

## Retinoic acid and limb regeneration -a personal view

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I did my PhD thesis on amphibian limb regeneration and subsequently continued to study this amazing phenomenon as a post-doc and then as a scientist at the National Institute for Medical Research, Mill Hill, London. Doing a PhD in developmental biology in those dim and distant times (early 1970's) was a considerably less intense experience than it is today because a typical experiment consisted of observations on the results of tissue extirpation or tissue transplantation (e.g. blastema rotations in limb regeneration, extirpation of parts of the developing limb bud, isolation of blastomeres in developing embryos etc.) whereas now a typical experiment involves a high degree of molecular genetic analysis (which genes are induced by such a manipulation, what are the pathways of genes involved, how do they interact, what is the effect of over-expressing one of these genes, what is the effect of down-regulating one of these genes etc.).

This, of course, is an inevitable consequence of an explosion in our knowledge of the genes involved in development. As I have pointed out before (Maden, 1994) we then knew of *no* genes involved in limb development whereas now we must know about several hundreds. Compare, for example, a recent paper from Juan Carlos Izpisua Belmonte's lab (Kawakami *et al.*, 2001) in which 3 genes are described (*Wnt-2b*, *Wnt-8c*, *Wnt-3a*), their expression patterns, the time course of their induction by *Fgf-10* and *Fgf-8*, their role in the *Fgf-10/Fgf-8* loop, the role of  $\beta$ -catenin in this process, the effect of over-expression of *Wnt-2b*, *Wnt-8c*,  $\beta$ -

*catenin*, *Fgf-10* and AXIN, with papers from the early 1970's which described cell division and cell densities in the limb (Summerbell & Wolpert, 1972) or measured the lengths of the cartilage elements (Summerbell & Wolpert, 1973) or were more theoretical in nature (Summerbell, Lewis & Wolpert, 1973).

Anyway, the result of having less experimental pressure in one's research was that there was much more time for reading around the subject and for going back in the literature further than 10 years, as is now the norm. During my PhD period I read everything I could get my hands on to do with regeneration starting with Aristotle's 'Generation of Animals' which referred to the regrowth of the tails of lizards and serpents if they were cut off and the regeneration of young swallows' eyes, through Spallanzani's 'An Essay on Animal Reproductions' (1769) which described experiments on tadpole leg and tail regeneration and salamander jaw, leg and tail regeneration, Todd's (1823) experiments on the role of nerves in salamander limb regeneration which were conducted while he was a doctor at the British naval base in Naples and then on to the proliferation of publications in regeneration towards the end of the 19<sup>th</sup> century and into the early part of the 20<sup>th</sup> century, many of which were in German or French. These papers contain fascinating insights and conjectures, many of which are pertinent today and have by no means been answered and they made highly absorbing reading. Continuing with this tradition of reading all the literature I could while at NIMR, the library provided computer literature

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searches (before it was available to everybody on their own PCs) and I would send off to interlibrary loans for photocopies of these papers.

At the time I was doing experiments to try to understand what the cellular basis of positional information was in the regenerating amphibian limb. I still think the regenerating limb is one of the finest and clearest examples of positional information that there is – if a limb is cut through the mid-humerus then the distal humerus, elbow, forearm and hand regenerates, but if the limb is cut through the mid-radius and ulna then only the distal radius and ulna and hand regenerates. That is, the blastemal cells within the cut stump must have positional information because they clearly know what has been cut off in order to begin to replace the correct structures. I had hypothesised that the nerves played some part in the assignment of positional information and I had done experiments to change the level of the nerves at the cut stump – pulling forearm level nerves up through the limb to the humerus level and then amputating at that level. I predicted the regenerate should be missing the intervening elements (distal humerus, elbow, proximal radius and ulna), but what happened was that the regenerates were complete, but slightly smaller. Presumably this was due to a decreased supply of neurotrophic factor either caused by the damage done or because there were fewer nerve fibres at distal levels compared to proximal levels.

So imagine my amazement when I read that the precise experimental result I had been trying to obtain had already been done. The papers which I read as interlibrary loan photocopies were from the lab of I.A. Niazi in Jaipur, India and were published in the *Indian Journal of Experimental Biology* and *Folia Biologica (Krakow)*. Niazi had long been interested in the fact that frog tadpoles could regenerate their limbs soon after they had developed them, but that this ability was lost as the tadpoles approached metamorphosis (e.g. Dent, 1962). Niazi and colleagues reasoned that by inhibiting or delaying metamorphosis one would be able to prolong the ability to regenerate limbs. This could be done with vitamin A, indeed, giant tadpoles can be produced by treating premetamorphic tadpoles with various retinoids. In addition, they were also testing whether compounds that have deleterious effects on limb development are equally harmful to the limb regenerate and vitamin A was known to cause limb malformations when applied in excess to embryos.

