Ontogeny and localization of the lens crystallins in *Xenopus laevis* lens regeneration

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SUMMARY

Ontogeny and localization of the lens crystallins, especially the γ-crystallins were investigated in *Xenopus laevis* lens regenerating system by the 'indirect' immunofluorescence staining method. Antibodies directed against *Rana pipiens* γ-crystallin antigen were used for the detection of this crystallin; the validity of such an experiment has been shown in a previous report. To detect total lens proteins we used *X. laevis* anti-total lens protein antibody.

The regenerates were staged according to Freeman (1963) and the first positive reaction with both the two antisera was observed in an early stage-4 regenerate. The site of the immunofluorescence reaction was nearly identical in both, suggesting that γ-crystallins are one of the first, if not the first of the lens crystallins to appear during lens regeneration.

The secondary fibres, when developed, showed less immunofluorescence than the primary fibres with *R. pipiens* anti-γ crystallin antibody, though the reaction was intense in the secondary fibres with *X. laevis* anti-total lens protein antibody.

The intensity and distribution of immunofluorescence increased with the growth of the lens. With the *R. pipiens* anti-γ crystallin antibody, the lens epithelium did not show any immunofluorescence reaction at any stage of lens regeneration. With *X. laevis* anti-total lens protein antibody, the epithelium showed an immunofluorescence reaction earlier than in the normal lens development. With the two antisera we used, we did not observe any immunofluorescence outside the lens tissue.

INTRODUCTION

Lens crystallins of various amphibian species are classified into α-, β-, and γ-crystallins (McDevitt, 1967; Campbell, Clayton & Truman, 1968; Brahma & van Doorenmaalen, 1969; Polansky & Bennett, 1970; Brahma & Bours, 1971; McDevitt, 1972; Polansky & Bennett, 1973). Biochemical and immunofluorescence studies have shown that in bovine and amphibian lenses, the γ-crystallins can be detected only in fibre cells or in those cells which are in the process of developing into fibres and have entered into a permanent stationary phase. These cells thus become biochemically specialized for the

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synthesis of tissue specific macromolecules, the \( \gamma \)-crystallins (Papaconstantinou, 1965; Takata, Albright & Yamada, 1965; McDevitt, Meza & Yamada, 1969; McDevitt & Brahma, 1973).

In most of the urodele amphibian species it has been shown that lens regeneration takes place from the dorsal iris all through the life of the animal (Reyer, 1954; Stone, 1959; Reyer, 1962); while in the South African clawed toad, \textit{Xenopus laevis}, an anuran amphibian, lens regeneration has been reported to take place from the corneal epithelium only, and to be restricted to the larval stages (Overton & Freeman, 1960; Freeman & Overton, 1961; Freeman, 1963; Waggoner, 1973). However, Campbell (1963) reported lens regeneration from dorsal iris in adult \textit{X. laevis} although Brahma & van Doorenmaalen (1968) failed to observe lens regeneration from dorsal iris in adults of the same species.

Campbell (1965) made immunofluorescence studies of the total lens crystallin antigens in a \textit{X. laevis} lens regenerating system by the 'direct' method; but no reports are available on the ontogeny of the \( \gamma \)-crystallins during lens regeneration in this species, though experiments have been described on the ontogeny and localization of \( \gamma \)-crystallins during lens regeneration in urodeles and in the normal lens development of \textit{R. pipiens} and \textit{X. laevis} (Takata et al. 1964a, b, 1965, 1966; McDevitt et al. 1969; McDevitt & Brahma, 1973).

In this article results are presented regarding the ontogeny and localization of lens crystallins, especially the \( \gamma \)-crystallins, in the \textit{X. laevis} lens regenerating system, studied by the 'indirect' immunofluorescence staining method.

We used \textit{X. laevis} anti-total lens protein antibodies for the detection of total lens crystallins and \textit{R. pipiens} anti-\( \gamma \) crystallin antibodies for the detection of \( \gamma \)-crystallins. The validity of using heterologous anti-\( \gamma \) crystallin antibody against \textit{X. laevis} lens has been confirmed previously (McDevitt & Brahma, 1973). Moreover, it has recently been shown that \textit{R. pipiens} anti-\( \gamma \) crystallin antibody can be used successfully to detect \( \gamma \)-crystallin antigens in newt lens regenerates both \textit{in vivo} and \textit{in vitro} (Nöthiger, McDevitt & Yamada, 1971; Yamada, Reese & McDevitt, 1973).

