Kawasaki Med. J. Vol. 2: No. 4, 165-174, 1976

STUDIES ON THE EFFECTS OF LENS ANTISERUM IN THE CHICK EMBRYO

Juzo SAWANO* and Akira IKEDA**

* *Department of Anatomy, Hiroshima University, School of Medicine, 1-2-3 Kasumicho, Hiroshima, Japan*

** *Department of Anatomy, Kawasaki Medical School, 557 Matsushima, Kurashiki, Okayama, Japan*

Accepted for Publication on Jun. 23, 1976

Abstract

This work was undertaken to examine the penetration of the immunoglobulins of antiserum to chick lens crystallins into chicken embryos, by the immunofluorescence method. The effects of lens antiserum on the embryos were also studied.

White Leghorn embryos were harvested at various times for 24 hours up to 55 hours of incubation. To examine the process of penetration of the immunoglobulin into the embryo 0.1 ml antiserum was deposited over the embryos through a small hole in the shell. After an additional incubation period of 1 or 12 hours the embryos were fixed with Carnoys and examined by the fluorescence obtained with fluorescent goat gamma-globulin directed against rabbit gamma-globulin (Difco).

After observation of a sample from a 55 hour embryo, the Elvanol was removed and the section was treated with antiserum to crystallins, followed by treatment with fluorecent goat anti-rabbit globulin, in order to obtain the normal pattern of crystallin distribution for comparison.

The anti-lens crystallins immunoglobulins deposited on the embryos could not penetrate into the lens cells where the lens crystallins were synthesized, but merely entered the intercellular sapces.

Furthermore, the gamma-globulin itself was not likely to be the cause of the cytotoxicity observed with the antisera because the lens crystallins antibody could not enter the cells to a precipitin reaction against the lens crystallins in these cells.

INTRODUCTION

There were numerous reports on cytotoxic action of tissues antibodies on developing embryos.[1~6,8~10,14~17,20]

Several authors did not doubt that tissue specific antibodies were

valuable experimental tools in the study of normal and abnormal development in chick and amphibian embryos.

The tissues specific antibodies when applied direcdtly to the embryo in proper dose and stage could induce the specific alteration just as surgical procedures could result in predictable malformation.[4]

By the immunofluorescence technique employing anti- lens serum, Clarke et al. found a shift in the intensity of fluorescence from the optic cup in early stages to the lens spithelium after the lens induction. They suggested that the presence of a lens-like antigen in the optic vesicle prior to lens induction and the antibodies to crystallins were responsible for the epecific malformations.[5,6,9,10]

In our data, the initiation of crystallin synthesis after formation of the placode, the gradual activation of more cells, and complete absence of fluorescence in organs other than the developing lens are striking findings. They differ from earlier results by the same system, but agree well with the immunofluorescent studies on lens formation in the newt, as well as with our immunochemical data.[11,12,23,24]

The questions have been raised why the effect of antibodies was limited in the early developmental stages where the lens crystallin was not yet synthesized in the eye.

To solve the questions we tried to follow the penetration of the immunoglobulins of antiserum to chick lens crystallins into chicken embryos by the immunofluorescence method.

MATERIALS AND METHODS

Antisera: Lenses of 8 to 9 month-old chicken were homogenized at 3°C. after the addition of 2 ml. of distilled water (brought to pH 7 with 0.001 M phosphate) per 1 gr. of lens. The homogeneate was then centrifuged at 20,000 r.p.m. for 15 minutes at 3°C. The supernatant fiuid, containing 8 to 9 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 7 day intervals for the periods up to several months. Sera were collected repeatedly throughout the course of immunization and used immediately for the experimental work.

The antisera were tested for their specificity by immunodiffusion and immunoelectrophoresis against lens extract, extracts of other organ (brain, skin, liver, kidney) and serum.

Animals and preparation of tiseues: Fertilized White Leghorn eggs

were incubated at 38°C. Embryos were harvested at various time during the 24 to 55 hours (23 somite embryo) of incubation. To examine the penetration of the immunoglobulin of antiserum to chick lens crystallins into chicken embryos, 0.1 ml. antiserum was deposited over the embryos through a small hole in the shell. After the additional 1 or 12 hour incubation period, the embryos were fixed for 2 hours with Carnoys which was directly deposited from shell hole at 3°C, and the embryos were removed from the eggs.

