Secondary lens formation from the cornea following implantation of larval tissues between the inner and outer corneas of *Xenopus laevis* tadpoles

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SUMMARY

Secondary lens formation from the cornea of larval *Xenopus laevis* has been used as a measure of the lens-inducing capacities of various larval *Xenopus* tissues. The experimental design employed involved implantation of selected body tissues between the inner and outer corneas of stage-50 tadpole eyes, in such a way that the integrity of the inner cornea and eye cup was not disrupted.

Implantation of retina, pituitary, limb blastema or limb bud resulted in secondary lens formation from the outer cornea. Such lenses were similar in appearance to stage-5 lens regenerates described by Freeman (1963). No secondary lenses were observed in eyes receiving either heart or hind brain implants or in eyes which underwent corneal separation but which received no implant.

It is concluded that the retina is the natural source of a stimulatory factor which initiates and maintains corneal transformation to lens during lens regeneration following lensectomy. Influences emanating from pituitary, limb blastema and limb bud, but apparently not from heart or hind brain, are able to act on cornea in a way similar to the retinal factor. Furthermore, our findings support the contention that in the normal eye, the inner cornea is a barrier to the passage of retinal factor and so maintains the single lens structure of the eye. When this barrier is by-passed by lens-inducing tissue, as in the present experimental design, lens formation from the cornea is able to take place. Electronmicroscopical studies have shown that the inner cornea, in the stage-50 tadpole eye, consists of a dense meshwork of collagen fibrils and a basal layer of cohesive elongated mesenchymal cells well suited for this barrier function.

INTRODUCTION

Lensectomy of *Xenopus laevis* tadpoles in many cases is followed by regeneration of a new lens from the inner layer of the outer cornea (Freeman, 1963). However, we have previously shown that lens regeneration from the cornea can proceed in the presence of the lens if the integrity of the inner

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cornea is effectively disrupted (Reeve & Wild, 1978). From these findings we postulated that contrary to earlier suggestions (Campbell & Jones, 1968; Manwaring, 1972) an inhibitory lens factor does not exist \textit{in vivo} in \textit{Xenopus laevis} but rather, a stimulatory factor necessary for the initiation and maintenance of lens regeneration emanates from the eye cup; upon wounding of the inner cornea this factor is able to reach the inner cell layer of the outer cornea and exert its effect.

The present study attempts to determine whether or not retina is a source of the stimulatory eye cup factor, as it is in the case of Wolffian lens regeneration (reviewed by Yamada, 1977) and explores the possibility that in the normal \textit{Xenopus laevis} tadpole eye the inner cornea prevents this factor from gaining access to the inner cell layer of the outer cornea and thus maintains the single lens structure of the eye. Studies on Wolffian lens regeneration have shown that both pituitary (Powell & Segil, 1976) and limb blastema (Reyer & Wolfitt, 1969; Reyer & Withersty, 1971; Reyer, Woolfitt & Withersty, 1973) can stimulate secondary lens formation from the dorsal iris of \textit{Notophthalmus viridescens}. Also, larval \textit{Xenopus laevis} cornea is able to undergo lens formation when implanted into the stump of an amputated hind limb (Waggoner, 1973). These observations clearly demonstrate that influences present in extra-ocular tissues are capable of inducing lens formation. Therefore in this study we have also investigated the lens-inducing capacity of larval \textit{Xenopus laevis} tissues other than those derived from the eye by examining their ability to induce secondary lens formation from \textit{in situ} cornea.

MATERIALS AND METHODS

Source of tissue implants

Retina, pituitary, limb bud, heart and hind brain were removed from stage-50 tadpoles that had been anaesthetized in 1:3000 MS 222 in 10% Holtfreter's solution. To obtain retina, whole eyes were first removed, an incision made in the outer and inner cornea, and the lens extracted. Neural retina was then dissected from pigmented epithelium and sclera. Heart tissue comprised the ventricle. To obtain limb blastemas the hind limbs of stage-52 donor tadpoles were amputated between the presumptive knee and presumptive ankle. Blastemas were allowed to develop for five days and then removed. Retina, pituitary, heart and hind brain were cut into fragments of approximately equal size and kept in sterile 10% Holtfreter's solution prior to implantation. One whole limb bud or limb blastema was used as an implant, and these approximated in size to the other tissue fragments.

