Folia Biol. (Kraków)
Vol. 26 (1978) No 1

Abnormal Hind Limb Regeneration in Tadpoles of the Toad, *Bufo andersoni*, Exposed to Excess Vitamin A

I. A. NIAZI and SAROJ SAXENA

Department of Zoology, University of Rajasthan, Jaipur, India

Accepted for publication July 15, 1977

Young tadpoles of the toad, *Bufo andersoni*, at a very early stage of hind limb development underwent amputation through the shanks and were exposed to 15 IU of vitamin A palmitate per 1 ml of water.

Whereas controls regenerated normal limbs, in the treated tadpoles the regenerates were retarded and highly abnormal. In 6 out of 14 cases multiple regenerates developed from the stump. In 5 cases the regenerated portion consisted of rudiments of the whole limb including thigh, shank, and foot regions, i.e. also from parts already present in the stump.

It is suggested that exposure to excess vitamin A increases the potencies of the blastemas thus enabling them to overcome the restrictive influences of the stump and to form a whole limb instead of only the distal parts actually removed by amputation.

Since the initial finding of COHLAN (1953) it has become well established that in mammals maternal hypervitaminosis A at critical stages of organogenesis causes in the foetuses a variety of malformations including those of limbs (TAKEKOSHI, 1964; MORRIS, 1973). In anurans excess vitamin A retards ontogenetic development of hind limbs, suppresses thyroid activity (NIAZI and SAXENA, 1972), and affects tail regeneration (NIAZI and SAXENA, 1968).

The aim of the present study was to investigate the effects of excess vitamin A on hind limb regeneration in toad tadpoles. The results demonstrate that hypervitaminosis A not only retards the regeneration process, but also causes malformations and reduplications.

MATERIAL AND METHODS

Young tadpoles of the toad, *Bufo andersoni*, were used in the experiments. At the time of amputation the three primary segments, thigh, shank and foot, were just recognizable in the developing hind limbs and on the margin of the foot-paddle the prominences of the 4th and 5th toe rudiments had become distinct. According to the Normal Table of SHIVPAL and Niazi (1977) for this species, the tadpoles were at about stages VII—VIII. Previous, unpublished observations had shown that at this stage regenerative capacity in the hind limbs is quite high.

The tadpoles were anaesthetized with MS 222, „Sandoz", (1 : 4000) and both hind limbs of each individual were amputated through the shank. One group of 5 tadpoles was then reared in ordinary tap water and served as control. The experimental group, consisting of 7 tadpoles, was placed in a large bowl containing vitamin A palmitate („Arovit", Roche, India), at a dose of 15 IU per 1 ml water. To this bowl more water, but no vitamin A was gradually added to maintain the level of the medium. The animals were regularly fed fresh boiled spinach. The experiments were carried out at room temperature (30—32°) and lasted for 28 days from the day of amputation.

For histological examination the tadpoles were fixed in Bouin's fluid, embedded in paraffin, sectioned at 7 μm, and stained with haematoxylin and eosin.

RESULTS

By the end of the 4th week all 5 control tadpoles were metamorphosing with both fore-limbs emerged and tails in the process of resorption. Treated tadpoles had lagged far behind and none of them showed any metamorphic changes.

Regeneration had occured in all 10 limbs of the 5 control tadpoles. The regenerates were completely normal and well developed (Fig. 1), contained normally differentiated skeletal elements and striated muscle bundles and their epidermis was of adult type with numerous, flask-shaped, multicellular mucous glands.

Regeneration had also occured in all 14 limbs of the 7 treated tadpoles but they differed in the type of regenerates produced. However, all regenerates of this group were retarded in growth and differentiation and were abnormal in shape. The type of abnormality and the degree of retardation in regenerates of the right and left limbs of each individual were more or less identical or very similar. Histologically, skeletal and muscle differentiation was very poor and in most cases the mesenchyme was almost completely undifferentiated. The skin was of larval type with in no case any multicellular mucous glands. The type of regeneration which occured in the experimental tadpoles is described below.

Case 1. The regenerated shank region was normal but morphogenesis of the foot was defective in both regenerates. The feet were swollen and oligodactylous (Fig. 2).

