A Monoclonal Antibody Specific to Müller Cells and Selective Synaptic Sites in the Retina

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We have produced a monoclonal antibody which stains the Müller cells in the region of the photoreceptors, nerve terminals surrounding the horizontal cells, and nerve terminals in the inner plexiform layer in carp, goldfish, and white perch retinas. Electron microscopy showed that the staining in the outer and inner retina was confined to Müller cells and presynaptic terminals, respectively. In the teleost brain, the antibody stained only the optic tectum and efferent fibers from the stratum album centrale. Biochemical characterization by immunoblotting showed that this monoclonal antibody recognizes an approximately 70-kD molecule in both whole retina and brain homogenates, suggesting that the antibody recognizes an identical molecule. No staining was noted in the spleen or the liver. This monoclonal antibody appears to be specific to a molecule common to the Müller cells and presynaptic terminals in the teleost retina, and although it is present in other parts of the central nervous system, it is confined to the visual pathway. Invest Ophthalmol Vis Sci 31:607-616, 1990

The development of the hybridoma technique¹ has provided the neurobiologist with a powerful tool to dissect and analyze the nervous system. With this technique, monoclonal antibodies have been raised against both simple and complex neural antigens, and as a result, many antigens specific to the nervous system have been identified that otherwise would have remained obscure.²⁻⁶ In our laboratory, we have shown that retinal cells can be distinguished antigenically.⁷ We have found surface antigens specific to subclasses of cells and even to certain parts of a particular subclass of retinal cells. Having established that there are surface antigens specific to 1) a class of retinal neuron, 2) subclasses, and 3) parts of retinal cells, we have continued to search for interesting retinal antigens. We describe in this report a novel molecule associated with the distal part of Müller cells and selective junctional sites in the fish retina.

Materials and Methods

A female Balb/c mouse was immunized three times with dissociated retinal cells from the carp (Cyprinus carpio) at 2-3-week intervals and was given a booster injection 3 days prior to the fusion.⁷ The animals were maintained and handled in accordance with the ARVO Resolution on the Use of Animals in Research. The hybridoma was raised from a fusion by using a modification of the method of Gefter et al.⁸ The dissociated carp retinal cell mixture was prepared by incubating isolated retinas in Ringer’s solution (130 mM NaCl, 2.8 mM KCl, 1.23 mM MgCl₂, 2.11 mM CaCl₂, 25 mM glucose, 8.4 mM Heps, 0.75 mM Na₂HPO₄) containing collagenase (1.5 mg/ml; Sigma, St. Louis, MO) for 10-15 min at room temperature (RT). After this treatment, the retinas were washed twice in Ringer’s solution and then dissociated by drawing the retinas up into a 10-ml serologic pipette several times.

Initial screening was done by a solid-phase radioimmunoassay by using 96-well flexible microtiter plates coated with dissociated carp retinal cells. Hybridomas considered positive by this assay were re-

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Supported in part by National Institutes of Health grants EY-00824 and EY-00811. LHYY is a Heed and National Research Service Award (EY-06145) fellow.

Submitted for publication: June 23, 1989; accepted August 15, 1989.

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screened, by using an immunofluorescence staining technique.

**ABC-Immunoperoxidase and Immunofluorescence Staining**

Retinas or brains from the carp (Cyprinus carpio), white perch (Roccus americana), or goldfish (Carassius auratus) were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.6, for 1 hr at RT and then overnight at 4°C. Cryostat sections (10-12 μm thick) were cut and dried onto gelatin-coated slides, preincubated with 15% normal goat serum (NGS) in phosphate-buffered saline (PBS; Sigma) for 1 hr at 4°C, and incubated with the monoclonal antibody overnight at 4°C. After washing for 1 hr, the slides were incubated with either biotin-conjugated goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) overnight at 4°C for immunoperoxidase staining or with fluorescein-conjugated goat anti-mouse IgG (Boehringer Mannheim) for 1 hr at 4°C for immunofluorescence staining. The slides were washed for 1 hr. Those prepared for immunoperoxidase staining were incubated with avidin-biotinylated peroxidase complex (ABC, prepared according to the manufacturer’s instructions; Vector Labs, Burlingame, CA) for 1 hr at 4°C, washed, and reacted with diaminobenzidine tetrahydrochloride (DAB, 0.5 mg/ml; Polysciences, Worthington, PA). Those prepared for immunofluorescence staining were mounted in glycerol and viewed with fluorescence optics.

