Histol Histopath (1993) 8: 437-442

Histology and Histopathology

Practical applications of some antibodies labelling the human retina

P. Chévez[1,2] and R.L. Font[1]

[1]Department of Ophthalmology, Cullen Eye Institute, Baylor College of Medicine, Houston, Texas and [2]Hermann Eye Center, Department of Ophthalmology, The University of Texas Health Science Center, Houston, Texas, USA

Summary. Eight enucleated eyes were promptly fixed in 10% formalin and processed for light microscopy. Employing the ABC technique, we used antibodies against neuron-specific enolase (NSE), synaptophysin (SY), neurofilament (NF), glial fibrillary acidic protein (GFAP), keratins, LEU-7 and S-100 protein. Using NSE we found immunoreactivity in all sensory layers except the outer segments of photoreceptors which were non-reactive. SY showed a striking immunoreactivity of the synaptic regions (outer plexiform and inner plexiform layers). NF labelled the axons of the ganglion cells. GFAP stained perivascular glial cells and Müller cells only in areas of peripheral microcystoid degeneration. Keratin was non-reactive in all layers. LEU-7 showed that all layers were moderately reactive while the Müller cell processes and the outer limiting membrane were strongly immunoreactive. S-100 protein stained the perivascular glial cells in the nerve fiber layer. We conclude that NSE and LEU-7 are helpful markers for most layers of the sensory retina; GFAP and LEU-7 clearly outline Müller cells and GFAP immunoreacts with astrocytes; NF is specific for axons and SY labels vividly the synaptic regions.

Key words: Human retina, Monoclonal antibodies, Glial fibrillary acidic protein, S-100 protein, neurofilament

Introduction

The pattern of cellular organization of the vertebrate retina was clearly defined by the classical studies of Santiago Ramón y Cajal in 1893 (Thorpe and Glickstein, 1972). Using the Golgi method, Cajal identified each of the major retinal cell types. They include photoreceptors, horizontal, bipolar, amacrine, ganglion cells and the glial cells (astrocytes and Müller cells). Currently, it is possible to differentiate specific functional classes of neuronal cells based on differences in their antigenic expression. In the past two decades, technical advances in the use of monoclonal and polyclonal antibodies have emerged as powerful and highly specific molecular probes that have both diagnostic and therapeutic implications.

There are well known commercially available antibodies that have been used for detection of neuronal and glial cells, neuroendocrine cells and their tumors. Some studies have labelled retinoblastoma cells, epiretinal membranes and a variety of intraocular lesions employing immunohistochemistry (Molnar et al., 1984a,b; Messmer et al., 1985; Kivelä, 1986a; Taylor et al., 1986; BenEzra et al., 1987; Erickson et al., 1987; Mancini et al., 1989; Nork et al., 1990).

The purpose of this study is to analyze the normal staining patterns in the human retina using seven commercially available antibodies: neuron-specific enolase (NSE), synaptophysin (SY), neurofilament (NF), glial fibrillary acid protein (GFAP), keratin, LEU-7 and S-100 protein.

Materials and methods

We studied normal retinas in eight eyes. Seven were postmorten enucleated eyes from HIV-positive patients (age range: 41 to 71 years) and one was a surgically enucleated eye from a two-month baby girl who had an undifferentiated retinoblastoma. The globes were promptly fixed in 10% buffered formalin with an average postmortem fixation time of 3:30 hours (range: 15 minutes to 7:30 hrs.). All specimens were embedded in paraffin and cut at five μm. The slides were stained with hematoxylin-eosin and the periodic-acid Schiff (PAS). Immunoperoxidase techniques using NSE, SY, NF, GFAP, Keratin, LEU-7 and S-100 protein were employed. When digestion was necessary we used 0.4% pepsin (Dako) in 0.01N hydrochloric acid at 37 °C for 15 minutes. Primary antibodies were obtained from Dako: NSE (monoclonal; prediluted and incubated per 60 min), GFAP (polyclonal; dilution 1:30 and incubated overnight), NF (monoclonal; prediluted and incubated

