

MONOCLONAL ANTIBODIES SELECTIVE FOR THE INNER PORTION OF THE CHICK RETINA¹

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Received October 28, 1981; Revised December 21, 1981; Accepted December 30, 1981

Abstract

Five monoclonal antibodies were selected by immunizing mice with embryonic chick optic nerve and screening the resultant hybridoma antibodies by immunofluorescence on sections of the retina. The optic nerve was chosen as the immunizing tissue since it is presumably enriched in ganglion cell-specific antigens. These antibodies bind to antigens which are largely localized to the inner part of the retina. Two of the antibodies, RET1 and RET2, show highly selective binding to the optic fiber layer. Another, RET3, stains the optic fiber layer, ganglion cell layer, and inner plexiform layer. Antibody RET4 stains the optic fiber layer, ganglion cell layer, inner plexiform layer, and the inner one-half of the inner nuclear layer; this antibody binds to an antigen on or associated with the plasma membrane. Antibody RET5 stains the retinal ganglion cell layer faintly and the inner and outer plexiform layers strongly. The potential utility of these antibodies for future studies on retinal development is discussed.

The embryonic chick neural retina is a widely used model for the early development of the central nervous system. Among the features which recommend it are its relatively large size and the ease of obtaining and surgically manipulating precisely staged embryos. The sequence of the major events in histogenesis has been determined (Coulombre, 1955) and extensive information on cell birth dates is available (Kahn, 1973, 1974). Particular attention has been paid to the development of the retinotectal projection. It has been demonstrated that ganglion cells have acquired their locus specificity by embryonic day 4 (Crossland et al., 1974). Ganglion cell axons first reach the optic tectum at day 6 of embryonic development and continue to grow over the surface of the tectum until about day 10 (Crossland et al., 1975; Rager, 1976; Rager and von Oeynhausen, 1979). Ablation of the early retina shows that some tectal neurons are trophically dependent on retinal input (Kelly and Cowan, 1972). Conversely, ablation of the tectum before it is

innervated by the retina causes massive death of retinal ganglion cells (Hughes and LaVelle, 1975), and section of the optic nerve in adult quail causes ganglion cell degeneration (Muchnik and Hibbard, 1980).

The developing chick retina also has been studied *in vitro*. Aggregate cultures of the retina show some of the morphological and biochemical features of early development (Seeds, 1971). Dispersed retinal cell cultures have been used to study the development of acetylcholine receptors and morphologically and functionally defined synapses (Vogel et al., 1976; Ruffolo et al., 1978). Finally, the chick retina has been widely used as a model system for cell-cell recognition in early development. The role of the cell-adhesive protein CAM was first elucidated in the chick retina (Brackenbury et al., 1977; Thiery et al., 1977; Buskirk et al., 1980). Topographic differences in the adhesive specificity of retinal cells have been demonstrated (Barbera et al., 1973; Gottlieb et al., 1976; Marchase, 1977). Growing axons of retinal cells in tissue culture have the ability to adhere preferentially to tectal cells over retinal or telencephalic cells (Bonhoeffer and Huf, 1980). A cell surface antigen whose concentration varies dramatically along the dorsoventral axis of the chick retina has been described and is possibly involved in encoding positional information (Trisler et al., 1981).

Monoclonal antibodies which are specific to certain cell types will be important for further studies in this system since they will greatly facilitate the study of the development of identified cell types both *in vivo* and *in vitro*. The pioneering study of Barnstable (1980) has

¹ It is a pleasure to acknowledge the excellent technical assistance of Ms. Mary Beth Eschman. Mr. George Hvostik participated in some of these experiments. We thank Dr. Joshua Sanes for the use of his fluorescence microscope and advice on immunohistochemistry and Dr. Mark Willard for helpful comments on the manuscript. D. I. G. was supported by National Institutes of Health Grant NS12867 and by a Research Career Development Award from the National Institute of Neurological and Communicative Disorders and Stroke.

