An immuno-fluorescent study of lens regeneration in larval Xenopus laevis

by JOHN C. CAMPBELL

From The Institute of Animal Genetics, University of Edinburgh

WITH TWO PLATES

INTRODUCTION

It is well known that several species of amphibia, especially those of the genus Triturus, can regenerate a lens after removal of the original lens from the eye. In most of these species the regenerate develops from the iris (Reyer, 1954), but in larval Xenopus laevis (Overton & Freeman, 1960; Freeman & Overton, 1961, 1962; Freeman, 1963; Campbell, 1963) and possibly in early embryonic stages of Hynobiusunnangso (Ikeda, 1936, 1939) the regenerating lens can be formed from corneal tissue. The morphological changes associated with regeneration of the lens from the cornea in X. laevis have been fully described by Freeman (1963), who has shown that the regenerate develops from the inner cell layer of the outer, or ectodermal, cornea, appearing initially as a small clump of cells in the mid-pupillary region. This aggregate organizes into a vesicle, from the posterior wall of which the primary lens fibres are formed. This is followed by the production of secondary lens fibres from the equatorial region of the vesicle epithelium, and detachment of the regenerate from the cornea into the pupillary space.

Since the differentiation of the regenerate must involve chemical as well as morphological changes, immunological studies of the regenerating lens were undertaken, in order to obtain information on changes in tissue antigens during regeneration. Fluorochrome-labelled antibodies against whole lens extract were used in order to demonstrate changes in distribution of lens antigens within the tissues involved in regeneration, and in order to be able to correlate these changes with morphologically evident events.

MATERIAL AND METHODS

Animals

Larval X. laevis from induced matings were used between developmental stages 55 and 58 (Nieuwkoop & Faber, 1956). The animals had been reared in tap water and fed on dried nettle powder.

1 Author's address: M.R.C. Epigenetics Research Group, Institute of Animal Genetics, West Mains Road, Edinburgh 9, Scotland, U.K.
Operation

The animals were anaesthetized by immersion in a 1/2000 solution of M.S. 222 (Sandoz, Ltd.), then transferred to tap water for the operation. The lens was removed through a corneal incision, by the application of slight pressure to the posterior part of the orbit. As the lens was extruded from the pupil, it was dissected free from the ciliary attachments. Each lens was examined on removal, and if any damage was noted, the animal from which it had been removed was discarded. The animals were kept in tap water for the duration of the experiment.

Antiserum preparation

Extracts of the saline-soluble components of adult *X. laevis* lens were prepared and adjusted to a concentration of 15 mg./ml. of protein. They were subsequently used to induce antibody formation in rabbits aged between 9 and 18 months. Each rabbit received at least ten intravenous injections, each containing 15 mg. of protein, over a period of 6 weeks, and was bled 10 days after the last injection.

Those antisera which proved immunologically satisfactory, giving positive reactions with antigen dilutions of higher than 1:1000, were fractionated by 40 per cent saturation of ammonium sulphate, and the globulins thus precipitated were retained and salted out twice more. Residual ammonium sulphate was removed from the globulin solution by dialysis against phosphate buffered saline, pH 7.2. The globulins were then coupled to either rhodamine sulphonyl chloride prepared from lissamine rhodamine B 200, (I.C.I. Ltd.) or fluorescein isothiocyanate (Borden Chemical Co.) by the techniques recommended by Nairn (1962). Uncoupled fluorochrome was removed from the globulin solutions by passage through a column of Sephadex G-25 (Pharmacia) and subsequent dialysis against phosphate buffered saline, pH 7.2, of the first eluted fraction. The globulin solutions were then concentrated to the original volumes by forced draught and dialysed against phosphate buffered saline, pH 7.2, to equilibrate the salt content of the solvent.

Tissue preparation

Animals were killed by decapitation at daily intervals after the operation. The heads were immediately snap-frozen in trichlorofluoromethane (Arcton 12, I.C.I., Ltd.) cooled by liquid oxygen to −183°C. The heads were then transferred to alcohol cooled by Drikold (I.C.I. Ltd.) to −76°C. and freeze substitution allowed to proceed. After bringing the heads in alcohol to room temperature, they were cleared in terpineol, embedded in paraffin wax and 6 μ sections were cut. The regenerates were staged according to Freeman (1963).