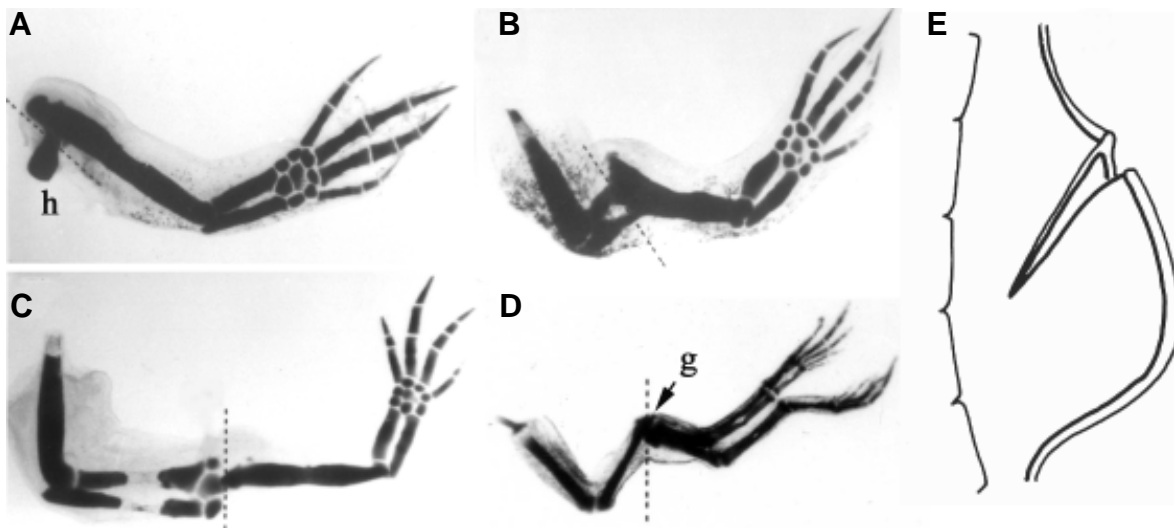
In the first paper (S. Saxena & I.A. Niazi, *Ind. J. Exp. Biol.* 15, 435-439, 1977) they used tadpoles of the toad, *Bufo andersonii*, when the hindlimbs had just developed and each of the segments were clearly demarcated. They amputated these hindlimbs through either the thigh or shank level and placed them in a 15 IU/ml solution of vitamin A palmitate for the duration of the experiment (7-13 days). In the control limbs amputated through the thigh the majority of them (72%) failed to regenerate anything after 13 days whereas in those amputated through the shank the majority (96%) regenerated. This revealed the typical proximodistal loss of regenerative ability in tadpole limbs. High amputations treated with vitamin A palmitate also mostly failed to regenerate (80%), but histological examination showed that most limbs had extensive dedifferentiation and had formed a blastema which did not progress any further. Some limbs formed two accumulations of blastemal cells. Shank amputations treated with vitamin A palmitate also mostly regenerated (99%), but the quality of the regenerates was very poor. They had fewer toes, they were twisted and smaller with 'peculiar swellings'. Histologically the vitamin A treatment had

intensified and accelerated early regenerative events to a greater extent at shank levels than at thigh levels and there was extensive dedifferentiation. In a few cases two blastemas seemed to be forming, although the experiment did not last long enough to determine what happened to these two blastemas.

I was particularly intrigued in this report by the presence of two blastemas instead of the expected one and in the presence of excessive dedifferentiation which I considered to be a reflection of the re-expression of positional information in blastemal cells (Maden, 1977). Could the two blastemas develop into two limbs from one stump? In the second paper (I.A. Niazi & S. Saxena, *Folia Biol. (Krakow)* 26, 1-8, 1978) these questions seemed to have been answered, although the experiments were only done on 5 control *Bufo andersonii* tadpoles and 7 experimentals, a rather small number considering the vast numbers of amphibian eggs that are normally laid in ponds. Amputation through the shank was followed by treatment of these 7 tadpoles with the same concentration of vitamin A palmitate (15 IU/ml) for 28 days. The actual concentration of vitamin A palmitate that the tadpoles received was impossible to know because it was administered only on the first day and the water in the bowl of tadpoles was topped up throughout the 28 days. Nevertheless, the results of each of the 7 cases were individually described. Three cases had swellings at the level of amputation, three cases had regenerated a thigh, shank and foot from the shank amputation plane and two cases had three regenerates growing from the amputation plane. Although there was no histology or cartilage stained wholemounts to confirm this dramatic respecification of amputation level, the pictures of the tadpoles certainly gave that impression.