**Double-Labeling Immunofluorescence**

Cryostat sections of white perch retina were preincubated with 15% NGS in PBS for 1 hr at 4°C and incubated with a mixture of 1D2.3 monoclonal antibody and rabbit anti-tyrosine hydroxylase antiserum (Eugene Tech International, Allendale, NJ) overnight at 4°C. After washing, the slides were incubated with fluorescein-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG (Boehringer Mannheim) for 1 hr at 4°C. The slides were washed, mounted, and viewed with fluorescence optics for fluorescein and rhodamine. To rule out any possibility of cross-fluorescence between the two filters or cross-reactivities between primary and secondary antibodies (mouse monoclonal antibody/rhodamine-conjugated goat anti-rabbit IgG, and rabbit anti-tyrosine hydroxylase serum/fluorescein-conjugated goat anti-mouse IgG), the following controls were done. Slides incubated with monoclonal antibody 1D2.3 alone and labeled with fluorescein-conjugated anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG showed no fluorescence when viewed with the rhodamine filter; slides incubated with rabbit anti-tyrosine hydroxylase serum and labeled with rhodamine-conjugated anti-rabbit IgG and fluorescein-conjugated anti-mouse IgG showed no green fluorescence when viewed with the fluorescein filter.

**ABC-Immunoperoxidase Staining for Electron Microscopy (EM)**

White perch retinas were fixed in 0.05% glutaraldehyde, 4% paraformaldehyde, PB, pH 7.6 for 1 hr at RT and subsequently in 4% paraformaldehyde, PB, pH 7.6 for 12 hr at 4°C. The retinas were cryoprotected in 30% sucrose, PB for 12 hr. To enhance penetration of reagents, the tissue was frozen in 30% sucrose with dry ice and thawed. The retinas were placed receptor side up onto a millipore filter on a Swinnex filter holder, adapted from Zucker and Yazulla. The flattened retinas were sliced into sections approximately 200 μm thick. Strips of retina were preincubated with 15% NGS, PBS for 1 hr at 37°C and then incubated with ascites fluid containing monoclonal antibody 1D2.3 or preimmune mouse serum for 36 hr at 4°C. The specimens were washed with six changes of PB for 3 hr at RT and then incubated in Vectastain biotinylated goat anti-mouse IgG (1:200, Vector Labs) for 24 hr at 4°C. The retinal strips were washed for 2 hr and subsequently incubated with avidin-biotinylated peroxidase complex (Vector, prepared according to the manufacturer’s instructions) for 6 hr at RT. After washing for 2 hr, the specimens were changed to 0.05 M Tris-HCl buffer, pH 7.6 and incubated with DAB (2 mg/ml). After 20 min, specimens were incubated in fresh DAB containing 0.01% H2O2 for another 30 min. The DAB reaction was stopped by three 10-min washes in 0.05 M Tris-HCl buffer followed by two washes in PB. The tissue was post-fixed in 2% osmium tetroxide in PB for 1 hr at 4°C and embedded in Epon 812 after dehydration in ethanol. Ultrathin sections were cut and stained with lead citrate and uranyl acetate and viewed with a JEOL 100B electron microscope at an accelerating voltage of 60 kV.