Case 2. The regenerates had not developed beyond the foot-paddle stage and the shank region was swollen (Fig. 3).

Case 3. The amputation plane had passed exceptionally through the thigh, above the knee, and not through the shank. The regenerate of each side consisted of two knob-like protuberances which, in sections, were found to contain only undifferentiated mesenchyme. The protuberances looked like persistent blastemas (Fig. 4).

Case 4. Although both the right and left regenerates were more or less normally shaped, the feet were oligodactylous, having three digits each. Both regenerates growing out of the shank stumps consisted of thigh, shank, and foot regions. Internally, the mesenchyme had not advanced beyond the very early stage of condensation (Fig. 5).

Case 5. The regenerates at both sides were trifurcated and each branch appeared to be an elongated conical blastema, except for one whose distal margin was flattened and somewhat spatulate. Internally, these regenerates contained only undifferentiated mesenchyme (Fig. 6).

Case 6. The right regenerated limb was swollen and the foot was oligodactylous. The regenerate of the left limb had a normally shaped foot with rudiments of all five toes. Moreover, this regenerate developing on the stump consisted of all the parts of a limb, i.e. a thigh, shank, and foot (Fig. 7).

Case 7. In this tadpole the regenerates of both right and left sides were very similar. Distally to each stump there had developed three regenerates which, however, exhibited different degrees of development. One regenerate of each stump was better formed with a normal type of foot, the other two being small with inhibited morphogenesis. The normal one of the three regenerates on each stump consisted of thigh, shank, and foot regions, similarly to those found in cases 4 and 6 (Figs 8 and 9).

DISCUSSION

The results obtained in the present study demonstrate that an excess of vitamin A causes very abnormal limb regeneration in anuran tadpoles. The abnormalities include retarded morphogenesis and growth and also reduplications. In the ontogenetic development of frog tadpole limbs under hypervitaminosis A only retarded growth was observed for which antithyroid action of the vitamin was postulated as the major cause (NIAZI and SAXENA, 1972; also see KOLLROSS, 1961). However, reduplications of regenerating limbs are not easy to explain on this basis alone. Probably some direct effects of vitamin A on the exposed stump tissues following amputation may also be involved.

As described above, in 6 out of 14 amputated limbs of treated tadpoles duplicated or triplicated regenerates developed on each stump. The available information on the role of tissue interactions during amphibian limb regeneration suggests that the formation of multiple regenerates on the same stump should be connected with the processes occuring during the very early phases of regeneration. As it is known, amputation is followed by the formation of a characteristic apical epidermal cap over the wound surface; subsequently, under this cap blastemal cells accumulate (for review see THORNTON, 1968; and GOSS, 1969). The role of the epidermal cap formed over the amputation surface in regenerating limbs of amphibians appears to be similar to that of the apical-ectodermal ridge in limb morphogenesis in chick embryos. The presence of multiple apical ectodermal ridges on chick embryo limb buds and of more than one apical epidermal cap on amphibian limb stumps result in reduplicated limbs and regenerates, respectively (ZWILLING, 1961; THORNTON and THORNTON, 1965; see also FABER, 1971, 1976; and STOCUM and DEARLOVE, 1972.) It is therefore possible that exposure to excess vitamin A during the period immediately following amputation results in the formation of multiple apical epidermal caps which, subsequently, lead to the accumulation of blastema cells at several centres, laying down the basis for the formation of more than one regenerate on the stump. However, in order to verify this hypothesis more detailed studies on the effect of hypervitaminosis A on early phases of regeneration are needed. One of the mechanisms involved in the formation of multiple regenerates could be increased cell death in certain regions of the wound epithelium.

A highly significant phenomenon was the formation of regenerates which appeared to consist of more than the distal parts of limbs actually removed by amputation. In 5 out of 14 regenerates of the treated tadpoles rudiments of whole new limbs had grown out of the stumps which themselves consisted of the thigh and proximal portion of the shank. The regenerates included a new thigh, shank and foot. By a different procedure DE BOTH (1970) also obtained in a few cases the formation of some proximal structures, though the blastemas were formed after amputation at a more distal level. DE BOTH (1970) amputated the forelimbs of axolotls through the wrist, removed the resultant blastemas from the stumps, denuded them of the epidermis, and transplanted the combined mesenchymal components of several such blastemas into the flank or the orbit from which the eyeball had been removed. The blastemal mesenchyme, formed at the wrist level and then transplanted, differentiated into not only hand but also forearm and even upper arm structures in some cases. Accordingly, the conclusion was drawn that blastemal mesenchyme derived from distal levels of a limb may be capable of forming the whole limb and not merely structures distal to the level of amputation. The results obtained in the present study support this conclusion.

