

# RUBELLA CATARACT IN VITRO: SENSITIVE PERIOD OF THE DEVELOPING HUMAN LENS\*

BY M. KARKINEN-JÄÄSKELÄINEN, L. SAXÉN, A. VAHERI, AND P. LEINIKKI

(From the Third Department of Pathology and the Department of Virology, University of Helsinki, Helsinki 29, Finland)

In 1941, Gregg (1) suggested a causal relationship between maternal rubella infection and congenital malformations, particularly those of the lens, inner ear, and heart. Since then, additional features have enlarged the clinical picture of the "congenital rubella syndrome". Isolation of the virus from fetal tissues months after maternal infection, first by Selzer (2) and later by others (3), has indicated that the fetal infection is persistent. The virus has been isolated repeatedly from cataractous lenses obtained both at autopsy (4, 5) and at operation (6). In autopsy material, Bellanti and his associates (7) found higher concentration of rubella virus in cataractous lenses than in any other tissue studied. The virus may persist in the lens for three years postnatally (8).

Another feature of the pathogenesis of the rubella syndrome is known from clinical studies: each of the various organs that may be affected has its own "sensitive period" to the viral infection, after which no gross malformations are produced. These sensitive periods are mainly confined to the first trimester of pregnancy. The narrowly limited periods of susceptibility suggest that restrictive mechanisms must operate in the fetal organism, but they are not understood (9, 10).

To explain these organ-specific sensitive periods, a hypothesis has been put forward (11) that certain viruses gain access to ectoderm-derived organs only as long as the invagination and detachment of these from the surface is incomplete. Upon losing open connection with the extraembryonic space they cease to be susceptible to the virus.

In the present in vitro study we investigated the possibility that closure of the lens vesicle, with formation of the lens capsule around it, is the mechanism temporally restricting the susceptibility of the human embryonic lens to rubella virus.

## Materials and Methods

*Embryos.* About 500 products of conception obtained at early therapeutic abortions at the Boije Hospital, Helsinki, were available. They were handled aseptically and placed in phosphate-buffered saline (PBS) containing penicillin and streptomycin. The tissues were rinsed and embryonic eye rudiments and other tissues were excised under the dissecting microscope and set up in culture within 6 h after curettage. Eye rudiments, altogether 79 pairs, were collected from embryos aged 4-10 wk. Of these, 12 pairs, aged 4-6 wk with crown-rump (C-R)<sup>1</sup> lengths of 4-6 mm, were in the lens placode or open-lens-vesicle stages (Fig. 1 A). In 40 pairs, aged 6-10 wk with C-R lengths of ca. 10-25 mm, the

\* This work was supported by the Finnish Cultural Foundation, the Sirgrid Jusélius Foundation, and the National Research Council for Medical Sciences, Finland.

<sup>1</sup>Abbreviations used in this paper: C-R, crown-rump; PBS, phosphate-buffered saline; PFU, plaque-forming unit.

