Permissive and directive interactions in lens induction

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SUMMARY

The interactive events leading to lens formation and the developmental potentialities of the presumptive lens ectoderm were examined in vitro. The presumptive lens ectoderm of both mouse and chick embryos was capable of forming a lens even when isolated from the optic vesicle before the two tissues reach the stage of close association. This lens-forming bias can be released with favourable culture conditions and by various heterotypic mesenchymes. The same permissive, unspecific conditions or heterotypic tissues failed to trigger lens formation in trunk ectoderm. The directive effect of the optic vesicle was demonstrated in experiments where it was grown in contact with the trunk ectoderm. The latter developed distinct lentoid bodies synthesizing lens proteins. The origin of the lentoid was confirmed in interspecies combination of chick and quail tissues. It is concluded that lens formation is governed by a series of interactive events consisting of both directive and permissive influences.

INTRODUCTION

According to the classic concept, induction of the lens takes place during the interaction between the retinal anlage, i.e. the optic vesicle, and the head ectoderm. Spemann showed this interdependence in Rana fusca by removing the prospective sensory retina in the open medullary plate stage. As a consequence, lenses failed to form and he concluded that the inductive influence of the retinal anlage was essential for lens formation (Spemann, 1901). His discovery was soon followed, however, by reports that similar experiments in other amphibian species gave apparently contradictory results (Lewis, 1904; King, 1905). Most of the experiments were reproduced, and a clear picture has emerged of the steps leading to lens induction in amphibians (for reviews, see Spemann, 1912; Mangold, 1931; Twitty, 1955; Grobstein, 1956; Lopashov & Stroeva, 1961; Coulombre, 1965 and Jacobson, 1966).

1) The prospective retina is not the only lens inductor, but merely the last in a series of tissues that exert this inductive influence in head ectoderm during early embryonic development. (2) Ectoderm from other sites besides the head is capable of forming a lens. Initially, all the cells from the upper hemisphere of the amphibian embryo possess this potentiality. With advancing development the capacity becomes spatially restricted, the extent of the competent tissue

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varying from one species to another, and also depending on the temperature at which the embryos are reared. At lower temperatures, chemical differentiation proceeds more rapidly than morphogenesis (Twitty, 1928). Embryos kept in the cool before experiments will form lenses much more readily than those kept at room temperature throughout development (Ten Cate, 1953).

Several researchers failed to obtain differentiation from the presumptive lens ectoderm in vitro on species where it is possible even in the absence of the retinal anlage in vivo (Perri, 1934; Woerdeman, 1941; de Vincentiis, 1949; Jacobson, 1958). It was suggested that lens differentiation depends on the inductive influence of some other tissue besides the optic vesicle (Mangold, 1931; Liedke, 1951, 1955). Okada & Mikami (1937) substituted several tissues in the place of the optic cup in Triturus pyrrhogaster and were able to induce a lens with the following: nose anlage, ear vesicle, brain, heart, liver, and from younger embryos dorsal archenteron wall, neural plate, ectoderm, mesoderm, and entoderm of the head region. Lens formation may also be elicited by unspecific triggers, such as salamander liver, boiled salamander heart (Holtfreter, 1934) or alcohol-treated liver, a known inductor of anterior central nervous system structures (Toivonen, 1949), even by treatment with acetone or alcohol, as in Fundulus embryos (Werber, cited in Twitty, 1955). During normal development, the first tissue to underlie the amphibian presumptive lens is the entodermal wall of the future pharynx. While gastrulation proceeds the edge of the mesodermal mantle, the future heart, extends to the posterior margin of the lens ectoderm. During the neurula stage the neural folds lift the lens ectoderm from contact with the mesoderm and the future retina evaginates from the wall of the neural tube as the optic vesicles, which approach the ectoderm, making contact with the presumptive lens cells. All three tissues, the pharyngeal entoderm, the heart mesoderm and the optic vesicle, are potent lens inductors (for review, see Jacobson, 1966). The entoderm and mesoderm gradually lose their inductive capacity, but the retina does not. As the lens grows throughout life, it continuously requires the presence of the inductor. All ‘free’ lenses, which differentiate for some time even in the absence of a retina, eventually degenerate.

Although Jacobson (1956) predicts that the process of lens induction is similar in all vertebrates, the only difference being in timing of the response of the target ectoderm and hence in the degree of dependence upon the neural inductor, our knowledge concerning the higher vertebrates is fragmentary.