**Gel Electrophoresis and Immunoblotting**

Perch retina and brain were homogenized in 1% NP-40 (Calbiochem-Behring, La Jolla, CA), 1 mM ethylenediamine tetraacetic acid (EDTA), PBS, pH 7.4. The homogenates were centrifuged at 3000 g for 10 min to sediment cell debris and nuclei. The nuclei-cleared supernatants were microfuged at 10,000 g for 15 min at 4°C. The protein samples were diluted to a final concentration of 1% sodium dodecyl sulfate (SDS), reduced and alkylated with 50 mM dithiothreitol (DTT) and 100 mM iodoacetamide. After...
boiling for 5 min, the samples were applied to mini-
gels (Model 360, Bio-Rad Labs, Richmond, CA) with
gradient polyacrylamide of 4–20% and run according
to Laemmli. After electrophoresis, the proteins
were transferred to nitrocellulose membranes (Bio-
Rad) at a constant 400 mA for 2.5 hr as described by
Towbin et al. Membranes were blocked with 1%
Carnation milk/2% glycine, Tris-buffered saline for 5
hr at 37°C. Mouse ascites fluid containing the anti-
body 1D2.3 was used at a dilution of 1:500 and 125I-
labeled goat F(ab')2 anti-mouse IgG (5.6 μCi/μg, New
England Nuclear) was used as the secondary label at
0.12 μCi/ml. Membranes were washed with 20 mM
Tris buffer, 0.4 M NaCl, 0.25% Tween-20 (Bio-Rad),
0.25% NP-40, 2 mM EDTA, pH 7.4. Autoradiogra-
phy was performed for 8–16 hr with intensifying
screens. The molecular weight (M_r) standards used
for the blots consisted of prestained protein markers
obtained from Bethesda Research Labs.

A separate experiment (data not shown) was car-
ried out in the attempt to determine the precise local-
ization of the antigen 1D2.3 in the retinal cells. Dis-
sociated retinal cells in the absence of any detergent
were frozen and thawed three times, and the cell ly-
sate was centrifuged at 100,000 g for 1 hr at 4°C to
sediment all membrane fractions. The pellet was
washed three times, resuspended with 2% SDS and
applied to gel electrophoresis along with the super-
nantant. Both lanes showed immunologic reactivity with
the antibody 1D2.3.

Results
Staining Pattern with Monoclonal Antibody 1D2.3
by Light Microscopy

In carp, goldfish, and white perch retinas, the
monoclonal antibody 1D2.3 stained prominently in
the region of the outer limiting membrane, outer
plexiform layer, processes surrounding the horizontal
cells, and inner plexiform layer, in a laminated fash-
on as shown in Figures 1 and 2. In addition, there
was light staining in the outer nuclear layer which
delineated the photoreceptor perikarya.

Two types of staining were seen by light micros-
cope: diffuse staining in the region of the outer nu-
clear layer, and more intense, particulate staining in
the inner nuclear layer and inner plexiform layer.
These differences in staining were particularly clear
in immunofluorescent preparations, as shown in Fig-
ure 2. The staining at the level of the outer limiting
membrane was very prominent but fuzzy, and it ex-
tended slightly beyond the outer limiting membrane.
Similarly, the staining in the region of the receptor
terminals and outer plexiform layer also was prominent but diffuse. In the outer nuclear layer, the staining was light and diffuse.

In the inner nuclear layer, the labeling around the horizontal cells was much more particulate, as it was in the inner plexiform layer. In the inner plexiform layer, the monoclonal antibody 1D2.3 stained several strata. As shown in Figures 1 and 2, four strata were distinctly labeled, and in addition, there was lighter staining between these strata.

The particulate staining in the inner nuclear and inner plexiform layers suggested nerve terminal staining. In the fish inner nuclear layer, the terminals surrounding the horizontal cells were predominantly dopaminergic processes from the interplexiform cells. To test whether the staining by antibody 1D2.3 corresponded to these processes, a double-labeling experiment with antibody 1D2.3 and rabbit anti-tyrosine hydroxylase antiserum (Eugene Tech) was carried out. The controls were prepared as described in Methods to rule out any possibility of cross-fluorescence between the filters or species-cross-reactivities between primary and secondary antibodies. As shown in Figure 3, the staining in the horizontal cell layer was virtually identical with the two antibodies. Every staining site showed colabeling, and the only difference in staining pattern was the intensity, which varied slightly between the two antibodies; more intense staining of individual processes usually, although not always, was seen with anti-tyrosine hydroxylase antiserum. Our observations indicate that the two antibodies stain the same processes surrounding the horizontal cells, and that the particulate staining is related to synaptic sites.

