Immunofluorescence studies on induction and differentiation of the chicken eye lens

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The synthesis of crystallins was studied in chicken embryos of 48 hours up to 8 days of incubation, by means of the indirect fluorescent antibody technique. The first positive reactions were observed in 23 somite embryos (50 to 53 hours of incubation). A few cells in the morphologically most advanced area of the lens placode showed fluorescence. With the deepening of the placode the reaction gradually spread to more exterior parts, concomitant with signs of morphodifferentiation. At 3 to 3.5 days of embryonic life both anterior epithelium and fibers were positive. Intra- and extra-embryonic ectoderm, neural tissues, and optic cup and its derivatives did not show fluorescence in any of the stages studied. On the basis of these and of earlier results current theories regarding lens induction in the chicken embryo are rejected. It is assumed that the onset of crystallin synthesis may be dependent on gene activation in the lens cell nuclei under the influence of retinal factors. Some other theoretical aspects of lens differentiation are discussed.

The onset of crystallin synthesis is often considered as one of the crucial steps in the differentiation of the lens, and has thus extensively been investigated, mainly in chicken embryos. Yet no uniform opinion regarding first appearance, localization, and even character of these proteins has been reached.

Burke and co-workers did not demonstrate lens antigens until the morphologic development of this organ was already well advanced, at 96 hours of incubation. Others, however, found positive reactions as early as 40 to 44 hours, before the lens rudiment can even be distinguished from the surrounding head ectoderm. Most authors hold an intermediate position and report the first specific lens antigens at 50 to 60 hours of embryonic life. Even in this short time span of 10 hours, however, tremendous changes take place in the morphologic characteristics of the lens. It progresses from a slightly thickened lens placode, still part of the surface ectoderm, to a well-defined vesicle which is detached from the superficial layers. And for a better understanding of the processes underlying differentiation, it is essential to know whether lens antigens are produced in the first or only in the later part of this period.

In both embryonic and adult animals other organs than the lens may contain crystallins, although it still remains to be proved that these proteins are actu-
ally synthesized in situ. Indeed, we have reason to believe that these findings may be due to artifacts.4

The described contradictory experimental results have led to a number of contradictory theories on induction, differentiation, and regeneration of the lens, and a reinvestigation of the problem with a view to elimination of some of these seemed indicated.

Most immunologic techniques necessitate the use of extracts derived from many pooled embryos, which automatically leads to the investigation of material from different developmental stages. We selected the fluorescent antibody method,13 which enabled us to demonstrate antigens at the cellular level in individual, exactly staged embryos. Careful control of the specificity of our reagents was thought necessary.

**Experimental**

**Antisera.** Lenses of 8- to 9-month-old chickens were homogenized at + 3°C after addition of 2 ml. of distilled water (brought to pH 7 with 0.001M phosphate) per 1 Gm. of lens. The homogenate was then centrifuged at 20,000 r.p.m. for 15 minutes at + 3°C. The supernatant fluid, containing 8 to 10 per cent protein, was mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). A 2 ml. portion of the emulsion was injected subcutaneously into each of a number of albino rabbits at 14 day intervals for periods of up to several months. Sera were collected repeatedly throughout the course of immunization and stored individually in small aliquots at − 20°C until used. The antisera were tested for their specificity by immunodiffusion and immunoelectrophoresis according to methods previously described4 against lens extract, extracts of other organs (brain, skin, liver, kidney), and serum. All antigen solutions were used in a wide range of dilutions, from 2 to 50 mg of protein per milliliter.

**Animals.** Fertilized White Leghorn eggs were obtained commercially from one single source and incubated at 38°C. Embryos were harvested at various times from 48 hours up to 8 days of incubation. They were washed in buffered saline (0.15M NaCl, buffered at pH 7.1 with 0.01M Na-phosphate), the number of somites was carefully counted under a dissecting microscope, and the embryos were staged according to Hamburger and Hamilton.14 Embryos younger than 3.5 days were fixed in toto; from the older ones only the eyes were taken.

**Preparation of tissues.** To select the best procedure, a large number of eyes of 5 day embryos were processed in different ways. Special attention was given to fixation. Carnoy’s solution and 95 per cent ethanol gave excellent results with a minimum of autofluorescence and aspecific reactions. Acetone and acid fixatives such as formaldehyde–ethanol–acetic acid (10:85:5) or ethanol–acetic acid (95:5) induced very strong autofluorescence. Lillie’s neutral buffered formalin apparently affected some cell components more than others, resulting in a very specific localization of the immunofluorescence. Cryostat sections were not tried, because previous work on the ontogeny of the pituitary (Zwaan, unpublished data) showed it to be very difficult to obtain sections thin enough for observation of cellular details. Furthermore, with this technique, leakage of soluble antigens from the tissues is a distinct possibility, complicating the study of their localization.