Preparation of recipients

Tadpoles at stages 49 to 51 were used as recipients of tissue transplants. They were anaesthetized in 1:3000 MS 222 in 10% Holtfreter's solution and
operated on in full-strength Holtfreter's solution. Figure 1 depicts the operative procedure diagrammatically. A small incision was made with iridectomy scissors through the outer cornea along the corneal-epidermal junction of the right eye. The attachment between the inner and outer cornea was then severed by means of a small scalpel blade inserted through the incision: the inner cornea itself remained intact. Implants were then manipulated into the space between the inner and outer corneas. The recipient tadpoles were allowed to recover in full-strength Holtfreter's solution for 24 h and were then subsequently reared in 10% Holtfreter's solution. All animals were killed 14 days after the operation and processed for histological examination as previously described (Reeve & Wild, 1978).

Controls consisted of tadpoles which had undergone separation of the inner and outer corneas but which received no implants, and of contralateral left eyes that remained untouched in the experimental tadpoles.

**Electronmicroscopy**

Eyes, including the outer corneas, were removed from stage-50 tadpoles and fixed for 5 h at room temperature in 3% glutaraldehyde in 0.1 M phosphate buffer containing 2 mM-CaCl₂ (pH 7.4). Post-fixation was carried out for 1 h at room temperature in 1% osmium tetroxide made up in the same buffer solution. Eyes were dehydrated in ethanol and embedded in Spurr epoxy resin. Sections were cut on an LKB ultramicrotome, stained with saturated uranyl acetate solution and counterstained with lead citrate (Reynolds, 1963) in a carbon-dioxide-free atmosphere.
Table 1. Wounding of the outer cornea only (A) followed by implantation of retina (B) between the inner and outer corneas

<table>
<thead>
<tr>
<th>Operation</th>
<th>No. of operations</th>
<th>No. of tadpoles and grafts recovered</th>
<th>No. of secondary lenses (all stages)</th>
<th>No. of secondary lenses reaching stage 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>30</td>
<td>30(^1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>6(^2)</td>
<td>4</td>
<td>4</td>
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Stages referred to are those of Freeman (1963) for lens regeneration.

Table 2. Wounding of the outer cornea only (A) followed by implantation of pituitary (B), limb blastema (C), limb bud (D), heart (E) and hind brain (F) between inner and outer corneas

<table>
<thead>
<tr>
<th>Operation</th>
<th>No. of operations</th>
<th>No. of tadpoles and grafts recovered</th>
<th>No. of secondary lenses (all stages)</th>
<th>No. of secondary lenses reaching stage 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>45</td>
<td>45(^1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>15(^2)</td>
<td>8</td>
<td>4(^*)</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>13(^2)</td>
<td>9(^\dagger)</td>
<td>7</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>13(^2)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
<td>20</td>
<td>14(^2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>20</td>
<td>16(^2)</td>
<td>0</td>
<td>0</td>
</tr>
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* Remainder at stage 3.
\(^\dagger\) Six tadpoles had undergone secondary lens formation; in two cases more than one secondary lentoid was present.

RESULTS

In these experiments secondary lens formation from the inner layer of the outer cornea, in the presence of the existing lens and an intact inner cornea, has been used as a measure of lens-inducing factor present in different tissues. Results obtained are summarized in Tables 1 and 2.

No secondary lentoids were observed in any of the 75 control tadpoles which received no implant (Table 1, A and Table 2, A). However, secondary lentoids were present in four out of six eyes which had retained retinal implants (Table 1, B). All lentoids had undergone primary and secondary lens fibre differentiation and were similar in appearance to stage-5 regenerates (Freeman, 1963) that form from cornea in lensectomized eyes of *Xenopus laevis* tadpoles (Fig. 2a, b). In all cases the secondary lentoid was in close proximity to the retinal implant and in one case (Fig. 2b) appeared to be detaching from the inner cell layer of the outer cornea as evidenced by a bridge of cells, thus confirming its corneal origin. The polarity of the developing lentoid appeared to be influenced by the bulk of the implant and not by the underlying eye cup. Thus
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Fig. 2. (a) Secondary lentoid (SL) developing between the inner and outer cornea following implantation of retina (R). L, Host lens; EC, eye cup.

(b) Higher-power view of the secondary lentoid and retinal tissue shown in Fig. 2a. Note what appears to be a bridge of cells (arrowed) connecting the lens and inner cell layer of the outer cornea (OC). Nerve fibres (NF) are evident in the retinal implant. IC, Inner cornea.

the area of primary transformation to lens fibres (the primary fibrogenic centre) was directed towards the retinal implant and the developing lens epithelium away from the implant (Fig. 2b).

Secondary lens formation also occurred in eyes where pituitary had been implanted between the inner and outer corneas (Fig. 3). Of the fifteen tadpoles which had received the pituitary implant eight showed secondary lentoid formation (Table 2, B). Four of the developing lentoids had not undergone fibrogenesis, and corresponded to stage-3 regenerates: the other four all showed primary lens fibre formation characteristic of stage 5 (Fig. 3). As deduced from their close association with the inner cornea, some secondary lentoids forming
Fig. 3. Secondary lentoid (SL) developing between the inner cornea (IC) and the outer cornea (OC) after implantation of pituitary (P) between the two corneas. The lentoid may have developed from cells which originally comprised the point of attachment (PA) between the two corneas. AC, Anterior eye chamber, L, host lens.