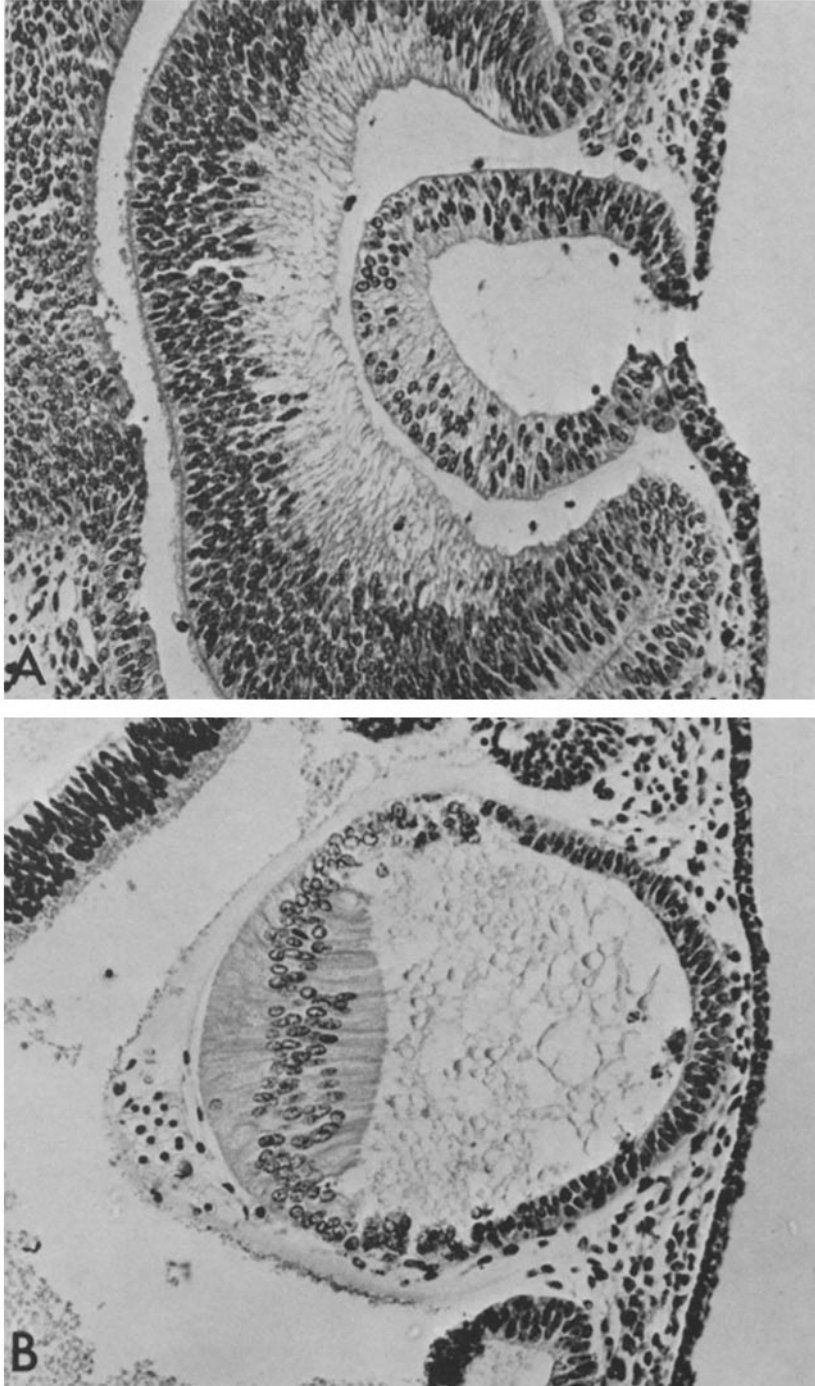


FIG. 1. The starting situations seen in histological sections. (A). Open-lens-vesicle stage of a human embryo aged 4-5 wk with C-R length ca. 6 mm. (B). Closed-lens-vesicle stage of a 6-wk old embryo with C-R length ca. 10mm.

lens vesicle was closed (Fig. 1 B). Another 27 pairs of closed-stage eye rudiments were used for the microsurgical approach. 42 cultures of other embryonic organs, consisting mainly of heart, kidney, and lung tissues from 4- to 10-week embryos, were treated similarly. The embryonic age was determined by C-R length and several external morphological criteria (12).

*Culture of Embryonic Organs.* A modified Trowell-type organ culture set-up was used. The tissues were grown on a piece of Millipore filter supported at a liquid-gas interphase on a stainless steel grid in 20 ccm<sup>2</sup> glass dishes containing 10 ml of medium in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C (13). The medium consisted of BME basal medium supplemented with double the usual amount of amino acids (Orion, Helsinki) and 15% inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland). Streptomycin and Penicillin G (Hoechst Pharmaceutical Co., Kansas City, MO.) were added in concentrations of 50 µg and 100 IU/ml, respectively. Medium was added, if necessary, but not changed.

*Rubella Virus Strains and Infection.* The RA 27/3 strain of rubella virus (14) was used in most experiments. The stock virus preparations were grown in BHK 21/13S cell suspension cultures (15) and had titers ranging from 10<sup>7.3</sup> to 10<sup>7.8</sup> plaque-forming units (PFU) per milliliter when assayed in BHK 21/WI-2 cells (16). The Judith strain of rubella virus (17) was grown in RK13 cells (18) and had a titer of 10<sup>4.5</sup> PFU/ml. The Laakso strain was isolated from a therapeutic rubella abortion and was grown only in human embryonic Wi-38 fibroblasts (19) before use in the present study. The inoculum of the strain had a titer of 10<sup>3.3</sup> PFU/ml.

The tissues to be infected were immersed in undiluted virus preparations at 37°C for 2-8 h, rinsed repeatedly, and placed in culture dishes. In 27 pairs of closed-stage eye rudiments an incision was made through the lens capsule before immersion and was kept open with a piece of membrane filter to allow the virus to enter the lens during the infection period.

*Antisera.* For preparation of antiviral antibodies the Judith strain of rubella virus was grown in cultures of rabbit kidney cells (RK 13 line) maintained in 2% rabbit serum. Virus, partially purified by ultracentrifugation from the extracellular medium of the infected cultures, was mixed with Freund's incomplete adjuvant and used to prepare hyperimmune serum in rabbits. The rubella hemagglutination inhibition titer (20) of the antiserum was 1:10,240.