The following method was finally adopted: The tissues were fixed for 2 hours in Carnoy’s solution in the cold (+ 3°C), dehydrated, and embedded in paraffin in the usual way. Sections were cut at 3 μ and deparaffinized in 2 changes of xylene (15 minutes) followed by 3 changes of ethanol 95 per cent (15 minutes). Finally, they were rinsed twice for 10 minutes in buffered saline.

**Immunofluorescence.** Originally the direct method was used, but later we preferred the indirect method ("sandwich" technique) because of its far greater sen-
sitivity, assessed by Coons as tenfold. Goat or sheep gamma-globulin solutions, directed against rabbit gamma-globulin and conjugated with fluorescein-isothiocyanate, were bought commercially (Difco, Detroit, Mich., or Roboz, Washington, D.C.). Immunoelectrophoretically they reacted only with the gamma-globulin fraction of rabbit serum. To reduce the possibility of aspecific staining, the solutions were absorbed with mouse tissue powder (Difco). Per milliliter of labelled gamma-globulin, 100 mg. of powder, slightly wetted with saline, was added. The mixture was stirred occasionally for 2 hours, left overnight, and centrifuged at 12,000 r.p.m. for 60 minutes at +3° C. This was repeated with 50 mg. of tissue powder.

Sections were exposed to rabbit lens antiserum for 20 minutes in a moist chamber at +20° C. They were washed twice for 10 minutes with buffered saline and then covered with fluorescent goat or sheep globulin for 20 minutes. After rinsing twice with buffered saline the sections were mounted in a buffered aqueous solution of polyvinyl alcohol (Elvanol, grade 51-05; DuPont Electrochemicals Department, Wilmington, Del.).

Slides were examined under a Reichert Zetopan fluorescence microscope equipped with a high-pressure mercury vapor lamp HBO-200, with primary filters (Schott) UG 1 and BG 12 and barrier filter GG 9. Photographs were made on 35 mm. Tri-X Pan film (Kodak), which was developed with Rodinal (Agfa) 1:75 for 15 minutes at +20° C. for high contrast, or on High-speed Ektachrome film (Kodak).

Controls. The specificity of the observed reactions was controlled at all three levels of the “sandwich.” At the tissue level the reactions of organs other than the lens were observed. Brain, skin, kidney, liver, and heart of the adult chicken were processed as described for the lens. Furthermore, complete horizontal sections were made through head, neck, thorax, and abdomen of embryos, incubated for 2 to 5 days, and studied for possible fluorescent reactions. In the middle layer, the immune serum against lens proteins was replaced with normal rabbit serum, with preimmunization serum, with sera to other antigens, with saline, or with absorbed antisera. The latter was prepared by addition of small amounts of lens protein and incubation for 30 minutes at 37° C. The resulting precipitate was removed by centrifugation and these steps were repeated until no more precipitate was formed. The completeness of the absorption was tested by immunoelectrophoresis, which showed the absence of reacting antibodies and the presence of an antigen excess in the absorbed antisera for each of the 3 crystallin fractions. At the upper level the fluorescence-labeled sheep or goat gamma-globulin was replaced with fluorescent normal serum or with a solution of fluorescent dye in saline.

Results

Immunoelectrophoretic tests showed that all rabbit antisera reacted with chicken lens proteins, while some also gave precipitin lines with the extracts of other organs. For the present work four antisera were selected which did not react with tissues other than the lens, and which with the latter showed precipitin lines to all three main crystallin fractions: alpha-, beta-, and FISC or delta-crystallin (Fig. 1). Minor differences in the pattern, which occur very often and have some significance for studies on the structure of the lens proteins, were regarded as unimportant for the present series of experiments. And indeed, in the immunofluorescence work the staining patterns given by all antisera specific for the crystallins were identical.

The immunofluorescent tests showed no crystallins at the earlier developmental stages up to the 21 somite embryo (48 to 52 hours of incubation). Neither lens primordium nor future retina showed any fluorescence (Fig. 2). A short time later, in the 23 somite embryo (50 to 53 hours) a few cells of the lens placode had started.
Fig. 1. Immunoelectrophoretic demonstration of antibodies to the three main protein fractions of the chicken lens. Alpha-crystallin is located at the anodic (left) side, the beta-crystallins give a very long line, divided in arcs with spur formation, delta-crystallin has an intermediate mobility. Protein concentration of lens extract 12 mg. per milliliter (upper reservoir) and 7 mg. per milliliter (lower reservoir).

