Exptl Eye Res. (1966) **5**, 151–155

Immunohistological Demonstration of Adult Lens Antigens in the Embryonic Chick Lens (II)

WILLEM J. VAN DOORENMAALEN

Department of Medical Anatomy and Embryology, State University, Utrecht, The Netherlands

(*Received 3 March 1966*)

γ-Globulins against total-chick-lens-homogenate were conjugated with fluorescein isothiocyanate and chromatographically fractioned on DEAE cellulose. The specific fractions obtained, were applied to histological sections of acetone-fixed and -dehydrated embryos. The earliest stage at which lens antigens could be demonstrated in the retinal wall of the lens vesicle, was just before fibre formation at an age of 66–68 hr (about 32 somites). The γ-globulins used carried a high content of antibodies against the so-called First Important Soluble Component (FISC). This suggests that FISC is the first antigen to appear. It is assumed that the antigens are not present during lens induction and do not take part in this process. They apparently do so in the differentiation of the lens under the influence of the retina.

1. Introduction

By the application of several immunochemical methods it was found that adult lens antigens appear at early stages of lens development, before differentiation has taken place (Ten Cate and van Doorenmaalen, 1950; Flickinger, Levi and Smith, 1955; Beloff, 1959; Langman, 1959; Konyukov and Lishtvan, 1959; Maisel, 1961; Rabaey, 1962; Zwaan, 1963; and Van Dam, Schalekamp, Schalekamp-Kuyken and Ten Cate, 1963). The antigens were demonstrated in the supernatant of homogenates of lens anlagen by the precipitin reaction or by immunoelectrophoresis.

After culturing eye primordia on media containing lens antisera, or after injections of antisera in vivo, pathological changes occurred in the tissues of the developing eye and in some other tissues. The alterations were assumed to be evoked by the binding of antibodies to lens antigens, present as early as at the stage of lens induction (Langman, 1956, 1962; Clarke and Fowler, 1960).

The site of the adult lens antigens in the cells of the developing lens was demonstrated histologically, both with the aid of autoradiography (Clayton and Feldman, 1955 [mouse]) and by immunohistology (van Doorenmaalen, 1958; Clarke and Fowler, 1960; Flickinger, 1955 [chick]; and Takata, Albright and Yamada, 1964 [salamander]). For a detailed review see van Doorenmaalen (1966) and Ten Cate (1966).

Applying the immunohistology to early stage of the embryonic chick lens, the above mentioned authors observed a non-specific staining. This staining can be interpreted as the binding of conjugated antilens-globulins to cell- and tissue-constituents other than those of the lens and may be due to:

(a) non-immunological binding of the conjugated γ-globulins to tissue proteins as a result of physico-chemical forces and caused by the excessive conjugation of fluorescent dye molecules to one γ-globulin molecule;

(b) an immunological reaction of antibodies against antigens present not only in the lens but also in other organs and which, therefore, are not specific for the lens. By injection of total-chick-lens-homogenates into rabbits several antibodies are produced, among which are those against common antigens, such as enzymes.

In the present study concerning the adult lens antigens at the youngest developmental stages of lens development any non-immunological, non-specific staining has been eliminated.

2. Methods

Antisera were produced by injecting total-chick-lens-extract combined with Freund's adjuvant. γ-Globulins were prepared by precipitation with ammonium sulphate at pH 7·4, followed by careful dialysis. The protein solution was brought to pH 9 by adding twice the volume of carbonate buffer (0·5 M). The γ-globulins were conjugated to fluorescein-isothiocyanate (Baltimore, Biol. Lab.) by adding a solution of the dye in dry acetone to a γ-globulin solution in a ratio of 3·5 g dye (in 3 ml acetone) to 10 ml of a 1% protein solution. The dye was added gradually, in small portions, in the course of 1 hr, at 4°C. The mixture was stirred during the addition of the dye and for 2–8 hr after conjugation. Then dialysis against 0·005 M phosphate buffer was performed for several days till no free fluorescein was seen outside the dialysis tube (McDevitt, Peters, Pollard, Haster and Coons, 1963; Wood, Thompson and Goldstein, 1965). DEAE-cellulose was prepared for column chromatography according to McDevitt et al. (1963) and the conjugated protein solution was applied onto it. Three fractions were obtained by stepwise elution at the following buffer solutions: fraction (A) at 0·05 M phosphate buffer; fraction (B) at 0·1 M phosphate buffer pH 6·3 and finally fraction (C) at 1 M NaCl, pH 7·3. About 20 different samples of antisera from 3 rabbits were conjugated and fractionated as described above. Several samples of normal sera were treated simultaneously and identically.