Antirabbit IgG swine antibodies conjugated with fluorescein isothiocyanate (fluorescein/protein molar ratio 2.5) were from the Institute of Sera and Vaccines, Prague, Czechoslovakia.

Both antisera were absorbed with embryonic tissue homogenate consisting mainly of eye tissue, the conjugate once, and the antiserum twice.

Specificity of the antirubella virus antibodies was evaluated with immunofluorescence in rubella virus-infected BHK21/13 S cells. A serum dilution of 1:50 was used; a fairly clear specific fluorescent staining was detectable with dilutions of 1:30 to 1:60. The conjugate was diluted to 1:10 with PBS and fetal calf serum (1:1).

*Indirect Immunofluorescence.* Frozen sections were cut at -20°C, placed on slides cleaned with methyl alcohol, air-dried, fixed for 10 min in methyl alcohol at -20°C, and rinsed in PBS at -4°C. Paraffin sections were prepared by the technique of Sainte-Marie (21). Sections were stained with diluted antiserum in a humidified chamber for 30 min at 37°C and rinsed twice in PBS. They were then exposed to the diluted conjugate mixed with traces of amido-black (Amidoschwartz, Merck Inco, Darmstadt, West Germany) counterstain (22), for another 30 min at 37°C, rinsed as before, dipped in distilled water, and mounted in glycerol-PBS (3:1). In each experiment, parallel preparations were made from an uninfected control; both test and control sections were also stained with control rabbit serum and with conjugate alone.

*Microscopy and Photography.* The explants were fixed in Carnoy, sectioned serially at 4 µ, and stained with hematoxylin-eosin.

The slides were examined with a Leitz Orthoplan microscope, (E. Leitz, Inco., Rockleigh, N.Y.) and micrographs were taken on Ilford FP-4 Panchromatic 22 Din. plates. For a study of fluorescence, a high-pressure mercury vapor lamp (Philips 200 W/4, Philips Electronic Instrument, Mount Vernon, N.Y.) was used, fitted to incident light equipment (Fluoreszenz-Auflicht-illuminator nach Ploem) with a transmission filter 2 + 3 mm BG 12 and a suppression filter K 4-595. An inbuilt dichromatic combination of mirror 495 nm and suppressor filter K 495 was chosen, a shortwave pass-interference filter KP 490 was employed. The exposure time was 2-4 min. The exact co-ordinates of the fluorogram were recorded and the same area was rephotographed after staining with hematoxylin-eosin.

## Results

*Lenses Infected in the Open-lens-vesicle Stage.* Uninfected eye rudiments in which the lens vesicle was still open (Fig. 1 A) grew well in vitro, undergoing normal invagination of the lens placode, closure of the lens vesicle, and differentiation of the lens fibers. The infected rudiments developed normally at first but the differentiating lens fibers underwent vacuolar degeneration after about 10 days. Frequently, the damage was first detectable in the equatorial zone where terminal mitosis takes place and prolonged cultivation leads to destruction of the fibers (Fig. 2 A). The lens capsule remained intact. The anterior lens epithelium neither showed damage nor gave any hypertrophic response, but appeared quite normal. Eosinophilic granulation, although reported to appear in some virus-induced cataracts (23), was never seen except within the cell debris, where it was probably attributable to denatured lens crystallins. These damaged lenses closely resembled the histological picture of congenital rubella cataract (24, 25).

*Lenses Infected after Closure of the Lens Vesicle.* The lenses infected in the closed-lens-vesicle stage (Fig. 1 B) showed no sign of damage, but continued to differentiate well in vitro. For periods of up to 4 wk degenerative changes were minimal and similar to those detectable in uninfected controls, being attributable to organ culture conditions (Fig. 2 B).

*Immunofluorescence Study of the Infected Eye Rudiments.* With immunofluorescence methods the viral antigens were clearly detectable as cytoplasmic granulation, mainly concentrated in the perinuclear area. Unlike Cotlier et al. (26), we did not see any sign of specific fluorescence inside the cell nuclei. In the open-stage rudiments the viral antigens were detectable within the lens itself (Fig. 3 A) as well as in the surrounding tissues. In the closed-stage explants the lens itself was negative and fluorescence was confined to the cytoplasm of cells outside the lens, in the outer ectoderm, mesenchymal cells, and retina (Fig. 3 B). No specific fluorescence was seen in the uninfected controls.