Offprint requests to: Dr. Ramon L. Font, M.D., Cullen Eye Institute, Baylor College of Medicine, One Baylor Plaza, NC-200, Houston, TX 77030, USA

per 60 min), S-100 protein (monoclonal; dilution 1:300 incubated overnight); from Boehringer Mannheim Biochem: SY (monoclonal; dilution 1:10 and incubated overnight using predigested sections), keratin (monoclonal; dilution 1:100 and incubated overnight employing predigested sections) and from Becton Dickinson: LEU-7 (monoclonal; prediluted and incubated per one hr). Monoclonal antibodies were made in mouse cells and polyclonal antibodies were raised in rabbits. The secondary antibody was goat anti-mouse for monoclonals and goat anti-rabbit for polyclonal antibodies. Incubation was always performed in a moist chamber for one hour at 37 °C. Overnight incubation was done at 4 °C. For visualization of the antigen-antibody binding sites we used the avidin-biotin complex method (Taylor, 1986) (Vectastain) and for chromogen we selected 3,3'-diaminobenzidine tetrahydrochloride (Fluka, Buchs, Switzerland). As control tissues we employed sections of brain and optic nerve for NSE, SY, NF, and GFAP, sections of tonsil for LEU-7, sections of skin for keratin and sections from a cutaneous malignant melanoma for S-100 protein.

Results

Neuron-Specific Enolase (NSE)

We found intense immunoreactivity for the inner segments of photoreceptors but the outer segments were non-reactive. The remaining layers of the sensory retina were moderately immunoreactive (Fig. 1).

Synaptophysin (SY)

The synaptic regions showed striking immunoreactivity. The outer plexiform layer displayed a vesicular pattern clearly outlining the cone pedicules and the rod spherules, best seen adjacent to the macular region (Fig. 2).The inner plexiform layer displayed a

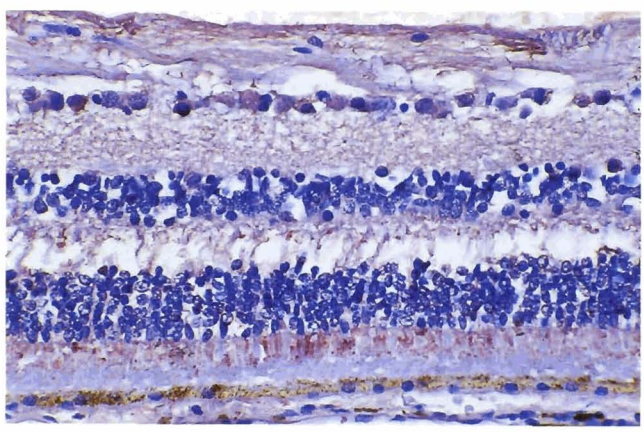

Fig. 1. Section of retina (posterior pole) shows neuron-specific enolase immunoreactivity in all sensory layers except the outer segments of photoreceptors which show negative staining. x 250

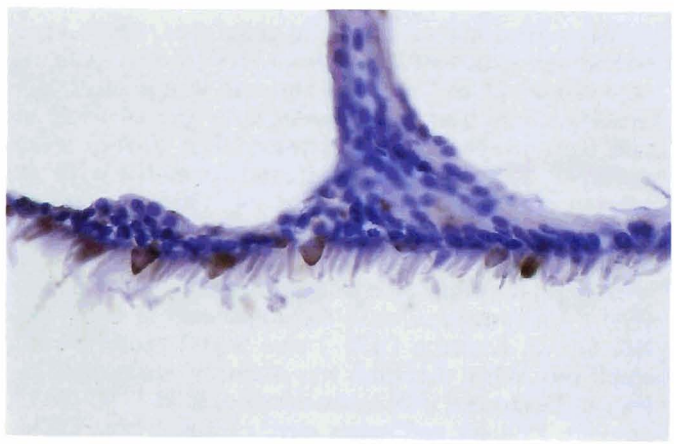

Fig. 3. Peripheral retina whithin areas of microcystoid degeneration displays synaptophysin immunoreactivity for the inner segments of scattered cones found in this region. x 300