I assumed that I was one of the few people working in limb regeneration who would have read either of these papers and I considered this result to be one of the most exciting I had ever seen particularly since I had been trying to get this precise result for years. I immediately purchased all the vitamin A compounds I could find in various catalogues and did hundreds of experiments using axolotls as well as *Xenopus* (because they were being used by others in the lab for studies on the retinotectal system), *Rana temporaria* and *Bufo bufo* because I could easily obtain their eggs in the spring in local ponds. The results were amazing: a concentration dependent proximodistalisation of the blastema from each of the three limb levels, stylopod (Fig. 1A), zeugopod (Fig. 1B), autopod (Fig. 1C) and I published these initial findings in *Nature* (Maden, 1982), a far cry from *Folia Biol. (Krakow)*. All the retinoids gave this effect at differing potencies, presumably according to their rate of conversion to retinoic acid which can interact with the nuclear retinoic acid receptors (molecules which were unknown at the time of these experiments). The relative potencies did not correlate with their binding to the cytoplasmic protein cellular retinoic acid binding protein which was known about at this time. Synthetic retinoids used in dermatological research and for treatment also did this respecification and they also worked when administered in different ways (i.p., local implants into the blastema etc.). Other non-retinoid compounds which I used had no effect on regeneration such as all the other vitamins or molecules of similar structure to retinoic acid such as undecylenic acid (the side chain of retinoic acid without the ring terminus).

The reason for performing the experiments on other methods of administration was because there was considerable scepticism of the results when the retinoids, which are insoluble in water, were



**Fig. 1. Examples of the effect of retinoids on regenerating limbs.** (A-C) Complete limbs of axolotls regenerating from each limb level – stylopod (A), zeugopod (B) and autopod (C). The amputation plane is marked by a dashed line. In (A), the amputation was through the mid-humerus leaving a small part of the humerus remaining in the stump (h). In (B) the amputation was through the mid-radius and ulna. In (C) the amputation plane was through the carpals. (D) The effect of retinoids in a *Rana temporaria* hindlimb amputated through the shank level (dashed line). A complete pair of limbs including the pelvic girdle (g) has regenerated. (E) Drawing of a chick wing bud with a slit cut in the anterior part into which was inserted a piece of newspaper soaked in retinoic acid dissolved in DMSO.

simply placed into the dishes of water that the animals were kept in. For example, some suggested that bacteria in the water converted the retinoids into other compounds or it was due to a pH change in the water and there were strange reports that the effects on regeneration were different according to whether the animals were kept in the dark or in the light (Lheureux, Thoms & Carey, 1986). However, I noticed that even if an insoluble compound like retinoic acid was sonicated into the water, after 24 hours crystals of retinoic acid could be seen *within* the regeneration blastema under the wound epidermis. I have no idea how they got there (directly through the epidermis?), but the net result, surprisingly, was a local administration to the blastema.

There were some interesting species specificities in these studies which were to be of significance subsequently. The respecification of regenerating limbs worked superbly in axolotls where only proximodistal specification was observed (Fig. 1 A-C) and in *Rana temporaria* where both proximodistal and anteroposterior respecification occurred resulting in a complete pair of limbs including the pelvic girdle regenerating from the shank level (Fig. 1D), it worked less well in *Xenopus* and it did not work at all in *Bufo bufo*. Subsequently others reported success in *Notophthalmus viridescens*, *Pleurodeles waltli*, *Triurus vulgaris*, *Bufo melanostictus* and *Rana breviceps* and it does not seem to work in *Rana pipiens*.

This species specificity of effect was a lesson that I failed to learn in other experiments that I performed. At this time I also tried the effects of vitamin A on tail amputation predicting that if it was a proximalising agent then a whole new animal should be regenerated from the tail amputation plane. I did these experiments on axolotls and also on *Xenopus* tadpoles as there were lots of spare tadpoles around at that time. Neither of these experiments produced any results since at the concentrations of vitamin A which would give limb respecifications the tail regenerated normally. Ten years later it was reported in *Nature* that the same experiment on

a species of Indian frog, *Uperodon systoma*, gave the dramatic homeotic transformation of tails into limbs (Mohanty-Hejmadi *et al.*, 1993). I subsequently showed that this also worked on *Rana temporaria*, but it did not work on *Xenopus*. So I should have continued with the failed tail experiments, using this third species. This is a good example of choosing the right experiment, but on the wrong species, as indeed would the limb experiments had they only been conducted on *Bufo bufo*, for example.

