An immunofluorescent study of cornea development in the chick

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SUMMARY

The developmental pattern of appearance of a structural corneal antigenic determinant was studied by immunofluorescent methods. It was first detected in the primary corneal stroma at stage 23, and thereafter spread throughout the primary and secondary stroma. This antigen was absent from Bowman's membrane at its initial stage of development, suggesting that this membrane is a new structure in corneal morphogenesis, and not simply a modification of the primary stroma. This study also suggests that the antigenic determinant is a carbohydrate and is secreted by the corneal epithelial cells.

INTRODUCTION

The development of the cornea is of interest with respect to several biological problems such as the basis of transparency, the epithelial secretion of connective tissue, and the phenomenon of epithelial–mesenchymal interaction. Considerable attention has been paid to the organization of the corneal stroma, and to its biochemical structure (Anseth, 1961; Coulombre, 1965; Hay & Revel, 1969; Trelstad, 1970). However, one of the specific problems of corneal morphogenesis that has not yet been solved concerns the formation of Bowman’s membrane. The antigenic properties of corneal proteins are also of interest in view of the clinical importance of heterologous corneal transplantation in man.

In the present study the developmental pattern of appearance of a chick corneal antigenic determinant has been analysed by immunofluorescent methods. The results obtained provide information on the development of the cornea with particular reference to Bowman’s membrane.

MATERIALS AND METHODS

Animals

Fertilized White Leghorn eggs were obtained from a single commercial source and incubated at 38 °C. Embryos were harvested at various times from 3.5 days up to 19 days of incubation and staged according to Hamburger &

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Hamilton (1951). Chicks at 5 and 6 days after hatching were also used in the study.

Preparation of tissue antigens

Corneas were excised from adult White Leghorn chickens and homogenized in phosphate buffered saline pH 7-2. The homogenate was centrifuged at 20000 at 4 °C for 20 min. The supernatant was used for immunological tests and for antibody production in rabbits. Protein concentration of the supernatant was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Soluble protein extracts were similarly obtained from adult chick liver, heart, skin, and kidney.

Preparation of antibodies

Three ml of corneal soluble protein (9.5 mg protein/ml) was emulsified in 2 ml Freund’s adjuvant and injected subcutaneously at 10-day intervals into rabbits in multiple sites. After the 3rd series of injections the rabbits was bled and the immune sera collected. Aliquots of the antiserum were also absorbed with lyophilized preparations of chick serum, soluble liver protein, soluble skin protein and soluble chick corneal stromal protein. The stromal protein was prepared by scraping off the epithelium and endothelium from the adult cornea and homogenizing the remaining stroma. The supernatant after centrifugation was lyophilized.

Immunologic methods

(a) Agar gel

Immunologic reactions were performed in agar using the double diffusion technique. Immunoelectrophoresis in agar was performed at pH 8.2 using 0.4 M sodium barbital-sodium acetate buffer. Amido black was used for protein stain, and periodic acid and Schiff reagent for carbohydrate (Uriel & Grabar, 1956).

(b) Immunofluorescent methods

To determine the best conditions for immunofluorescence, a large number of eyes from stage-35 embryos were processed in different ways. Fixation with 95 % ethanol produced the best results. Carnoy’s solution and Lillie’s neutral buffered formalin destroyed the antigenity of the tissue. The following method was finally adopted. The tissues were fixed for 12 h in 95 % ethanol in the cold (+ 3 °C), dehydrated, and embedded in paraffin in the usual way. Sections were cut at 3 or 5 μm and deparaffinized in two changes of xylene followed by three changes of 95 % ethanol. Finally, they were rinsed twice for 20 min in phosphate buffered saline pH 7.1. The indirect method of immunofluorescence was used.

Goat IgG fraction containing antibodies against rabbit gamma-globulin and conjugated with fluorescein-isothiocyanate was obtained from Miles Laboratories, Kankakee, Illinois.
Fig. 1. Immunoelectrophoretic analysis of tissue extracts. A, Cornea (10 mg protein/ml); B, liver (13 mg protein/ml); C, skin (5 mg protein/ml); D, heart (15 mg protein/ml); E, serum (23 mg protein/ml); F, serum (23 mg protein/ml); G, cornea (10 mg protein/ml); H, cornea (10 mg protein/ml). \textit{UCA}, unabsorbed chick cornea antiserum; \textit{CSA}, rabbit antiserum against chick serum protein; \textit{ACA}, chick cornea antiserum absorbed with chick serum protein and soluble liver protein.