*Lenses Infected in the Closed Stage after an Incision in the Lens Capsule.* If an incision was made in the lens capsule, some fiber material was seen to extrude from the lens. If uninfected, however, such rudiments grew well in vitro, and cell damage was minimal. In infected explants vacuolar degeneration was seen in the fibers bordering the incision and viral antigens were detectable within 24 h (Fig. 4). The reaction spread to adjacent fibers and within 2 wk the entire lens was fluorescent. As destruction of fibers progressed, the lens became identical with those infected in the open-lens-vesicle stage. Again, the capsule was intact and the anterior lens epithelium appeared normal.

*Other embryonic Organs.* The embryonic lung, heart, and kidney differentiated well in vitro for periods of 4-6 wk. Immunofluorescence revealed viral antigens in isolated cell groups for a few days after the infection. During the following 2 wk the antigens slowly spread over the entire tissues. In histological examination, however, the infected rudiments were indistinguishable from the uninfected controls, which is in accord with the findings of other investigators (27, 28).

*Different Virus Strains and Virus Production.* Most experiments were carried

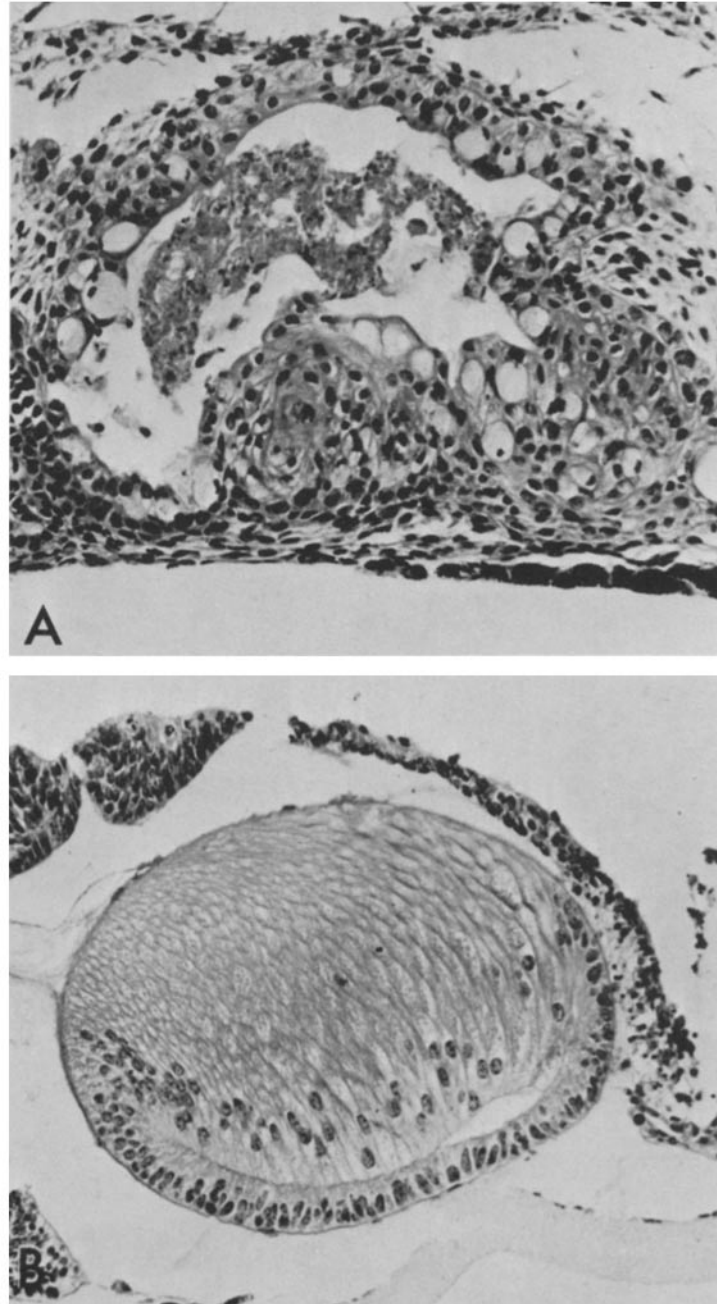


FIG. 2. Histological sections of two human embryonic eye rudiments grown in vitro for 2 wk. (A). Infected with rubella virus in the open stage. (B). Infected in the closed stage.

out with the RA27/3 strain, but the Judith and Laakso strains were also tested, with essentially identical results. Virus was only occasionally detected in the medium. The amount of tissue was probably insufficient to produce a detectable level of virus in the 10 ml of medium in which they were grown, unless at least