Fig. 4. Secondary lentoid (SL) developing from the inner cell layer of the outer cornea (OC) after implantation of blastema (BL) between the inner cornea (IC) and outer cornea. Note that this lentoid appears to have two primary fibre nuclei (arrowed).

in the presence of pituitary may have developed from cells originally constituting the attachment of the inner and outer corneas. Such lentoids generally lacked an epithelium and were relatively smaller than those developing from the inner cell layer of the outer cornea.

Of the thirteen tadpoles which had retained the blastema implant, six had undergone secondary lentoid development as evidenced in most cases by the presence of well-developed lentoids corresponding in appearance to stage-5 regenerates (Table 2, C and Fig. 4). In two cases more than one secondary
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Fig. 5. Secondary lentoids (SL) developing between the inner cornea (IC) and outer cornea (OC) after implantation of blastema (BL) between the two corneas. One lentoid (A) appears to be derived from the inner cell layer of the outer cornea and the other (B) from the bridge of cells that had formed the point of attachment between the inner and outer corneas.

Fig. 6. Secondary lentoid (SL) developing between the inner cornea (IC) and the outer cornea (OC) after implantation of limb bud (LB) between the two corneas. In one region (arrowed) shattered lens tissue overlies the inner cornea, giving the false impression of continuity with the implanted limb bud.

lentoid was present: in one of these, lentoids had formed from the inner cell layer of the outer cornea and also apparently from cells attached to the inner cornea which probably comprised the attachment between inner and outer corneas (Fig. 5); in the other, three small lentoids had developed within the inner cell layer of the outer cornea. In two cases secondary lentoids failed to undergo complete differentiation and had the appearance of small lens vesicles.

Implantation of limb bud between the inner and outer corneas also induced secondary lentoids in five out of the thirteen tadpoles which had retained the
implant (Table 2, D). All corresponded to stage-5 lens regenerates (Fig. 6) and all had apparently developed from the inner layer of the outer cornea.

No secondary lentoids were observed in the eyes where either heart or hind brain had been implanted (Table 2, E and F) apart from the fact that in a few cases, concerning hind brain, cells which originally formed the point of inner and outer cornea attachment gave rise to a small vesicle which may or may not have been a secondary lentoid.
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Examination in the electron microscope of sections cut perpendicular to the corneal surface revealed that the inner cornea was composed of layers of densely compacted collagen fibrils in close association with mesenchymal cells (Fig. 7a, b). Collagen fibrils were arranged in an orthogonal fashion and had an approximate diameter of 25 nm. No cell junctions were seen between the mesenchymal cells, so that the mesenchymal layer had the appearance of a syncytium.

DISCUSSION

Previous findings have shown that the presence of the eye cup (Freeman, 1963; Waggoner, 1973; Filoni, Bosco & Cioni, 1979) and effective wounding of the inner cornea (Freeman, 1963; Reeve & Wild, 1978) are important factors in the expression of lens regeneration from the cornea. Lenscetomy itself appears not to be a prerequisite for corneal lens regeneration, since secondary lens formation can be initiated by merely wounding the outer and inner cornea or by dislocating the lens, and can result in fully differentiated lenses if the original lens is removed and then re-implanted (Reeve & Wild, 1978). On the basis of these findings we suggested that a factor responsible for the initiation and maintenance of lens regeneration emanates from the eye cup, and that in the normal eye this factor is prevented from gaining access to the inner cell layer of the outer cornea by the intervening inner cornea. A similar conclusion has recently been reached by Filoni, Bosco, Paglioni & Cioni (1980). These workers showed that after incision of the integument of the dorsal region of the eye and of the eye wall, but without lens removal, secondary lens formation occurred from the pericorneal epidermis in a high proportion of cases.