The fractions were dialysed against bi-distilled water to which was added ammonium acetate (10 ml of a 0·1 M solution to 1 litre), and lyophilized. Powders were dissolved again in the elution buffer to 1% and used for immunohistology. Fraction (A) and in most cases fraction (B) was specific.

Sections were prepared from embryos fixed in acetone (during a period the length of which varied with the size of the embryo). An embryo, 70 hr old, was fixed in a cold (4°) mixture of acetone and bi-distilled water (1 : 1) for 45 min. Dehydration was done by transferring the specimen subsequently to acetone with bi-distilled water in a ratio of 3 : 1 (45 min), then to pure acetone (45 min) and to acetone, dried with $CaCl_2$ (1 hr). Via methylbenzoate it was embedded in paraffin. Sections were stretched and glued with the aid of a gelatin solution (1%) and dried during about 10 hr at 37°.

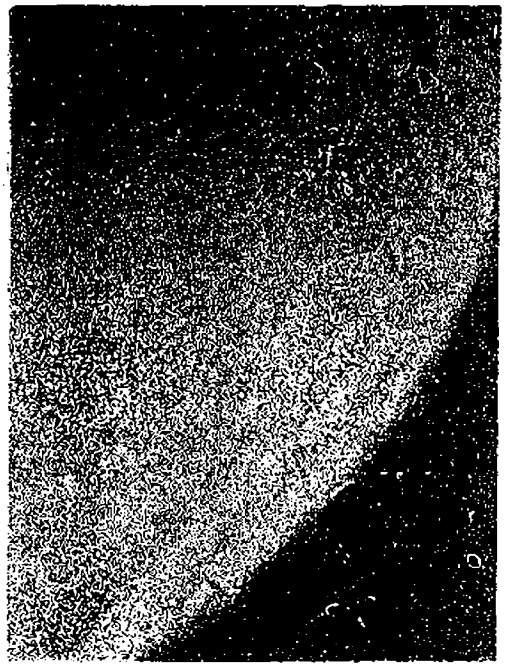
In later experiments the fixation was improved by adding acetic acid to the acetone mixture used for the fixation (0·2 ml glacial acid to 10 ml of the mixtures of acetone and bi-distilled water).

Specific, chromatographic fractions of conjugated γ-globulins were applied onto the sections at 18° in a moist chamber for 15–20 min. Then the sections were carefully washed with buffered saline (7·4) or with elution buffer.

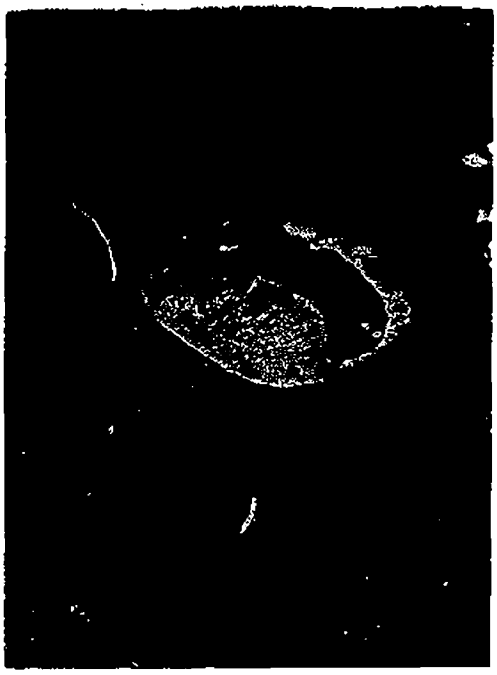
Control experiments were conducted with conjugated, normal γ-globulins, prepared as described above. Staining of either the lens or any other embryonic tissue was never brought about by fraction (A) and hardly ever by fraction (B).

The *blocking* test for specific staining was performed according to Nairn (1962). The sections were treated at first with unlabelled antibody and after incubation washed carefully. Then the labelled antiserum was put onto the sections. Failure of the lens tissue to be stained was sufficient evidence of the immunological nature of the binding of the conjugated antisera.

(a)



(b)



(c)

(d)

PLATE 1. Sections of acetone-fixed chick-eyes of 15 days at the marginal zone (a) and lens fibres (b), of 3½ days (c) and 68 hr (d) treated with fluorescent anti-lens serum.