The tissues were dehydrated at 3°C. After being embedded in paraffin, they were sectioned at 3 μ. The sections were deparaffinized, rinsed twice for 10 minutes with buffered saline, and covered with fluorescent goat globulin against rabbit gamma-globulin (Difco) for 20 minutes in a moist chamber at 20°C. After another rinse with saline, the section were mounted in a buffered aqueous solution of polyvinyl alcohol (Elvanol, grade 51-05; duPont Electrochemicals Department, Wilington, Delaware). After observation of a sample from 55 hour embryo, the Elvanol was removed and the sections have been bone by the indirect method according to the previous published papers on the lens crystallins which were synthesized in the lens placode[11,12], in order to obtain the normal pattern of crystallin distribution for comparison.

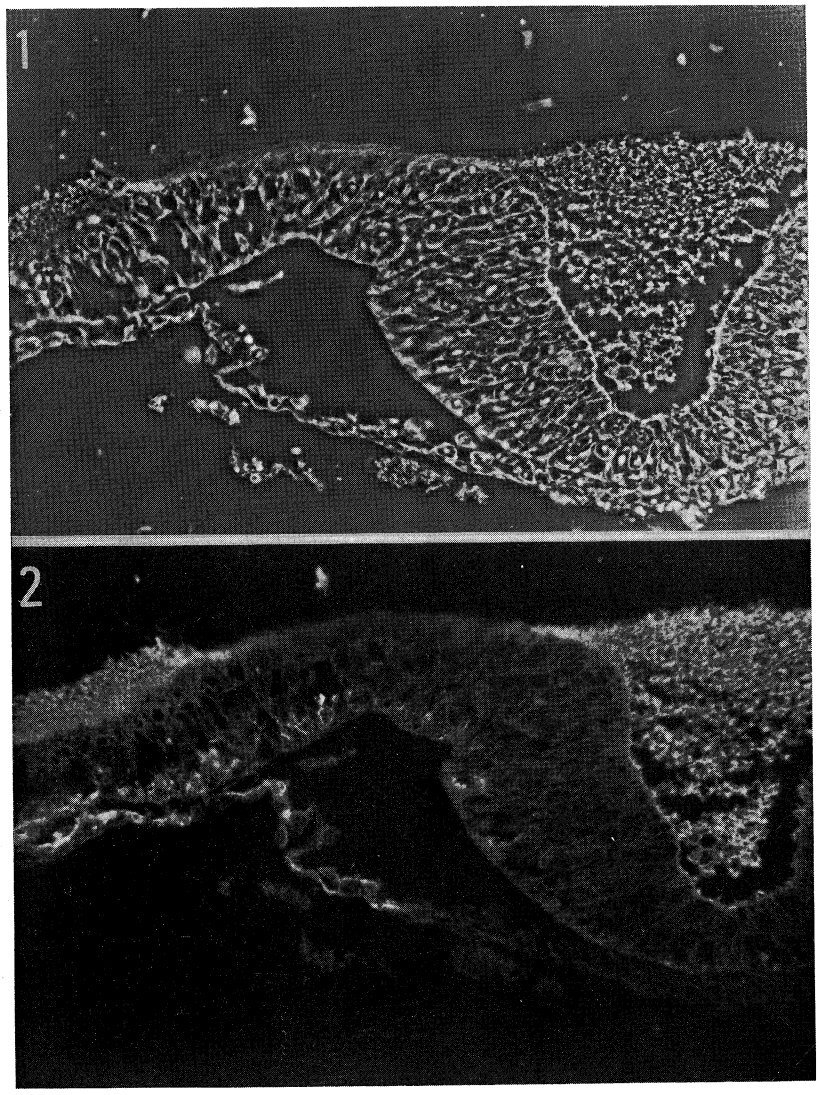
Slides were examined under a Reichert Zetopan fluorescence microscpe equipped with a high-pressure mercury vapor lamp HBO-200, with primary filters (Schott) UG 1 and BG 12 and barrier filter GG 9. Photographs were taken on 35 mm Tri-X Pan film (Kodak), which was developed with Rodinal (Agfa) 1: 75 for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

RESULTS

For the present work the antisera were selected which did not react to tissues other than the lens and which showed precipitin lines to three main crystallins: alpha-, beta- and delta-crystallins.