**MATERIALS AND METHODS**

The collection and rearing of fertilized eggs from \textit{X. laevis} up to larval stages has already been described (McDevitt & Brahma, 1973). When the larvae reached stages between 52 and 54 (Nieuwkoop & Faber, 1956) they were used in the present experiment. From any single clutch, larvae with identical growth rates were selected for the operation described below.

Before the operation, the larvae were anaesthetized with MS 222 as suggested by Freeman (1963). They were transferred to a Petri dish with a base of 3\% agar and containing conditioned tap water. A groove was cut in the agar base to hold the animals in position during the operation. Lenses were removed from the left eye with sharp tungsten needles under a dissecting binocular
Crystallins in Xenopus laevis lens regeneration

microscope, and each lens after lentectomy was carefully examined under the microscope, with high magnification, to check the completeness of removal. Then the larvae were returned immediately to an aquarium with constant aeration.

Animals with regenerates from 2nd day till 18th day after lens removal were anaesthetized, decapitated and the heads washed in water at 4 °C. The heads were fixed either in cooled Carnoy (absolute alcohol:chloroform:glacial acetic acid, 6:3:1) or in 95% ethanol at 4 °C, processed and sectioned at 5 µm thickness according to McDevitt et al. (1969).

Regenerates were stained according to the methods described earlier (McDevitt et al. 1969; McDevitt & Brahma, 1973) and identified according to the five stages of Freeman (1963). The antibodies used in the present series were from the same stock used in our previous experiment (McDevitt & Brahma, 1973). The X. laevis anti-total lens protein antibody was diluted to remove any non-specific staining (Goldman, 1968); this was not necessary with the anti-γ crystallin antibody.

After staining, sections were mounted in ‘Mowiol’ N-30-38 extra (Hoechst AG, Frankfurt/M, Germany) according to the methods of Thomason & Cowart (1967). Lenses were examined with an E. Leitz fluorescence microscope and photographed under identical conditions to those described previously (Brahma & van Doorenmaalen, 1971; Brahma, Rabaey & van Doorenmaalen, 1972). From the same regenerate, phase contrast pictures were taken to demonstrate the site of the immunofluorescence.

Staging of the lens regenerates

Freeman (1963) described the process of lens regeneration from the cornea in X. laevis larvae in detail and he divided the entire process into five arbitrary stages. A running summary of all these stages as described by Freeman (1963) is presented here to facilitate understanding of the stages mentioned in this article.

Stage 1. This is the stage in which, immediately after lens removal, cells of the inner layer of the outer cornea change from squamous to cuboidal shape.

Stage 2. The cells of the inner layer of the outer cornea at this stage form a mass, two to three cell layers thick, in the pupilary space.

Stage 3. The loose cells of the aggregate become oriented with regard to each other and tend to separate from other corneal cells and form a rudiment which grows in size into the space occupied by the original lens.

Stage 4. At the beginning of this stage some of the cells farthest from the cornea show enlargement of their nuclei and nucleoli. Mitotic activities are restricted to the periphery of the vesicle and cells with large nuclei produce irregular fibres, the first sign of fibre formation. From this stage, the regenerate is generally found to be separated from the cornea.
Crystallins in *Xenopus laevis* lens regeneration

**Stage 5.** The secondary fibres begin to form the equatorial zone of the lens epithelium and the nuclei of the primary fibres begin to disappear. From this stage onwards, no major histological changes are observed in the lens except that it continues to grow in size.

Freeman (1963) also sub-divided some of the stages i.e. 3, 4 and 5 into early, middle and late stages for a more precise categorization of the process of lens regeneration.

**RESULTS**

**Immunofluorescence with anti-total lens protein antibody**

The first positive immunofluorescence reaction observed under our experimental conditions was at *early stage 4*. It was found to be localized in a number of cells in an area of the lens rudiment farthest from the cornea, where future lens fibres would develop (Fig. 1). With further differentiation of the regenerate, more and more cells of the primary fibre cell area showed positive immunofluorescence and in the *mid stage-4 regenerate*, the external layer of the vesicle started to show weak immunofluorescence (Fig. 2). By *early stage 5* when secondary fibres began to develop from the equatorial zone, both primary and secondary fibres showed more intense immunofluorescence than the epithelium (Fig. 3). In the *mid stage-5 regenerate* the secondary fibres showed more fluorescence than the primary fibres, and the epithelium showed less fluorescence than both the primary and secondary fibres. But in general, intensity of immunofluorescence reaction was enhanced (Fig. 4).