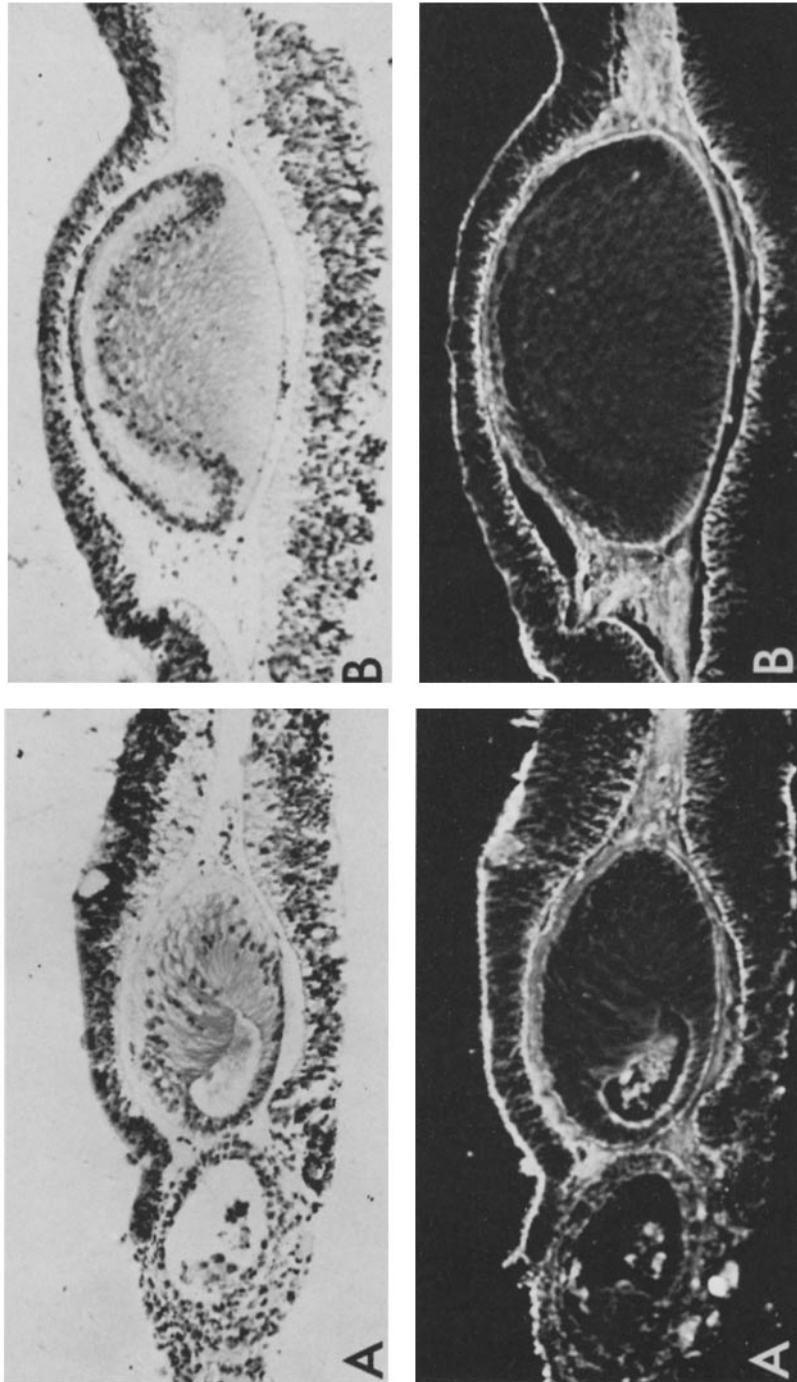


FIG. 3. Paraffin sections showing the distribution of viral antigens visualized by the indirect immunofluorescence technique. (A). In the open stage the fluorescence is seen as cytoplasmic granulation in the lens fibers. (B). In the closed stage no fluorescence is seen within the lens.