Immunological testing

After removal of wax a small amount of labelled globulin solution was placed on each section and left for at least 30 min. in a moist chamber. In general,
Photomicrographs of lentectomized eyes of *Xenopus laevis* tadpoles between developmental stages 55 and 58 (Nieuwkoop & Faber, 1956). The eyes were fixed by freeze-substitution, embedded in paraffin wax, sectioned at 6 μ, and treated with fluorochrome labelled anti-lens serum. The sections were photographed with Kodak High Speed Ektachrome Film and the transparencies were copied on Ilford Pan F. Film. In each case the scale mark represents 0.05 mm.

**PLATE 1**

**Fig. A.** Anterior part of unoperated eye. Note labelling over lens fibres and unlabelled cornea. Rhodamine coupled antiserum.

**Fig. B.** Cornea 15 hr. after lentectomy. No labelling. Rhodamine coupled antiserum.

**Fig. C.** Cornea 1 day after lentectomy, showing labelling of cytoplasm of cells of inner layer of epithelium. Rhodamine coupled antiserum.

**Fig. D.** Anterior part of eye 2 days after lentectomy. Note labelling of corneal epithelium and neural retina. Rhodamine coupled antiserum.

**Fig. E.** Cornea 4 days after lentectomy, showing a small clump of cells over the pupillary space. Labelling is present throughout the epithelium. Rhodamine coupled antiserum.

**Fig. F.** Cornea and lens vesicle 5 days after lentectomy. Note labelling over the cytoplasm of the cells of the corneal epithelium and vesicle. Rhodamine coupled antiserum.
rhodamine-labelled reagents were preferred to fluorescein-labelled ones, because of the blue-green auto-fluorescence of tissues. After the globulin solution was removed the section was washed in several changes of phosphate buffered saline, pH 7.2, over a period of at least 30 min. Sections were examined under a Reichert Zetopan fluorescent microscope, incorporating an HBO-200 mercury vapour lamp, and using primary filters (Schott) UG 1 and BG 12 and secondary filter GG 9. Photographs were taken on Kodak High Speed Ektachrome film and copied on Ilford Pan F film.

Controls

In order to demonstrate the specificity of the reactions observed control sections were examined after treatment with each of three reagents, fluorochrome-labelled non-immune serum, a solution of fluorochrome, or non-labelled antilens serum followed by fluorochrome-labelled anti-lens globulin solution.

RESULTS

In the unoperated eye there is intense fluorescence over the whole of the lens, with the exception of the epithelium. In general the younger fibres at the periphery of the lens fluoresced slightly less brightly than the older fibres. In such eyes the cornea is unlabelled (Plate 1, Fig. A).

Within 12 to 15 hr. after removal of the lens, the wound in the cornea has healed over, and the cells of the inner layer of the corneal epithelium have started to change from squamous to cuboidal—regeneration stage 1. At this point there is no fluorescence detectable in the cornea (Plate 1, Fig. B), though by the end of the first day of regeneration lens antigens have appeared in the cells of the corneal epithelium (Plate 1, Fig. C). The labelling, which is relatively weak, is largely restricted to the cells of the inner layer of the epithelium in the mid-pupillary region, and is confined to the cytoplasm. The fluorescence at the outer margin of the cornea which is first apparent at this stage is due to strong blue-white auto-fluorescence of the tissue. Auto-fluorescence in this region is found throughout the period of regeneration until stage 5. By the end of the second day after lentectomy, specific labelling is seen in all cell layers of the epithelium over an area larger than the pupillary space (Plate 1, Fig. D). The fluorescence, which is still cytoplasmic, is strongest in the inner cell layer over the pupillary space.

During regeneration stage 2, the third and fourth days after lens removal, when a loose clump of cells is formed from the inner layer of the epithelium over the pupillary space, fluorescence is found throughout the cornea. Although there is no concentration of label over the clump of cells, the greatest intensity is found over the pupillary space (Plate 1, Fig. E) and at the periphery of the cornea. The intense fluorescence visible at the outer margin of the cornea is due to auto-fluorescence.

