An Immunoembryological Study of the Chick Iris

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WITH THREE PLATES

Using immunological techniques it has been shown that in all major vertebrate species the lens, iris and pigment retina of the eye share common antigens (Langman & Prescott, 1959; Maisel & Langman, 1961; Flickinger & Stone, 1960; Maisel, 1962). These data are of particular interest since in certain animals the iris has the ability to form a lens or lens cells in the absence of the original lens. Indeed, lens regeneration from the dorsal iris is well documented in the genus Triturus (Stone, 1952; Reyer, 1954). Although McKeehan (1961) and Woerdeman (1962) did not record lens regeneration in the chick embryo in vivo, other observers have reported on the formation of lens cells from embryonic chick pigment retina and iris explanted in vitro (Dorris, 1938; van Deth, 1940; Reinhold, 1958).

Immunological analysis of the chick iris and pigment retina using anti-lens serum has revealed that these tissues contain proteins with antigenic (surface reactive) groupings similar to lens alpha, beta and gamma crystallins. It has therefore been suggested that under normal conditions these antigens are integrated into the cellular structure of the iris and retina, while in abnormal circumstances, such as removal of the original lens, the same antigens may become the building stones for the formation of lens cells (Langman & Prescott, 1959). However, since antigenic specificity is a function of only part of a molecule, one must be cautious of equating substances, or their biological roles, on the basis of antigenic similarity alone.

In view of the unique biochemical relationship proposed for iris and lens tissue, it was felt necessary to analyze further the nature and number of antigens present in the iris tissue. The following investigation was therefore undertaken in order to determine the electrophoretic, ultracentrifugal and immunological properties of the iris antigens, and their sequence of appearance during embryogenesis. Since these properties have previously been established for the chick

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lens (Maisel & Langman, 1961), it would be possible to compare the structure of immunologically identical macromolecules in iris and lens.

MATERIALS AND METHODS

Antigens

Lenses from adult fowl were carefully dissected free from the capsule and surrounding tissues and homogenized in 0.9 per cent. saline. After centrifugation for 10 min. at 3000 r.p.m. the supernatant was removed and the protein content determined by the biuret method. In each instance test solutions were adjusted to concentrations of 10 mg. protein per ml. Extracts of adult chick iris were prepared in a similar manner.

The outer layer of the optic cup (presumptive pigment retina and iris) was dissected from 200 white Leghorn chick embryos at each stage of 72, 80 and 96 hr. incubation, and iris tissues from batches of 75–120 embryos of 5, 8, 10, 12, 16 and 22 days incubation. The tissue obtained from 72-, 80- and 96-hr. embryos was tested at concentrations of 2-1–3-5 mg. protein/ml., while those of the remaining extracts varied from 5-1–8-9 mg. protein/ml. At least two extracts of each stage prepared from different batches of embryos were used for immunological analyses.

Antibodies

Adult lens extract containing 25 mg. protein/ml. was suspended in Freund’s adjuvant in a ratio of 3:2 and injected subcutaneously in five widely separated sites into four rabbits. The amount injected at each site was 1 ml. and was repeated three times at intervals of 10 days. The serum of the rabbits was obtained 10 days after the last injection. The titres of the antisera, determined after Boyd’s method (1956), by testing against serial dilutions of lens extract (the initial concentration being 10 mg. protein/ml.), varied from 1:8000 to 1:16,000. Antibodies to chick iris were similarly prepared in six rabbits using an emulsion containing 4 ml. iris (10 mg./ml. protein) and 1 ml. adjuvant. Four of the sera had antibody titres ranging from 1:6000 to 1:12,000, while two were less potent, the titres being 1:600 and 1:1000 respectively. Each antilens serum, and the more potent four anti-iris sera were used for immunological tests.

Agar diffusion precipitation technique

The double diffusion technique of Ouchterlony (1953) and immuno-electrophoresis was used. The details of these procedures have been previously described (Maisel, 1962).