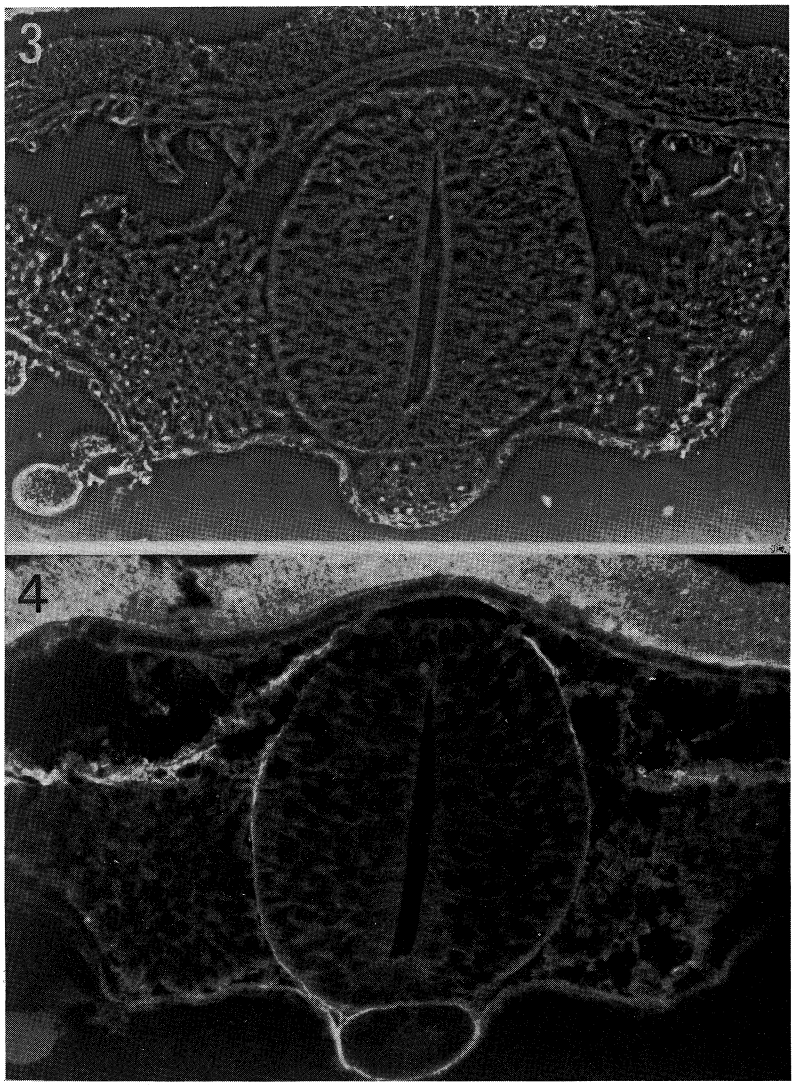
Immunofluorescent tests of the immunoglobulin showed no fluorescence at 12 hours additional incubation period in all stages, but it was possible to show the immunoglobulin which was deposited to the embryos after an additional 1 hour incubation period.

In the one somite embryo (24 hours), the neural plate and neural groove stages, the basal cells of neural fold and neural crest, the splanchnic layer of mesoderm and the entoderm were damaged by the antiserum and the fluorescence of immunoglobulin has been found in that areas. (Fig. 1, 2)



Figs. 1~2. Immunofluorescence and phasecontrast microscope pictures of the transverse section of the 24 hrs. embryo. The basal cells of the neural fold were damaged by the antiserum and the fluorescence of immunoglobulin was found in that area. (×400)

In the ten somite embryo (36 hours), the ectodermal cells were damaged and the positive reaction was mainly localized in the ectoderm. In addition, the fluorescence has been found on the membranous structure of the external limiting membrane of spinal cord and notochord. (Fig. 3, 4)