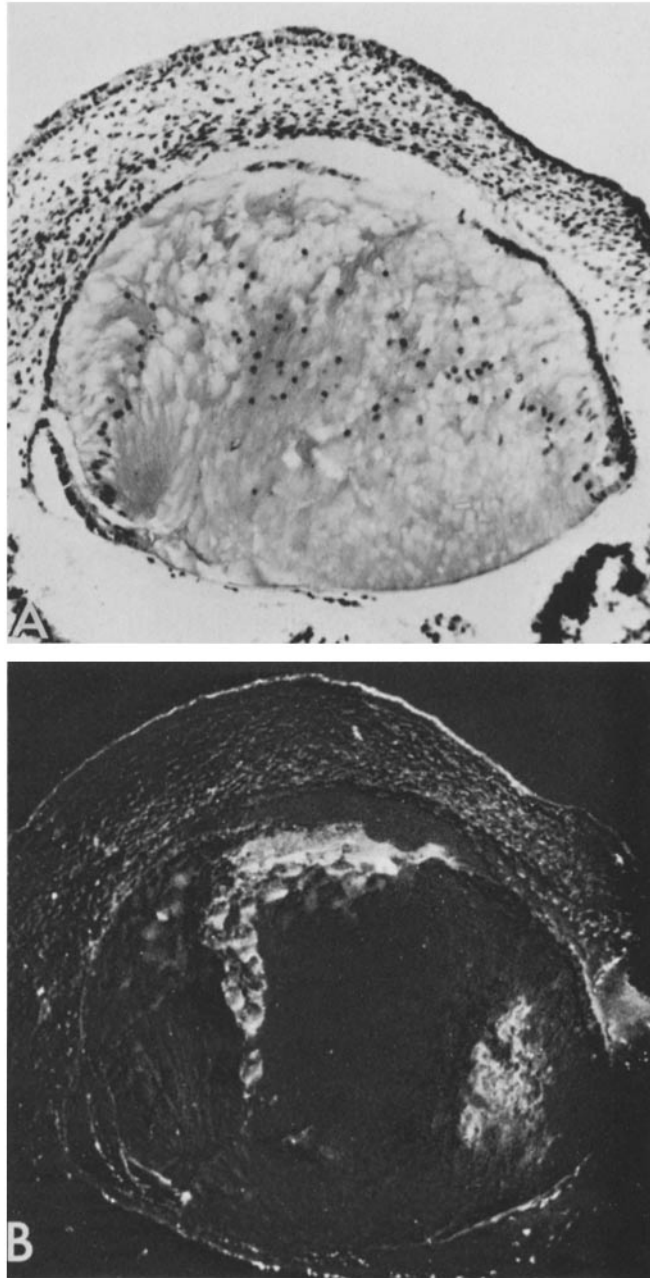


FIG. 4. Paraffin section of a closed-stage rudiment infected through an incision in the lens capsule. Immunofluorescence revealed viral antigenicity in adjacent lens fibers, later spreading over the entire lens and accompanied with cytopathic changes.

four eye rudiments and several pieces of other tissues had been grown in the same dish.

#### Discussion

According to the hypothesis of Robertson, Blattner, Williamson and their associates (11), ectodermal organs such as the lens, inner ear, olfactory epithelium and visceral arch ectoderm cease to be vulnerable to viral infection when, one at a time, they invaginate, becoming detached from the ectoderm, and are no longer in connection with the exterior. Cataract formation in chick embryos infected with mumps virus (one of the three myxoviruses these workers studied) was examined in detail by one of us (23). Infection in the open-lens-vesicle stage caused cataract formation, as they had shown (29, 30), *in ovo*, but also *in vitro*. The cataracts were confirmed to be of viral origin by electronmicroscopic demonstration of replicating virus in the cytoplasm of the fibers (31). Fiber destruction was accompanied by the appearance of specific antiviral immunofluorescence in the cataracts. The sensitive period terminated when the lens vesicle closed. If an incision was made in the lens capsule, the infection produced lesions of the same type as in the open stage. Closed-stage lenses with intact capsules never showed any sign of infection.

The results of the present study confirm that the lens capsule acts as a protective barrier around the human embryonic lens at the time of closure of the lens vesicle. The time coincides with the limits of the sensitive period observed in clinical studies (32). Rubella virus is unable to penetrate into the lens from the amniotic fluid, where it has been isolated (33), after the capsule is formed by thickening of the basement membrane immediately after closure of the lens vesicle. On the other hand, if the virus is already inside the lens tissue, it probably cannot traverse the capsule to the other direction either; it continues to replicate inside the lens, building up the high concentrations observed in congenitally cataractous lenses.

The mode of action of the cytopathic effect of the virus in lens tissue is unknown. Rubella virus has been shown to exert an inhibitory effect on cell division (34). The early cytopathic effect which was found in the mitotic zone of the lens is probably due to this effect. Other mechanisms must be involved, however. The lens fiber cells lose their nuclei during differentiation (35). This was observed also in our oldest rudiments from 9-10-wk old embryos. If infected after the capsule had been incised, viral infection was initiated in such maturing, nondividing cells bordering the incision, leading to accumulation of viral antigen and cytopathic effect. Virus replication seems to be entirely cytoplasm-dependent in them. It may be postulated that the replication cyclis differs from that in normal, nucleated cells and this altered cyclis may allow the persistence of infectious virus in the lens tissues ever for years after the initiation of infection.

