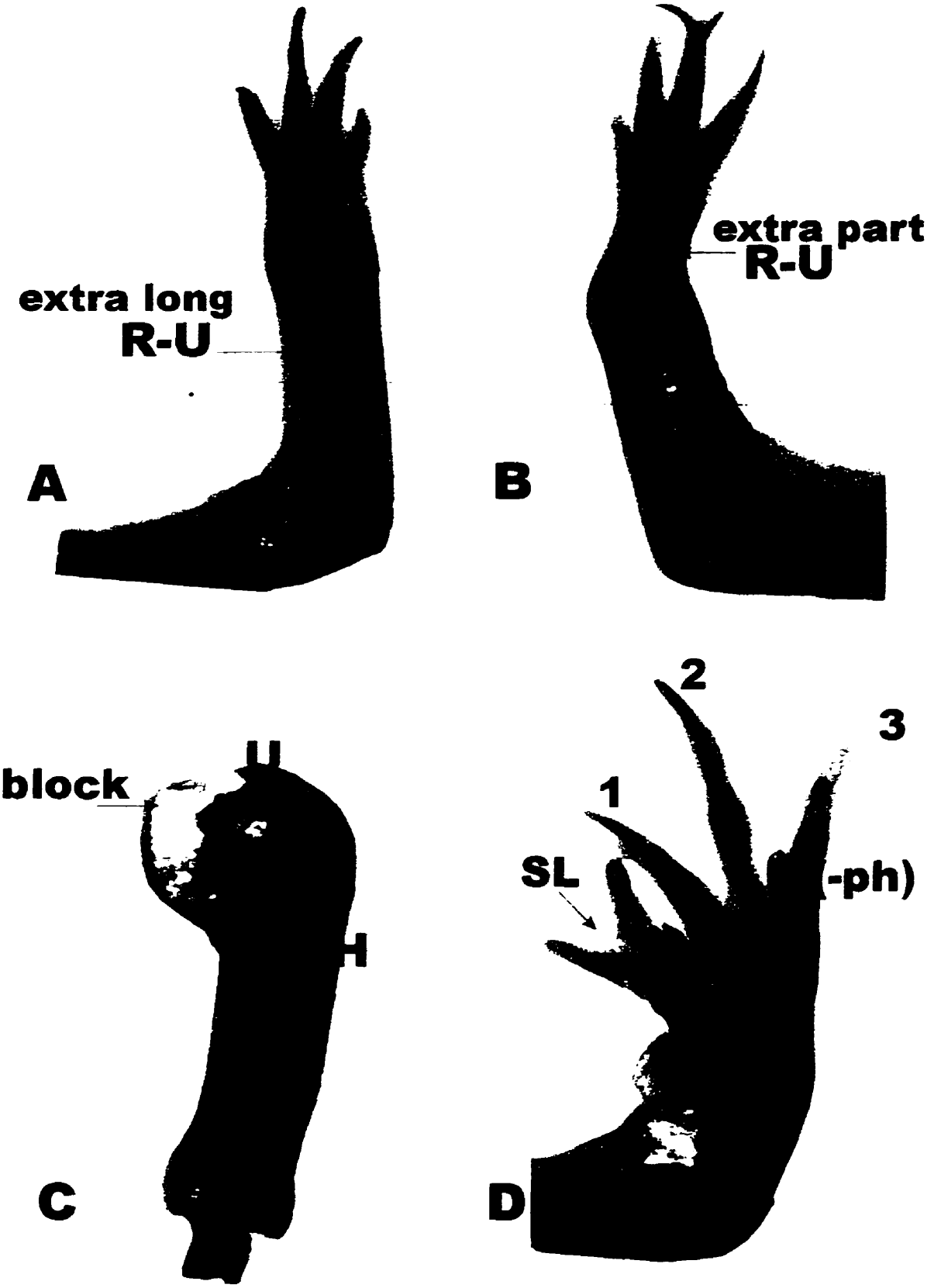
Figure 10: All limbs were treated with 9.37 µg/limb RA.

A) This right forelimb reveals an extra long radius and ulna (R-U). ×11.

B) This left forelimb reveals an extra part radius and ulna (R-U). ×12.

C) An inhibited limb. No regeneration occurred. ×18.

D) This forelimb shows a lack of skeletal elements on digit 4 (-ph). In addition, there is a two-digit supernumerary limb (SL) projecting from the posterior axis, and extending toward the anterior axis. ×17.


extra long
R-U
A
extra part
R-U
B
block
U
H
C
2
3
1
SL
(-ph)
D

the regenerate is missing digit 4, and there are approximately 4 supernumerary digits stemming from the anterior margin. The contra-lateral limb also revealed 2 supernumerary digits, digit 2 and digit 3, on the anterior margin. Furthermore this regenerate also possessed a supernumerary limb in the pallette to notch stage (Figure 11D). Another type of supernumerary limb, growing from the anterior margin of the limb, appeared to have a partial humerus, very short radius and ulna bones, well formed digit 1 and 2, and two not well developed digits (Figure 11E). The original regenerate was unaffected, except for a reduction in carpal number from eight to six. The contra-lateral limb has regenerated an extra humerus, the radius and ulna appear very small, and there are seven carpals (Figure 11F).

The defects varied extensively among contra-lateral limbs, when 1 small and 1 medium block of Ro61, and 1 medium block of RA were implanted. Therefore, I have summarized the results in Table 6B.

Figure 11: A) Left forelimb was treated with 6.25 µg/limb RA and 6.25 µg/limb Ro 41-5253, shows incomplete development of phalanges (*ph). The radius and ulna bones have also regenerated abnormally. ×17.