Normally, the stump influences the morphogenetic potentialities of the blastema, permitting the latter to form only distal parts of the limb which are actually removed or lost (GOSS, 1961). In the opinion of FABER (1971, 1976), although this restrictive influence of the stump certainly operates, it is not indispensable. According to him such influence is superimposed upon the self-organizing properties of the blastema. The present authors fully agree with this hypothesis on the basis of the observations described above. It may be recalled that DE BOTH (1970) obtained the formation of proximal parts from distal level blastemas by transplanting them into the flank or the orbit and thus liberating them from the influence of the stump. In the present experiments whole limbs were regenerated in continuity with the stump, in spite of the latter's so-called restrictive influence. These results suggest that an actual physical separation of the blastema from the stump may not be an essential pre-requisite for the blastema to manifest its full morphogenetic potentialities. The additonal factor in the present experiments was the exposure of the tadpoles and their amputated limbs to a large amount of vitamin A in the rearing medium, at least during the period of wound healing, dedifferentiation, blastema formation, and beginning of morphogenesis of the regenerates. These early processes of limb regeneration in tadpoles of *Bufo andersoni* are completed in about 3 days after amputation at 30—32°C. It may be that exposure to excess vitamin A facilitated the acquisition by blastema cells of greater than usual potencies (perhaps through a greater degree of dedifferentiation), thus enabling the blastema to overcome the restrictive influence of the stump and to form all the parts of a limb instead of only the distal ones.

EGUCHI and WATANABE (1973) were able to obtain newt lens regeneration from ventral iris by treatment of the latter with a carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine. Lens regeneration has never been obtained from the ventral iris under normal circumstances. The results of the quoted study indicate, therefore, that certain experimental conditions can facilitate reawakening of developmental potentialities in some tissues which otherwise remain suppressed. An excess of vitamin A may have similar properties.

STRESZCZENIE

J. A. NIAZI i S. SAXENA: *Nietypowa regeneracja tylnych kończyn kijanek, Bufo andersoni, poddanych działaniu witaminy A*

Młodym kijankom ropuchy, *Bufo andersoni*, amputowano tylne kończyny na poziomie podudzia, po czym poddano je działaniu palmitynianu witaminy A (15 j.m./ml wody).

Podczas gdy kijanki kontrolne regenerowały prawidłowe kończyny, regeneraty kijanek, na które działano witaminą A były zahamowane w rozwoju i anormalne. W 6 przypadkach na 14 na kikutach wytwarzało się po kilka regeneratów. 5 regeneratów składało się z zawiązków całych kończyn, tj. z uda, podudzia i stopy, a więc także z tych części, które pozostały w kikucie.

Autorzy sugerują, że witamina A zwiększa potencjał rozwojowy blastemy regeneracyjnej i umożliwia jej pokonanie ograniczających właściwości kikuta. Dzięki temu regenerat różnicuje się w całą kończynę, a nie tylko w części dystalne usunięte podczas amputacji.

СОДЕРЖАНИЕ

J. A. NIAZI, S. SAXENA: *Нетипичная регенерация задних конечностей головастиков Bufo andersoni, подверженных действию витамина A*

Малым головастикам жабы *Bufo andersoni*, ампутировали заднюю конечность на уровне голени, затем головастики подвергали действию пальмитиниана витамина A (15 м.е./ml воды).

В то время, как у контрольных головастиков происходила регенерация нормальных конечностей, регенераты головастиков, подверженных действию витамина A были анормальны и наблюдалось торможение развития. На 14 случаев в 6 случаях на обрубках образовалось по несколько регенератов. 5 регенератов состояло из зачатков всей конечности, то есть, из бедра, голени и ступни, таким образом, из тех частей, которые оставались в обрубке.