3. Results

Immunoelectrophoretic characteristics of the antisera used are presented in Fig. 1(a) showing a pattern of 7–8 lines, each line representing at least one separate component. One line is the so-called First Important Soluble Component (FISC) as designated by Rabaey (1962), who detected this protein as being the first component appearing in lens development. This component is found in birds and reptiles. Its concentration in homogenates of the chick lens is high (Hoenders, 1965) and the same was found to hold for the concentration of antibodies as was seen in immunoelectrophoresis and by diluting the antisera.

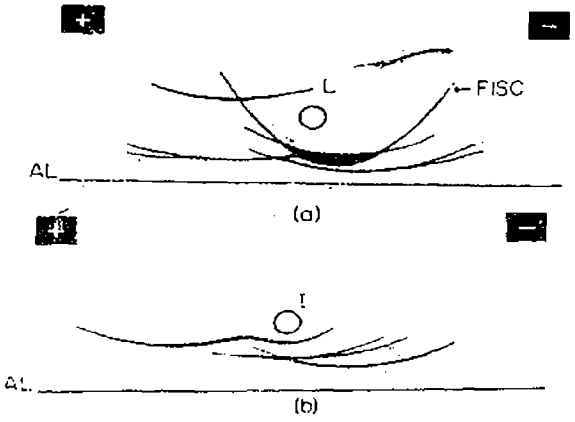


FIG. 1. (a) Immunoelectrophoresis pattern of anti-adult-lens antigens serum (AL) against adult lens extract (L).
(b) Immunoelectrophoresis pattern of anti-adult-lens antigens serum against adult iris extract (I).

However, the antisera, used in this investigation do react also with iris extracts as is seen in Fig. 1(b) and therefore they are not specific for the lens. Yet in the immunohistological technique, these antisera did not react with iris or with other tissues of the embryo. It is still uncertain why these antibodies fail to combine with iris antigens in sections. Possibly other tissues substances covered or masked the iris antigens as a result of the fixation and dehydration, preventing the iris antigens to combine with the antibodies.

In earlier work (van Doorenmaalen, 1958) it was shown that the lens is most intensively stained by fluorescent antibodies in the cells of the marginal zone (Plate 1(a)), the part of the lens where the fibres originate and where antibodies only combine with protoplasm of the cells. Newly formed fibres are brightly stained but as they become older and reach the lens centre their staining reaction decreases. Furthermore, the fibres show an intense, massive staining at the retinal ends while at the superficial ends the staining is cloudy (Plate 1(b)). The lens epithelium never stains (Plate 1(c)). Before fibre formation, however, only the retinal layer of the lens vesicle is greenly fluorescent (Plate 1(d)).

Still earlier, at 68 hr (30–32 somites), the staining decreases abruptly to zero. This stage appears to be the earliest at which lens antigens can be shown during lens development in the present experiments. At this stage the lens anlage consists of an epithelial vesicle separated from the ectoderm. No histological difference is seen as yet between the superficial and deep layer of the lens, apart from a green fluorescence, seen in the immunohistological procedure. At stages earlier than 68 hr of incubation no fluorescence could be observed in any tissue of the embryo.

4. Discussion

Failure of the immunohistological reaction method to reveal the presence of adult lens antigens at stages younger than 68 hr may not be explained only by the absence of lens antigens, since the antigens may be present but have become immunologically inactive on account of fixation and dehydration (van Doorenmaalen, 1957). Furthermore, a low concentration of these antigens at early stages may be responsible for the absence of antibody precipitation. However, in that case one should expect a gradual decrease of the fluorescent staining.

The earliest stage at which the immunohistological method allowed the detection of lens antigens differs by 10 hr of incubation from that stage (58 hr) at which Ten Cate and van Doorenmaalen (1950) could demonstrate them with the precipitin reaction. However, for the preparation of a homogenate of lens anlagen it is very difficult to secure specimens of exactly the same age, as the age can be determined only be means of a high power microscope. The authors may have included some older lenses.

The results of the present investigation also contradict those of Langman, Maisel and Squires (1956) and of Clarke and Fowler (1960), obtained in organ cultures. The action of antibodies in tissue culture, however, is not clarified, and its specificity not yet accepted. Moreover, Flickinger et al. (1955), Ten Cate, Dandrieu and Gortzak (1960) and Vyazow (1962) could not confirm the results of both Langman and Clarke.