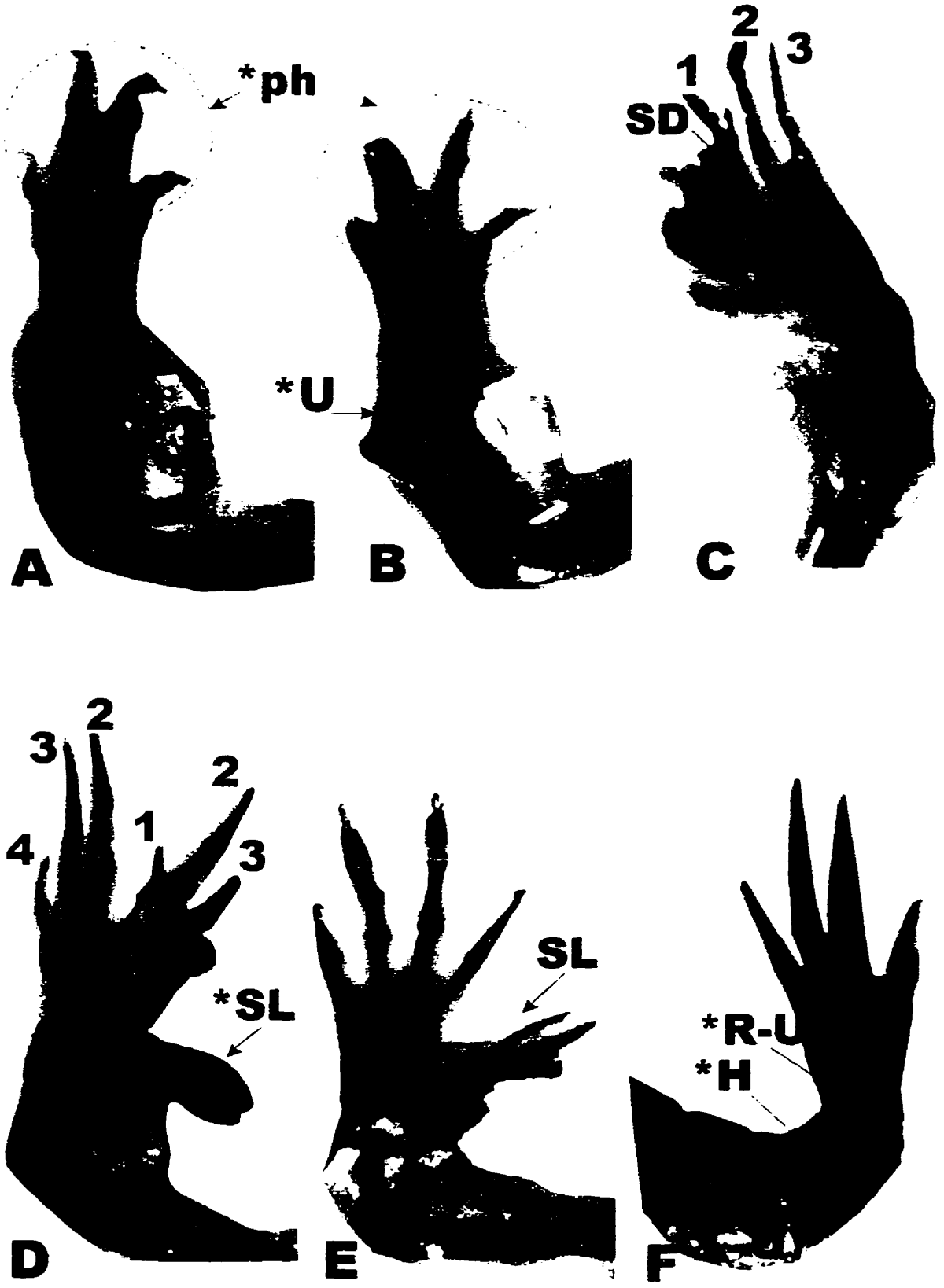
B) Left forelimb was treated with 6.25 µg/limb RA and 6.25 µg/limb Ro 61-8431, shows incomplete development of phalanges (*ph). In addition, the ulna has developed a small bulge not seen in control limbs (*U). ×19.

C) Right forelimb was treated with 6.25 µg/limb RA and 9.37µg/limb Ro 41-5253, shows digit 4 completely missing from the original limb. In addition, there are three supernumerary digits (SD) present on the anterior margin of the original limb. It is difficult to detect the radius and ulna of the original limb. ×16.

D) Forelimb was treated with 6.25 µg/limb RA and 9.37µg/limb Ro 41-5253, shows supernumerary digits 2, 3, and digit 1 seems to be shared with the original limb. In addition, there is a supernumerary limb in the pallette to notch stage (*SL) on the anterior margin of the original limb. ×16.

E) Left forelimb was treated with 6.25 µg/limb RA and 9.37µg/limb Ro 41-5253, shows a normal looking original regenerated limb. In addition, there appears to be a supernumerary limb extending from the posterior margin of the original limb (SL). The supernumerary limb appears to have a partial humerus, radius, ulna, and two well developed digits 1 and 2. ×16.

F) Right forelimb was treated with 6.25 µg/limb RA and 9.37µg/limb Ro 41-5253, shows that the distal end of the humerus has regenerated first, followed by the distal radius and ulna (*R-U). ×15.


*ph
*U
2
3
1
SD
A
B
C
3
2
2
4
1
3
*SL
SL
*R-U
*H
D
E
F

The following notes apply to Table 6B:
D = digit, and (-phalanges) = phalange loss.
L = Left forearm, and R = Right forearm

TABLE 6B - Ro61(9.37 µg/limb) + RA (6.25 µg/limb)						
PATTERN		AXOLOTL #				
		1	2	3	4	5
inhibited	L	+				+
	R					
7 carpals	L					
	R		+			
6 carpals	L		+	+		
	R				+	
5 carpals	L					
	R			+		
4 carpals	L				+	
	R					+
D4 (-phalange)	L				+	
	R	+				
D3 (-phalange)	L				+	
	R			+		
D2 (-phalange)	L					
	R	+				
D1 (-phalange)	L					
	R	+				
extra cartilage element	L	+				
	R					

The following notes apply to Table 6B continued:
L = Left forearm, and R = Right forearm

TABLE 6B - Continued

Table 6 B - Ro61(9.37 µg/limb) + RA (6.25 µg/limb)						
PATTERN	AXOLOTL #					
		1	2	3	4	5
supernumerary digits	L					
	R				+	+
supernumerary process	L			+		
	R			+		
Radius-Ulna abnormal	L			+	+	
	R	+		+	+	

The next experiment looked at implanting 1 large block of Ro41 and 1 medium block of RA. There were no supernumerary limbs or digits, however, there was one incidence of a supernumerary process (Figure 12A). The radius and ulna bones appeared frequently abnormal (bent or curved, Figure 12B), but all digits possessed the proper phalange number. Two of the ten limbs had eight carpals, but there was a decrease in carpal number to seven (4/10) and six (1/10) carpals. Furthermore, there was an increase in carpal number from eight to nine (2/10), and ten (1/10).

Implanting 1 large block of Ro61 and 1 medium block of RA, there were two incidences of supernumerary digits (Figures 12C, D), as well as two cases of supernumerary limbs. The two supernumerary limbs differ in appearance, one is growing towards the anterior margin, while the other is extending toward the posterior margin (Figures 12E, and 12F, respectively). These defects represented the most severe in this experiment. Two of the twelve limbs were complete, while others had carpal reductions from eight to seven (5/12), or six (2/12). Two limbs had carpal numbers of nine, and eleven, however, this was associated with the presence of supernumerary digits. The radius and ulna bones appeared largely normal; in five limbs the bones were bent or curved, and all limbs possessed the proper phalange number (except when supernumerary digits were present).

Figure 12: Each forelimb was treated with 6.25 µg/limb RA and either 12.5 µg/limb Ro 41-5253 or Ro 61-8431.

A) Digit 4 is missing a phalange, and there is a supernumerary process (SP) extending from the anterior margin of the original limb. ×16.