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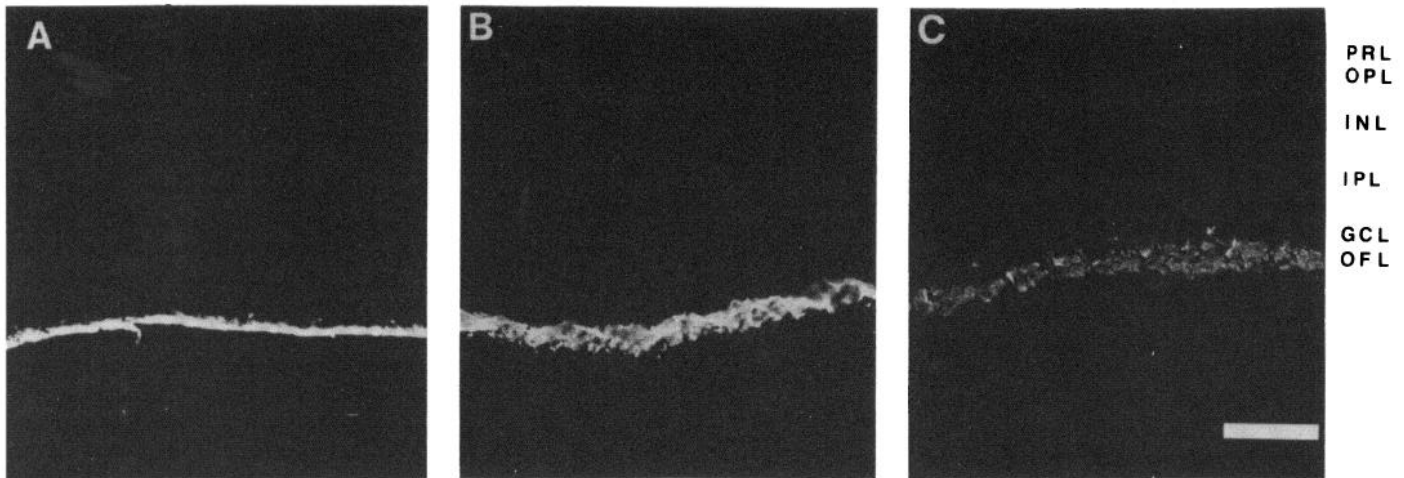


Figure 1.⁴ Monoclonal antibodies RET1 and RET2 are specific for the optic fiber layer. Primary incubations were with ascites fluid at 1:100 dilution. *Panel A* shows the pattern of staining obtained with RET1 on the E9 retina. The corresponding pattern for the E19 retina is shown in *panel B*. In *panel C*, staining of the E19 retina by antibody RET2 is shown. Calibration marker, 100 μ m.

demonstrated the feasibility of obtaining cell-specific monoclonal antibodies directed to retinal neurons. With this in mind, we have obtained a set of five monoclonal antibodies which bind selectively to the inner portion of the chick retina. These antibodies were obtained by the novel approach of immunizing mice with a long tract, the optic nerve, in the hope of raising monoclonal antibodies to the tract's cells of origin. The results show that this approach is a useful one for selecting monoclonal antibodies with restricted binding to the retina. Furthermore, the approach may be applicable in other systems for selecting monoclonal antibodies to neurons which contribute their axons to long tracts.

Materials and Methods

Animals. All tissues were from embryonic or newly hatched white Leghorn chickens. Fertile eggs were obtained from Spafas, Inc., Roanoke, IL and incubated in a forced draft incubator at 39°C. Mice were of the Balb/c strain obtained from Cumberland View Farms, Clinton, TN.

Immunization and cell fusion. Mice were immunized with optic nerves from embryonic day 14 chicks. This choice was made because the optic nerve at this stage consists predominantly of ganglion cell axons, which should be a rich source of proteins synthesized in ganglion cells. The nerve at this stage is practically devoid of myelin (Rager, 1976). Each mouse received an intravenous injection of approximately five optic nerves homogenized in 0.3 ml of phosphate-buffered saline (PBS). Mice were boosted 4 weeks later with the same amount of antigen injected either intravenously or intraperitoneally. Three days later, spleens were removed and fused with the NS-1 myeloma line by standard procedures (Galfré et al., 1977). Cells from each fusion were distributed to two Costar 96-well dishes (Corning, Inc.). Ap-

proximately 12 days after fusion, the wells were tested for antibodies to the retina.

