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DEVELOPMENT OF APICAL ECTODERMAL RIDGE DURING EARLY HINDLIMB MORPHOGENESIS IN THE TADPOLES OF *RANA TIGRINA* DAUD.

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SUMMARY

The hindlimb buds of the tadpoles of Indian bullfrog, *Rana tigrina* were investigated for the development of an apical ectodermal ridge (AER). At stage 34, when the limb buds are approximately as long as broad, the AER appears as a slight thickening at the apex of the limb bud due to the cells of the basal bayer of two layered epidermis becoming columnar. At stage 35, when the limb buds are slightly longer than broad, the AER becomes a three layered prominent conical cap extending over the apex of the limb bud. Its basal layer is columnar. The marginal vein in the mesenchyme is very close to the AER and there seems to be no basal membrane below the ridge. The mesenchymal cells immediately below the epidermis are aligned in a row. From stage 36, when the limb bud has attained maximum length, the AER begins to regress and by stage 38 when digit formation begins, the AER is no more discernible.

INTRODUCTION

Convincing evidence is available that there exists a necessary relationship between the epidermis and mesenchyme of a developing limb bud (Zwilling, 1955; Kieny, 1971; Saunders and Ruess, 1974). The development of apical epidermal ridge (AER),

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capping the limb buds apically, has been reported by several investigators in reptiles (Milaire, 1957; Raynaud, 1972; Vasse, 1973; Mathur and Goel, 1976), birds (Saunders, 1948; Jurand, 1965; Saunders and Gasseling, 1968) and mammals (O'Rahilly, Gardener and Gray, 1956; Milaire,1965 ; Jurand, 1965; Kelley, 1973). Though Balinsky (1965) denied the formation of AER on amphibian limb buds, Tarin and Sturdee (1971), Dawd and Nawar (1972), Stebler (1973) and Shivpal (1976) have reported its presence in anuran amphibian species. The inductive role has been widely ascribed to the apical ectodermal ridge in limb morphogenesis (see Kelley and Fallon, 1981)..

The present study was conducted to investigate histologically the developing hindlimb buds of the tadpoles of Indian Bullfrog, *Rana tigrina* for the presence of apical ectodermal ridge.

MATERIALS AND METHODS

The tadpoles of *Rana tigrina*, used in this study, were reared in the laboratory from a single spawn of eggs collected from a rain-water pool in Meerut (Western Uttar Pradesh, India). The investigations were made on the developing hindlimbs of tadpoles of stages 32-38 (Agarwal and Niazi, 1977). A brief description of morphological features of developing hindlimbs in tadpoles of these stages is given below :

Stage 32 : Initial hindlimb buds as circumscribed elevations.

Stage 33 : Hindlimb buds broader than long.

Stage 34 : Hindlimb buds as long as broad.

Stage 35 : Hindlimb buds longer than broad.

Stage 36 : Hindlimb buds elongated; distal end spatulate.

Stage 37 : Distal end of hindlimbs paddle-shaped; knee bend distinct.

Stage 38 : Presumptive thigh, shank and ankle+foot segments well demarcated; indentation between rudiments of toes 4 and 5.

The tadpoles were narcotized in 1 : 4000 solution of MS 222 (Sandoz) in tap water and then the intact limb buds/limbs were removed under stereoscopic binocular microscope.

For histological studies the limb buds/limbs were fixed in Bouin's solution and processed for paraffin embedding and sectioning. The serial longitudinal sections cut at 6 or 7 microns thickness and stained with haematoxylin and eosin were studied under light microscope. Selected sections were photomicrographed at suitable magnifications.

OBSERVATIONS

The longitudinal sections of hindlimb buds/limbs examined in this investigation reveal the appearance of the apical ectodermal ridge at stage 34, presence of well-formed ridge at stage 35, beginning of ridge regression at stage 36 and its disappearance at stage 38. An account of the histological picture of AER is presented here.

The earliest hindlimb bud at stage 32 consists of mesenchymal cells covered by epidermis. The epidermis consists of two layers of cells of which the outer layer appears to be irregular (Fig. 1). By stage 33 the cells of both epidermal layers become cuboidal (Fig. 2). At stage 34 the epidermis is two layers of cuboidal cells and the basal layer is mitotically very active. A slight thickening at the apex of the limb bud indicates a developing apical ectodermal ridge (AER). In this region the cells of the basal layer of epidermis have become columnar. The basement membrane underlying the epidermis is absent in the apical region of the bud. A marginal vein appears in the mesenchyme below the epidermis (Fig. 3).