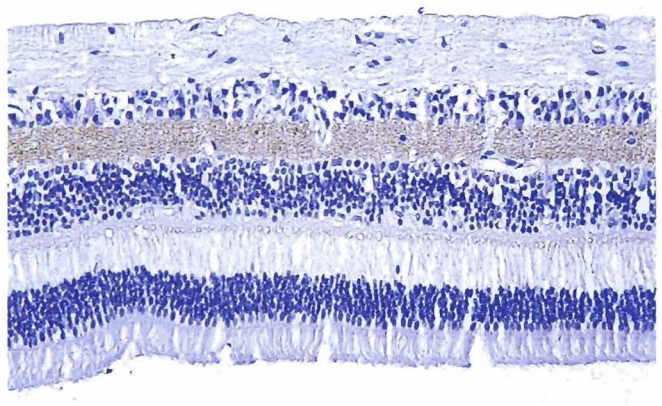

Fig. 2. Section of perimacular retina shows positive anti-synaptophysin staining of synaptic regions with a granular pattern for the inner plexiform layer (above) and a vesicular pattern for the innermost portion of the outer plexiform layer (below). The later region clearly outlines synapses between photoreceptors and horizontal and bipolar cells. x 150

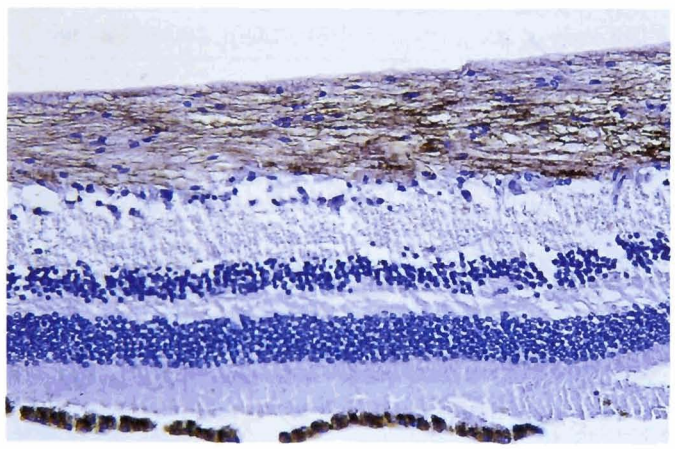

Fig. 4. Peripapillary retina with positive staining for neurofilament of axons in the nerve fiber layer. x 150

distinct granular pattern. Scattered inner segments of cones found at the periphery were positively stained (Fig. 3).

Neurofilament (NF)

The axons of ganglion cells in the nerve fiber layer were immunoreactive mainly in the peripapillary region (Fig. 4). An interesting finding was the positive staining of corpora amylacea in the nerve fiber layer in the peripapillary region and in the optic disc.

Glial Fibrillary Acidic Protein (GFAP)

The majority of the retina showed positive staining of perivascular glial cells and scattered astrocytes contained within the ganglion cell and nerve fiber layers (Fig. 5). Additionally, we observed positive staining of cytoplasmic processes of Müller cells only in the areas of peripheral microcystoid degeneration (Fig. 6).

Keratin

We found that all layers of the sensory retina were non-reactive.

LEU-7

The outer limiting membrane showed a discontinuous, linear staining pattern that contrasted with the negatively stained cell bodies of the photoreceptors (Fig. 7). The remainder of the retinal layers displayed a moderately immunoreactivity for cell membranes of Müller cells. In the inner layers, foot processes of Müller cells stained strongly positive standing out among the negatively stained ganglion cells (Fig. 8).