Screening procedure. Immunohistochemistry of the retina was utilized to determine which primary wells contained the monoclonal antibodies of interest. Eyes were taken from embryos and the lens and vitreous were removed; the remaining portions of the eye were immersed in freshly prepared 0.01 M periodate, 0.1 M lysine, and 0.05% paraformaldehyde (McLean and Nakane, 1974) at 4°C for 30 min and then frozen and sectioned at 20 μ m in a cryostat. Sections were mounted on gelatin/chrome alum-coated slides. One hundred microliters of hybridoma supernatant was puddled onto each section and incubated at room temperature for 60 min and the sections were rinsed with three 2-min washes of PBS. Bound mouse antibody was detected by incubating sections with fluorescein-conjugated rabbit anti-mouse IgG (Cappel Laboratories) at 1:2000 dilution in PBS/10% normal goat serum for 60 min at room temperature. Slides then were washed three times in PBS, fixed in 2% paraformaldehyde/PBS, washed twice in PBS, mounted in 50% glycerol/PBS, coverslipped, and examined with a Leitz microscope equipped for epifluorescence.

Hybridoma cloning and monoclonal antibody production. Hybridomas from primary wells containing interesting antibodies were cloned in soft agar (Coffino et al., 1972) using 3T3 cells as a feeder layer. Clones were tested and positive clones were recloned. Permanent lines were established, ascites tumors were induced in Balb/c mice, and ascites fluid was collected and stored.

Localization of binding sites for monoclonal antibodies from isolated clones. This report describes the retinal binding pattern for five monoclonal antibodies produced by cloned hybridoma lines. These were determined exactly as described under "Screening procedure" above. In some cases, the primary antibody was in the form of culture supernatant and the negative control consisted of fresh medium. In other cases, ascites fluid at the indicated dilution was used and normal mouse serum at the same dilution was used as a negative control.

Photomicrography. Photographs were made using Ko-

⁴The abbreviations for retinal layers used on the figures are: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OFL, optic fiber layer; OPL, outer plexiform layer; PRL, photoreceptor layer.

dak Tri-X film exposed for 30 sec. Film was developed with Diafine developer.

Results and Discussion

Incidence of lamina-specific monoclonal antibodies. In two of the fusions in this series, observations were made on the frequency of lamina-specific antibodies in the supernatants of primary hybridoma wells. In the first fusion, the primary immunization was given intravenously and the boosting dose was given intraperitoneally. One hundred eighty-nine wells were surveyed of which 16% had antibodies which bound to the entire retina and 7% had antibodies which were largely restricted to cells or processes in the inner retina. In a second fusion, the primary dose and the boosting dose were both given intravenously. One hundred ninety primary wells were surveyed of which 22% had antibody binding to the entire retina and 27% had antibodies in which binding was largely restricted to inner portions of the retina. Hybridoma production is notoriously variable and any attempt to rigorously establish the numbers and types of antibodies produced in a particular scheme would be prohibitively time consuming. The limited survey that we have

cited here suffices to make a point of practical importance: these protocols yield a large enough number of hybridomas with restricted binding to make the approach attractive.