At stage 35 the AER is a well formed, distinct and prominent conical ridge capping the apex of the bud. The ridge consists of three well defined layers of epidermal cells of which the basal layer is columnar, the outer layer consists of cuboidal cells and the cells of middle layer are irregularly disposed. A few dark staining and apparently pycnotic nuclei are seen in the AER. The marginal vein is present in the inner mesenchymal zone, two or three cell layers beneath the epidermis. No basement membrane is seeen underlying the ridge. At this stage mesenchymal cells immediately below the basal layer of limb bud epidermis are seen to be arranged in a single row very close to the surface. Mitotic cells are abundantly seen in the epidermis

as well as in the mesenchyme. Beginning of chondrogenesis of femur is indicated by condensation of mesenchyme in the proximal region and sciatic nerve fibres are seen in the basal region of the bud (Figs. 4, 5 & 6).

At stage 36 the AER is still present but is not conical any more. Instead, it is now flattened and extends around the entire tip of the spatulate distal end of the bud. Its continued presence is indicated by the observation that only in this apical region the epidermis is three layered. At stage 37 the AER is seen to persist around the tip of the foot paddle while at stage 38 the AER is no more discernible.

DISCUSSION

A general feature of the early stages of limb development in all amniotes has been found to be the thickening of the epidermis covering the apical part of the limb bud. This thickening has been termed the apical ectodermal ridge or AER. First clearly described for the chick (Saunders, 1948), it has been observed in the early limb buds of mouse (Milaire, 1965), man (O'Rahilly, Gardener and Gray, 1956; Kelley, 1973), turtle (Vasse, 1973), lizard (Mathur and Goel, 1976). The actual form of this ridge differs from species to species. It may be keel shaped, nipple-like or dome-shaped (Zwilling, 1961; Stocum, 1975). A large number of experimental studies initiated by Saunders (1948) and carried forward and extended on chick embryos by Saunders, Zwilling and many others (Saunders, Cairns and Gasseling, 1957; Gasseling and Saunders, 1961; Goetnick, 1964; Saunders and Gasseling, 1968; Fraser and Abott, 1971 a, b; Errick and Saunders, 1973, 1974; Zwilling, 1961, 1974; Cairns, 1975; Globus and Globus, 1976) have established that AER plays a very active role in limb morphogenesis and influences the development of the distal parts of the limb. The occurrence of AER on the limb buds in amphibians was denied by Balinsky (1965). However, Tschumi (1957) had reported the presence of AER in the developing limb bud of *Xenopus laevis* although the AER in this species was not found to be so pronounced as in the chick embryo. Perhaps because of this difference Dober and Tschumi (1969) have asserted that a well developed crest or ridge similar to that of

AER of the chick or mammalian embryos in the sense used by Saunders does not develop in *X. Iaevis* although Tschumi (1957) had earlier shown that the formation of distal structures in its limbs also depends on the presence of a ridge-like structure in the apical ectoderm. However, Tarin and Sturdee (1971) have demonstrated the presence of AER in *X. laevis* limb buds by light as well as scanning electron microscopy. Since then the presence of AER in the developing limb buds has been reported in a number of anuran species including *Bufo viridis* (Dawd and Nawar, 1972), *Alytes obstetricans, Bombina variegata, R. temporaria, R. esculenta, B. bufo, B. calamita* (Stebler, 1973) and *B. andersonii* (Shivpal, 1976). The present observations show that a definite AER also develops in the hindlimb buds of *R. tigrina.* In this species the AER appears like a conical cap at the apex of the limb bud. It is first observed at stage 34 when the limb bud is as broad as long and persists up to stage 37 when the prospective foot region becomes paddle-shaped. This observation generally agrees with those of Tarin and Sturdee (1971), Dawd and Nawar (1972) and Stebler (1973) on the anurans studied by them. However, it may be mentioned that although the conical shape of the ridge disappears with early digitation of the foot, the epidermis at the tips of differentiating digits in *R. tigrina* is seen to remain relatively thicker until the digits are fully formed (Agarwal, 1978).

Structurally, the AER in *R. tigrina* consists of three layers of epidermal cells while the epidermis around the rest of the bud is only two layered; and cells of its basal layer are columnar. This structure agrees well with the description of AER given by Tarin and Sturdee (1971) for *X. laevis* and Dawd and Nawar (1972) for *B. viridis.* According to Stebler (1973) while the AER in *Alytes obstetricans, R. temporaria* and *R. esculenta* is formed by increase in cell layers and the basal cells becoming columnar, this ridge in *B. bufo, B. calamita* and *Bombina variegata* is the result only of the basal cells becoming tall without increase in number of cell layers.

A number of authors have observed the presence of pycnotic cells or eosinophilic bodies in the AER of amphibian and chick embryo limb buds (Tarin and Sturdee, 1971; Amprino, 1965; Dober, 1968; Stebler, 1973). Pycnotic cells were also observed

in the AER of *R. tigrina* limb buds and, in agreement with Tarin and Sturdee (1971), it is suggested that these bodies may be products of cellular degeneration.

The initial limb bud in *R. tigrina* tadpoles is found to be without blood supply. The first sign of vascularization is seen at stage 34 when a marginal vein appears in the mesenchyme running just below the epidermis. At stage 35 the marginal vein is seen in close proximity to the AER as is reported consistently in most amniotes and also in amphibians studied so far.