Electrophoretic analysis

Comparative analysis of iris and lens extract was done using the combined agar gel-paper electrophoresis technique (Zak et al., 1960). In order to collect
IMMUNOEMBRYOLOGICAL STUDY OF THE CHICK IRIS

separated fractions, iris extract containing 7·5 mg. protein/ml. was run in the Spinco Model C.P. continuous flow electrophoresis apparatus for 36 hr. The analysis was made at 4°C. in Veronal buffer, at pH 8·6 and ionic strength 0·02. The current used was 65 mA. and sample flow adjusted to deliver 3·2 ml. per hr. (At completion of the separation, the curtain was dried at 120°C, washed, and stained with 1 per cent. Bromphenol blue for 30 min.). Appropriate fractions were collected and dialyzed against 0·9 per cent. saline for 24 hr., and then concentrated by ultrafiltration or precipitation by 1/2 saturation with ammonium sulphate (for alpha crystallin).

Ultracentrifuge studies

Isolated iris fractions were tested in the ultracentrifuge. All centrifuge runs were made in a double sector cell, with a rotor speed of 50,740 r.p.m. The rate of movement of the maximal ordinate was determined from measurements on a Gaertner microcomparator, with the measurements being to 0·001 mm. The temperature of the ultracentrifuge was maintained at 20°C. by the RTIC thermistor control system.

RESULTS

Immuno-electrophoretic analysis

The results obtained when iris and lens extracts were tested simultaneously with anti-iris and anti-lens serum (titres 1:12,000 and 1:16,000 respectively) are shown in Plate 1, Fig. 1. Iris extract formed ten precipitin bands when tested with the homologous antiserum (Plate 1, Fig. 1a), while only five were formed when tested with anti-chick lens serum (Plate 1, Fig. 1c). The precipitin reactions of the lens anti-lens system have previously been identified as representing alpha, beta and gamma crystallins and are so labeled in the figure (Maisel, 1962).

Comparing the precipitin reactions of lens and iris tested with anti-iris and anti-lens serum, it can be seen that in each instance precipitin bands were formed which corresponded in position to those of lens alpha, beta and gamma crystallin. Applying the technique of Wadsworth & Hanson (1960) to the iris–anti–lens system, it has been shown that those iris and lens antigens which are electrophoretically similar in position are also identical immunologically (Maisel, 1962). However, three precipitin bands of the iris–anti-iris system did not correspond immunologically to any of the lens fractions. In order to test for the nature of these components iris antiserum was tested with normal chick serum (Plate 1, Fig. 1d). One precipitin band formed corresponding in position to iris antigen (S), suggesting that the corresponding antigen is probably a serum protein present in the iris extract.

In order to test for the presence of tissue specific antigens, iris antiserum was first absorbed with 5 mg. chick serum and then 10 mg. lyophilized chick lens.
This absorbed antiserum tested with iris extract formed two faint bands with iris but did not react with lens or serum (Plate 1, Fig. 1e, f). It was thus concluded that these antigens are specific for iris tissues only.

The findings reported above represent the most consistent observations in 131 tests using four different iris extracts and six lens extracts. Each anti-lens serum detected alpha, beta and gamma crystalline antigens in the iris, similarly the four anti-iris sera cross-reacted with the corresponding lens proteins. The serum specific antigen was observed with each anti-iris serum (both unabsorbed and absorbed), two of the sera each with titres 1:12,000, detected both iris specific antigens, while only the more negatively charged component was formed by the remaining sera (titres 1:8000 and 1:6000).

**Electrophoretic and ultracentrifugal analysis**

Analysis of iris and lens by electrophoresis in agar is recorded in Plate 2, Fig. 3. It is evident that iris tissue is deficient in that component corresponding to lens beta crystalline. Similar patterns were recorded by continuous flow electrophoresis. Iris fractions corresponding to zones A, B, and C were collected and tested for immunological identity using anti-lens and anti-iris sera. Zone C was found to contain mainly alpha crystallin antigen with a small amount of gamma crystallin. The protein was precipitated by half-saturation with ammonium sulphate and then dissolved in a minimum amount of distilled water. Some protein remained insoluble and was removed by centrifugation. The supernatant after dialysis against normal saline was found to contain only alpha crystallin antigen (Plate 2, Fig. 3b). Ultracentrifugal analysis revealed a single peak with a sedimentation coefficient of \( S_{20} = 15.1 \) (Plate 1, Fig. 2a).