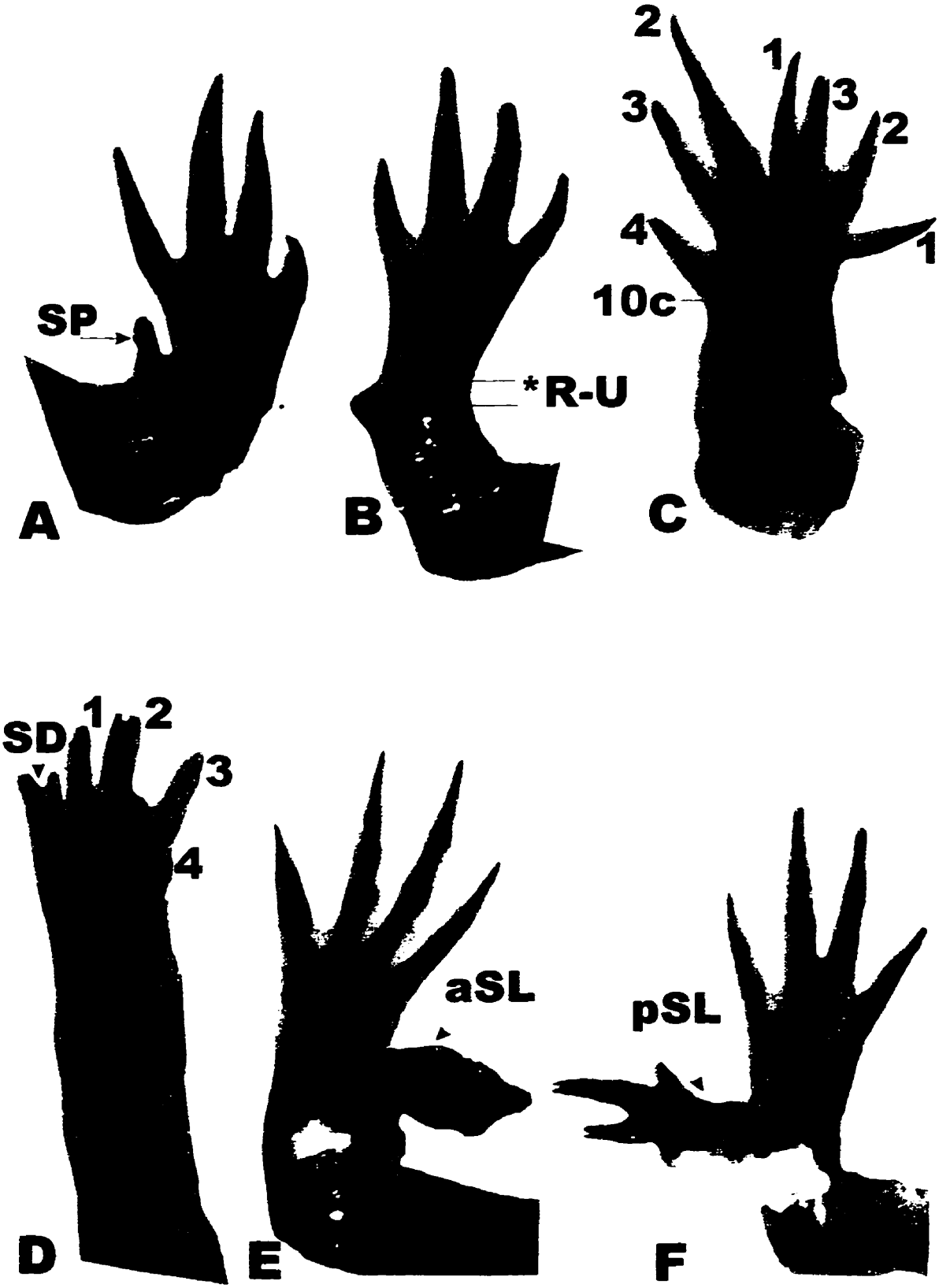
B) All digits had complete phalange development, but the radius and ulna are abnormally bent (*R-U). In addition, the ulna has developed a small bulge not normally seen in control limbs. ×15.

C) The regenerated limb possesses supernumerary digits with phalanges (ph) arranged in a 2-2-3-2-2-3-2 pattern on digits 1, 2, 3, 1, 2, 3, and 4 respectively. In addition, there was an increase in carpal number from eight to ten (10c). ×18.

D) The regenerated limb possesses supernumerary digits (SD). Digits 1, 2, and 3 have the correct phalange number, but digit 4 is lacking all skeletal elements. In addition, it is difficult to observe the correct carpal number, and the radius and ulna appear abnormally short.

E) This shows a normal looking regenerated limb. However, there appears to be a supernumerary limb extending from and towards the anterior margin of the original limb (aSL). The supernumerary limb appears to have a partial humerus, radius, ulna, and digits have begun developing. ×14.

F) This limb shows a normal looking original regenerated limb. However, there appears to be a supernumerary limb extending from the anterior margin of the original limb, but extending towards the posterior axis (pSL). The supernumerary limb appears to have a partial humerus, and two well formed digits. ×10.


SP
A
*R-U
B
2
1
3
3
2
4
1
10c
C
SD
1
2
3
4
D
aSL
E
pSL
F

Experiment 5 - Combined Effect of Ro41 and Ro61

To observe the combined effect of Ro41 and Ro61 on patterning, 1 medium and 1 large block and 1 small block (18.75 µg/limb) of both Ro41 and Ro61 were implanted. At the combined antagonist concentration of 37.5 µg/limb, there were no noticeably different skeletal defects (58% of limbs were complete). The only variation observed was a reduction in carpal number from eight to seven (34%) or six (8%), but this was the usual carpal number (seven or six) for regenerated limbs.

Experiment 6 - Preliminary Experiments using LE135 and LE540 (Table 7A to 7C)

These preliminary experiments looked at implanting retinoid antagonists LE135 and LE540. The amount of either LE135 or LE540 implanted was approximately equivalent to the amount of drug contained within one large silastin block. The results obtained using LE135 and LE540 alone, and in combination were very different. Generally, there was an inhibition of regeneration when LE135 was implanted alone (8/12 limbs treated were affected)(Table 7A). The carpal number never exceeded four, and the inhibited limbs possessed digit-like outgrowths (Figure 13A). When more than one digit was present they all lacked phalanges (Figure 13B).

The majority of limbs implanted with LE540 alone appeared as did controls (9/12 limbs treated were normal)(Figure 13C). The usual carpal number (seven) for regenerated limbs was observed. There was only one incidence of a complete digit missing, and only three limbs lacked phalanges (Table 7B).

The following notes apply to Tables 7A and 7B:
• The numbers do not necessarily add up to 100%, since both reduction of carpals, and missing phalanges may have been observed in the same limbs.
** D4 = digit 4, and (-phalanges) = phalange loss.

TABLE 7A

Experiment 6 - LE135 (about 12.5 µg/limb)	
PATTERN	**No. of limbs = 12 (100%)**
complete	0 (0%)
inhibited	8 (67%)
4 carpals	3 (25%)
3 carpals	2 (17%)
2 carpals	1 (8%)
0 carpals	6 (50%)
D4 (-phalanges)	4 (33%)
D3 (-phalanges)	4 (33%)
D2 (-phalanges)	4 (33%)
D1 (-phalanges)	4 (33%)

TABLE 7B

Experiment 6 - LE540 (about 12.5 µg/limb)	
PATTERN	**No. of limbs = 12 (100%)**
complete	9 (75%)
inhibited	0 (0%)
7 carpals	6 (50%)
D4 (-phalanges)	3 (25%)
D1 (-phalanges)	1 (8%)

When limbs were treated with both LE135 and LE540 simultaneously, there were no incidences of inhibition of regeneration, and no complete/normal regenerates (Table 7C). The carpal number was reduced to five, four, three, or two, and phalanges were consistently missing (Figures 13D, E). Furthermore, 60% of treatment limbs regenerated incomplete digits at six weeks post-amputation, that is, metacarpals and phalanges did not stain up well (Figure 13F).