Optic vesicle-dependent lens formation has been described in experiments both in chick (Waddington & Cohen, 1936; Alexander, 1937; van Deth, 1940; McKeenhan, 1951; Langman, 1956) and in mouse (Muthukkaruppan, 1965). Alexander (1937) and van Deth (1940) also reported that body ectoderm from avian embryos 2 days old or younger responded to the inductive stimulus of the optic vesicle by forming a lens. With advancing age of the host embryos, the capability for lens formation was gradually lost as the cells of the posterior body ectoderm lost their responsiveness early, while those of the head ectoderm
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retained it for some time. McKeehan (1951) reported lens formation in 4-somite embryos, not only in the head region ectoderm but also in the extra-embryonic ectoderm.

The inductive influence of the cephalic endomesoderm of early chick embryos was studied by Mizuno (1970, 1972). He suggested that lens induction is a two-step process, in which the hypoblast with some mesoblast cells first acts upon the undermined epiblast until the streak stage, whereafter lens formation can be experimentally triggered by several other tissues than the optic vesicle, such as embryonic dorsal skin dermis, mesonephros, sclerotome, liver, and gizzard mesenchyme, whereas retina from slightly older embryos was only a weak inductor. Those lenses obtained in vitro were shown with immunohistological methods to be capable of synthesizing lens-specific proteins (Mizuno & Katoh, 1972). This synthesis is detectable even before full differentiation of the lens has taken place, which is in accordance with the development in vivo (Ikeda & Zwaan, 1967).

In the present study, the lens forming capacity of the presumptive lens ectoderm of mouse and chick embryos was studied in vitro. The isolated ectoderm was grown in various culture conditions and combined with heterotopic tissues. Such permissive factors allowed lens differentiation even when the ectoderm was isolated before making contact with the optic vesicle. The inductive effect of the optic vesicle was studied by growing it in combination with the trunk ectoderm of 2-day avian embryos. In the lentoids formed, the synthesis of lens crystallins was demonstrated by immunofluorescent methods. The possibility of cell contamination was excluded by using chick-quail chimaeric combinations.

MATERIALS AND METHODS

Embryos

Eggs of White Leghorn chicks and Japanese quail were obtained from a local poultry farm, and incubated in forced draft incubator at 37.5–38.5 °C. Inbred strains A/Sn and CBA and hybrids A/CBA were used to obtain mouse embryos. Human embryonic material was obtained from early therapeutic abortions (Boije Hospital, Helsinki).

Preparation and culture of the tissues

All tissues were handled aseptically, rinsed and dissected in saline (mouse and human in PBS, chick and quail in Tyrode solution) prepared with disposable needles and grown on Millipore membrane filters (Millipore Corporation, Bedford, Mass.). Both agarose (L’Industrie Biologique Française S.A., Gennevilliers, Seine) and 0.5–2.0 % agar (Difco) made up in basic salt solution proved less favourable. The filter was placed over a hole in stainless-steel screen in a modified Trowell-type culture (Saxén et al. 1968). When necessary, the tissue
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fragments were coated with agar (1%). Mouse and human embryonic tissues were cultured in Eagle's minimal essential medium (MEM) (Gibco, New York, cat. no. H-15), and chicken and quail embryos in BME diploid medium (Gibco, New York, cat. no. G-13). Various other media, including McCoy's 5a medium (modified) (cat no. H-15), Nutrient mixture F-12 (cat. no. H-17), Medium 199 (cat. no. E-12), Waymouth's chemically defined medium (Waymouth, C.: J. natn. Cancer Inst. 22, 1003–1017, 1959) (according to prescription: Orion Pharmaceuticals, Helsinki), brought no obvious improvement in growth, differentiation or survival. Although Armstrong & Elias (1968), culturing rat eye rudiments in Waymouth's medium, observed good differentiation on adding glutamine in concentrations of 0.1–100 mg/l., no such effect was noted in this study, nor did addition of insulin or glucose in various concentrations improve the results.

Addition of protein supplement to the nutrient medium proved vital for differentiation of the lens. We used an extract of eye-free embryos of chick or quail. In the final medium, 20% was embryo extract and 20% was foetal calf serum (Microbiological Associates Inc., Bethesda, Md., U.S.A.) inactivated at 56 °C for 1 h. Plain human amniotic fluid from 6-week embryos allowed good differentiation and so did the basic salt solutions supplemented with 40% of chick or quail egg yolk only. Doubling the amino acid concentration (Eagle's essential and non-essential, Orion Pharmaceuticals, Helsinki) gave healthier cultures, and differentiation of the avian lenses later proved to benefit when inactivated chicken serum was substituted for foetal calf serum (Orion Pharmaceuticals, Helsinki). Streptomycin (50 µg) and Penicillin G (100 i.u./ml) (Hoechst) were added. Cultures were grown in a humified incubator at 37 °C in an atmosphere of 5% CO₂ in air. Cultivations seldom exceeded 6 days.