Our experiments demonstrate one of the mechanisms restricting embryonic susceptibility to many viral infections during development. All the cells of a very young embryo seem to be highly sensitive, but gradually a certain compartmentalization takes place, limiting the damage to a few organs and cell populations. Finally, when organogenesis is complete, the fetus shows limited sensitivity comparable to that of the adult organism, the target site depending on the



“tropism” of the virus involved (36). Our present limited understanding of these phenomena is mainly due to lack of good animal models for studying viral teratology. The “rubella syndrome” seems to be especially difficult to simulate in experimental animals (37). In animals, even when transplacental infection occurs, embryonic mortality is high, and the results are not applicable to human embryos. Employment of human embryo organ culture (38) offers a good approach. It seems to be the only way to study human target cells and eliminate species-specific differences in virus susceptibility. In addition, it allows exact timing of the infection and unique possibilities for experimental manipulations, as shown by the present study.

### Summary

The clinically known sensitive period of rubella cataract was studied in vitro by infecting 79 human eye rudiments from embryos aged 4–10 wk with rubella virus. The course of the infection was followed by histological and indirect immunofluorescence methods. Of the rudiments, 12 pairs were in the lens placode or open-lens-vesicle stage, 40 already had closed lens vesicles and in another 27 closed-stage pairs an incision was made in the lens capsule before infection to allow the virus to enter the lens. Uninfected controls differentiated well in vitro for 4–6 wk. The eye rudiments infected in the open-lens-vesicle stage showed lens fiber destruction and viral antigens within the lens. No damage or viral antigens were detected in rudiments infected in the closed stage unless the lens capsule was incised. When this was done, however, fiber damage ensued and viral antigens appeared. The lens capsule was concluded to form a protective barrier around the sensitive fibers at the time of closure of the lens vesicle, confirming the earlier hypothesis and clinical findings.

*Received for publication 20 January 1975.*

### References

1. Gregg, N. M. 1941. Congenital cataracts following German measles in the mother. *Trans. Ophthalmol. Soc. N. Z.* 3:35.
2. Selzer, G. 1963. Virus isolation, inclusion bodies, and chromosomes in a rubella-infected human embryo. *Lancet.* 2:336.
3. Monif, G. R. G., G. B. Avery, S. B. Korones, and J. L. Sever. 1965. Postmortem isolation of rubella virus from three children with rubella-syndrome defects. *Lancet.* 1:728.
4. Monif, G. R. G., J. L. Sever, G. M. Schiff, and R. G. Traub. 1965. Isolation of rubella virus from products of conception. *Am. J. Obstet. Gynecol.* 91:1143.
5. Cotlier, E., J. Fox, and M. Smith. 1966. Rubella virus in cataractous lenses of congenital rubella syndrome. *Am. J. Ophthalmol.* 62:233.
6. Reid, R. R., A. M. Murphy, A. M. Gillespie, D. C. Dorman, M. A. Menser, R. Hertzberg, and J. D. Harley. 1966. Isolation of rubella virus from congenital cataracts removed at operation. *Med. J. Aust.* 1:540.
7. Bellanti, J. A., M.S. Artenstein, L. C. Olson, E. L. Buescher, C. E. Luhrs, and K. L. Milstead. 1965. Congenital rubella: clinico-pathologic, virologic and immunologic studies. *Am. J. Dis. Child.* 110:464.
8. Menser, M. A., J. D. Harley, R. Hertzberg, D. C. Dorman, and A. M. Murphy. 1967. Persistence of virus in lens for three years after prenatal rubella. *Lancet.* 327.