A–E and F–H represent two separate experiments. After absorption, the cornea antiserum formed one precipitin band with cornea G and no reaction with chick serum F. The cornea pattern with two separate unabsorbed antiserum is shown in H for comparison. Note that one antiserum (I) formed fewer bands with the cornea. The arrows indicate the serum albumin reaction.

Sections were exposed to rabbit anti-cornea serum (unabsorbed or absorbed) for 20 min in a moist chamber at +20 °C. They were washed twice for 10 min with buffered saline pH 7.1 and then covered with fluorescent goat-gamma globulin for 20 min. After rinsing twice with buffered saline the sections were mounted in phosphate buffered aqueous solution of polyvinyl alcohol pH 7.1 (Elvanol, grade 51-05; Du Pont Electrochemicals Department, Wilmington, Delaware). Slides were examined under Reichert Zetopan-Binolux fluorescence microscope. Photographs were made with 35 mm Tri-X Pan film (Kodak).
Controls

The specificity of the immunofluorescent reactions was determined with the following experiments. The reaction of the cornea antiserum with other organs was studied. In separate experiments normal rabbit serum preimmunization serum, serum to lens protein or 0.9% saline was substituted for the cornea antiserum. The fluorochrome-labeled goat gamma-globulin was replaced with fluorochrome-labeled normal serum or with a solution of fluorescent dye in saline.

RESULTS

(a) Immunoelectrophoretic analysis

The precipitin pattern obtained when adult chick corneal extract was tested against corneal antiserum is shown in Fig. 1. Cross-reactions were obtained with cornea, and a wide variety of other tissues including liver, kidney, heart, skin, and serum. At least six precipitin areas were formed by the cornea. After absorption with chick serum and liver the rabbit antiserum formed only one precipitin band against cornea.

(b) Immunofluorescent analysis

The results reported below were all obtained with the cornea antiserum absorbed with chick serum protein and soluble liver protein.

Immunofluorescence which was first noted in stage-23 embryos (4 days of incubation) in the primary corneal stroma (Figs. 1A, B, 5A, B) faded out at the corneoscleral junction. No immunofluorescence was observed in other tissues of the eye. At stage 26 (4½–5 days) the reaction was more intense throughout the primary stroma, and extended for a short distance beyond the corneoscleral junction (Figs. 2C, D, 5C, D).
At the end of stage 27 (5½ days of incubation) presumptive fibroblasts originating in the area overlying the vascular mesenchyme of the limbus begin to invade

Figure 2

Immunofluorescent analysis of chick embryonic cornea, using cornea antiserum absorbed with chick serum and liver.

(A, B) Stage 23 (4 days). Note the intense immunofluorescence in the primary stroma. A × 80; B × 320.

(C, D) Stage 26 (4½–5 days). Immunofluorescence is present throughout the primary stroma and fades distal to the cornea–scleral junction. C, × 80; D, × 320.

(E, F) Stage 28 (5½ days). The wavy nature of immunofluorescence is noted in the stroma, and epithelial perinuclear immunofluorescence is seen. E, × 80; F, 320.

(G, H) Stage 35 (8–9 days). The immunofluorescence remains intense in the subepithelial stroma and fades just distal to the cornea–scleral junction. G, × 80; H, × 80.
Corneal development in chick
the primary corneal stroma reaching its centre toward the end of stage 28 (Hay & Revel, 1969). These cells penetrate the middle and posterior layers of the primary stroma and begin producing the adult or secondary stroma. Endothelial cells appear at the corneal periphery at stage 22 (4 days of incubation) and at stage 28 (5½–6 days of incubation) the endothelial lining of the cornea is complete.

At stage 28 there is an intense wavy pattern of immunofluorescence in the stroma and some evidence of perinuclear fluorescence in the epithelial cells. The endothelium showed no fluorescence (Fig. 2E, F).

At stage 35 (8–9 days) immunofluorescence was distributed throughout the stroma, and found in the epithelial cells in a perinuclear position. Immunofluorescence was most intense in the primary acellular stroma (subepithelial), and faded distal to the corneoscleral junction (Figs. 2G, H, 3A, 5E).