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Fig. 1. The forebrain and the optic vesicles of an 14-somite chick embryo after their separation from the overlying ectoderm and the head mesenchyme. Short treatment with trypsin-pancreatin followed by mechanical separation.

Fig. 2. Cross-section of the forebrain region of a 22-somite mouse embryo showing optic vesicles in close apposition to the lens placodes.

Fig. 3. Section of a well-developed lens after 5 days’ cultivation of the optic rudiment from a 22-somite mouse embryo. H & E stain.

Fig. 4. Cross-section of the forebrain region of a 16-somite mouse embryo showing the optic vesicles separated from the presumptive lens ectoderm by a narrow inter-space. The ectoderm shows no sign of placode formation.

Fig. 5. Section of an explant of the presumptive lens ectoderm cultured after removal of the optic vesicle. The ectoderm shows a well-shaped lentoid body. Culture time 5 days. H & E stain.

Fig. 6. Section of a lentoid body developed during 5 days’ cultivation of the presumptive lens ectoderm separated from the optic vesicle before the establishment of contact with the ectoderm. Chick embryo, 7 somites. H & E stain.
Isolation of the tissue components

Heads of the embryos were severed and kept separately from the bodies to avoid cellular contamination. The tissues were kept at +4 °C for 45 min in a solution consisting of three parts of trypsin (1:250 Difco) and one part of pancreatin (NF, Difco), freshly prepared. In this solution the tissues were separated from each other by gentle manipulation and placed in serum-containing medium (Fig. 1).

Histological methods

The explants were fixed in Carnoy, embedded in paraffin, sectioned serially at 4 μm, and stained with haematoxylin-eosin. Quail tissues were fixed in Zenker, and Feulgen stain was applied with light green counterstain (see Le Douarin, 1969). For immunofluorescent staining the tissues were fixed in cold Carnoy and embedded in paraplast (Sherwood Medical Industries, Miss.). The paraplast was removed with cold xylene, and the sections were processed through alcohols in the cold and rinsed repeatedly at room temperature with Ca- and Mg-free PBS. The antiserum was kindly provided by Dr Ruth Clayton as rabbit anti-total lens antiserum lyophilizate. Normal rabbit serum in similar dilutions was used as a control. Commercial fluorescein isothiocyanate-conjugated anti-rabbit (swine) serum globulin was used (Sevac, Prague, Czechoslovakia) with traces of amido black as counterstain (Amidoschwarz 10 B, Merck). The indirect method of Weller & Coons was employed (1954), and the slides were incubated at 37 °C for 30 min with antiserum, rinsed and incubated similarly with the conjugate, rinsed and mounted with glycerol-PBS. The slides were examined with a Leitz Orthoplan microscope, and micrographs were taken on Ilford FP-4 Panchromatic 22 din. plates. Fluorograms were examined with a high-pressure mercury vapour lamp (Philips 200 W/4), fitted with incident light equipment of the microscope with inbuilt dichromatic combination of mirror (495 nm) and suppressor filter (K 510) (Fluoreszenz-Auflichtilluminator nach Ploem). Transmission filter 3 mm BG 12 and suppressor filter K 510 were used with additional shortwave pass-interference filter KP 490. Exposure time was 2 min. Co-ordinates of fluorograms were recorded and the slides rephotographed after staining with haematoxylin-eosin.

RESULTS

Establishment of contact between the ectoderm and the optic vesicle

To establish a background for the experimental studies on the various steps of lens determination, the normal development of the interacting optic vesicle and ectoderm was studied in the strains of mouse and chick used in our laboratory. In a 15-somite mouse embryo the optic vesicle is approaching the ectoderm, and a close apposition of these tissues is achieved at the 19-somite stage. At the
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32-somite stage the lens vesicle is already closed and becomes detached from the ectoderm. Similarly, close contact (at the light microscope level) between the optic vesicle and the overlying ectoderm is established in the chick embryo at the 10–11 somite stage, and formation of the lens vesicle is completed around the 27–29 somite stage. Variations in rate of development were noted between individuals and even between the eyes of the same embryo but, though frequent, they were mostly slight and of no importance for the experiments to follow. Our strains did not show any marked differences in the development of the optic apparatus described earlier in mouse (Muthukkaruppan, 1965) or in chick (McKeehan, 1951).