Immunological analysis of Zone B showed that it contained a mixture of beta and gamma crystallin antigens in low concentration. One hundred ml. of fluid collected from two analyses of iris tissue was concentrated by ultrafiltration to a volume of 2.5 ml. Ultracentrifugal analysis showed the presence of two peaks with sedimentation coefficients of \( S_{20} = 5.5 \) and 3.5 respectively (Plate 1, Fig. 2b). These components were separated in the ultracentrifuge using a separation cell designed by Yphantis & Waugh (1956). The fraction with sedimentation coefficient \( S_{20} = 3.5 \) was found to be identical to lens gamma crystallin using anti-lens serum (Plate 1, Fig. 2c). Immuno-electrophoretic analysis with anti-iris serum showed the presence of the more positively charged tissue specific antigen in addition to the gamma fraction. By serially diluting at first the antigen and in a second experiment the iris antiserum, it was concluded that the tissue specific fraction was present in small amounts so that the peak as seen in the ultracentrifuge was considered to represent mainly gamma crystallin. Ultracentrifugal fraction of \( S_{20} = 5.5 \) was found to consist only of beta crystallin, when tested with anti-iris and anti-lens sera (Plate 1, Fig. 2d).
EXPLANATION OF PLATES

PLATE 1

Fig. 1. Immuno-electrophoretic analysis of iris (I), chick lens (L), and chick serum (CS) tested with anti-lens and anti-iris serum. All antigens were tested at concentrations of 10 mg. protein/ml. The precipitin reactions of the lens anti-lens system correspond to alpha, beta and gamma crystallins and are so labeled. Iris extract tested with anti-lens and anti-iris serum formed precipitin bands which correspond with those of the lens proteins. One band, formed by chick serum tested with anti-iris serum, corresponds to reaction S of the iris-anti-iris system. Anti-iris serum absorbed with lens and serum formed two bands with iris extract. The more positively charged component would correspond to the band indicated by the arrow in c, while the other is located close to the antigen well.

Fig. 2. Ultracentrifugal analysis of isolated iris fractions. (a) Iris alpha crystallin antigen (6·1 mg. protein/ml.) (S_{20}) = 15.1. (b) Iris beta (β) and gamma (γ) crystallin antigens (10 mg. protein/ml.), gamma crystallin (S_{20}) = 3.5, beta crystallin (S_{20}) = 5.5. (c) Iris gamma crystallin antigen (5·1 mg. protein/ml.) (S_{20}) = 3.5.
**PLATE 2**

**FIG. 3.** Phrogram, (a) Total lens extract (10 mg. protein/ml.). (b) Total iris extract (7 mg. protein/ml.).

**FIG. 4.** Immuno-electrophoretic analysis of total lens extract and isolated iris fractions using anti-lens serum. (a) Total lens extract (10 mg. protein/ml.). (b) Iris alpha crystallin antigen (6·1 mg. protein/ml.). (c) Iris gamma crystallin antigen (5·1 mg. protein/ml.). (d) Iris beta crystallin antigen (3 mg. protein/ml.).
Fig. 5. Immuno-electrophoretic analysis of embryonic extracts using anti-iris serum. (a) 72-hr. pigment retina and iris extract (2.3 mg. protein/ml.), showing the presence of the alpha crystallin antigen. (b) 8-day iris extract (7 mg. protein/ml.). The serum specific antigen is present, as well as the beta and gamma crystallin antigens. (c) 10-day iris extract (8.6 mg. protein/ml.). The arrow points to the iris specific antigen. (d) 14-day iris extract (8.9 protein/ml.). The second iris specific antigen is indicated by the arrow. S = antigen with serum specificity.
Appearance of lens antigens in the iris during development