Monoclonal antibodies specific for the optic fiber layer. All of the monoclonal antibodies described in this paper are produced by cell lines which have been cloned. Two monoclonal antibodies, RET1 and RET2, exhibit a high degree of selectivity for the optic fiber layer as shown in Figure 1. The optic fiber layer is stained intensely in both day 9 embryo (E9) and day 19 embryo (E19) retina with no stain, except a slight background fluorescence, visible in any other portion of the retina. That other portions of the retina are not intrinsically refractory to staining is shown by the patterns obtained with other monoclonal antibodies in this study. For example, the antibody shown in Figure 3A has been shown previously to stain all levels of the retina approximately equally and thus seems to be directed against a common neural antigen (Lemmon et al., 1982). The optic fiber layer does not have a high intrinsic fluorescence or ability to bind antibody nonspecifically since many antibodies in our collection fail to stain this layer. Figure 2D shows

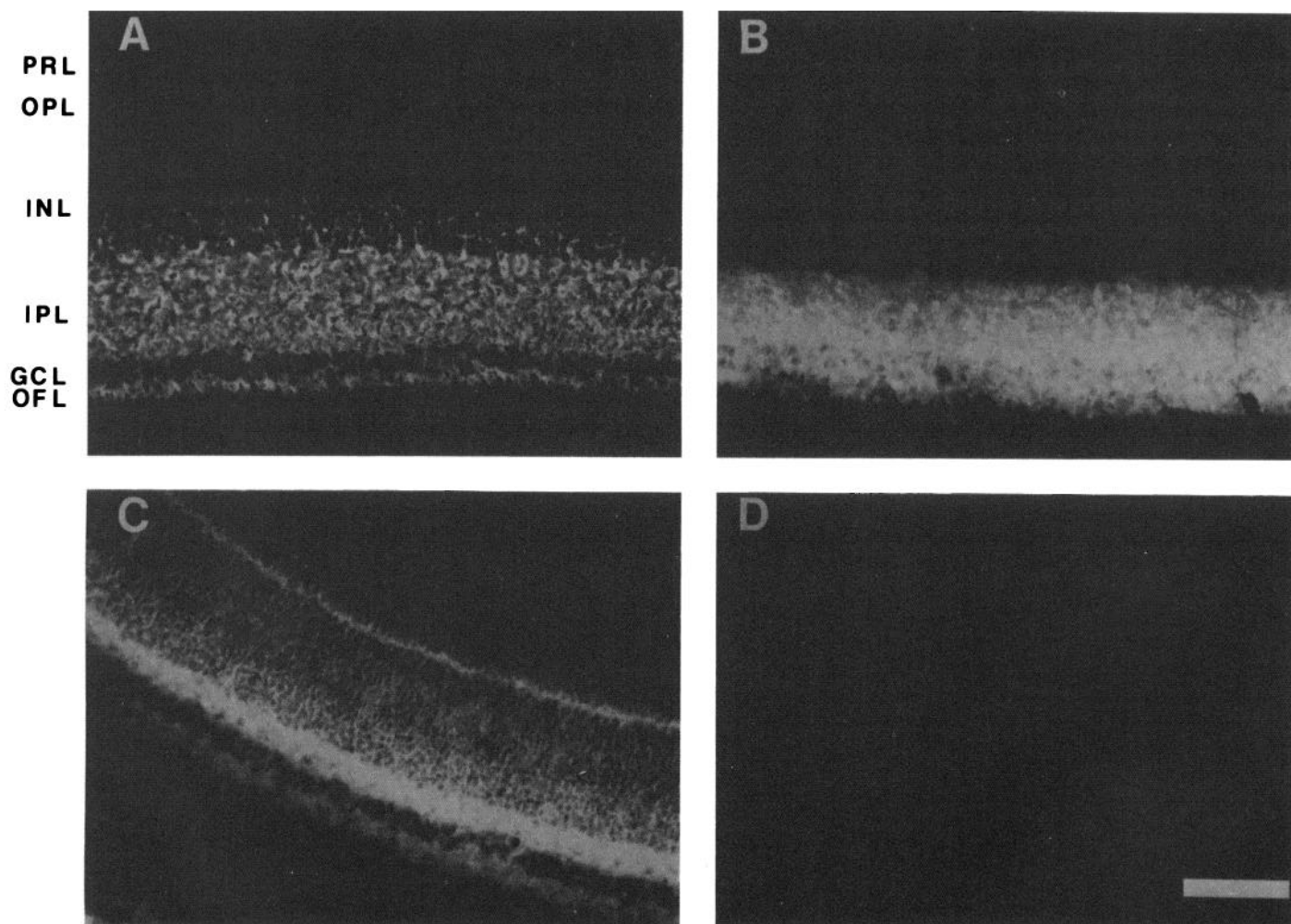


Figure 2. Binding patterns of RET3 and RET4. A, RET4 staining of E19 retina; B, RET3 staining of E19 retina; C, RET4 staining of E9 retina; D, RET3 staining of E9 retina. The primary antibody for RET3 was the cell culture supernatant; for RET4, it was 1:100 ascites fluid. Calibration marker, 100 μ m.

that antibody RET3 does not stain the optic fiber layer of the E9 retina. Antibodies RET1 and RET2 are produced by hybridomas that arose from independent cell fusion events. Their patterns of binding to the retina are indistinguishable from each other. In addition, both of these antibodies stain extraocular muscle fibers with a pattern of ring fluorescence which strongly suggests that they bind to the plasma membrane or basal lamina. It would be tempting to conclude from this data that RET1 and RET2 recognized an antigen localized to ganglion cell axons since they are the dominant structure of the optic fiber layer. However, other interpretations of the localization are possible although less likely. The antigen(s) could conceivably be on Müller cells and be highly restricted to their vitreal terminal portions. They also might be a component of an extracellular matrix restricted to the optic fiber layer. The exact cellular localization of these antigens will have to be settled by EM analysis. In spite of this uncertainty, antibodies RET1 and RET2 will be useful probes for studying the development of the chick retina *in vitro*.