Fig. 2. Lens primordium and optic cup of a 21 somite embryo, shortly after the beginning of the contact period. Absence of fluorescence shows that crystallin synthesis has not yet started. (×900.)

to produce crystallins (Fig. 3), a granular fluorescence appearing in their cytoplasm while the cell nuclei were negative. The positive cells were localized in that area of the future lens which first established contact with the outgrowing optic cup and which had invaginated most. The surrounding cells of the lens placode and the optic cup were negative. In subsequent stages, the fluorescent reaction gradually spread to other cells and increased in intensity in the region destined to differentiate first in primary lens fibers.

In Hamburger-Hamilton stage 16 (27 to 28 somites, 52 to 57 hours), the placode has deepened to form a lens pit, but the
cavity still communicates with the exterior through a pore, which is excentrically placed due to differential growth rates. In these embryos the complete inner wall of the pit was positive and the reaction was extending around the equatorial zone to the anterior epithelium (Fig. 4). The least differentiated part, surrounding the lens pore, was still completely negative. Fig. 5 gives an example of a control reaction carried out on an embryo of the same stage as that in Fig. 4. The replacement of the antiserum with serum harvested from the same rabbit before the start of the immunization resulted in the absence of staining reactions. This gives an indication of the specificity of the fluorescence observed in Fig. 4. The reaction was highly specific in its spatial distribution, and fluorescence was never observed in any parts of the embryos other than the lens. Particular attention was given to optic cup and stalk, neural epithelium, and intra- and extra-embryonic ectoderm, but they never showed any staining (Fig. 6). With the closure of the lens pit the anterior epithelium gradually became more involved and in embryos of 3 to 3.5 days (Hamburger-Hamilton stage 20 to 21) the entire lens—anterior epithelium, equatorial zone, and fibers—was synthesizing crystallins (Fig. 7). Iris, cornea, and retina, on the other hand, remained negative. This picture was essentially the same in all later stages investigated.

All control experiments were entirely satisfactory; replacement of any of the specific components of the fluorescent “sandwich” with nonspecific reagents resulted in absence of staining. No reactions were observed outside the lens in either adult or embryonic animals. Replacement of the lens antiserum with normal serum, preimmunization serum, or absorbed serum abolished the fluorescent reaction. Similarly, the goat or sheep antiserum to rabbit gamma-globulin was essential for a positive reaction.
Fig. 4. Fluorescent photomicrograph of the lens pit of a 28 somite embryo. All cells of the posterior wall produce crystallins and the fluorescence is spreading to the anterior part. The surroundings of the lens pore are still negative. (×730.)

Fig. 5. Similar embryo as in Fig. 4, control section. Lens antiserum was replaced with pre-immunization serum. There is no fluorescent reaction. (×730.)
Fig. 6. Similar embryo as in Fig. 4, low power view. The strongly staining lens pit stands out against a dark background: intra- and extra-embryonic ectoderm, optic cup, and neural epithelium are negative. (x230.)

Fig. 7. Lens epithelium and fibers of a 3.5 day embryo show fluorescence, but cornea and iris are negative. (x500.)
Thus it can be concluded that the observed fluorescent staining was a specific result of antigen-antibody interaction and did indeed reflect the presence of crystallins in the cytoplasm of the cells. The highly specific localization of the fluorescence and the logical sequence of developmental changes corroborates this view.

Discussion

The initiation, of crystallin synthesis after formation of the placode, the gradual activation of more and more cells, and the complete absence of fluorescence in organs other than the developing lens are three striking findings. They differ from earlier results with the same system, but agree well with recent immunofluorescent studies of Takata and colleagues on lens formation in the newt, as well as with our immunochemical data.

On the other hand, several authors, making use of immunodiffusion or of the cytotoxic action of lens antisera on living cells, found lens antigens in the lens primordium before any morphodifferentiation, as well as in embryonic and adult tissues apart from the lens.

It may be argued that these discrepancies are due to technical shortcomings of the fluorescent antibody technique. Campbell, studying corneal lens regeneration, explained the absence of staining in most ocular tissues by the decreased sensitivity of immunofluorescence compared with double diffusion techniques. The use of the highly sensitive "sandwich" method and the demonstration of only traces of antigen in single cells at a time, when immunoelectrophoretic and immunodiffusion tests are still negative, make this improbable in our case.

Conversely, the differences may be based on limitations of the other techniques. Immunodiffusion tests can show antigens in organ extracts, but they give no information on their spatial distribution, and we have indications that the occurrence of crystallins in intra-ocular tissues may be due to artifacts such as rapid postmortem diffusion of material from the lens, rather than to synthesis of crystallins by these tissues.