The following notes apply to Table 7C:
* The numbers do not necessarily add up to 100%, since both reduction of carpals, and missing phalanges may have been observed in the same limbs.
** D4 = digit 4, and (-phalanges) = phalange loss.

TABLE 7C

Experiment 6 - LE135 (about 12.5 µg/limb) + LE540 (about 12.5 µg/limb)	
PATTERN	**No. of limbs = 10 (100%)**
complete	0 (0%)
inhibited	0 (0%)
5 carpals	3 (30%)
4 carpals	4 (40%)
3 carpals	2 (20%)
2 carpals	1 (10%)
D4 (-phalanges)	4 (40%)
D3 (-phalanges)	4 (40%)
D2 (-phalanges)	4 (40%)
D1 (-phalanges)	4 (40%)
incomplete digit regeneration	6 (60%)

Figure 13: Forelimbs were treated with LE135, LE540, or both.

A) When this limb was treated with LE135 alone, there was an inhibition of regeneration. The radius and ulna can be seen, followed by only about three carpals (3c), and a digit-like process (dlp). ×10.

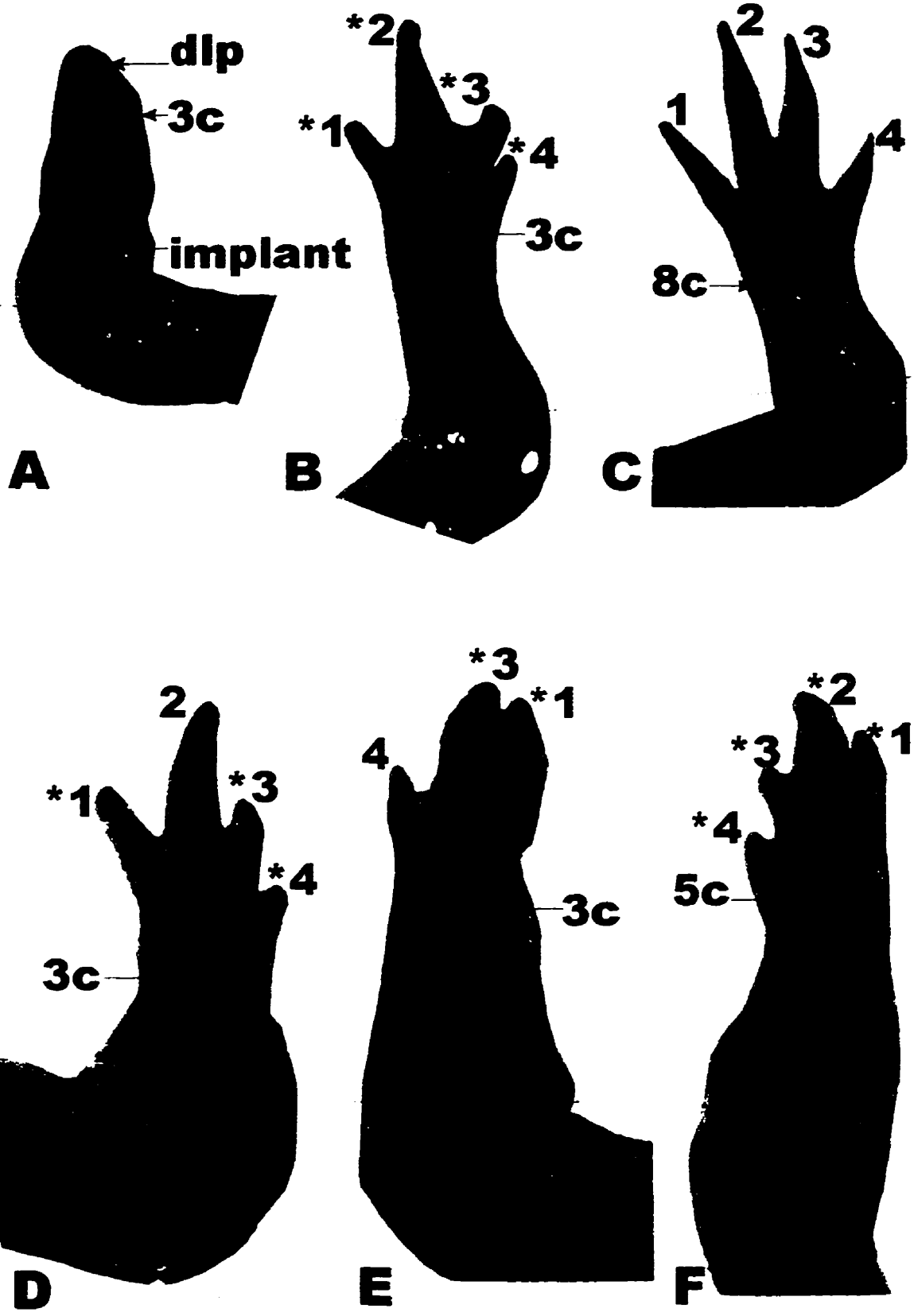
B) When this limb was treated with LE135 alone, there was a lack of proper phalange number on digits 1*, 2*, 3*, 4*. The radius and ulna can be seen, followed by only about three carpals (3c). ×9.

C) When this limb was treated with LE540 alone, an intact limb regenerated; possessing eight carpals (8c), and the correct phalange arrangement of 2-2-3-2 on digits 1 to 4 respectively. ×11.

D) When this limb was treated with both LE135 and LE540, a limb regenerated possessing three carpals (3c), and digits *1, *3, and *4 lacked phalanges. Digit 2 possessed the proper number of phalanges. ×19.

E) When this limb was treated with both LE135 and LE540, a limb regenerated possessing three carpals (3c), and only three very short digits regenerated. Digit *1 and digit *3 were missing phalanges, while digit 4 regenerated two phalanges. ×15.

F) When this limb was treated with both LE135 and LE540, a limb regenerated possessing five carpals (5c), and incomplete digits lacking metacarpals and phalanges on digits *1 to *4. ×13.


dlp
3c
implant
A
*2
*3
*1
*4
3c
B
2
3
1
4
8c
C
2
*3
*1
*4
3c
D
*3
*1
4
3c
E
*2
*1
*3
*4
5c
F

Discussion

This thesis examined if endogenous retinoic acid (RA) is essential for patterning of the axolotl (*Ambystoma mexicanum*) regenerating limb. Using retinoid antagonists Ro41-5253 (Ro41), and Ro61-8431 (Ro61), a RARα and RARβ knockout was created to block RAR/RXR-induced transactivation. The results reveal that Ro41 and Ro61 do not induce noticeably different malformations during patterning of the regenerating limb, in spite of being able to inhibit RARα- and RARβ-induced transactivation *in vitro*. Lack of major skeletal defects may have been due to the inability of these retinoid antagonists to inhibit retinoid-retinoid receptor transactivation *in vivo*. It can also be speculated that patterning was not affected by the administration of these antagonists because they specifically target RARα and RARβ. Other retinoid receptors may be substituting for these temporarily inaccessible receptors, thereby maintaining proper retinoid signaling. It is difficult to accurately state why limb skeletal pattern was not disrupted because of the recent results obtained from preliminary studies using the vitamin A antagonists LE135 and LE540. I found LE135 inhibited limb regeneration, while LE540 did not noticeably affect limb regeneration.