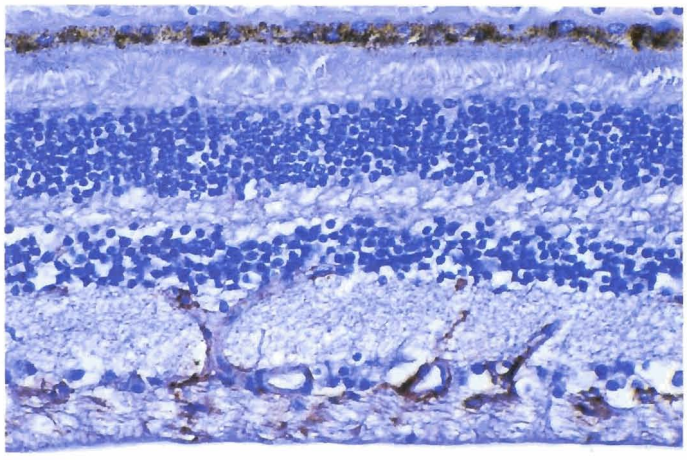

Fig. 5. Section of retina (posterior pole) showing glial fibrillary acidic protein immunoreactivity for perivascular glia in the nerve fiber and ganglion cell layers and scattered astrocytes located in the inner plexiform layer. x 250

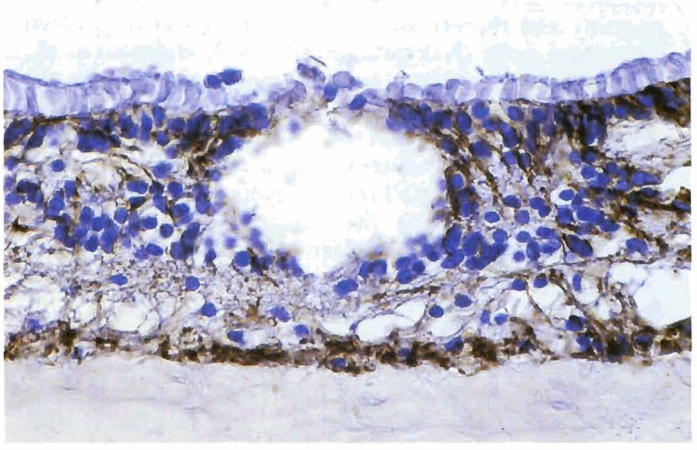

Fig. 6. Area of peripheral microcystoid degeneration of retina shows positive staining for glial fibrillary acidic protein of processes of Müller cells. x 300

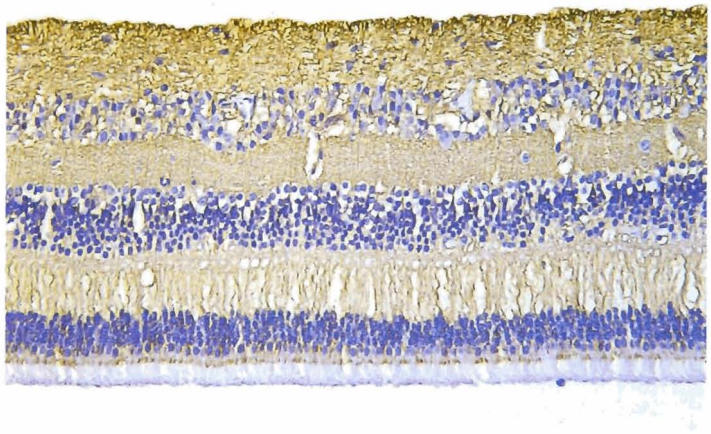

Fig. 7. Macular region shows anti-Leu-7 staining of the cytoplasmic membranes of Müller cells throughout the sensory layers, with a linear, discontinuous pattern for the outer limiting membrane. Notice that the photoreceptors are non-reactive. x 250