Figs. 3~4. Immunofluorescence and phasecontrast microscope pictures of 36 hrs. embryo (10 somites). Transverse section at the third somite of the embryo. The ectodermal cells were extremely damaged and the fluorescence was mainly localized in that area. In addition, the fluorescence was found to be the membranous structure of the external limiting membranes of spinal cord and notochord. (×400)

In the twenty-three somite embryo (55 hours) in which a few cells of the lens placode had started to produce delta-crystallin, the histo-

logical structure was not so damaged by the antiserum and the external limiting membrane of the lens placode was fluorescent-positive. The cells of the lens placode and the optic cup were fluorescent-negative. (Fig. 5) In the same sections which have been sumbitted to the indirect method after the Elvanol was removed, a few cells in the morphologically most advanced areas of the lens placode showed fluorescence. (Fig. 6)

Thus it can be concluded that the deposited immunoglobulin against the lens crystallins could not penetrate into the cells where the lens crystallins were synthesized, and it only passed through the intercellular space of the cells.

In addition, the immunoglobulin against lens crystallins was not the component of the cytotoxic factor in any developmental stage, because the lens crystallins antibody could not enter the cells to a precipitin reaction against the lens crystallins in these cells.

DISCUSSION

Several authors did not doubt that the antibody to lens crystallins applied directly to the embryo or the cells of tissue culture in proper does and stage could induce specific alterations and were responsible for the induction of the specific malformation. They made two mistakes in drawing their conclusions on the basis of assumptions as follows:

1) The eye vesicle may contain lens antigens prior to lens induction which transport from the retina to lens rudiment.

2) Antibody molecules in the anti-lens crystallin serum can possibly penetrate into lens cells and conjugate with intracellular lens crystallins. This specific immunological reaction may be responsible for the induction of the specific malformation.

On the first problem, we have a contradictory data reported in the previous papers.[11,12,23,24] In our data, the first positive reaction was 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 the immunofluorescent reaction with anti-chicken lens crystallins serum. At the 3 to 3.5 days of embryonic life both anterior epithelim and fibers were fluorescent 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, the earlier results of current theories regarding induction in the chicken embryos may be rejected.