Sometimes the character of precipitin lines is not easy to interpret, and in several cases their formation by aspecific rather than specific lens antigens has not been properly excluded.

Action of antibodies against living cells is difficult to evaluate and the cytotoxicity of lens antisera for lens primordium, ectoderm, and optic cup is not necessarily caused by crystallin-antibody interaction, even when precipitins to the crystallins are present. An example clearly illustrating this for another tissue can be taken from the extensive literature on autoimmune diseases, and concerns the effect of serum from patients with Hashimoto's disease (autoimmune thyroiditis) on cells of thyroglobulin in tissue culture. Originally the lethal effect was thought to be due to precipitating antibodies to thyroglobulin, but further investigations showed these to be harmless, while the cell damage was caused by a second group of antibodies directed against the microsomal fraction of the thyroid and demonstrable only by complement fixation.

Thus the differences between the present results and those of earlier authors can easily be explained by the use of different techniques. Under carefully controlled experimental conditions the fluorescent antibody technique is in our opinion one of the best methods currently available for this type of investigation. It is very sensitive, it is based on direct observation of the cellular localization of the antigens, and it allows the use of single embryos of exactly known developmental horizons.

Crystallins are apparently not produced until palisading of the placode cells is well under way. The type of protein involved in these early reactions cannot be deduced from the present data. Investigations with antisera to purified fractions, currently in progress, should provide an answer to this question. Immunoelectrophoretic analysis suggests that FISC or
delta-crystallin\textsuperscript{1, 6, 16} rather than alpha-crystallin\textsuperscript{8, 10} is involved.

In a logical sequence of events, the fluorescence spreads to other cells of the placode, following signs of cytologic changes. There seems to be a definite temporal relationship between the establishment of proximity between the individual lens cells and the optic cup on one hand, and the onset of crystallin synthesis on the other hand, although there is a lag of a few hours between the two events. This time may be needed for a sufficient activation of the protein synthesizing machinery of the lens cells. Alternatively the cells may have to be exposed for a certain time to retinal factors before transcription of genetic information can take place.

Many theories of lens induction and regeneration have been proposed in the past. The capacity for lens formation has been ascribed to the a priori presence of lens antigens in the competent tissues.\textsuperscript{11, 10, 12, 23} Related theories hold that crystallins are produced throughout the early embryo.\textsuperscript{3, 21} Woerdeman\textsuperscript{8} assumed the presence of a mother substance in the entire ectoderm, which could be changed either into lens proteins or into keratins, depending on the underlying mesoderm and other inductors. Vyasov and Averkina\textsuperscript{22} suggested a gradual concentration of lens antigen production in the eye forming area, based not on a de novo beginning of biosynthesis but on the appearance of conditions favorable for the selection of one of the many possible types of synthesis. Perlmann and De Vincentiis,\textsuperscript{8} finding lens antigens in the microsomal fraction of both anterior and posterior part of the early embryo, thought that microsomal templates might be blocked by their own product. Induction would then come about by solubilization of preformed proteins from the microsomes, possibly combined with activation of the ribosomes to produce more.

Transfer of lens proteins from the optic cup to the lens rudiment, either as initiators or as precursors, has also been described.\textsuperscript{7, 27}

Our results do not fit into the framework of any of the proposed theories; above all, it is clear that the presence of crystallins is not a prerequisite for lens formation, but rather that their appearance follows induction of the lens with a certain time interval.

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Fig. 8. Diagram showing the proposed mechanism of induction of the synthesis of lens crystallins, based on activation of genes in the lens cell nuclei (right side of the figure). This may come about under direct or indirect influence of factors, produced by the retina (left side of figure).
Scores of investigators have shown that the (future) retina plays an essential role in the induction and differentiation of the lens, probably based on the transport of certain factors from the neural part of the eye to the developing lens. This influence may continue for a considerable time during the differentiation and growth of the lens. If the embryonic mouse lens is transplanted into the embryonic chick eye, the crystallins synthesized after the operation are mouse, rather than chicken proteins. This may indicate that the genes of the lens cells rather than those of the retina determine the type of protein synthesized by the lens. Thus the retinal influence, although essential, seems to be permissive and not instructive.

Stable messenger-RNA's have been shown to be involved in crystallin synthesis. During differentiation of the lens there is a gradual increase in the number of different crystallins.

The data described above and our present and earlier immunochemical results lead us to conclude that crystallin synthesis is a highly specific process, and that its induction may be dependent on gene activation at the level of the lens cell nuclei. Retinal factors may be involved in this process, either directly as depressors or indirectly through changes in the cytoplasm of the lens epithelium.

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REFERENCES