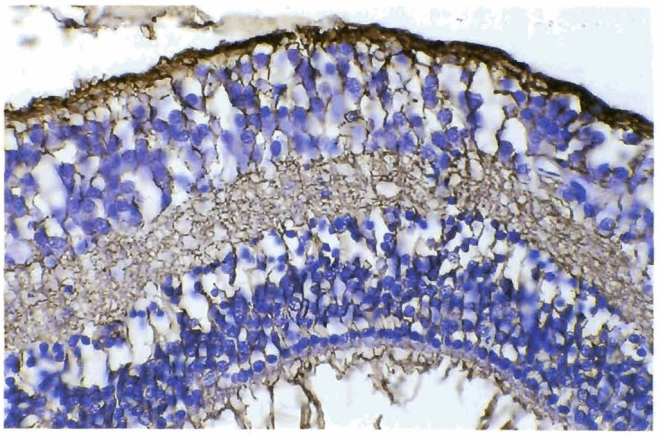

Fig. 8. Inner layers of retina (macular region) show strong Leu-7 immunoreactivity for cytoplasmic membranes of Müller cells traversing among the negatively stained ganglion cells. x 250

S-100 Protein

We found immunoreactivity of the glial cells in the perivascular regions of the nerve fiber layer. Müller cells and their processes failed to stain with S-100 protein.

Discussion

We have evaluated a panel of commercially available monoclonal antibodies to study the human retina.

Neuron-specific enolase (NSE) is composed of three major subunit types - alpha, beta and gamma-encode by different chromosomes (Abramson et al., 1989) and each type has been found in different cells and tissues. NSE is an isoenzyme of the glycolytic enzyme enolase, a cytoplasmic enzyme involved in glycolysis (Kivelä, 1986b; Jennette, 1989). Gamma-enolase (NSE) has a unique resistance to chloride-induced inactivation and it has been found to have a specific immunoreactivity not only for cells of neuroectodermal origin but also for primitive cells with the capability of differentiating into neurons and therefore, it labels neoplasms originated from these cells (Nakajima et al., 1983; Haimoto et al., 1985). NSE is present in high concentrations in the retina using radioimmunological and biochemical methods (Marangos et al., 1979). These results correlated nicely with our immunohistochemical findings showing that all retinal layers reacted positively with NSE and only the outer segments of the photoreceptors and the Müller cells were non-reactive. Our results clearly demonstrate that all neuronal cells in the human retina are expressing NSE antigen.

Synaptophysin is an integral membrane protein of unknown function purified from presynaptic vesicles of neurons from the central nervous system (Wiedenmann and Franke, 1985). Antisynaptophysin (SY) reacts with synaptic regions of the mammalian brain, retina and spinal cord and with neuroendocrine cells and tumors derivated from them (Kivelä et al., 1989). Our results with synaptophysin show a clear correlation with the synaptic regions of the human retina. In the innermost portion of the outer plexiform layer the staining pattern was vesicular outlining vividly the synapses among cone pedicules and rod spherules with bipolar and horizontal cells. The inner plexiform layer displayed a granular pattern which correlated with the fine network formed by the multiple synapses occurring in this layer. An interesting finding was the positive staining of cone inner segments found in the periphery of the retina. Kivelä et al. (1989) using SY for labelling 54 retinoblastomas found positive staining mostly confined to the apical cytoplasmic processes mainly in those tumors containing rosettes and fluerettes. They have postulated that this finding might result from a defective transport of synaptophysin-containing vesicles due to lack of processes corresponding to synaptic pedicles in well differentiated retinoblastomas. The same explanation can be applied to our findings since the cystic cavities observed in typical peripheral microcystoid degeneration of the retina are located in the outer plexiform layer causing compression and interruption of cellular transport and lack of detection of the antigen.