Control Groups

To examine how common carpal variants were in limbs which had never regenerated (native limbs), I analyzed the skeletal pattern of native limbs, and compared them to the regenerated limbs with and without blocks (see Tables 2A to 2C). Native limbs were found to have no skeletal defects, and were reported as being complete. Regenerated limbs with and without blocks showed more

variation in skeletal pattern than did native limbs. Scadding (1989) has also shown that native limbs do exhibit a complete skeletal pattern in the majority of cases, while regenerated limbs are more variable. The reason for examining the skeletal patterns in the autopodium of regenerated limbs with and without silastin implants was to examine whether pattern changes were caused by amputation and regeneration alone or by the implantation of silastin blocks during regeneration. All control limbs regenerated exhibited skeletal patterns (carpal reductions) comparable to native limbs. These results are consistent with the control studies performed by Maden et al. (1985).

Experiment 1 - Varying Implantation Time

Ro41 and Ro61 were administered at different implantation times to assess the optimum implantation time after amputation at which these retinoid antagonists would have the most profound effect on pattern formation (see Table 3). Using 25 µg/limb of either Ro41 or Ro61, there was no difference in the results (minor skeletal defects observed). Over the range of implantation times used (days 2, 4, 5, and 6) the defects observed were primarily carpal variants resulting from carpal fusion, and there were very few incidences of phalange losses (1-3%). Furthermore, regenerated limbs treated with Ro41 or Ro61 exhibited similar effects on pattern formation at this dose level.

In addition to the above, knowing that extensive dedifferentiation occurs at about day 4 post-amputation, and the most effective time of administration for 100mg/ml RA blocks is at this stage (Maden et al., 1985), 100mg/ml antagonist blocks were implanted on day 4 in experiment 2, 4, 5, and 6.

Experiment 2 - Varying Concentration

The effect of increasing the dose of Ro41 or Ro61 (doses = 31.25µg/limb, 37.5 µg/limb, 50 µg/limb, and 75 µg/limb) on day 4 post-amputation was examined. Over this dose range there were no observable differences in the results, and there were no noticeable differences between the two treatment groups (Ro41 or Ro61). The greatest variation in skeletal pattern of the four treatment doses was observed at the lowest dose, 31.25 µg/limb (see Table 4A). At this dose there were many digits with incomplete phalange numbers on digit 3 (26%), and digit 4 (61% affected). Less defects were observed with increasing antagonist dose, and at the highest dose (75 µg/limb) the incidence of phalange losses on digit 3 and digit 4, were 5% and 9%, respectively (see Table 4D).

Ro41 and Ro61 were used to create a receptor knockout and inhibit RAR-induced gene transcription. I expected to observe regenerated limbs with severe skeletal malformations as a result of disrupting retinoid mediated signaling, but this clearly was not the case (refer to Figures 7, 8, and 9A, B). One reason why patterning was not severely affected using Ro41 and Ro61 might be the antagonists selectivity for specific RAR subtypes (α and β). Ro41 was first described as an RARα-selective antagonist (Apfel et al., 1992), however based on the concept of functional redundancy, this receptor-subtype-selectivity has been criticized by Standeven et al. (1996). The subtype selectiveness of Ro41 may be limiting its ability to examine the role of RARs *in vivo*, since the RAR subtypes and isoforms may overlap in function. However, it is debatable whether Ro41 is in fact α-selective. This synthetic retinoid was tested for its antagonism in human promyelocytic (HL-60) cells which Apfel et al. (1992)

stated as expressing only α-subtype RARs. However, HL-60 cells have been shown to contain RARα and RARβ, first by Hashimoto et al. (1989), and later this idea was supported by Eyrolles et al. (1994). Furthermore, Moroni et al. (1993), and Apfel et al. (1992) have shown that addition of 50- to 100-fold excess of Ro41 does antagonize RARβ, whereas RARγ mediated signaling is unaffected.

In addition, Ro41 has been used to inhibit retinoid mediated signaling during early *Xenopus* and chick embryogenesis (López et al., 1995), and has been proposed to inhibit both RARα and RARβ transactivation. López et al. (1995) observed heart and foregut malformations when *Xenopus* and chick embryos were treated with the Ro41. These structures were not malformed in RARα or RARβ null mutations, but were malformed in RARαβ double mutant mouse embryos (Lohnes et al., 1994). Thus, the heart and gut defects observed in *Xenopus* and chick embryos may have resulted from Ro41 blocking not only RARα, but both RARα and RARβ simultaneously. Lohnes et al. (1994) did not observe any skeletal limb defects in RARαβ double mutants, but limb defects were evident in RARαγ double mutant mice. Therefore, RARβ may not be needed in patterning of the limb; since skeletal limb defects were not reported in Ro41 treated embryos, and in RARαβ double mutant mice (although limbs were abnormal in RARαγ double mutant mice). From this, Ro41 can not be precisely labeled α-selective, because it may be functioning as Ro61 in antagonizing both RARα and β. If this is the case, the results I obtained in the axolotl using Ro41 and Ro61 support the current literature, that inhibiting RARα and RARβ simultaneously does not affect patterning of the limb in a major way.

Experiment 3 and Experiment 5 - Extended Treatment Time & Combined Effect

Experiment 3 examined extended treatment times at 75 µg/limb, and experiment 5 was a brief look at the combined effect of Ro41 and Ro61. No observable differences were reported in extending the treatment time of either antagonist (refer to Tables 5A and 5B), or in administering Ro41 and Ro61 in combination. Again, these results suggest that blocking RARα and β has no drastic effect on patterning of the regenerating limb.

Experiment 4 - Ro41 or Ro61 Antagonism of Exogenous RA

Experiment 4 examined the ability of increasing concentrations of Ro41 or Ro61 to antagonize the exogenous effects of RA. Implanting 9.37 µg/limb of RA, there was predominantly almost 50% inhibition of regeneration, phalange loss on all digits, extra long radius-ulna bones, and supernumerary digits/limbs (refer to Figure 10). Subsequently, I examined the ability of Ro41 and Ro61 to counteract exogenous RAs effects *in vivo*. Increasing the dose of Ro41 and Ro61 (6.25 µg/limb, 9.37 µg/limb, and 12.5 µg/limb), these antagonists were only partially successful in antagonizing some of the effects induced by exogenous RA (6.25 µg/limb). All regenerating limbs treated with either 6.25 µg/limb or 9.37 µg/limb of Ro41 or Ro61 in combination with 6.25 µg/limb RA, revealed incidences of inhibited regeneration, missing phalanges, abnormal radius-ulna bones, and abnormal carpals (see Figure 11). Supernumerary digits or limbs were not seen in the limbs of animals treated with the lowest antagonist dose, but were observed in limbs treated with 9.37 µg/limb of Ro41 or Ro61 in combination with RA (6.25 µg/limb) (see Figures 11C , D, E). The incidence of supernumerary limbs and digits was also observed in limbs treated with 12.5

µg/limb Ro61 + 6.25 µg/limb RA, but not in those treated with 12.5 µg/limb Ro41 + 6.25 µg/limb RA. The regeneration of supernumerary limbs is thought to develop as a result of the method of retinoid application. Maden et al. (1985) have shown that delivering RA by implantation of silastin blocks can induce varying degrees of supernumerary limbs, and this induction is unrelated to RA concentration. There were no cases of supernumerary limbs in control or antagonist treatment groups, thus it is possible that RA is causing local tissue irritation (not the block itself) which induces the formation of an accessory blastema.