A distinct feature of corneal morphogenesis is the appearance of Bowman’s membrane (anterior limiting lamina) at day 13 (stage 39). Immunofluorescent analysis of stage 36 to hatching showed no change from that observed at stage 35 except for the region of Bowman’s membrane. Perinuclear epithelial and stromal fluorescence in the 17-day-old embryo (stage 43) is shown in Fig. 3B. No reaction was noted in Bowman’s membrane. There is evidence of spotty immunofluorescence in the endothelium in Fig. 3B but this was not seen in later stages.

In chickens 6 days after hatching immunofluorescence was noted in the epithelial perinuclear position and throughout the stroma including Bowman’s membrane but was absent from the endothelium (Figs. 3C, 4C).

The appearance of immunofluorescence in Bowman’s membrane was studied

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**Figure 3**

*Bm*, Bowman’s membrane; *Ep*, epithelium; *P*, perichondrium.

Immunofluorescent analysis of chick embryonic cornea, using cornea antiserum absorbed with chick serum and liver.

(A) Stage 35 (8–9 days). The intense subepithelial immunofluorescence is seen. There is wavy fluorescence in the remainder of the stroma. × 320.

(B) Stage 43 (17 days). Immunofluorescence is seen in the stroma and epithelial cells. Bowman’s membrane is negative. × 320.

(C) Six days after hatching. There is immunofluorescence throughout the stroma, in Bowman’s membrane and in the basal epithelial cells. × 320.

(D) Stage 41 (15 days). No fluorescence is seen in Bowman’s membrane. × 640.

(E) Six days after hatching. Fluorescence is less intense in Bowman’s membrane in the peripheral cornea. × 640.

(F) Six days after hatching. Note the fluorescence in the region of Bowman’s membrane in the central cornea. × 640.

(G) Stage 35 (8–9 days). Note fluorescence in the peribronchial mesoderm. × 160.

(H) Stage 35 (8–9 days). Note fluorescence in the wall of the aorta. × 160.

(I) Stage 35 (8–9 days). Cross-section through the thigh. Note the fluorescence in the subepidermal mesoderm and the perichondrium. × 400.

(J) Stage 35 (8–9 days). Mesonephros. Note the fluorescence in the basement membrane of the tubules. × 640.
Fig. 4. Immunofluorescent analysis of chick embryonic cornea, using cornea antiserum absorbed with chick serum and liver. (A) 19-day embryonic cornea (× 640); (B) 6-day post-hatching cornea (× 640); (C) 6-day post-hatching cornea (× 640); (D–F) represent skin, renal tubule and liver from a chicken 6 days after hatching (D, × 320; E, 640; F, 320). Bowman's membrane with some immunofluorescence is seen in the 19-day embryo. Note the perinuclear fluorescence. Intense staining in Bowman's membrane and the perinuclear region of the epithelial cells is seen in Fig. 4B. Note the absence of fluorescence in the endothelial cells in C. Immunofluorescence is noted in the connective tissue of the skin, renal tubule basement membrane, and portal tract. Fluorescence is absent from tubule epithelial cells, hepatocytes, epidermis, and muscle fibers. Bm, Bowman's membrane; End, endothelium; Ep, epithelium.

In greater detail. No reaction was seen in Bowman's membrane at stage 41 (15 days), while some immunofluorescence was present at stage 45 (19 days) (Figs. 3D and 4A, respectively). However, at 6 days after hatching immunofluorescence was clearly noted in Bowman's membrane (Figs. 3F and 4B), most marked in the central cornea and reduced in intensity toward the periphery (compare Fig. 3E, F). The intensity of epithelial perinuclear fluorescence is clearly seen in Fig. 4B. The distribution of immunofluorescence also suggests localization at epithelial cell membranes.
Immunofluorescence in other tissues

Immunofluorescence was found in the stroma of a variety of tissues including the basement membrane of renal tubules, dermis, perichondrium, the walls of blood vessels, and in the portal tracts of the liver. It was not present within cells such as renal tubule cells, epidermal cells, hepatocytes, or lens fiber cells (Figs. 3G–J, 4D–F).

Control experiments

No corneal immunofluorescence was noted when the antiserum was replaced with saline, chick lens antiserum, or normal rabbit serum. Furthermore, when the antiserum was additionally absorbed with corneal stromal protein or skin protein all fluorescence was eliminated from the cornea.