By the fifth day of regeneration a vesicle has been formed—regeneration stage 3—and fluorescence is detectable in the cytoplasm of the cells of the vesicle as well
as in those of the cornea (Plate 1, Fig. F). The cells of the vesicle fluoresce less strongly than those of the cornea. In late stage-3 regenerates (Plate 2, Fig. G) the fluorescence in the vesicle is less strong than in earlier vesicles, though some cells at the posterior margin of the vesicle, from which the primary lens fibres are going to be formed, contain small areas of very intense fluorescence.

At regeneration stage 4, between the seventh and tenth days of development of the lens, the primary lens fibres become morphologically evident and the fluorescence in the vesicle is located mainly over the fibres (Plate 2, Figs. H & I), there being at most very weak fluorescence over the cytoplasm of the other cells. At the start of stage 4 the cornea is still fairly strongly labelled (Plate 2, Fig. H), but this fluorescence becomes less intense in successively later specimens in this stage until, by the start of stage 5 (Plate 2, Fig. J), there is little fluorescence left in the tissue.

Stage 5 of regeneration, which occurs 10 to 14 days after lentectomy, is the stage of the development of secondary lens fibres and growth of the lens. At the start of this stage, the labelling is most intense in the region of the primary fibres, which occupy the central position in the lentoid (Plate 2, Fig. J), and this distribution is sometimes reflected in almost complete regenerates (Plate 2, Fig. K), but in other cases fluorescence is distributed throughout the lens, the primary and secondary fibres being equally intensely stained (Plate 2, Fig. L). In none of the regenerates were the nuclei of the fibres stained, and as in the original lenses the epithelia were devoid of fluorescence. By this stage the cornea has apparently returned to its original condition and is not stained by the specific antiglobulin solution.

Of the other tissues of the normal eye, fluorescence was detectable only in the neural retina and within this tissue was restricted to the region of the rods and cones, and, less intensely, to the inner and outer fibrillar layers and to the pars ciliaris (Plate 1, Fig. D). There was no apparent change in the distribution or intensity of this labelling during the course of regeneration.

The control experiments established the specificity of the reactions observed. Control fluorescent serum and fluorochrome solution, when added to sections, gave no detectable reaction, and minimal fluorescence occurred with sections which were first treated with non-fluorescent specific antiserum and then allowed to react with labelled specific antiserum.

**DISCUSSION**

Before considering the results obtained, the limitations of the technique used should be discussed. The labelled antibody technique can give information on the spatial and temporal distribution of antigens more readily than any other immunological technique but, as used in the experiments described, it is apparently less sensitive in detection of antigens than several other methods. For example, using double diffusion techniques the antisera can detect small amount of lens
FIG. G. Cornea and lens vesicle 6 days after lentectomy. The labelling is distributed as in the earlier vesicle stage with the addition of small area of intense fluorescence in the cells at the posterior margin of the vesicle. Rhodamine coupled antiserum.

FIG. H. Cornea and lens vesicle 7 days after lentectomy. In the vesicle the intense fluorescence is over the area of the primary lens fibres. Fluorescein coupled antiserum.

FIG. I. Cornea and lentoid 8 days after lentectomy. In the lentoid the primary fibres are intensely labelled. Rhodamine coupled antiserum.

FIG. J. Cornea and lentoid 10 days after lentectomy. In the lentoid the most intense labelling is over the primary fibres, with slightly less intense labelling over developing secondary fibres. Labelling of the cornea is less intense than at earlier stages. Rhodamine coupled antiserum.

FIG. K. Regenerate 12 days after lentectomy. Note labelling over central part of lens only. Rhodamine coupled antiserum.

FIG. L. Regenerate 14 days after lentectomy. Note labelling over whole lens, with the exception of the epithelium. The cornea is unlabelled. Rhodamine coupled antiserum.

J. C. CAMPBELL

(Facing page 174)
An immuno-fluorescent study of lens regeneration

antigens in most eye tissues, but in the immuno-fluorescent technique described they react only with neural retina. There are several possible explanations of this difference, one being that there may be a slight loss of immunological reactivity of the antiserum during the coupling process, thus reducing the antibody titre to a level too low to detect small amounts of antigen. Another explanation is that there may be a loss of tissue antigenicity during the processes of fixation and embedding which precede the immunological testing. For each antigen system the appropriate histological techniques must be evolved, since it is known that many histological procedures destroy or reduce the reactivity of antigens (see Nairn, 1962, for full discussion). The methods described were adopted since they appeared to have little effect on the antigenicity of the lens and cornea, and caused minimal morphological disturbance. A third possible explanation is that in the other eye tissues the antigen molecules may be so dispersed that they could not be revealed by the fluorochrome labelled antisera.