Monoclonal antibodies RET3 and RET4. Antibody RET3 stains the inner part of the retina, including the optic fiber layer, the ganglion cell layer, and the inner plexiform layer (Fig. 2B). More distal layers of the retina are not stained. The staining does not show any ring fluorescence such as that seen with antibodies RET4 and 2241A6-6A1 (Lemmon et al., 1982) which are known to stain plasma membranes. Therefore, it is likely that RET3 stains some constituent of the cytoplasm rather than the plasma membrane. The portion of the retina stained by RET3 corresponds exactly to that occupied by the retinal ganglion cells and it is highly likely that the corresponding antigen is confined to the cytoplasm of these cells. Again, this conclusion must be tempered by the inherent limits of light microscopic localization. Unlike all of the other antibodies described in this study, RET3 does not stain the E9 retina (Fig. 2D). Since ganglion cells have been present for at least 3 days in the E9 retina (Kahn, 1973, 1974), it is likely that the antigen recognized by RET3 is acquired relatively late in the life of these cells.

Antibody RET4 stains the optic fiber, ganglion cell, and inner plexiform layers as well as the inner half of the inner nuclear layer of the E19 retina (see Fig. 2A). The

border between stained and unstained parts of the inner nuclear layer is sharp and reproducible from preparation to preparation. The staining in the ganglion cell layer and the inner nuclear layer is ring-like, suggesting that the antigen is either on or associated with the plasma membrane. Studies on living cultured dispersed retinal neurons show that about 30% of the cells are stained by RET4 (T. Usdin, V. Lemmon, and D. I. Gottlieb, unpublished results). Since the stained cells are living and therefore impermeable to antibodies, at least some of the antigen is found on the outer surface of the plasma membrane. Antibody RET4 clearly stains ganglion cells. It is not yet clear which class of cell from the inner half of the inner nuclear layer is being stained. When the E9 retina is stained with RET4, the labeling is more intense along the inner aspect of the retina but does clearly extend to the outer border of the retina (Fig. 2C). The mechanisms by which this more general pattern of labeling evolves into the E19 pattern shown in Figure 2A have yet to be studied.

RET5—An antibody selective for the plexiform layers. In the E19 retina, antibody RET5 shows very light staining of the ganglion cell layer and intense staining of the inner and outer plexiform layers. The inner and outer nuclear layers are not stained (Fig. 3B). In the E9 retina, a similar pattern is seen (Fig. 3C). A plausible explanation of this pattern of distribution is that RET5 recognizes a component found in retinal synapses.