The morphogenetic role of AER has been well established for the developing limbs in chick embryos as already mentioned before. In amphibians the only experimental studies have been those of Tschumi (1957) who showed that the formation of distal structures of the limb in *Xenopus* also depends on the presence of the apical ectodermal ridge. No such studies have been made on any other amphibians as yet. However, it may be pointed that in regenerating limbs of amphibians an apical epidermal cap (AEC) similar to AER of the embryonic limb buds develops on the blastema. The morphogenetic role of this cap in the regenerating limbs has been demonstrated to be more or less identical with that of the AER of the chick limbs during their ontogeny (Thornton, 1968; Faber, 1971).

Recently, Fallon and Kelley (1977), using transmission and freeze fracture electron microscopy, examined the fine structure of AER of some species of mammals and birds and observed the presence of numerous gap junctions. It is believed that these gap junctions play a vital role for inductive activity on the mesoderm for normal limb development. While the AER provides the conditions for axial elongation of the limb and for its progressive growth, shaping and orientation, the gap junctions in AER are thought to act as communicating structures to permit metabolic or electrotonic signalling between cells of AER and mesoderm (see Kelley and Fallon, 1981).

From the evidence so far accumulated it can be concluded that formation of AER in the early limb buds is not a feature of only amniotes but is found universally in all the tetrapods including amphibians. Moreover, such a structure serves a

universally similar morphogenetic role in limb ontogeny as well as regeneration.

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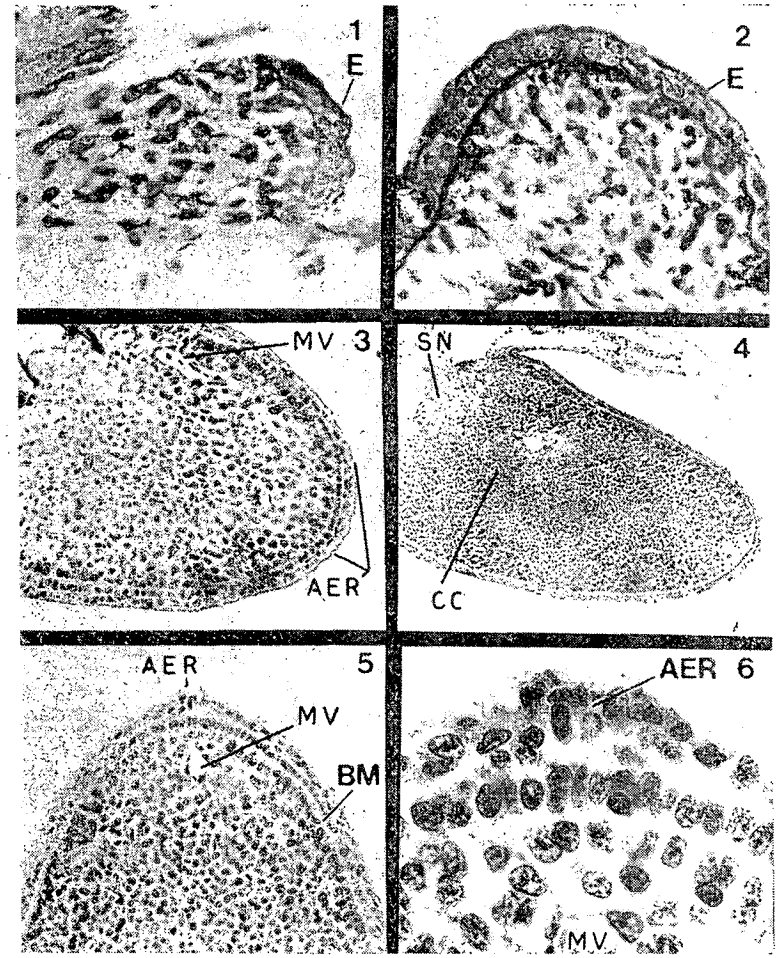
PLATE 1



Figures 1–6

1. L. S. of initial hindlimb bud (stage 32) showing mesenchymal cells covered by epidermis. E, epidermis. ×400. **2.** L. S. of stage 33 hindlimb bud. Note cuboidal cells of double layered epidermis. E, epidermis. ×400. **3.** L. S. of stage 34 hindlimb bud. Note AER as a slight thickening at the apex of limb bud. MV, marginal vein. ×200. **4.** L. S. of stage 35 hindlimb bud showing well formed AER. Note chondrogenic condensation (CC) and sciatic nerve (SN) in the proximal region. ×100. **5.** Apical part of limb bud (Fig. 4) in higher magnification. Note the marginal vein (MV) and mesenchymal cells arranged in a row below the epiderms. BM, basement membrane. ×312.5. **6.** Magnified view of AER of the section shown in Fig. 5. × Oil.