As an interesting postscript to these regeneration experiments, it transpires that Niazi had originally sent his paper reporting his vitamin A results to *Journal of Embryology and Experimental Morphology* (or *Development* as it now is) and it was rejected! This is why he published them in the relative obscurity of *Ind. J. Exp. Biol.* and *Folia Biol. (Krakow)*.

### Chick Experiments

At NIMR at this time I was in the Division of Developmental Biology and Dennis Summerbell who worked on the chick limb bud, was also a member of the division. When I got these results I obviously showed them to everybody and Dennis immediately set out to see what would happen in the developing chick limb bud. We had considerable amusement devising a method of administering the RA dissolved in DMSO, eventually settling upon small pieces of newspaper which were inserted into a slit in the limb bud (Fig. 1E). As far as newspaper quality went we found that the Richmond and Twickenham Times (a local newspaper) worked far better than the Evening Standard (a London evening newspaper). Dennis subsequently published his findings in *Journal of Embryology and Experimental Morphology* 78, 261-289 (1983) with what must surely be a world record number of 648.

There are many examples in the literature of back to back publications from two groups on the same subject. This was an

example of this same phenomenon as only a few miles away at the Middlesex Hospital Medical School, Cheryl Tickle was performing identical experiments on the chick limb bud, but came to be doing them for a completely different reason (see Tickle, 2002, this issue, pp. 847-852). Both groups were performing these experiments at the same time, but without any knowledge of each other's work.

## Conclusion

My lesson from this experience was clearly, read those papers in obscure journals as you never know what can be found there.

## Summary

Here I describe the situation which led up to the descriptions of the dramatic effects of retinoids on the regenerating amphibian limb. The original papers from an Indian group were published in relatively obscure journals but thanks to literature searches were made available for reading. Repetition of this work and its publication in widely read journals spawned the new field of research on the role of retinoids in developing and regenerating limbs.

**KEY WORDS:** *retinoic acid, retinoids, limb regeneration, limb development*

## References

- DENT, J.N. (1962). Limb regeneration in larvae and metamorphosing individuals of the South African clawed toad. *J. Morph.* 110: 61-77.
- KAWAKAMI, Y., CAPDEVILA, J., BUSCHER, D., ITOH, T., ESTEBAN, C.R. and IZPISUA BELMONTE, J.C. (2001). WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. *Cell* 104: 891-900.
- LHEUREUX, E., THOMS, S.D. and CAREY, F. (1986). The effect of two retinoids in limb regeneration in *Pleurodeles waltl* and *Triturus vulgaris*. *J. Embryol. Exp. Morph.* 92: 165-182.
- MADEN, M. (1977). The regeneration of positional information in the amphibian limb. *J. Theor. Biol.* 69: 735-753.
- MADEN, M. (1982). Vitamin A and pattern formation in the regenerating limb. *Nature* 295: 672-675.
- MADEN, M. (1994). The limb bud – part two. *Nature* 371: 560-561.
- MOHANTY-HEJMADI, P., DUTTA, S.K. and MAHAPATRA, P. (1992). Limbs generated at the site of tail amputation in marbled balloon frog after vitamin A treatment. *Nature* 355: 352-353.
- NIAZI, I.A. and SAXENA, S. (1978). Abnormal hind limb regeneration in tadpoles of the toad, *Bufo andersonii*, exposed to excess vitamin A. *Folia Biol. (Krakow)* 26: 3-8.
- SAXENA, S. and NIAZI, I.A. (1977). Effect of vitamin A excess on hind limb regeneration in tadpoles of the toad, *Bufo andersonii* (Boulenger). *Ind. J. Exp. Biol.* 15: 435-439.
- SPALLANZANI, A. (1769). 'An Essay on Animal Reproductions'. Translated from the Italian by M. Maty, in *Surgical Tracts* Vol. IV.
- SUMMERBELL, D. (1983). The effect of local application of retinoic acid to the anterior margin of the developing chick limb. *J. Embryol. Exp. Morph.* 78: 269-289.
- SUMMERBELL, D., LEWIS, J.H. and WOLPERT, L. (1973). Positional information in chick limb morphogenesis. *Nature* 244: 492-496.
- SUMMERBELL, D. and WOLPERT, L. (1973). Precision of development in chick limb morphogenesis. *Nature* 244: 228-230.
- SUMMERBELL, D. and WOLPERT, L. (1972). Cell density and cell division in the early morphogenesis of the chick wing. *Nature New Biol.* 239: 24-26.
- TICKLE, C. (2002). The early history of the polarizing region: from classical embryology to molecular biology. *Int. J. Dev. Biol.* 46: 847-852.
- TODD, T.J. (1823). On the process of reproduction of the members of the aquatic salamander. *Quart. J. Sci.* 16: 84-96.