Conclusion. The monoclonal antibodies described in this report are novel. Barnstable (1980) has described antibodies which are specific to photoreceptors and Müller cells and one which seems to bind to all neurons of the rat retina. The pattern of these antibodies clearly differs from those which we describe here. Eisenbarth et al. (1979) isolated a monoclonal antibody which binds to the tetrasialo ganglioside G_Q and showed that this antigen was present on all retinal neurons. Trisler et al. (1981) have studied a monoclonal antibody which recognizes a surface antigen found throughout the depth of the retina but which forms a gradient of concentration along the dorsoventral axis. By the criteria of binding localization, the antibodies that we have designated RET1 to RET5 differ from each of these.

Monoclonal antibodies RET1 to RET5 were selected by immunizing mice with the unmyelinated optic nerve

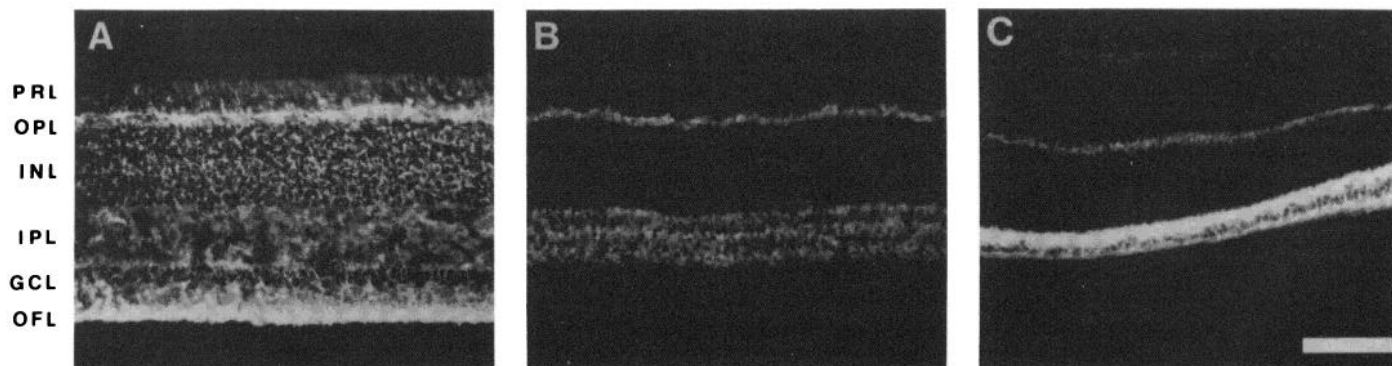


Figure 3. Binding of a common neural antigen and of RET5. A, This shows the binding of monoclonal antibody 2241A6-6A1 which binds to all layers of the retina. B, Binding of RET5 to the E19 retina; C, binding of RET5 to the E9 retina. The primary antibody in the case of RET5 is a 1:100 dilution of ascites fluid. Calibration marker, 100 μ m.

of the embryonic chick. Therefore, it is not surprising that antibodies specific for the inner aspect of the retina are seen when hybridomas from such animals are screened. Three of the antibodies that we describe are highly likely to be ganglion cell specific. RET1 and RET2 bind selectively to the optic fiber layer and RET3 stains exactly the portion of the retina occupied by the full extent of the ganglion cell. However, until EM analysis is performed, we cannot designate RET1 to RET3 definitely as ganglion cell-specific reagents. Antibody RET4 has a broader specificity binding to ganglion cells and cells of the inner half of the inner nuclear layer. Because the antibody recognizes a cell surface antigen, it will be a useful reagent for separating positive and negative cells from dispersed cell suspensions from the retina. All of the antibodies that we describe will be useful markers in further studies of the growth and development of retinal neurons. In particular, they will allow us to measure the growth and development of cells in the inner retina both *in vivo* and *in vitro* by simple, rapid radioimmune assays.

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