One other limitation of the technique as used in the experiments described is that it gives no indication of the behaviour of individual antigens during regeneration, since the antiserum was prepared against whole lens extract which is known to contain at least ten antigens (Campbell, unpublished). At present work is being undertaken to investigate, by double diffusion techniques, the order and times of appearance of individual antigens in lens regenerating from cornea.

The antiserum used was unabsorbed, although it was known to contain antibodies to antigens which are found in other eye tissues as well as the lens. This was done since it was considered that differentiation of the regenerate should be considered as the elaboration of all the antigens normally found in the lens, not only those which are wholly lens specific.

It is clear from the results described that extensive chemical changes take place in the cornea of the lentectomized eye before any morphological change occurs. It may be that the early appearance of lens antigens in the regenerating cornea is associated with the change in shape of the cells of the inner layer of the corneal epithelium from squamous to cuboid but, since a similar change occurs in a healing but not regenerating cornea (Freeman, 1963), it is unlikely that the two are causally related. It seems more likely that the appearance of lens antigens is related to the change in nucleolar ratio which occurs in the cells of the inner layer of the corneal epithelium in the mid-pupillary region within the first day after lentectomy (Freeman, 1963), since this may well reflect synthetic activity in these cells.

Although the presence of lens antigens is seen at the earliest stages of regeneration, it is not until lens fibres start to be formed that the most intense fluorescence occurs, thus suggesting that most lens antigens are associated with the fibres. With the antisera used there appeared to be a predisposition to react with older fibres, since the younger fibres at the periphery of the lens are less intensely stained (e.g. Plate 1, Fig. A). A similar observation has been made on the lens of *Triturus viridescens* by Takata, Albright & Yamada (1964a).

The association of increase in lens antigens and commencement of lens fibre
formation has been observed in other immunological studies of lens regeneration in amphibia, although in them the lens developed from the iris by Wolffian regeneration. Takata et al. (1964a), using techniques similar to those used in the present study but using antisera absorbed with mouse tissue powder, investigated regeneration in *T. viridescens* and showed that lens antigens were not detectable in the regenerate until the stage of early elongation of the primary lens fibres. However, as a result of the absorption, only those antibodies to wholly lens specific antigens were left in the antiserum, and this could account for the failure to detect lens antigens in the regenerate at earlier stages.

Ogawa (1963) studied lens regeneration in *T. pyrrhogaster* with both fluorescent antiserum and an immunodiffusion technique, using antiserum absorbed with iris or retina. In the study with labelled antiserum he excised irises containing regenerates and bathed them, unfixed, in the fluorescent reagent and, after washing, he was able to detect whether the regenerate had reacted with the antiserum or not. The earliest stage at which he was able to demonstrate specific labelling was vesicle formation, 10 days after lens removal. It is not clear from his illustrations whether fibre formation has started by this stage or not. It would seem that this technique is of value only in studying cell surface antigens, and thus it is possible that earlier stages may have contained intracellular lens antigens. In the immunodiffusion tests with absorbed antiserum, positive reactions were not obtained from regenerates younger than 10 days, but, with unabsorbed serum, positive reactions were obtained at all stages from the iris, though the intensity of reaction increases after 15 days of regeneration.

Information on the appearance of lens antigens during Wolffian regeneration in *T. taeniatus* has been obtained by two distinct methods. Titova (1957) used the anaphylactic reaction test, using, as sensitizing injections, extracts of irises 5 and 15 days after lentectomy, and, as challenge injections, an extract of lens of *Rana ridibunda*. The results showed that lens antigen was absent from the 5-day regenerates, but present in 15-day regenerates, by which stage lens fibres have formed. Vyasov & Sazhina (1961), using the capillary method of micro-double-diffusion and an antiserum against lens extract of *R. temporaria*, showed that on the fifth day of regeneration no lens antigens are present, but by the seventh day a vague positive reaction occurs and on the eleventh day one clear band is formed. Thus, in this species also, the lens-specific antigens appear at the time of fibre formation.