The data recorded below represent the findings in 140 immunological analyses of iris tissue. Extracts of presumptive pigment retina and iris dissected from the optic cup of 72- and 80-hr. embryos was found to contain alpha crystallin antigen when tested with anti-lens sera. Alpha and gamma antigens were consistently recorded in extracts of 7-day chick iris, while beta crystallin antigen was first detected in 8-day chick iris. Using anti-iris sera one band was formed by 72-hr. pigment retina and iris and six bands by 8-day iris (Plate 3, Fig. 5a, b). Of the latter reactions one band corresponded to the antigen with serum specificity, one to alpha crystallin, one to beta crystallin and the remainder to gamma crystallin. Extracts of 10-day iris formed eight bands (Plate 3, Fig. 5c). At this stage the first tissue specific iris antigen was detected, while the second one was apparent in 14-day iris extracts (Plate 3, Fig. 5, 5d). The latter observations were confirmed in eleven tests using two absorbed anti-iris sera and two different extracts of 10-day and 14-day iris, each containing 8.6–9.1 mg. protein/ml.

DISCUSSION

Analysis of the antigenic components of the chick iris reveals the presence of three groups of proteins. The first group consists of antigens which are tissue specific for the iris when compared with lens and serum, the second consists of antigens which are shared by iris and lens, while the third has the specificity of a serum component. Studies on the appearance of these antigens during development showed that the lens alpha crystallin antigen was the first to appear, being detected in 72-hr. iris extracts. Antigens corresponding to lens beta and gamma crystallin were apparent in 8-day extracts, while the first iris specific antigen was recorded in 10-day extracts. At this time differentiation of iris musculature is in progress (Lewis, 1903) and may represent the site of synthesis of the latter iris antigen.

The time of appearance of lens antigen during the development of the iris differs somewhat from the pattern in which they are detected during lens formation. In previous investigations (Maisel & Langman, 1961) it was found that alpha crystallin is the first protein to arise during lens development, at the time of invagination of the lens placode (60-hr. embryos), while beta crystallin appears at the onset of active fiber formation (72-hr. chick embryos). In the iris, however, beta crystallin antigen was not detected until the 8th day of development. This does not necessarily imply that its synthesis in the iris commences at a considerably later stage of development than in the lens. Indeed, beta crystallin is present in very low concentration in the iris of the adult chick relative to the other lens antigens, suggesting a low level of synthesis of this protein. Under such circumstances the antigen may be formed early, but can only be detected at a later stage of development when an amount sufficient for immunological identification has accumulated in the cells.
The immunological data concerning the nature of the lens antigens in adult chick iris show that they are identical immunologically and electrophoretically to alpha, beta and gamma crystallins. Furthermore, from a comparison of their sedimentation coefficients, it may be concluded that the molecular size of iris alpha and gamma crystallin antigen closely approximates that of the corresponding lens proteins. Thus the values of the above iris components are $S_{20}=15.1$ and $3.5$ respectively, while those of the lens are $S_{20}=17$ and $4$. With regard to the beta crystallin antigen, however, it appears that the iris protein ($S_{20}=5.5$) is a significantly different molecule in size and/or shape from the corresponding lens fraction ($S_{20}=9$).

Although one must necessarily be cautious in interpreting these data, the iris cells appear unique in that they contain the necessary enzyme systems for the formation of proteins which so closely resemble those of the lens. Indeed the presence of a relatively high concentration of alpha crystallin antigen in this tissue is of particular interest. This protein, the first to appear during lens formation, is also the least species specific component of the chick lens. Furthermore, when 32-hr. chick embryos were treated with antisera, degeneration and abnormal growth of the optic vesicle and lens was found only in those exposed to anti-alpha crystallin serum, but not in embryos treated with anti-beta or anti-gamma crystallin serum (Langman et al., 1962). It has therefore been suggested that alpha crystallin plays an essential role in the early differentiation of the lens, and it would be reasonable to suggest that the presence of this component in the iris would facilitate the initiation of lens formation should environmental conditions be appropriate.

In order to gain a better understanding of the biological role of tissue macromolecules, Nace et al. (1960) proposed that they be divided into three types: identical, requiring that molecules differ only with regard to their source; analogous, requiring that the normal biological functions of the molecules being compared have gross similarity; and homologous. Molecules of the last type, independent of their origin or normal function, would have in common at least one structural configuration which is capable of acting as a site for some specific biological activity. Such molecules may differ in size and other physicochemical properties but possess at least one functionally identical reactive group. Applying these criteria, lens antigens in the iris might be considered to be homologous to the lens proteins. (To prove identities of these proteins would require a detailed analysis of their primary, secondary and tertiary structures.) Under normal conditions no functional similarity of these molecules would be observed since they exist in different milieus. However, under abnormal conditions the iris components would express their activity in the formation of a lens.