9. Saxén, L., and J. Rapola. 1969. *Congenital Defects*. Holt, Rinehart and Winston, Inc., New York.
10. Catalano, L. W., and J. L. Sever. 1971. The role of viruses as causes of congenital defects. *Ann. Rev. Microbiol.* **25**:255.
11. Blattner, R. J., and F. M. Heyes. 1961. Role of viruses in the etiology of congenital malformations. *Prog. Med. Virol.* **3**:311.
12. Blechschmidt, E. 1963. *Der Menschliche Embryo. Dokumentation zur Kinetischen Anatomie*. Friedrich-Karl Schauttauer-Verlag, Stuttgart.
13. Saxén, L., O. Koskimies, A. Lahti, H. Miettinen, J. Rapola, and J. Wartiovaara. 1968. Differentiation of kidney mesenchyme in an experimental model system. *Adv. Morphog.* **7**:251.
14. Plotkin, S. A., D. Cornfield, and T. H. Ingalls. 1965. Studies of immunization with living rubella virus. Trials in children with a strain cultured from an abortus foetus. *Am. J. Dis. Child.* **110**:381.
15. Vaheri, A., W. D. Sedwick, S. A. Plotkin, and R. Maes. 1965. Cytopathic effect of rubella virus in BHK21 cells and growth to high titers in suspension culture. *Virology.* **27**:239.
16. Vaheri, A., C.-H. von Bonsdorff, T. Vesikari, T. Hovi, and P. Väänänen. 1969. Purification of rubella virus particles. *J. Gen. Virol.* **5**:39.
17. McCarthy, K., C. H. Taylor-Robinson, and S. E. Pillinger. 1963. Isolation of rubella virus from cases in Britain. *Lancet.* 593.
18. McCarthy, K., and C. H. Taylor-Robinson. 1965. Growth and cytopathic effects of rubella virus in primary rabbit tissue culture. *Arch. Gesamte. Virusforsch.* **16**:415.
19. Hayflick, L., and P. S. Moorhead. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**:585.
20. Stewart, G. L., P. D. Parkman, H. E. Hopps, R. D. Dougals, J. P. Hamilton, and H. M. Meyer. 1967. Rubella-virus hemagglutination-inhibition test. *N. Engl. J. Med.* **276**:554.
21. Sainte-Marie, G. A. 1962. Paraffin embedding technique for studies employing immunofluorescence. *J. Histochem. Cytochem.* **10**:250.
22. Leinikki, P. 1971. Immunofluorescent assay of herpes virus type 1 and type 2 antibodies in rabbit and human sera. *Arch. Gesamte. Virusforsch.* **35**:349.
23. Karkinen-Jääskeläinen, M. 1973. Spatial and temporal restriction of mumps virus induced lesions in the developing chick lens. *Acta Pathol. Microbiol. Scand. Suppl.* **243**:1-52.
24. Zimmerman, L. E. 1968. Histopathologic basis for ocular manifestations of congenital rubella syndrome. *Am. J. Ophthalmol.* **65**:837.
25. Töndury, G. 1962. *Embryopathien. Über die Wirkungsweise, Infektionsweg und Pathogenese von Virien auf den menschlichen Keimling*. Springer-Verlag KG, Berlin.
26. Cotlier, E., and J. Fox. 1968. Cataracts induced by rubella virus in humans and in experimental animals. *Biochemistry of the Eye Symp.* 1966. Karger AG, Basel.
27. Best, J. M., and J. E. Banatvala. 1971. Further studies on the growth of rubella virus in human embryonic organ cultures: preliminary observations on interferon production in the cultures. *J. Hyg.* **69**:223.
28. Woods, W. A., R. T. Johnson, D. D. Hostetler, M. L. Lepow, and F. C. Robbins. 1966. Immunofluorescent studies on rubella-infected tissue cultures and human tissues. *J. Immunol.* **96**:253.
29. Williamson, A. P., R. J. Blattner, and L. Simonsen. 1957. Cataracts following mumps virus in early chick embryos. *Proc. Soc. Exp. Biol. Med.* **96**:224.
30. Robertson, G. G., A. P. Williamson, and R. J. Blattner. 1964. Origin and development of lens cataracts in mumps infected chick embryos. *Am. J. Anat.* **115**:473.

31. Virtanen, I., M. Karkinen-Jääskeläinen, and L. Saxén. 1973. Ultrastructural characteristics of mumps virus replication in the cataracts of chick embryonic lens. *Exp. Eye Res.* **17**:463.
32. Mayes, B. 1957. La rubéole et le foetus. *Triangle.* **3**:10.
33. Alestig, K., F. K. Bartsch, L.-A Nilsson, and O. Strannegard. 1974. Studies of amniotic fluid in women infected with rubella. *J. Infect. Dis.* **129**:79.
34. Plotkin, S. A., and A. Vaheri. 1967. Human fibroblasts infected with rubella virus produced a growth inhibitor. *Science (Wash. D.C.)* **156**:659.
35. Modak, S. P., and S. W. Perdue. 1970. Terminal lens cell differentiation. I. Histological and spectrophotometric analysis of nuclear degeneration. *Exp. Cell Res.* **59**:43.
36. Ebert, J. D., and F. H. Wilt. 1960. Animal viruses and embryos. *Q. Rev. Biol.* **35**:261.
37. Elizan, T. S., A. Fabiyi, and J. L. Sever. 1969. Study of rubella virus as a teratogen in experimental animals: a short review. *J. Mt. Sinai Hosp.* **36**:108.
38. Best, J. M., J. E. Banatvala, and B. M. Moore. 1968. Growth of rubella virus in human embryonic organ cultures. *J. Hyg.* **66**:407.