In the inner plexiform layer, the double-labeling experiment showed strata distinctly stained with
monoclonal antibody 1D2.3 and scattered dopaminergic processes stained with anti-tyrosine hydroxylase antiserum. The majority of dopaminergic processes colabeled with 1D2.3 antibody; however, much of the staining with monoclonal antibody 1D2.3 did not colabel with anti-tyrosine hydroxylase antiserum (data not shown). This experiment suggests that the 1D2.3 molecule is localized in synaptic sites and may be common to a number of different neurotransmitter–neuromodulator strata in the inner plexiform layer.

Staining Pattern by EM

White perch retinas were processed for EM–immunoperoxidase staining and sectioning. The diffuse staining shown in Figures 1 and 2 was localized to the Müller cells. These cells extend throughout the retina, and distally they form gap junctions with each other and desmosomelike junctions with the photoreceptors. These junctional complexes are seen as the outer limiting membrane at the light microscopic level. The Müller cells also extend microvillous processes beyond the outer limiting membrane; these processes surround the inner segments and form the so-called fiber basket. As shown in Figure 4a, the staining beyond the level of the outer limiting membrane is associated with the Müller cell microvilli; the outer limiting membrane itself, i.e., the row of junctional complexes, was not stained. In the outer nuclear layer, there was Müller cell staining surrounding the photoreceptors (Fig. 4a), and in the outer plexiform layer, similar staining was interspersed between unstained photoreceptor terminals (Fig. 4b). No obvious Müller cell staining was found in the inner retina, proximal to the photoreceptors. That is, Müller cell processes were typically paler in appearance with our fixation method than were neuronal processes. Numerous Müller cell profiles were observed proximal to the photoreceptors, and invariably these were devoid of staining. It is possible that a small percentage of Müller cell processes were stained, but none of the stained processes we observed below the level of the photoreceptors could be identified positively as Müller cell processes.

As shown in Figure 5a, in the distal inner nuclear layer, staining was localized to interplexiform cell terminals found between the horizontal cells and in many cases presynaptic to them. In the inner plexiform layer, staining was found in terminals presynaptic to amacrine cells and ganglion cells (Fig. 5b). Most of the stained processes contained synaptic vesicles, and many showed synaptic sites of conventional morphology (Fig. 5a, b). Some of the labeled terminals contained large dense-core vesicles, as shown in Figure 5c. No staining was found in the bipolar cell terminals. EM thus shows that the 1D2.3 antigen is localized to the Müller cells in the outer retina and to the presynaptic terminals in the synaptic layers in the inner retina.

Specificity of the Monoclonal Antibody 1D2.3

The tissue-specificity of antibody 1D2.3 was tested in spleen, liver, and brain. No staining was noted in the spleen or liver. In the perch brain, the antibody stained only limited regions of the optic tectum and the efferent fibers from the stratum album centrale. The staining of layers stratum fibrosum et griseum superficiale, stratum griseum centrale, and stratum album centrale is shown in Figure 6. There was no staining in the spinal cord.

As to species-crossreactivity, identical staining in the retina was seen in carp, goldfish and white perch. No staining was seen in the turtle, toad, rabbit, or human retina.