In vitro differentiation of the post-contact lens ectoderm

In the first set of experiments, the differentiative capacities of 'induced' lenses were followed during culture in vitro. For this purpose, lenses with or without their adjacent tissues were dissected from embryos that had passed the stage of contact between the interacting tissues (above). Four types of culture were prepared:

- The optic bud with the overlying ectoderm dissected out and cultured en bloc.
- The presumptive lens ectoderm separated and cultured alone.
- The optic bud isolated and cultured alone.
- The presumptive ectoderm combined with optic and through a Millipore filter (transfilter culture).

With mouse tissues beyond the 19-somite stage (Fig. 2), altogether 62 cultures were successful. Of the whole eye rudiments (optic bud plus ectoderm), 85% developed lenses in our conditions (Fig. 3). The corresponding figure for the transfilter cultures was 79% and for the cultures of isolated ectoderm 80%. None of the isolated optic buds showed any signs of lens differentiation.

The results with the chick tissues were basically similar (Jääskeläinen & Saxén, 1972), except that ectoderm rarely formed a lens if isolated soon after the stage of first contact and only acquired the capacity for independent development around stage 19.

Experiments on presumptive lens ectoderm isolated before it established contact with the optic vesicle

A series of experiments was next performed with donor embryos well before the stage at which contact between the interactants is normally established (Figs. 4 and 5). Stages close to that age were not used in the final experiments, because isolation and separation of the components during these stages proved difficult even after prolonged enzyme treatment, and consequently the risk of cellular contamination increased. The four types of experiments listed in the previous paragraph were again performed on mouse and chick tissue, and the results are listed in Table 1.
Table 1. Results of various types of in vitro experiments with mouse and chick ocular tissue dissected before the optic bud had made contact with the presumptive lens ectoderm

<table>
<thead>
<tr>
<th>Type of explant (N = 30)</th>
<th>Lens differentiation</th>
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<tbody>
<tr>
<td></td>
<td>Mouse</td>
</tr>
<tr>
<td>Optic vesicle + ectoderm (remaining in contact)</td>
<td>25</td>
</tr>
<tr>
<td>Optic vesicle + ectoderm (in transfilter contact)</td>
<td>18</td>
</tr>
<tr>
<td>Isolated lens ectoderm</td>
<td>7</td>
</tr>
<tr>
<td>Isolated optic vesicle</td>
<td>0</td>
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Effect of heterotypic inducers and culture conditions

The demonstration that mouse lens would differentiate in ectoderm that had been isolated well before contact with the optic vesicle was followed by various experiments to confirm and analyse further the process. In transfilter experiments, presumptive lens ectoderm was isolated from mouse embryos at the 12- to 16-somite stage and combined with various heterotypic or heterospecific tissues. Positive results with clearly distinguishable lenses on the ectodermal side of the filter were obtained with the following tissues:

- Head mesenchyme from 11- to 12-day mouse embryos (9/15).
- Metanephric mesenchyme from 11-day mouse embryos (5/10).
- Salivary mesenchyme from 12-day mouse embryos (3/10).
- Pulmonary mesenchyme from 11-day mouse embryos (2/10).
- Optic bud from 15-somite stage chick embryo (7/10).
- Retinal tissue from 5-week human embryo (6/15).

In all these experiments, as well as in the experiments with pre-contact ectoderm, the results were greatly influenced by the culture conditions, especially by the protein concentration of the culture medium. When cultured alone both mouse and chick pre-contact ectoderm developed lenses in about 50% of cases when the sole culture medium used was human amniotic fluid. Similarly, when foetal calf serum was replaced by chick serum, isolated pre-contact stage chick ectoderm developed clearly distinguishable lenses in more than 50% of the explants (Fig. 6).

Experiments with chick trunk ectoderm as target tissues

Preliminary experiments in which the trunk ectoderm of a 4- to 14-somite chick embryo was combined with an optic vesicle at the same stage revealed frequently lens differentiation in the explants. To establish the origin of these lenses and to exclude cellular contamination of either of the tissue components, further experiments were made with reciprocal chick/quail combinations. Here, use was made of the nuclear marker of the quail cells (see Le Douarin, 1969). With these two species 112 successful reciprocal combinations were made between
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Fig. 7. (A) Sections showing the result of the experimental combination of quail trunk ectoderm and chick optic vesicle of 8-somite embryos. (B) The cells of the lentoid body show the quail nuclear marker. Culture period 6 days. H & E stain.