Of the six intermediate filaments, three are related to neuronal tissue (DeLellis and Kwan, 1988; Nagle, 1988). Neurofilament (NF) is a family of three polypeptides with molecular weights of 68, 150 and 200 Kd. Each of the polypeptides is derived from a distinct mRNA population (Nagle, 1988). Antibodies against the 200 Kd subunit label mature neurons of the central and peripheral nervous system, neoplasms of neural origin or showing neuronal differentiation and primitive neuroectodermal tumors of CNS as well as oat cell carcinomas of the lung and other neuroendocrine tumors (Kivelä et al., 1986). Using NF we stained the axons located in the ganglion cell and nerve fiber layers as well as in nerve bundles within the optic nerve properly. An interesting finding was the positive staining of corpora amylacea within the optic disc and peripapillary retina. Other species (rabbit and guinea pig) displayed positive staining of nerve fiber and ganglion cell layers, inner plexiform layer, inner nuclear layer, and outer plexiform layer (Shaw and Weber, 1984). These species are expressing different subunits of neurofilaments in each layer. The antibody that we used recognizes the subunit 200 Kd and therefore, we believe it might be the reason for the selective staining of the axons from the ganglion cells and the negative reaction in the other retinal layers. The positive staining of corpora amylacea with NF support the hypothesis regarding the axonal origin of corpora amylacea sustained by Wolter and Liss (1958) and later by Avendaño et al. (1980).

Another form of 10 nm filament is glial fibrillary acidic protein (GFAP) which is the major subunit of the intermediate filaments of astroglia. It is unique for glia but not all glia cells react positively with GFAP; some examples are the astrocytes from newborn rats and Müller cells from the retina in many species which stain positively only after suffering some kind of injury (Molnar et al., 1984a; Björklund et al., 1985; Kivelä et al., 1986; Erickson et al., 1987; Lewis et al., 1988). GFAP is useful for the identification of glial tumors, tumors with glial component and in reactive gliosis of the central and peripheral nervous system (Taylor, 1986). Previous studies (Eng, 1985; Taylor, 1986) have reported immunoreactivity for mature astrocytes in the CNS and occasional ependymal cells. Within the developing CNS, GFAP is expressed by immature astrocytes, ependymal cells and oligodendrocytes. Employing GFAP we stained astrocytes normaly located in the inner retinal layers including the perivascular astrocytes but not the Müller cells in the posterior pole and equatorial retina. Müller cells were reactive only in the peripheral retina where microcystoid degeneration was observed. It is likely that Müller cells from the human retina do not express GFAP in normal conditions unless they are activated by some kind of insult. This may occur in peripheral microcystoid degeneration where the cystic spaces in the outer plexiform layer cause mechanical stretching of Müller

cells. Several studies have used GFAP in a variety of human retinal disorders demonstrating than human Müller cells become immunoreactive for GFAP following different types of injury (Molnar et al., 1984a,b; Hiscott et al., 1984; Björklund et al., 1985; Messmer et al., 1985; Nork et al., 1986, 1987, 1990; Erickson et al., 1987; Lewis et al., 1988; Mancini et al., 1989).

Keratin is a family of 20 different polypeptides (40 Kd to 68 Kd) they label keratinizing and nonkeratinizing cells and epithelial tumors (Kasper et al., 1988). Using anti-keratin we observed a lack of immunoreactivity in all layers of the sensory retina.

LEU-7 is a monoclonal antibody originally raised against a membrane fraction of the HSB-2 human T-lymphoblastoid cell line and selected for labelling human natural killer (HNK) cells (Abo and Balch, 1981). LEU-7 detects a carbohydrate epitope on myelin-associated glycoprotein, murine neural cell adhesion molecules, pheripheral nerves, central and peripheral nervous system, neuroendocrine granules, and the cells of the amine precursor uptake and decarboxylation (APUD) system (McGarry et al., 1983; Schuller-Petrovic et al., 1983). Using LEU-7 we found a strong immunoreactivity of Müller cells. In our study, LEU-7 appears to be primarily associated with cell membranes rather than cytoplasmic components of Müller cells. Ultrastructural studies using the immunogold technique are necessary to confirm our findings that LEU-7 stains the cell membranes rather than cytoplasmic processes of Müller cells.