Since the concentration of FISC in lens homogenates is very high, just as with the titre of antibodies in our sera, one may presume that the fluorescence seen in the lens anlage is due to the presence of the FISC. This view is supported by the experiments of Rabaey with immunoelectrophoresis, where a FISC-line could be demonstrated at 70 hr of incubation. If the idea is accepted that FISC and the other immunoelectrophoretical components are absent at stages younger than 68 hr, it implies that these components are absent during the induction of the lens anlage. Hence they are of no significance in that process. However, since they appear at the onset of lens fibre differentiation, they apparently do play a role at that stage of lens development. Moreover, as they are first found in the deep layer of the lens vesicle, and later occur at high concentration in that part of the growing lens which is adjacent to the retina, it may be the manifestation of the well-known influence of the retina on lens differentiation. Clear evidence of the retinal influence was obtained in the chick embryo by the investigations of Coulombre and Coulombre (1963) and also of Genis-Galvez (1965). These authors extirpated the presumptive retina, after the lens vesicle had been formed, and found that no lens fibres developed in the deep layer of the lens vesicle. These and similar experiments in other animal species convincingly showed the important role of the retina in lens fibre differentiation.

ACKNOWLEDGMENTS

The author is grateful to Miss C. M. J. Stuifsand and Mr. G. B. Meeuwissen for their capable assistance.

REFERENCES

Beloff, R. H. (1959). *J. Exp. Zool.* **140**, 493.
Clarke, W. M. and Fowler, I. (1960). *Develop. Biol.* **2**, 155.
Clayton, R. M. (1954). *Nature* **174**, 1059.
Clayton, R. M. and Feldman, M. (1955). *Experientia* **11**, 29.

Coulombre, J. and Coulombre, A. (1963). *Science* **142**, 1489.
Flickinger, R. A., Levi, E. and Smith, A. E. (1955). *Physiol. Zool.* **28**, 79.
Genis-Galvez, J. M. (1965). *Compt. Rend. Assoc. Anat.* **125**, 642.
Hoenders, H. J. (1965). Thesis, University of Amsterdam.
Konyukov, B. V. and Lishtvan, L. L. (1959). *Zh. Obsch. Biol.* **20**, 299.
Langman, J., Schalekamp, M. A. D. H., Kuyken, M. P. A. and Veen, R. (1956). *Acta Morphol. Neerl. Scand.* **1**, 142.
Langman, J. (1959). *J. Embryol. Exp. Morphol.* **7**, 264.
Langman, J., Maisel, H. and Squires, J. (1962). *J. Embryol. Exp. Morphol.* **10**, 178.
McDevitt, H. O., Peters, J. H., Pollard, L. W., Harter, J. G. and Coons, A. H. (1963). *J. Immunol.* **90**, 634.
Maisel, H. (1961). *Anat. Record* **139**, 25.
Nace, G. W. and Clarke, W. M. (1958). *A Symposium on the Chemical Basis of Development*, ed. by W. D. McElroy and B. Glass, p. 546. Johns Hopkins Press, Baltimore.
Nairn, R. C. (1962). *Fluorescent Protein Tracing*. E. and S. Livingstone Ltd, London.
Rabaey, M. (1962). *Exptl Eye Res.* **1**, 310.
Takata, C., Albright, J. F. and Yamada, T. (1964). *Exptl Cell Res.* **34**, 207.
Ten Cate, G. and Van Doorenmaalen, W. J. (1950). *Proc. Koninkl. Ned. Akad. Wetenschal.* **53**
Ten Cate, G., Dandrieu, M. and Gortzak, E. (1960). *Vth Intern. Embryol. Conf., Pallanza.*
Ten Cate, G. (1966). *New Methods in Embryology*. Hermann, Paris, in press.
Van Dam, A. F., Schalekamp, M. A. D. H., Schalekamp-Kuyken, M. P. A. and Ten Cate, G. (1963). *VIth Intern. Embryol. Conf., Helsinki.*
van Doorenmaalen, W. J. (1957). Thesis, University of Amsterdam.
van Doorenmaalen, W. J. (1958). *Acta Morphol. Neerl. Scand.* **2**, 1.
van Doorenmaalen, W. J. (1966). *New methods in Embryology*. Hermann, Paris, in press.
Vyazow, O. E. (1962). *Immunology of Embryogenesis*. Medgiz, Moscow.
Wood, B. T., Thompson, S. H. and Goldstein, G. (1965). *J. Immunol.* **95**, 225.
Zwaan, J. (1963). Thesis, University of Amsterdam.