From this stage on, the lens did not show any major structural change, except that it increased in size and the intensity of immunofluorescence reaction was increased throughout the area of the lens.

Figs. 5, 6, 7 and 8 are the phase contrast pictures of the tissues shown in Figs. 1, 2, 3 and 4.

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Fig. 1. Immunofluorescence photomicrograph of an early stage-4 regenerate exposed to *X. laevis* anti-total lens protein antibody ×281. The regenerate was fixed in cooled Carnoy.

Fig. 2. Immunofluorescence photomicrograph of a mid stage-4 regenerate exposed to *X. laevis* anti-total lens protein antibody ×243. The regenerate was fixed in cooled 95% ethanol.

Fig. 3. Immunofluorescence photomicrograph of an early stage-5 regenerate exposed to *X. laevis* anti-total lens protein antibody ×206. The regenerate was fixed in cooled 95% ethanol.

Fig. 4. Immunofluorescence photomicrograph of a mid stage-5 regenerate exposed to *X. laevis* anti-total lens protein antibody ×172. The regenerate was fixed in cooled Carnoy.

Figs. 1–4 are dark field photomicrographs.

Figs. 5–8 are the phase contrast photomicrographs of the above lenses with identical magnifications and shown as histological reference.
Crystallins in Xenopus laevis lens regeneration

Immunofluorescence with anti-γ crystallin antibody

With this antiserum we also observed that the first positive immunofluorescence reaction appeared in an early stage-4 regenerate. It was localized at approximately the same area which showed the earliest positive reaction with *X. laevis* anti-total lens protein antibody (Fig. 9). As the cells in this region started to differentiate into fibres, more and more cells became involved in the synthesis of this crystallin. In the mid stage-4 regenerate it became quite evident that only fibre cells were involved in the synthesis of this crystallin class, and no reaction could be observed in the external layer of the vesicle which would ultimately develop into lens epithelium (Fig. 10). In the early stage-5 regenerate, the secondary fibres were almost negative, while the fluorescence was restricted only to the primary fibres (Fig. 11). In the mid stage-5 regenerate, the secondary fibres showed fluorescence, though less than primary fibres, while the total pattern of intensity of immunofluorescence increased (Fig. 12). This state of fluorescence prevailed in regenerates from mid stage-5 regenerate onwards. In no case could we detect immunofluorescence in the lens epithelium, and no immunofluorescence in the cornea was observed with either antibody.

Figs. 13, 14, 15 and 16 are phase contrast pictures of the tissues shown in Figs. 9, 10, 11 and 12.

In a few cases we observed double lenses and in one case multiple lens formation. Two such regenerates were treated with anti-γ crystallin antibody. It was observed that the stages of differentiation of the double lenses were identical, and their immunofluorescence patterns were also identical (Fig. 17), while in the other with multiple lenses, only one showed fibre differentiation and the expected positive immunofluorescence reaction with anti-γ crystallin antibody (Fig. 18).

Figs. 19 and 20 are phase contrast pictures of the tissues shown in Figs. 17 and 18.

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Fig. 9. Immunofluorescence photomicrograph of an early stage-4 regenerate exposed to *R. pipiens* anti-γ crystallin antibody × 271. The regenerate was fixed in cooled Carnoy.

Fig. 10. Immunofluorescence photomicrograph of a mid stage-4 regenerate exposed to *R. pipiens* anti-γ crystallin antibody × 234. The regenerate was fixed in cooled 95% ethanol.

Fig. 11. Immunofluorescence photomicrograph of an early stage-5 regenerate exposed to *R. pipiens* anti-γ crystallin antibody × 199. The regenerate was fixed in cooled 95% ethanol.

Fig. 12. Immunofluorescence photomicrograph of a mid stage-5 regenerate exposed to *R. pipiens* anti-γ crystallin antibody × 164. The regenerate was fixed in cooled Carnoy.

Figs. 9–12 are dark field photomicrographs.

Figs. 13–16 are the phase contrast photomicrographs of the above lenses with identical magnifications and shown as histological reference.
Reaction with serum from non-immunized rabbits

In this series we determined the specificities of the immune antibody types by replacing them with serum from non-immunized rabbits. Immunofluorescence reaction in both cases were negative, indicating specificities for total lens and lens fibre cells (anti-total lens protein and anti-\( \gamma \) crystallin antibodies, respectively).