There were no phalange losses or complete inhibition of regeneration in limbs treated with 12.5 µg/limb of Ro41 or Ro61 + RA (6.25 µg/limb). These results suggest that 12.5 µg/limb of Ro41 and Ro61 may be antagonizing exogenous RA's ability to produce limbs lacking phalanges, thereby producing digits with complete phalange numbers. At 12.5 µg/limb, either antagonist may also be able to effectively block RA-induced inhibition of regeneration. This concentration, also induced an increase in the number of limbs with extra carpals (from eight to nine, ten or eleven). There were no cases of increased carpal number in animals treated with RA alone, or at the lowest antagonist dose used in combination with RA. Maden (1982) reported the induction of carpals in the presence of low levels of RA. Perhaps the antagonists effectively lowered the concentration of exogenous RA to a level where extra carpals were induced.

The nature of the limb defects showed considerable variation, making it difficult to assess how exogenous RA interacted with retinoid antagonists *in vivo*. Administering both to the regenerating limb, may have unbalanced the retinoid

signaling pathway, such that diverse skeletal malformations arose. Is it possible that Ro41 and Ro61 are not capable of antagonizing exogenous RA *in vivo*? Are these antagonists functioning differently *in vivo*, in the presence and absence of exogenous RA? Ro41 has been shown to counteract the teratogenic effects of a RARα-selective agonist (Ro 40-6055) *in vitro* (Eckhardt and Schmitt, 1994). However, *in vivo* studies (in mice) have revealed that simultaneous administration of Ro41 and teratogenic doses of the Ro 40-6055 did not completely counteract the teratogenic effects, but reduced the frequency and/or severity of major malformations. The results obtained in experiment 4 partially correlate with the mouse study, since Ro41 or Ro61 only reduced the frequency of some RA-induced limb malformations. The major malformations detected in the Eckhardt and Schmitt (1994) study were cleft palate, and ear abnormalities. Interestingly, they did not observe any significant incidences of limb malformations after administration of Ro41 alone, RARα-selective agonist alone, or Ro41 and this agonist in combination. Thus, this again (see discussion - experiment 2) suggests that blocking RARα and RARβ transactivation does not necessarily affect patterning of the limb.

From the results obtained, it is not surprising that single agonist/antagonist combinations can provide only a fraction of the picture of the complex retinoid control of gene expression *in vivo*, despite clear cut results in *in vitro* systems. However, it is important to attempt to show how these novel retinoid receptor antagonists function in the whole animal. Significant alterations in patterning during limb regeneration resulting from the treatment of retinoid antagonists *in vivo* should provide some evidence as to what the RARs function

is in the intact animal as compared to *in vitro*. Are RARαβ antagonists functioning differently in the presence of exogenous RA during limb regeneration as compared to limb development? Part of the answer may lie in the fact that amphibians are the only vertebrates capable of limb regeneration, and it should be expected that the results obtained in a regenerative system would differ in some way from those obtained in developing mouse or chick embryo limbs. Furthermore, previous studies suggest the existence of significant differences in the response to vitamin A between developing and regenerating limbs in both the axolotl and *Xenopus* (Scadding and Maden, 1986a and b). This may also be true of an antagonists ability to function effectively in antagonizing endogenous RA in developing versus regenerating systems.

Experiment 6 - Preliminary Work using LE135 and LE540

Preliminary studies were started which looked at the effects of retinoid antagonists LE135 and LE540, alone or in combination, on the regenerating limbs of axolotl larvae.

LE135 and LE540 have similar binding affinities for RARα and RARβ, however, 1000-fold excess of LE135 does not affect the binding of RA to RARγ, RXRα, RXRβ, or RXRγ. Furthermore, LE540 was found, *in vitro*, to be the more potent antagonist when compared to LE135 (Umemiya et al., 1997). This difference in potency is thought to be the ability of LE540 to antagonize; in addition to RARα and RARβ; RARγ, RXRα, RXRβ, and RXRγ. Based on this knowledge, I expected limb regeneration to be inhibited when LE540 was administered to the axolotl, and that the axolotls treated with LE135 would regenerate relatively normal limbs, with defects similar to those seen in the limbs

of animals treated with Ro41 and Ro61. This was clearly not the case (see Tables 7A, B, C). LE135 inhibited regeneration of almost 70% of regenerating limbs, while LE540 revealed normal looking regenerates (75%)(see Figures 13A, B). Moreover, when LE135 and LE540 were implanted into the regenerating limb in combination, regeneration was not inhibited, but it also did not yield normal looking limbs (see Figures 13D, E, F). Used together, the regenerating limbs possessed abnormal phalange and carpal number, and 50% of regenerates showed incomplete digit development after six weeks of regeneration. Therefore, it appears that the inhibitory effects of LE135 on regeneration were counteracted by the presence of LE540. Could LE540 be acting as a retinoid agonist in the presence of a retinoid antagonist?

LE135 Inhibits Limb Regeneration

Ro41, Ro61, and LE135 are compounds thought to antagonize only RARα and RARβ, however the results obtained using LE135 alone, contradict the results obtained using either Ro41 or Ro61 alone. Why this discrepancy? This could have been caused by several factors. First, LE135 and LE540 were implanted as a thick paste and the amount of these antagonists was estimated to be about the amount of Ro41 or Ro61 in one to two large silastin blocks (12.5 µg/limb to 25 µg/limb). This could have resulted in LE135 leaking out of the silastin paste more efficiently and more rapidly than Ro41 or Ro61 could leak out of the cured silastin block, subsequently targeting more redifferentiating cells of the regenerating limb. However, I believe this would have had a minimal effect on the drugs capacity to reach the cells, because more of the test compound (LE135) would also have had an easier chance of leaking out of the open wound

made as a result of the implantation technique. Furthermore, in the experiments using Ro41 and Ro61, six large silastin blocks were implanted at one time, while the amount of LE135 implanted could not have exceeded the amount contained in more than two large silastin blocks. We do not know if the various RARα and RARβ isoforms; RARα1 and α2, and RARβ1, β2, β3, β4; serve different functions, and if these isoforms function interchangeably. nor do we know what isoforms retinoid antagonists are targeting, and how they function *in vivo*? In light of these uncertainties, attempts to interpret these results can only be speculative.