The results obtained from the present study, in which retina was implanted between the inner and outer corneas, indicate that the neural retinal component of the eye cup is responsible for providing the stimulus needed for transformation of cornea to lens. Retention of implanted retina was rather low, this most likely being due to the fact that the tissue readily fragments, but where it had been retained, secondary lentoids similar in appearance to stage-5 lens regenerates were present in a high proportion of cases. Sclera, it should be mentioned, has by itself no lens-inducing capacity (Reeve, unpublished observations). Cornea transplanted to the posterior eye chamber forms secondary lenses in the presence of the lens in a high proportion of cases (Reeve & Wild, 1978) indicating that the retina produces inducing factor in situ. However, it could be argued that the physical insult to the lens resulting from its dislocation or removal and re-implantation removes constraints that the lens normally holds over cornea in terms of its differentiation to lens. This argument clearly does not hold in the present experimental design, since the lens is not touched; furthermore the suggestion that the ventral pole of the lens might be different from the dorsal pole in terms of its inhibition (Filoni et al. 1980) is clearly obviated. The absence of secondary lentoids in control eyes which
underwent separation of inner and outer corneas, but which received no retinal implants, indicates that influences emanating from the eye cup itself were in no way responsible for the observed secondary lentoid formation. Thus these results confirm the importance of the integrity of the inner cornea as a barrier and show that the existing lens exerts no control over the inner layer of the outer cornea in terms of its lens-forming capacity. If this barrier is by-passed, as in the present experimental design, lens induction can take place. It seems reasonable to conclude that in the normal eye, what maintains the single lens state is simply the physical barrier to retinal factor provided by the inner cornea. Although from light microscopical studies the inner cornea has the appearance of a thin, delicate structure, preliminary electron-microscopical investigations show that at stage 50 at least, the inner cornea consists of a dense meshwork of collagen fibrils together with a basal layer of very cohesive elongated mesenchymal cells (Reeve, 1978). No direct tests have been carried out to determine the permeability of this tissue, especially to macromolecules, but it is conceivable from the morphological evidence that it could form an effective barrier between the eye cup and outer cornea. This is an area where further investigation is now required.

Our finding that retinal factor is important for initiating and maintaining secondary lens formation from cornea is in agreement both with in vivo (Reyer, 1966) and in vitro (Yamada, Reese & McDevitt, 1973) studies showing dependency of iris on neural retina in Wolffian lens regeneration. The results obtained from experiments involving implantation of larval *Xenopus laevis* tissues other than retina clearly demonstrate that pituitary, limb blastema and limb bud are also capable of inducing and maintaining the development of secondary lentoids from cornea in the presence of the original lens. Whilst influences emanating from these tissues are capable of acting on cornea in a way similar to those emanating from retina, no such influences apparently emanate from heart and brain. This could be attributable to either qualitative or quantitative differences between the tissues. Blastema, unlike the other tissues studied, gave rise to multiple lentoids, perhaps indicating that this is a potent lens inducer. These findings with extraocular tissues are in good agreement with studies made on Wolffian lens regeneration. Thus pituitary (and retinal) implants into the anterior chamber of the eyes of *Notophthalmus viridescens* have been shown to stimulate secondary lens formation from the dorsal iris epithelium in the presence of an existing lens (Powell & Segil, 1976). Furthermore, dorsal iris epithelium implanted into the blastema of a regenerating limb of *Notophthalmus viridescens* underwent transformation into lens (Reyer & Woolfitt, 1969; Reyer, Woolfitt & Withersty, 1973). The latter finding and those made in the present study are paralleled by the finding that corneal implants into the stump of an amputated hind limb of *Xenopus laevis* tadpoles are similarly able to undergo transformation into lens in some cases (Waggoner, 1973). It has been suggested that neurotrophic factors, present in the environment of
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a regenerating limb, may be responsible for the transformation of iris to lens (Reyer & Withersty, 1971). However, it seems unlikely that corneal transformation to lens is due to special properties existing in the regenerating limb since, in the present study, normal limb bud was able to support differentiation from cornea.

The molecular nature of the lens-inducing influence(s) emanating from retina, limb blastema and limb bud is unknown. All appear to act directly on the cells of the cornea rather than through some type of systemic interaction since implants had no positive influence on contralateral control eyes. It is possible that either a common inducing substance is present in retina, pituitary, limb blastema and limb bud, or that various factors present in these different tissues have the same capacity to support lens differentiation from the cornea. The transforming substance could also be a general metabolic product produced by many tissues in differing amounts. The observations made by Campbell & Jones (1968) that cornea could transform to lens in culture without the presence of other eye tissues can also be interpreted in this way, since the culture medium would provide a rich source of amino acids and other growth factors which might in themselves be sufficient to alter the developmental pathway of cornea. It has been suggested that since urodele iris epithelial cells can now be grown in vitro and have been shown to convert to lens in some cases (Eguchi, Abe & Watanabe, 1974), in vitro culture offers the best system for testing lens-inducing capacity of retinal fractions (Yamada, 1977). In the light of our findings we suggest that implantation of retinal fractions (on appropriate inert carriers) between the inner and outer cornea of Xenopus laevis tadpoles could provide a highly satisfactory alternative in vivo approach.

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REFERENCES


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