DISCUSSION

Campbell (1965), with his unabsorbed X. laevis anti-total lens protein antiserum, found a positive immunofluorescence reaction in the corneal epithelium before the lens regenerate was developed and this situation persisted till the
regenerate reached stage 5. He observed positive immunofluorescence in the regenerate from stage 3; in late stage 3 its intensity was increased and some cells of the posterior margin of the lens vesicle showed strong fluorescence.

In the present series of experiments with *X. laevis* anti-total lens protein antibody made specific by dilution, we observed immunofluorescence only from the early stage-4 regenerate and not in the cornea.

Campbell (1965) also reported that with his antiserum he did not observe any immunofluorescence in the lens epithelium of the regenerates and he suggested that 'most lens antigens are associated with fibres'. With our antibody (total lens protein) we, on the other hand, could detect positive immunofluorescence in the epithelium, and, in contrast to the situation in *X. laevis* normal lens development (McDevitt & Brahma, 1973), the fluorescence appeared quite early during lens regeneration. This difference between the present observations and those of Campbell (1965) could be due to differences in the specificities of the antisera and/or the techniques employed.

The appearance of γ-crystallins in the prospective fibre cells as they begin to elongate is well established in urodele regenerates both in vivo and in vitro; also in normal anuran and mammalian lens development (Takata et al. 1966; McDevitt et al. 1969; Yamada et al. 1973; Schubert, Trevithick & Hollenberg, 1970).

The results with *X. laevis* lens regenerates differ from the above in the presence of a positive immunofluorescence reaction with *R. pipiens* γ-crystallin antibody in the prospective fibres cells earlier in the histogenesis of the regenerate. Similar results have also been recorded in *X. laevis* normal lens development with *R. pipiens* anti-γ-crystallin antibody (McDevitt & Brahma, 1973), thus reinforcing the concept of *X. laevis* as an organism with aberrant lens differentiation. In both cases, γ-crystallins are one of the first, if not the first, of the lens crystallins to appear.

McDevitt (1972) reported that in *R. pipiens* normal lens development, both β- and γ-crystallins, could be detected simultaneously as the first to appear by classical immunological methods. Yamada (1967) reported in his review article that in the newt lens regenerate, α-, and β-crystallins appear first in the cells before they enter the phase of fibre differentiation; and as soon as these cells enter the terminal cell cycle, γ-crystallins appear. This difference could be a species difference or a difference in the histogenesis of the lens regenerates. In addition, as stated by Yamada (1966) results obtained with his α- and β-crystallin antisera should be evaluated with reservations because of the antigen contamination he reported.

The importance of γ-crystallins in relation to fibre differentiation is once again demonstrated in our experiments. However, some difference could be observed between lens regeneration in urodeles and *X. laevis*. In the former, the lens regenerates from the dorsal iris after lentectomy, though it can also develop from the ventral iris after the administration of *N*-methyl-*N'*-nitro-*N*-
nitrosoguanidine (Eguchi & Watanabe, 1973), and these are known to be the cases of metaplastic changes since the iris is already a differentiated tissue, and all epithelial cells are terminally differentiated (Yamada, 1972). In X. laevis, however, lens regeneration appears to be restricted to larval stages and to come only from the corneal cells. At these stages, the development of the cornea is not complete (Freeman, 1963) and therefore, this does not appear to be a case of metaplastic change.

Amongst the vertebrates, either a normally developing lens or a lens regenerate pass through a typical vesicle stage where the external cell layer that will give rise to the lens epithelium can be distinguished from internal cell layer that would develop into primary fibres. In X. laevis, this vesicle is short-lived both in normal lens development (McDevitt & Brahma, 1973) and also in the regenerates. In addition, there is no such clear cut difference between external and internal layers in normal X. laevis development, and the vesicle stage appears after fibre formation. Moreover, reaction to anti-γ crystallin antibody appears long before any sign of fibre formation and in this respect, X. laevis lens development, both normal and regenerating, differs from that of lenses of other amphibian species studied so far. The developmental cause for such deviation is not known.

There are also other differences between corneal regeneration and normal lens development in X. laevis. In the former, the lens vesicle appears much earlier and the epithelium shows a positive immunofluorescence reaction with anti-total lens protein antibody earlier than in normal lens development, indicating a comparatively precocious synthesis of α- and/or β-crystallins in this cell type.

In addition, results from multiple lens regenerates, the occurrence of which ensures an identical milieu for differentiation, show strikingly that γ-crystallins are associated only with the process of fibre differentiation.

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Crystallins in Xenopus laevis lens regeneration


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