Авторы предполагают, что витамин A вызывает увеличение потенциала развития регенерационной бластемы и даёт возможность ей преодолеть ограничивающие свойства обрубка. Благодаря этому регенерат дифференцируется в полную конечность, а не только в дистальные её части, удалённые путём ампутации.

LITERATURE CITED

BOTH N. J., DE (1970). The developmental potencies of the regeneration blastema of the axolotl limb. W. Roux' Arch. Entwicklungsmech. Organ., **165**: 242—276.

COHLAN S. Q. (1953). Excessive intake of vitamin A as a cause of congenital anomalies in the rat. Science, **117**: 535—536.

EGUCHI G., WATANABE K. (1973). Elicitation of lens formation from the ventral iris epithelium of the newt by a carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine. J. Embryol. Exp. Morph., **30**: 63—72.

FABER J. (1971). Vertebrate limb ontogeny and limb regeneration: Morphogenetic parallels. Advances Morph., **9**: 127—147.

FABER J. (1976). Positional information in the amphibian limb. Acta Biotheor. (A) (Leiden), **25**: 44—65.

GOSS R. J. (1961). Regeneration of vertebrate appendages. Advances Morph., **1**: 103—152.

GOSS R. J. (1969). Principles of Regeneration. Academic Press, New York.

KOLLROSS J. J. (1961). Mechanisms of amphibian metamorphosis: Hormones. Amer. Zool., **1**: 107—114.

MORRIS G. M. (1973). The ultrastructural effects of excess maternal vitamin A on the primitive streak stage rat embryos. J. Embryol. Exp. Morph., **30**: 219—242.

NIAZI I. A., SAXENA S. (1968). Inhibitory and modifying influence of excess vitamin A on tail regeneration in *Bufo* tadpoles. Experientia, **24**: 852—853.

NIAZI I. A., SAXENA S. (1972). The influence of excess vitamin A on the growth of frog tadpoles with particular reference ot thyroid glands. Rev. Canad. Biol., **31**: 89—96.

SHIVPAL A., NIAZI I. A. (1978). A table of the normal developmental stages of larvae of the toad, *Bufo andersoni* Boulenger *(Bufonidae, Anura, Amphibia)*. Rajasthan University Studies in Zoology, (in press).

STOCUM D. L., DEARLOVE G. E. (1972). Epidermal-mesodermal interaction during morphogenesis of the limb regeneration blastema in larval salamanders. J. Exp. Zool., **181**: 49—62.

TAKEKOSHI S. (1964). The mechanism of vitamin A induced teratogenesis. J. Embryol. Exp. Morph., **12**: 263—271.

THORNTON C. S. (1968). Amphibian limb regeneration. Advances Morph., **7**: 207—242.

THORNTON C. S., THORNTON M. T. (1965). The regeneration of accessory limb parts following epidermal cap transplantation in urodeles. Experientia, **21**: 146.

ZWILLING E. (1961). Limb morphogenesis. Advances Morph., **1**: 301—330.

Address for reprints:

I. A. Niazi,
Department of Zoology, University of Rajasthan,
Jaipur 302004, India

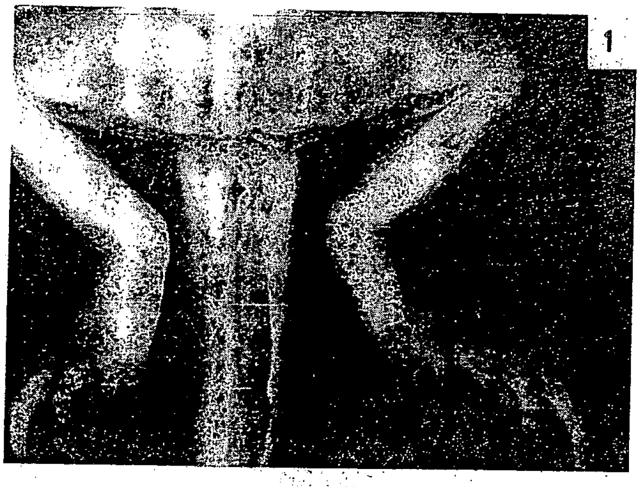
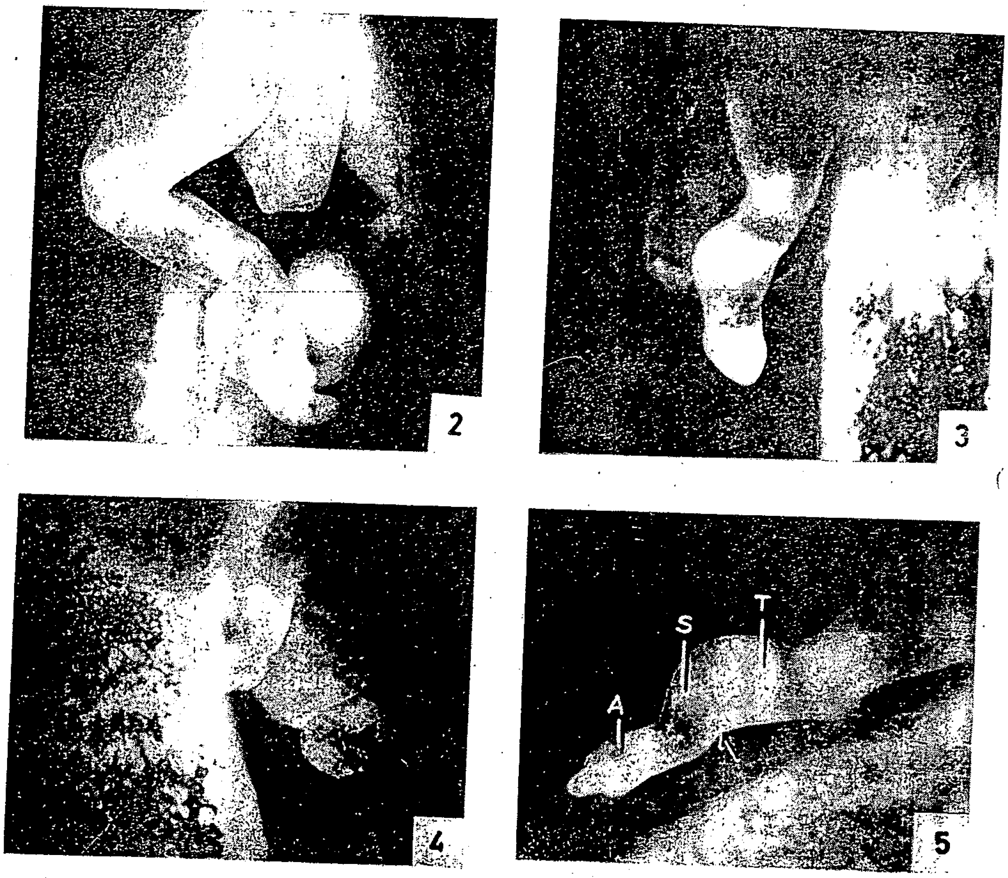


Fig. 1. Right and left regenerated hind limbs of a control tadpole of *Bufo andersoni*. Both limbs were amputated through the shanks.



Regenerates of hind limbs of tadpoles of *Bufo andersoni* exposed after amputation to excess vitamin A. Fig. 2. Case 1. Fig. 3. Case 2. Fig. 4. Case 3. In this tadpole the amputation planes passed exceptionally through the thighs. Fig. 5. Case 4. Note that the regenerate growing out of the shank consists of thigh (T), shank (S), and foot (A) regions. The arrow indicates the knee region of the regenerate.

Regenerates of hind limbs of tadpoles of *Bufo andersoni* exposed after amputation to excess vitamin A. Fig. 6. Case. 5. The regenerates are trifurcated. The distal margin of one of the branches (arrow) is flattened. Fig. 7. Case 6. Note that the regenerate of the left limb (growing out of the shank) consists of thigh (T), shank (S), and foot A regions. The arrow indicates the knee of the regenerate. Fig. 8. Case 7, right limb. Fig. 9. Case 7, left limb. Note that on both stumps in this tadpole three regenerates are visible, one of them being better developed than the other two. The "normal" regenerate consisted of thigh (T), shank (S), and foot (A) regions, though the limbs were amputated through the shank.