We conclude that NSE and LEU-7 are helpful markers for depicting most layers of the sensory retina; LEU-7 clearly outlines cell membranes of Müller cells and GFAP reacts with transformed Müller cells and with normal perivascular astrocytes while S-100 protein labels selectively the perivascular glial cells in the inner retina but does not immunoreact with Müller cells and their cytoplasmic processes. NF is specific for axons and corpora amylacea; SY labels vividly the synaptic regions and keratin is not expressed in the sensory retina. We believe this selective immunohistochemical panel is useful in delineating the normal architecture of the human retina and may be helpful, as a baseline pattern, in future studies of many pathologic conditions involving the retina.

Acknowledgements. The authors appreciate the excellent technical assistance provided by Mrs. Mary J. Keener. Supported in part by grants from the Retina Research Foundation, the Lions Eye Bank, Houston, Texas, Research to Prevent Blindness, Inc., New York, New York and The William Stamps Farish Fund and Abe and Rae Weingarten Fund.

References

Abo T. and Balch C.M. (1981). A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). J. Immunol. 127, 1024-1029.

Abramson D.H., Greenfield D.S., Ellsworth R.M., Fleisher M., Weiss R., Haik B., Schwartz M.K. and Bhalla R. (1989). Neuron-specific enolase and retinoblastoma. Clinicopathologic correlations. Retina 9, 148-152.

Avendaño J., Rodrígues M.M., Hackett J.J. and Gaskins R. (1980). Corpora amylacea of the optic nerve and retina: A form of neuronal degeneration. Invest. Ophthalmol. Vis. Sci. 19, 550-555.

BenEzra D., Chan C.C., Maftzir G., BenBassat H. and Hemo I. (1987). Heterogeneous expression of antigenic markers on retinoblasts. Opthalmic Pediatr. Genet. 8, 27-34.

Björklund A., Bignam A. and Dhal D. (1985). Immunohistochemical demonstration of glial fibrillary acidic protein in normal rat Müller glia and retinal astrocytes. Neurosci. Lett. 54, 363-372.

Cajal, Santiago Ramón; compiled and translated by Thorpe S.A. and Glickstein M. (1972). The structure of the retina. Charles C. Thomas. Publisher. Springfield, Illinois, USA.

DeLellis R.A. and Kwan P. (1988). Technical considerations in the immunohistochemical demonstration of intermediate filaments. Am. J. Surg. Pathol. 12, 17-23.

Eng L.F. (1985). Glial fibrillary acidic protein (GFAP). The major protein of glial intermediate filaments in differentiated astrocytes. J. Neuroimmunol. 8, 203-214.

Erickson P.A., Fisher S.K., Guérin C.J., Anderson D.H and Kaska D.D. (1987). Glial fibrillary acidic protein increases in Müller cells after retinal detachment. Exp. Eye Res. 44, 37-48.

Haimoto H., Takahashi Y., Koshikawa T., Nagura H. and Kato K. (1985). Immunohistochemical localization of gamma-enolase in normal human tissues other than nervous and neuroendocrine tissues. Lab. Invest. 52, 257-263.

Hiscott P.S., Grierson I., Trombetta C.J., Rahi A.H.S., Marshall J. and McLeod D. (1984). Retinal and epiretinal glia - an immunohistochemical study. Br. J. Opthalmol. 68, 698-707.

Jennette Ch.J. (1989). Markers associated with neuronal or glial differentiation. In: Immunohistology in diagnostic pathology. CRC Press, Boca Ratón, Florida. 234-256.

Kasper M., Moll R., Stosiek P. and Karsten U. (1988). Patterns of cytokeratin and vimentin expression in the human eye. Histochemistry 89, 369-377.

Kivelä T. (1986a). Expression of the HNK-1 carbohydrate epitope in human retina and retinoblastoma. An immunohistochemical study with the anti-Leu-7 monoclonal antibody. Virchows Arch. (A) 410, 139-146.

Kivelä T. (1986b). Neuron-specific enolase in retinoblastoma. An immunohistochemical study. Acta Opthalmol. 64, 19-25.

Kivelä T., Tarkkanen A. and Virtanen I. (1986). Intermediate filaments in the human retina and retinoblastoma. An immunohistochemical study of vimentin, glial fibrillary acidic protein and neurofilaments. Invest. Ophthalmol. Vis. Sci. 27, 1075-1084.