Thus it appears by analogy that the antigens which appear before fibre formation in *X. laevis* may be those which are not wholly lens specific but are nevertheless involved in the differentiation of the regenerate. However, the possibility is not excluded that lens specific antigens do appear before fibre formation in the species described, but are present in too low concentrations to be detectable by the techniques used. The appearance of lens antigens before the appearance of lens fibres is in accord with results of immunological studies on the normal development of the lens in embryos of various species, e.g. Ten Cate & Van Dooren-
maalen (1950), Flickinger, Levi & Smith (1955), Langman et al. (1956), Langman (1959), Maisel & Langman (1961) and Langman & Maisel (1962). Immuno-fluorescent studies of normal lens development have shown that in *Triturus pyrrhogaster* lens antigens are detectable in the cells of the inner wall of the lens vesicle at the stage when the vesicle is being formed from the placode and before any morphological sign of fibre formation is detectable (Takata et al., 1964b). Similar studies on the development of the chick lens (Van Doorenmaalen, 1958) show that specific labelling does not occur until the fifth day of development, some 20 hr. after lens fibres are apparent. However, other work using a similar technique (Campbell, unpublished) has shown the presence of lens antigens in the lens vesicle of 2½-day-old chick embryos. The difference between these two sets of results may be accounted for by differences in the specificities of the antisera used, and possibly partly by the use of different methods of tissue fixations: Van Doorenmaalen—cold acetone, Campbell—Carnoy’s fixative.

The results described demonstrate the rapid and extensive changes in the antigenicity of the cornea during the course of regeneration of the lens. However, they give no indication of the mechanisms of initiation and control underlying this metaplasia, and *in vitro* studies of the regeneration system have been started in order to investigate these more fundamental problems.

**SUMMARY**

1. The appearance and localization of lens antigens in lens regenerating from the cornea of larval *Xenopus laevis* were investigated by the fluorescent antibody technique.

2. The cornea of the normal eye was not stained by the antiserum used, but lens antigens were detected in the cytoplasm of cells of the inner layer of the corneal epithelium overlying the pupillary space within 24 hr. of lens removal.

3. In the cells of the lens vesicle, before fibre formation, the labelling, which was over the cytoplasm, was weak, except for small areas of cells at the posterior margin of the vesicle from which the primary lens fibres are formed.

4. The most intense fluorescence appears over the fibres and is stronger in older fibres, whether primary or secondary. The lens epithelium is always unlabelled.

5. The limitations of the technique are considered and the results are discussed in the light of other immunological data on normally developing and regenerating lens.

**RÉSUMÉ**

*Une étude par immuno-fluorescence de la régénération du cristallin chez la larve de Xenopus laevis*

1. L’apparition et la localisation des antigènes du cristallin, dans le cristallin en régénérescence de la cornée de la larve de *Xenopus laevis*, ont été étudiées par la technique des anticorps fluorescents.
2. La cornée de l'œil normal ne fixe pas l'antisérum utilisé, mais endéans les 24 hr. après la suppression du cristallin, les antigènes du cristallin ont été détectés dans le cytoplasme des cellules de la couche intérieure de l'épithélium de la cornée, au dessus de l'espace pupillaire.

3. Dans les cellules de la vésicule du cristallin, avant la formation de fibres, le marquage, qui se trouvait sur le cytoplasme, était faible, à l'exception de petites aires de cellules à la limite postérieure de la vésicule, d'où se forment les fibres primaires du cristallin.

4. La fluorescence la plus intense apparaît sur les fibres, et elle est plus forte chez les fibres plus âgées, que celles-ci soit primaires ou secondaires. L'épithélium du cristallin est toujours sans marquage.

5. Les limites d'application de la technique ont été considérées, et les résultats sont discutés en rapport avec d'autres données immunologiques chez le cristallin en développement normal ou en régénération.

ACKNOWLEDGEMENTS

The author wishes to thank Mrs R. M. Clayton for introducing him to the methods of immunology, and also Mr J. Wright and Mr A. Gillies for technical assistance with the experiments.

REFERENCES


An immuno-fluorescent study of lens regeneration


(Manuscript received 12th October 1964)