On the study of the development of anterior structures in the chick

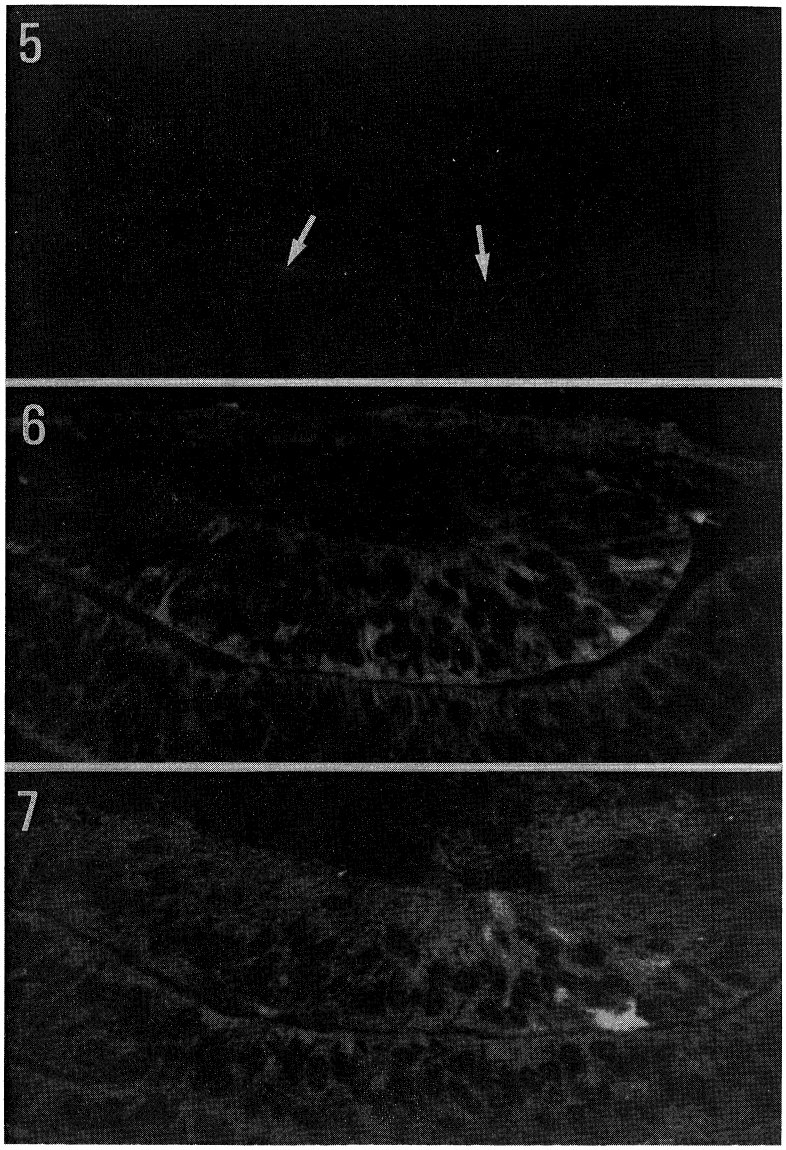


Fig. 5. Immunofluorescence picture of 55 hrs. embryo (23 somites). The section of the lens placode. The histological structure was so damaged by the antiserum and the external limiting membrane of lens placode was only fluorescent-positive. (arrow) (×400)

Fig. 6. The same section which has been prepared by the indirect method after the Elvanol was removed, in order to obtain the normal pattern of crystallin distribntuion for comparison. A few cells in the morphologically most advanced areas of the lens placode showed fluorescence. (×400)

Fig. 7. The section of the same stage embryo was done by the indirect immunofluorescence reaction with total lens crystallins antiserum. The immunofluorescence reaction was found fundamentally in the same area with Fig. 6. (×500)

after direct applications of antiserum against adult lens by Fowler et al.[9], 75 % of the embryos manifested the morphological defects which were correlated with the age of the embryos when treated. At the 10 somite stage, the embryos showed the gross abnormality in forebrain and the extreme reduction or complete absence of eye cup in which a lens-like antigen localized. At the present study, we also found the fluorescence in the basal cells of neural fold and neural crest, and also in the splanchionic layer of mesoderm and entoderm which were damaged by the antiserum.

However, this fluorescence did not seem to be due to the conjugation of antibody with the lens crystallins localization but to the passing immunoglobulin which was deposited to the embryo, because the immunofluorescence test of the immunoglobulin showed no fluorescence at the 12th hour of additional incubation period.

On the second problem, we were unable to confirm the specific cytotoxic action of the lens antisera on the chicken embryo. In our experience, the lens crystallin antibody used in the present experiment could not show precipitin reaction with lens crystallins which were synthesized inside the lens placode cells. If it was possible to make precipitin reaction, the fluorescence pictures of the immunoglobulin and the lens crystallins in the same section must be the same. (Fig. 5, 6, 7)

What is the factor to induce the abnormalities in the lens antisera? Mun reported the toxic effect of normal sera and homologus antisera on the chick embryo. His results suggested the interaction of complement and properdin or a properdin-like factor in the action of the antiserum on the chick embryos.[19] In our experience (unpublished), some normal sera were as active as the specific lens antisera. In both cases the toxicity could be virtually abolished by the heating of the fresh sera at 56°C for 30 min. This heat-lability indicated that the teratogenic factor could not reside in the antibody fraction, but consisted of anthor serum substance, possibly complement.

Dische showed that the glycoproteins and the lipoproteins of bovine lens were probably derived from the membranes, and an antigenic similarity between lens capsule and some another basement membranes was shown.[7]

Fluorescent antisera to total lens homogenates tended to be localized in connective tissues, basement membranes and in other membranous structures.[21] In our result, we also found the fluorescence at the membranous structures of the external limiting membranes of the spinal cord, notochord and lens placode. But these reactions were just temporal and complely disappeared at 12 hours additional incubation period. On the basis of our results, we had no clear-cut evidences on the membrane problem.

We wish to express our gratitude to Dr. J. Zwaan.