Fig. 8. Demonstration of the synthesis of lens proteins in a combination of trunk ectoderm and optic vesicle of 8-somite chick embryos. (A) U.v. photograph of a section of a lentoid body after treatment with fluorescein-labelled anti-total lens serum. (B) The same section rephotographed after subsequent staining with H & E.

optic bud vesicle and trunk ectoderm. In 67 cultures the explants formed a lentoid body or a typical lens. In the living cultures, this was visible within a day or two as a transparent sphere. Sections usually revealed a roundish, well-delineated body consisting of large, eosinophilic cells frequently showing elongation and parallel orientation (Fig. 7). All the lenses formed consisted predominantly, if not entirely, of cells with the nuclear characteristics of the donor of trunk
ectoderm. In addition, in two cases small lentoid bodies were seen with the nuclear structure of the donor of the optic vesicle, thus suggesting cellular contamination or lens formation from optic vesicle cells.

Every second combination experiment had as a control on the same screen a parallel culture of the isolated trunk ectoderm without additional tissues. None of these showed lens differentiation. The same was true when the ectoderm was combined with the heterotypic tissues listed in the previous paragraph or with the following tissues: spinal cord, notochord, spinal cord plus notochord, somites or trunk mesoderm (all from early chick embryos).

**Immunofluorescence**

To examine the occurrence of lens-specific antigens in lenses induced from heterotypic ectoderm, immunofluorescent localization of lens antigens was made as described in Methods. The antiserum stained normal embryonic chick lenses intensely, while controls treated with normal rabbit serum remained totally unstained. The same technique applied to lentoid bodies developed in the trunk ectoderm revealed a distinct fluorescence strictly localized to the lens (Fig. 8).

**DISCUSSION**

The present results seem to fit well into the general scheme proposed by Jacobson (1966) on the basis of his experiments on amphibian embryos. According to this, lens induction is a multiphasic process, in which the presumptive lens ectoderm is successively exposed to several inductor tissues of which the optic vesicle might be the last (see, however, Philpott & Coulombre, 1968). The present results show that the presumptive lens ectoderm is capable of forming a lens even when deprived of its ultimate inductor, the optic bud, before these interacting tissues reach the stage of close association in both avian and mouse embryos. The observations suggest that this lens-forming bias can be released with various, obviously ‘unspecific’ stimuli such as factors in chick serum, embryo extract and by various heterotypic mesenchymes.

We cannot, however, dismiss the possibility, although slight, that the results are due to cellular contamination. As shown in another system, kidney tubule induction, incomplete separation of the interactants, leaving a few cells contaminating the explant, may lead to erroneous interpretations (Saxén, 1971; Saxén & Karkinen-Jääskeläinen, 1975). In the experiments reported here, this seems a most unlikely explanation for several reasons. In the young, pre-contact stages especially, separation of the optic bud and ectoderm was rapid and easy, and in the acceptable cases no contaminant cells were seen under the microscope. Moreover, this explanation would imply that cells become detached from the optic vesicle and attached to the ectoderm, but at this stage the optic vesicle is already a coherent tissue and not easily damaged by enzyme treatment. Finally, the effect of the culture condition on this ‘spontaneous’ lens formation would
be difficult to explain by contamination. Thus the formation of lenses out of the presumptive ectoderm before the establishment of contact with the optic vesicle seems to represent permissive induction (Saxén, 1977). Accordingly, the target cells have already developed a lens-forming bias to be released by favourable, but unspecific permissive conditions. Here it should be emphasized that the same conditions and the identical heterotypic inductors failed to trigger lens differentiation in trunk ectoderm which evidently had not been through the preliminary lens induction process.

The results of this last series of experiments suggest the existence of another type of induction, recently named 'directive' (Saxén, 1977). Apparently, the cells of the trunk ectoderm remain multipotent with many developmental options (as also shown by previous workers dealing with the differentiation of the skin and the appendages; see Krafočhwil, 1972; Sengel & Dhouailly, 1977). The final lens inductor, the optic vesicle, is able to direct these cells towards lens formation and synthesis of lens-specific proteins without the early steps of normal lens induction. This directive influence cannot be replaced by non-specific, permissive conditions.

Our results and the scheme of multistep induction with permissive and directive influences seem to fit well with previous observations (Introduction). According to this view, Muthukkaruppan (1965) performed his experiments with already conditioned or predetermined ectodermal cells and the resulting lens differentiation was thus a consequence of permissive conditions. Experiments on very young tissues, amphibian gastrula ectoderm or chick epiblast, on the other hand, deal with multipotent cells with several developmental options, and need a directive induction to choose one of these, i.e. the influence of pharyngeal entoderm, heart mesoderm or optic vesicle in the amphibian development to form a lens and hypoblast with some mesoblast cells in the chick embryo. Coming back to the hypothesis by Mizuno, the present results suggest that the optic vesicle is a complete inductor, containing both first and second step factors.

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