Immunoblotting Analysis

Biochemical characterization of the antigen 1D2.3 with immunoblotting techniques also was carried out. Homogenates of whole perch retina and brain
Fig. 4. Electron micrographs showing immunoperoxidase staining of perch retina with monoclonal antibody ID2.3. (a) The outer limiting membrane (OLM), i.e., the row of junctional complexes, shows no staining. Note the dense Müller cell staining surrounding the photoreceptors (n, photoreceptor nucleus) and in the microvillous processes (arrowheads) distal to the OLM. (b) In a transverse section through the outer plexiform layer, Müller cell staining is seen interspersed among unstained photoreceptor terminals (arrows). Bar = 2 μm.
Fig. 5. Electron micrographs showing immunoperoxidase staining of perch retina with monoclonal antibody 1D2.3. Note the dense staining in the presynaptic terminals. (a) In the distal part of the inner nuclear layer, there is prominent staining in the interplexiform cell terminals presynaptic to the horizontal cells (H). (b) In the inner plexiform layer, dense staining is found in amacrine cell terminals (A) presynaptic to ganglion and amacrine cells. (c) This micrograph shows staining associated with large dense-core vesicles (LDV) in a terminal presynaptic to a ganglion cell (G). Bar = 0.5 μm.
were prepared in the presence of detergent, and both supernatants showed immunologic activity with the antibody 1D2.3 at an apparent M, of 70 kD, as shown in Figure 7. This suggests that the antibody recognizes an identical molecule in both organs. However, this experiment cannot determine the specific localization of the antigen in question.

In a further attempt to localize this antigen biochemically, we modified the above experiment by leaving out detergent in the initial step (data not shown). Dissociated retinal cells were frozen and thawed three times to achieve cell lysis. The cell lysate was centrifuged at high speed (100,000 g) for 1 hr to sediment all membrane fractions. The clear supernatant was aspirated and used for gel electrophoresis. Similarly, the pellet was resuspended with 2% SDS after 3 washes and applied to gel electrophoresis. Both pellet and clear supernatant showed immunologic activity, suggesting that the antigen 1D2.3 is a protein found both in membrane compartment and in cytosol.

Discussion

In this study, we describe the characterization of a mouse monoclonal antibody whose staining pattern by light microscopy suggests antibody specificity to Müller cells and to synaptic sites in the teleost retina. However, the staining pattern in the inner plexiform layer suggests that 1D2.3 antigen was not present in all synaptic sites. The inner plexiform layer of the vertebrate retina is divided into five sublayers or seven neurochemical strata, and as shown in Figures 1 and 2, only four strata—S1, S3, S4, and S7—were distinctly stained. The most distal and proximal sublayers (1 and 5) of the inner plexiform layer con-
Fig. 7. Immunoblot with monoclonal antibody 1D2.3 shows that the antibody is specific to a molecule of Mr 70 kD in both whole retina and brain homogenates. (Brain, 75 μg total protein; retina, 58 μg protein.)

The data from the biochemical analysis suggest that the antigen is present in both cytosol and membrane fractions. However, although the membrane fraction was washed several times prior to the SDS extraction, cytoplasmic contamination cannot be excluded entirely. The immunoblotting analysis also shows that the monoclonal antibody recognizes an identical molecule in both the retina and brain. Further immunoblotting analysis of the different brain regions should confirm the restriction of 1D2.3 antigen to the optic tectum, as demonstrated by immunocytochemical staining. No other tissue-cross-reactivity has been observed. Therefore, the antibody is specific to a molecule common to Müller cells and presynaptic terminals in the teleost retina, and it appears to be confined to the visual pathway. It is unclear what functional role this molecule may play.

The restriction of the 1D2.3 antigen to the distal part of the Müller cells suggests a potential role for this molecule in the interaction between Müller cells and photoreceptors. It will be of particular interest to look for the presence of this antigen in developing embryonic retinas and in newly formed synapses in perch horizontal cell cultures. The temporal relationship between the appearance of 1D2.3 antigen and the formation of synapses may provide clues to the functional role of this novel retinal molecule.

Key words: monoclonal antibody, retina, Müller cells, synaptic sites, visual pathway

Acknowledgments

The authors wish to thank Nancy Benegas for her excellent technical assistance and Dr. Charles Zucker for his early assistance with electron microscopy.

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