REFERENCES

1. Barrow, M. V. & Taylor, W. J.: The production of congenital defects in rats using antisera. J. exp. Zool. 176: 41-60, 1971
2. Braverman, M., Cohen, C., & Katoh, A.: Cytotoxity of lens antisera to disasociated chick neural retina cells in tissue culture. J. Embryol. exp. Morph. 21: 391-406, 1969
3. Brent, R. L.: The production of congenital malformations using tissue antisera. II. The spectrum and incidence of malformations following the administration of kindy antiserum to pregnant rats. Am. J. Anat. 115: 525-542, 1964
4. Breut, R. L.: Immunologic aspects of developmental biology, Advances in Teratology. vol. 1, ed. D. H. M. Wollam Logos, Academic press. 1966
5. Clarke, W. M. & Fowler, I.: The inhibition of lens-inducing capacity of the optic vesicle with adult lens antisera. Dev. Biol. 2: 155-171, 1960
6. Clarke, W. M. & Fowler. I.: The nature of the toxicity of aduly lens antisera. Anat. Rec. 139: 216, 1961 (abstract)
7. Dische, Z.: Glycoproteins of the lens. Invest. Opthalmol. 4: 592-606, 1965
8. Ehrlich, G., Halbert, S. P. & Manski, W.: Cytotoxity of anti-tissue antibodies. II. Effect of anti-lens sera in tissue culture. J. Immun. 89: 391-399, 1962
9. Fowler, I. & Clarke, W. M.: Development of anterior structures in the chick after direct application of adult lens antisera. Anat. Rec. 136: 194, 1960 (abstract)
10. Fowler, I. & Clarke, W. M.: The inhibition of lens-inducing capacity of the optic vesicle with adult lens antisera. Anat. Rec. 133: 277, 1959 (abstract)
11. Ikeda. A. & Zwaan, J.: Immunofluorescence studies on induction and differentiation of the chicken eye lens. Invest. Ophthal. 5: 402-412, 1966
12. Ikeda, A. & Zwaan, J.: The changing cellular localization of alpha-crystallin in the lens of the chicken embsyo, studied by immunofluorescence. Dev. Biol. 15: 348-367, 1967
13. Langman, J.: The first appearence of specific antigen during induction of the lens. J. Embryol. exp. Morph. 7: 193-202, 1959
14. Langman, J.: The effect of lens antiserum on chick embryos. Anat. Rec. 137: 139-140, 1960 (abstract)
15. Langman, J.: The effect of antibodies on embryonic cells. Canad. Cancer Conf. 5: 349-362, 1963
16. Langman, J.: The influence of lens antibodies on development of the eye in the chick embryo. Eye Structure, II. Symp., ed. by J. W. Rohen, Schattauer-Verlag, Stuttgt. 431-438, 1965

17 Licata, R. H., Lev, M. & Brown, E. R.: The production of congenital malformations utilizing specific anti-heart tissue antibodies. Anat. Rec. 142: 252-253, 1962 (abstract)
18. Maisel, H. & Langman, J.: An immunoembryological study on the chick lens. J. Embryol. exp. Morph. 9: 191-201, 1961
19. Mun, A. M.: Toxic effect on normal sera and homologous antisera on the chick embryo. Biol. Bull. 115: 239-247, 1958
20. New, D. A. T. & Brent, R. L.: Effect of yolk-sac antibody on rat embryos grown in culture. J. Embryol. exp. Morph. 27: 543-553, 1972
21. Perkins, E. S.: In "Biochemistry of the Eye". Symp., 1966, (M. U. Dardenne and J. Nordmann, ed.), p. 316, Karger Basel, 1968
22. Pomerat, C. M.: Morphogenetic effects of spleen antigen and antibody administrations to chick embryos. Exp. Cell Res., Suppl. 1: 578-581, 1949
23. Zwaan, J. & Ikeda, A.: An immunochemical study on the beta-crystallins of the chicken lens: ontogenetic and phylogenetic aspects. Eye Structure, II. Symp., ed. by J. W. Rohen, Schattauer-Verlag, Stuttgt. 412-429, 1965
24. Zwaan, J. & Ikeda, A.: Macronolecular events during differentiation of the chicken lens. Exp. Eye Res. 7: 301-311, 1958