While this treatment could readily apply to the alpha and gamma crystallins in the iris, it presents greater difficulty with regard to the beta fraction. Beta crystallin, which forms approximately 75 per cent of the total soluble proteins in the chick lens, is a complex and highly species specific molecule, suggesting
that it has become greatly specialised in structure and function (Maisel & Langman, 1961). Under such rigid circumstances it would not be surprising to expect that the biological role of this protein be determined both by its surface reactive groups, as well as by the remainder of the macromolecule. Indeed, the data of Dorris (1938), Van Deth (1940) and Reinhold (1958) show that well differentiated lens fibers were rarely found in the lentoid bodies regenerated from chick iris or pigment retina, suggesting inadequate synthesis of beta crystallin. This may well be related to the considerable difference in sedimentation constant (and thus molecular size and/or shape) of the lens beta crystallin, and the corresponding molecule present in the iris. Such a difference may make the iris molecule an inadequate substitute in the synthesis of the highly complex beta crystallin.

One of the ways to further the elucidation of this problem would be to examine the nature of the antigens found in lens cells regenerated in vitro from iris and pigment retina.

SUMMARY

1. Immunochemical analysis of the chick iris reveals three kinds of antigens: one group consists of proteins specific for the iris when compared to lens and serum, the second group of proteins are identical immunologically and electrophoretically to chick lens alpha, beta and gamma crystallins, while the third consists of a protein with serum specificity. On the basis of their sedimentation coefficients it was concluded that iris alpha and gamma crystallins closely resemble the corresponding lens proteins in molecular size. However, iris beta crystallin antigen appears to be a considerably different molecule in size and/or shape from the lens protein.

2. Analysis of the pattern of appearance of the iris antigens during development showed that the lens alpha crystallin antigen was the first to arise, being detected in the presumptive retina of 72-hr. chick embryos. Lens beta and gamma crystallins were apparent in iris extracts of 8-day embryos. One tissue specific iris antigen was detected in 10-day extracts, at a time when the iris musculature is established, while the other was noted in 14-day extracts.

3. The significance of these data is discussed in relation to the problem of lens regeneration from the embryonic chick iris.

RÉSUMÉ

Étude immunoembryologique de l’iris du poulet.

1. L’analyse immunochimique de l’iris du poulet révèle trois sortes d’antigènes: un premier groupe consiste en protéines spécifiques de l’iris quand on le compare au cristallin et au sérum, les protéines du second groupe sont identiques, par leurs propriétés immunologiques et électrophorétiques, aux cristallines alpha,
bêta et gamma du cristallin de poulet, tandis que le troisième groupe consiste en une protéine de spécificité sérique. D’après leurs coefficients de sédimentation, on a conclu que les alpha- et gamma-cristallines de l’iris ressemblent étroitement aux protéines cristalliniennes correspondantes, par leurs dimensions moléculaires. Néanmoins, l’antigène bêta-cristalline le l’iris se révèle être une molécule très différente de la protéine cristallinienne par ses dimensions et/ou sa configuration.

2. L’analyse des caractéristiques de l’apparition des antigènes de l’iris au cours du développement a montré que l’antigène alpha-cristalline du cristallin est le premier à se différencier, étant décelé dans la rétine présomptive d’embryons de 72 h. Les bêta- et gamma-cristallines du cristallin sont présentes dans les extraits d’iris d’embryons de 8 jours. Un antigène tissulaire spécifique de l’iris a été décelé dans les extraits de 10 jours, à un moment où la musculature de l’iris est formée, alors que l’autre n’a été noté que dans les extraits de 14 jours.

3. La signification de ces résultats est discutée dans leurs rapports avec le problème de la régénération du cristallin à partir de l’iris embryonnaire, chez le poulet.

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