Retinoid antagonists LE135, Ro41, and Ro61 may be antagonizing different RARα and RARβ isoforms, and may also be operating very differently *in vivo*. In discussing the results obtained with Ro41 and Ro61 (see above), I suggested that patterning was largely unaffected in regenerating limbs due to the possibility of different receptors being functionally redundant. Thus, although RARα and RARβ were being antagonized, other retinoid receptors could substitute for them, thereby avoiding disruption to the retinoid signaling pathway. However, LE135 which can antagonize RARα and RARβ did cause possible disruption to retinoid-mediated signaling, because of the inhibition of limb regeneration. The specific RAR isoforms targeted by Ro41 and Ro61 may differ from those being affected by LE135. LE135 may be knocking out RARα1, α2, RARβ1, β2, β3, and β4; while Ro41 and Ro61 only antagonize one RARα-isoform, and only one RARβ-isoform. The concept of functional redundancy would still apply. However, another possible explanation for the inconsistency of the results obtained may rest in the design of these synthetic retinoids. This

could ultimately affect the retinoids' metabolism, function, and receptor specificity *in vivo*. With the exception of Ro41 (Eckhardt and Schmitt, 1994; López et al., 1995), the majority of retinoid antagonists synthesized have only been tested for their antagonistic potential in cell culture systems, such as the HL-60 cell line. Thus, one can not be certain that these antagonists will not be metabolized into different compounds having reduced, or even lacking antagonistic activity in whole animal systems. For example, the antagonistic potential of dibenzodiazepine derivatives (LE135, LE540) depends largely on the nature of the substituents on the diazepine ring. LE135 has a hydrophobic, benzo group on the diazepene ring. Replacing this group with a hydrophillic amide group, completely abolishes the antagonistic activity of LE135 (Eyrolles et al., 1994); however replacing it with a naphtho group produced the more potent antagonist LE540 (Umemiya et al., 1997). Furthermore, various cells comprise the blastema once redifferentiation occurs, and specific cell types may be metabolically converting the implanted retinoid antagonists. This raises an important caution about interpreting *in vivo* experiments which use retinoid antagonists as specific ligands for RARs and RXRs. Therefore, it may be necessary to know the metabolic activities of specific cell types to better understand if and how retinoid antagonists act on the signal transduction pathway.

LE540 has Little Effect on Limb Regeneration

As stated earlier, the only difference between LE135 and LE540 is the ability of the latter to antagonize all RAR subtypes and all RXR subtypes. If RA is responsible for inducing pattern formation in the regenerating limb through

binding to and activating specific RAR/RXR heterodimer(s), then one would expect to see some limb skeletal defects as a result of implanting LE540 into the regenerating limb. This was not the case: the regenerating limbs treated with LE540 did not reveal any noticeable limb defects, they looked like control limbs. Why did LE540 not have an effect on pattern formation, given that it has been shown to be a more potent retinoid antagonist than LE135 *in vitro* (Umemiya et al., 1997)? The results obtained when LE135 and LE540 were implanted simultaneously may help decipher what may be occurring when LE540 was implanted alone.

How does LE540 Interact with LE135 during Limb Regeneration?

The RXR nuclear retinoid receptor subfamily selectively binds 9-cis-RA and not t-RA. Evidence suggests that the RXR subfamily has (a) role(s) in mediating the action of members of the RAR subfamily through heterodimer formation (Mangelsdorf et al., 1994; Mangelsdorf and Evans, 1995). RAR/RXR heterodimer formation is thought to be needed for the formation of the ligand-receptor DNA complex on RAREs, and only RAR or RXR of the heterodimer needs to be bound by its ligand to activate gene transcription. In the presence of high concentrations of 9-cis-RA, RXR can form RAR/RXR heterodimers but also RXR/RXR homodimers to activate RXR target genes. However, in the presence of low levels of 9-cis-RA or high levels of RAR, RXR homodimer formation is repressed. Subsequently, RAR/RXR heterodimer formation can suppress 9-cis-RA from binding to RXR thus rendering RXR a silent partner in the heterodimer (Mangelsdorf and Evans, 1995). However, Zhang et al. (1992) have shown that RXR homodimers can serve as ligand-dependant transcription

factors in the event of high levels of 9-cis-RA, thus inducing transcription of RXR target genes. Similarly, one might speculate that in the event of low levels of all-trans-RA, or low RAR concentrations, that the formation of RXR homodimers would also be stabilized. Some cells may have the ability to metabolically convert trans-RA to 9-cis-RA thus affecting heterodimer formation, and consequently the retinoid signal transduction pathway (Kurlandsky et al., 1994). Each cell type may be capable of controlling intracellular ligand levels to favor one retinoid pathway or another. These complex interactions may be governing the molecular basis of retinoid action.

If RXR is a silent partner in RAR/RXR heterodimers, how could an RAR/RXR heterodimer comprising an antagonist(LE135)-bound RAR and an antagonist (LE540)-bound RXR bind to the retinoid response element on the DNA and activate transcription? The results obtained with LE135 alone suggest that transcription can not be activated in the presence of this antagonist on the RAR partner. However, the results obtained using LE135 in combination with LE540 suggest that some transcriptional activation can take place in the presence of a ligand capable of binding to the silent RXR partner of an inactivated RAR/RXR heterodimer. LE135 may be antagonizing and thereby inactivating the RAR partner of the heterodimer, while LE540 binds to and activates the silent RXR partner in the event of high levels of inactivated RAR partners. Subsequently, the LE135-RAR/LE540-RXR heterodimer complex would bind to the retinoid response element on the DNA and induce transcription of RAR target genes.

In vivo the RXR partner of RAR/RXR heterodimers may be able to

respond to RXR ligands (LE540), bind to them, and become transcriptionally active. Chen et al. (1996) have shown that high concentrations of retinoid ligands for RAR alone are sufficient to induce transactivation, while ligands for RXR are inactive unless they are associated with an RAR agonist or certain RAR antagonists. Furthermore, Chen et al. (1996) have shown that RXR ligands can synergize with RAR ligands, inducing more effective transcriptional activation. Therefore, both partners of RAR/RXR heterodimers can bind their ligands and can be transcriptionally active. It has also been reported that the dibenzodiazepine derivative HX600 has a synergistic effect on retinoid activities (Umemiya et al., 1995). HX600 is a RXR panagonist which exhibits no retinoidal activity alone, but can function as an antagonist at high concentrations, or as a synergist in association with ligands for RAR. It is thought that its antagonistic ability is a result of HX600 binding to and inactivating RARs, but this is still being evaluated (Umemiya et al., 1997). The synergistic activities of HX600 have been attributed to its ability to bind to the RXR partner of RAR/RXR heterodimers, despite weak binding affinities for RXRs (Tashima et al., 1997). HX600 may be binding to RXR, and activating RAR/RXR heterodimers. It will be important to determine how RXRs function *in vivo* to fully assess the implications of using synthetic compounds which can target the RXR subfamily.

Perspectives

If endogenous retinoids are essential during amphibian limb regeneration and mediate their actions through RARs, it would be expected that blocking RARs would have some adverse effects on patterning of the regenerating limb. Ro 41-5253, Ro 61-8431, and LE135 presumably act as RARα- and RARβ-specific retinoid antagonists. Ro 41-5253 and Ro 61-8431 had little or no affect on patterning of the regenerating limb, even at the highest possible dose (75μg/limb). This finding suggests either that RARα and RARβ may be functionally redundant (see Discussion), or that RARα and RARβ are not essential transducers of the retinoid signal *in vivo*. Preliminary studies using LE135 complicated this issue because it did affect patterning of the regenerating limb, this compound caused an inhibition of regeneration. Therefore, RARα and RARβ may in fact be essential for transcription of retinoid target genes, and the notion of functional redundancy among members of the RAR subfamily becomes challenged.