Kivelä T., Tarkkanen A. and Virtanen I. (1989). Synaptophysin in the human retina and retinoblastoma. An immunohistochemical and Western blotting study. Invest. Opthalmol. Vis. Sci. 30, 212-219.

Lewis G.P., Erickson P.A., Kaska D.D. and Fisher S.K. (1988). An immunocytochemical comparison of Müller cells and astrocytes in the cat retina. Exp. Eye Res. 47, 839-853.

Mancini M., Kennedy A., Frank R., Trese M.T., Hartzer M., Hukku B. and Lin L.R. (1989). A cell line derived from non-neoplastic human neuroretinal cells. Invest. Ophthalmol. Vis. Sci. 30, 449-508.

Marangos P.J., Schmechel D., Zis A.P. and Goodwin F.K. (1979). The

existence and neurobiological significance of neuronal and glial forms of the glycolytic enzyme enolase. Biol. Psychiatry 14, 563-579.

McGarry R.C., Helfand S.L., Quarles R.H. and Roder J.C. (1983). Recognition of myelin-associated glycoprotein by the monoclonal antibody HNK-1. Nature 306, 376-378.

Messmer E.P., Font R.L., Kirkpatrick J.B. and Höpping W. (1985). Immunohistochemical demonstration of neuronal and astrocytic differentiation in retinoblastoma. Opthalmology 92, 167-173.

Molnar J.L., Stefansson K., Marton L.S., Tripathi R.S. and Molnar G.K. (1984a). Distribution of S-100 protein and glial fibrillary acidic protein in normal and gliotic human retina. Exp. Eye Res. 38, 27-34.

Molnar J.L., Stefansson K., Marton L.S., Tripathi R.S. and Molnar G.K. (1984b). Immunohistochemistry of retinoblastomas in humans. Am. J. Ophthalmol. 97, 301-307.

Nakajima T., Kameya T., Tsumuraya M., Shimosato Y., Isobe T., Ishioka N. and Okuyama T. (1983). Immunohistochemical demonstration of neuron-specific enolase in normal and neoplastic tissues. Biomed. Res. 4, 495-504.

Nagle R.B. (1988). Intermediate filaments: A review of the basic biology. Am. J. Surg. Pathol. (Suppl) 12, 4-16.

Nork T.M., Ghobrial M.W., Gholam A.P. and Tso M.O.M. (1986). Massive retinal gliosis. A reactive proliferation of Müller cells. Arch. Ophthalmol. 104, 1383-1389.

Nork T.M., Wallow I.H.L., Sramek S.J. and Anderson G. (1987). Müller's cell involvement in proliferative diabetic retinopathy. Arch. Ophthalmol. 105, 1424-1429.

Nork T.M., Wallow I.H.L., Sramek S.J., Stevens T.S. and De-Venecia G. (1990). Immunocytochemical study of an eye with proliferative witreoretinopathy and retinal tacks. Retina 10, 78-85.

Schuller-Petrovic A., Gebhart W., Lassman H., Rumpold H. and Kraft D. (1983). A shared antigenic determinant between natural killer cells and nervous tissue. Nature 306, 179-181.

Shaw G. and Weber K. (1984). The intermediate filament complement of the retina: A comparison between different mammalian species. Eur. J. Cell Biol. 33, 95.

Taylor C.R. (1986). Immunomicroscopy: A diagnostic toll for the surgical pathologist. Vol. 19 in the series: Major problems in pathology, W.B. Saunders Co. Philadelphia. pp 15-16.

Wiedenmann B. and Franke W.W. (1985). Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of presynaptic vesicles. Cell 41, 1017.

Wolter J.R. and Liss R.L. (1958). Hyaline bodies of the human optic nerve: Histopathologic study of a case of advanced syphilitic optic atrophy. Arch. Ophthalmol. 61, 780-788.

Accepted February 4, 1993