DISCUSSION

At least six antigens were detected immunoelectrophoretically by an antiserum produced against chick corneal soluble protein extract. Cross-reacting antigens were found in serum, skin, heart, liver, and kidney. After absorption of the antiserum with serum and soluble liver protein, only one precipitin reaction was noted with cornea. The shape of the precipitin arc extending for some distance toward the anode and its positive reaction with PAS suggests that the antigen is a carbohydrate present on a number of carrier molecules. This seems more likely than the alternate suggestion that there is one protein with a variable number of small, charged non-protein groups attached.

The antigen may correspond to the bovine keratoglycosaminoglycan reported by Robert & Robert (1968) and Phillipsen & Broekhuyse (1972). The latter authors used immunofluorescence to localize the glycoprotein in the cornea, iridal vascular walls and other connective tissues of the eye. The stromal lamellae of the cornea were strongly fluorescent, the epithelium showed only faint fluorescence while Bowman’s membrane was negative.

In this study the antigen was not restricted to the cornea, but was detected in the stroma of a wide variety of tissues. It can thus be considered as a stromal specific antigen.

Development analysis indicates that the antigen is present first in the primary stroma starting with stage 23 of development. Although not very distinct, there is already evidence of perinuclear localization in the epithelial cells at stage 28. With further development, the antigen is found throughout the stroma, both primary and secondary, but never in fibroblast cells, nor in endothelial cells. Furthermore, the antigen appeared most concentrated in the immediate subepithelial region until day 14 of development. At this stage a non-fluorescent region became apparent subepithelially, coinciding in position with Bowman’s membrane, which was first identified histologically by Meyer & O’Rahilly (1959).
on day 13 of development (stage 39). Immunofluorescence was first seen in Bowman's membrane at day 19 (stage 45), and increased in intensity thereafter.

It was concluded that the immunofluorescence noted in the corneal epithelial cells represents the same antigen as that found in the stroma. This conclusion is based on the observation that absorption of the antiserum with chick serum and liver followed by chick stromal protein, extinguished immunofluorescence throughout the cornea. If different antigens were involved then stromal absorption should not have eliminated the epithelial fluorescence. Furthermore, it has been shown that the chick corneal epithelium secretes sulphated mucopolysaccharides as well as collagen (Meier & Hay, 1973).

After absorption of the antiserum with serum and liver no immunological reaction was detected with these tissues by agar diffusion or immunoelectrophoresis. However, the absorbed antiserum still formed an immunofluorescent reaction with liver stroma. Since the stroma forms only a small portion of the liver mass, the soluble stromal protein would comprise a minor fraction of the total soluble liver protein. After absorption of the antiserum with serum and liver, this minor stromal component could easily escape immunological detection in gel, but might be detected by the more sensitive immunofluorescent method. On the other hand, absorption with serum and skin (containing a high proportion of stromal protein) eliminated all immunological reactions, whether in agar gel or by immunofluorescence.

The origin of Bowman's membrane remains a point of contention. Pouliquen, Faure, Bisson & Offret (1966) believed that Bowman's membrane derived from the acellular ectodermal stroma, while Redslob (1935) and Meyer & O'Rahilly (1959) claimed that the acellular stroma disappeared completely between 9 and 10 days of incubation and that Bowman's membrane arose independently on about the 13th day of incubation. Using the electron microscope, Hay & Revel (1969) found that the primary stroma decreases in width from stage 28 to stage 35–36, and does not thicken again until stage 40. From stage 28 through stage 40, however, it has essentially the same fine structure of orthogonally arranged layers of collagen fibrils. When the postepithelial layer thickens at day 14, the
number of orthogonal layers doubles, and the old layers seem to be displaced inward where they become thicker. These authors could not determine whether any of the original collagen fibrils persist in the postepithelial layer of the mature cornea, and concluded that Bowman’s membrane is laid down on the residue of the primary stroma and that the corneal epithelium is involved in the secretion of both structures. Trelstad (1970) also found evidence that the epithelium is involved in the production of the primary stroma and Bowman’s membrane.

The present study may shed some light on the formation of Bowman’s membrane. The initial immunofluorescent reaction was observed in the primary corneal stroma as a dense subepithelial reaction. However, the first appearance of Bowman’s membrane was characterized by an absence of immunofluorescence. Starting with day 19 (stage 45) immunofluorescence appeared in Bowman’s membrane and then became more intense, suggesting that Bowman’s membrane at its initial formation lacks the antigen present in the primary stroma. Only later in development does this antigen appear in the membrane, as a result of secretion by the epithelial cells.

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REFERENCES


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