The formation of RAR/RXR heterodimers is needed for highest binding affinity to specific retinoid response element sites on the DNA. Furthermore, recent studies have implicated RXR as being capable of inducing gene transcription, and thus can function as an active or silent partner in some systems (Chen et al., 1996). If this is the case, the RAR/RXR heterodimer is the functional unit, and knocking out both partners should result in loss of a given function. Thaller and Eichele (1996), and Helms et al. (1996) have shown that blocking both partners using RAR and RXR panspecific antagonists LG629 and LG754 does result in abnormal development of chick wing buds. In general

these studies revealed that treatment with RAR and RXR antagonists causes a loss of limb skeletal structures, suggesting a role for retinoids during early limb development. Patterning of the wing may have been disrupted because of simultaneously blocking RAR and RXR signaling, thereby inhibiting transcription of genes required for limb development. However, blocking both RAR and RXR subfamilies with LE540 in the regenerating limb did not result in abnormal patterning of the limb. The discrepancy in the results may be due to the difference in the retinoids used, as well as the difference between the developing and regenerating limb system.

The results obtained with LE540 and LE135 suggest the possibility of functional redundancy existing between the RAR and RXR subfamilies, and not within the RAR subfamily alone. In the absence of RAR function, RXR may be binding a ligand, in this case LE540, and this ligand bound-RXR can then mediate a retinoid response which is still efficient enough to perform the function of the RAR/RXR heterodimer. This hypothesis is supported by the results obtained when LE135 and LE540 were implanted in combination. Where once LE135 could inhibit limb regeneration by blocking RAR function, in the presence of LE540 there was partial patterning of the regenerating limb.

Future Research

In conclusion, retinoid antagonists can be a useful experimental tool for elucidating the role of RARs and RXRs *in vivo*. However, due to the fact that the function of most retinoid antagonists *in vivo* is largely unknown, one must be careful when interpreting the results obtained with them. Further studies need to be conducted to assess how binding affinities of these antagonists for the RAR and RXR subfamilies *in vivo* differ from the *in vitro* receptor binding affinities. Furthermore, although there are several reports on the binding affinities of new retinoid antagonists and agonists to RARs, the binding constants are highly variable and strongly depend on the source of the receptors and the experimental conditions. The retinoid signaling pathway is made complex by the existence of multiple RAR and RXR subtypes and isoforms, and by the various RAR/RXR heterodimer combinations which can exist. Future studies will no doubt try to understand why there exist multiple retinoid receptors, and how the RAR/RXR heterodimer functions *in vivo* when either partner is inactivated. Current experiments aimed at deciphering what role RXR plays *in vivo*, and how it interacts with RAR and various endogenous and synthetic ligands point to the many gaps existing in our knowledge of the retinoid signaling pathway. This study may help offer a new perspective into understanding the interaction between RA, RAR/RXR heterodimers, and retinoid antagonists.

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IMAGE EVALUATION
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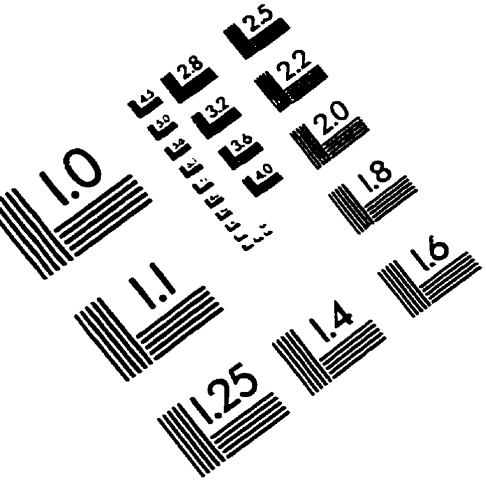

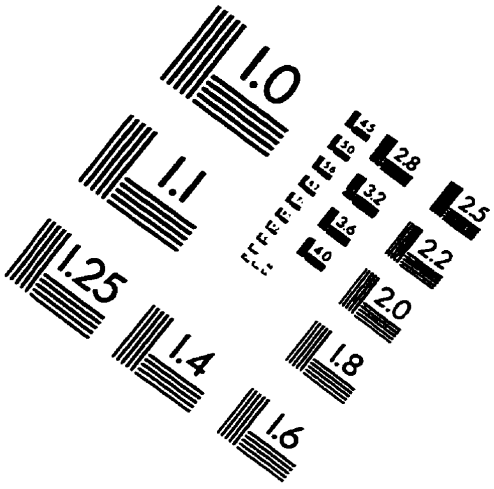

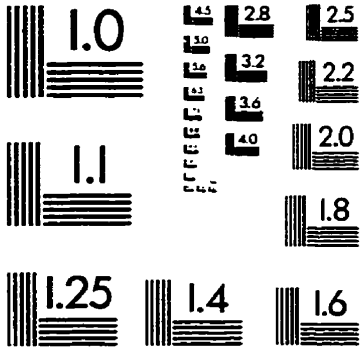

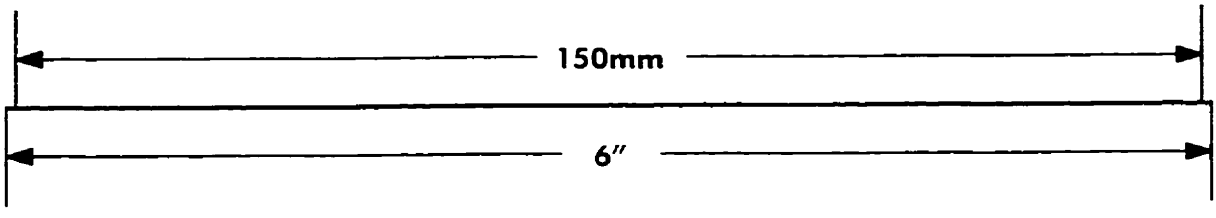

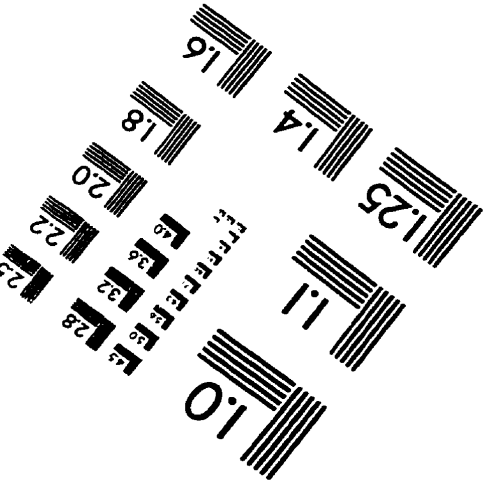

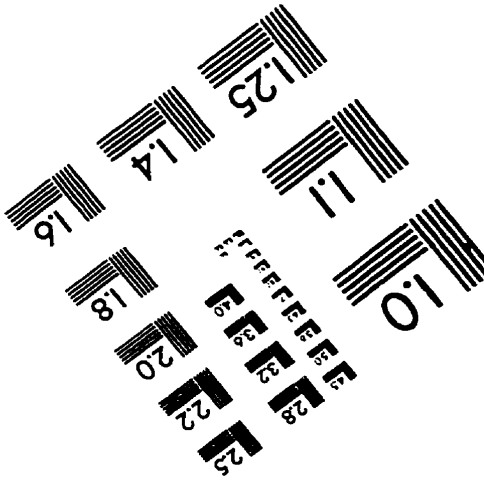

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