Monoclonal antibodies distinguish subtypes of retinal horizontal cells

(retina/hybridoma/cell culture)

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ABSTRACT Sixteen hybridomas have been identified that secrete antibodies specific to horizontal cells in the carp retina. The hybridomas have been classified into three groups based on their antibody staining patterns: group I, staining associated with all horizontal cells; group II, staining associated with the most abundant subtype of horizontal cell (CH1); and group III, staining associated with other subtypes of horizontal cells. Most of the hybridomas fall in group II; some of these antibodies stain the entire horizontal cell, but others are specific only to the cell perikarya and do not stain axonal processes. Our results suggest that there are surface molecules specific (i) to all retinal horizontal cells, (ii) to individual subtypes of horizontal cells, and (iii) to portions of horizontal cells. Furthermore, a group II antibody, which recognizes a 48- to 50-kDa membrane protein, has been found to provide a substrate selective for horizontal cell growth. Horizontal cells plated on coverslips coated with this antibody remain healthy in culture and extend long and elaborate processes for at least 3 weeks.

Horizontal cells are second-order neurons believed to mediate lateral inhibitory interactions in the outer plexiform layer of the retina (1-3). In fish, the unusually large size of these neurons has facilitated detailed investigations of their anatomy (4-10), physiology (1-3, 6, 9-13), and pharmacology (14-16). In cyprinids, four subtypes of horizontal cells have been identified on the basis of their shape, photoreceptoral and synaptic connections, and electrophysiological responses (4, 5, 7-10, 12-16). The possibility that there are, in addition, specific surface molecules associated with horizontal cells prompted a search for monoclonal antibodies that would serve as probes to detect such molecules and, hopefully, to enhance our understanding of the membrane properties of these neurons.

We report here the production and characterization of hybridomas that secrete antibodies that specifically stain horizontal cells in the carp retina. The results show that specific types and even subtypes of vertebrate central nervous system neurons can be distinguished antigenically and that a distinct complex mosaic of surface markers may be present on each cell type.

MATERIALS AND METHODS

Production of Horizontal Cell-Specific Monoclonal Antibodies. Female BALB/c mice were immunized 2-4 times with living horizontal cell preparations from carp (Cyprinus carpio) and were given booster injections 3 days prior to fusion. The hybridomas were raised from two separate fusions by using a modification of the method of Geffer et al. (17). Half of the hybridomas originated from a mouse immunized against purified horizontal cells obtained by velocity sedimentation gradients (15), and the other half originated from a mouse immunized against retinal cell mixtures enriched for horizontal cells. The enriched horizontal cell mixture was prepared by incubating isolated retinas in Ringer's solution (130 mM NaCl/2.81 mM KCl/1.23 mM MgCl2/2.11 mM CaCl2/25 mM glucose/8.4 mM Heps/0.75 mM Na2HPO4/0.44 mM KH2PO4) containing collagenase (1.5 mg/ml; Sigma) for 10-15 min at room temperature (RT). Following this treatment, the retinas were incubated in Ringer's solution containing cysteine-activated papain (DL-cysteine, 0.25 mg/ml; papain, 12.5 units/ml (Worthington)) for 15-20 min at RT. The retinas were washed twice in Ringer's solution and partially dissociated by gently drawing the retinas up into a 10-ml serological pipette. After one gentle passage through the pipette, the fragmented retina was transferred into fresh buffer, rinsed, and transferred into fresh buffer for final dissociation by more vigorous pipetting; enrichment of horizontal cells was obtained by discarding the large number of photoreceptors and ganglion cells separated in the initial dissociation phase.

Initial screening was by a solid-phase radioimmunoassay using 96-well flexible microtiter plates coated with enriched horizontal cell mixtures. Hybridomas considered positive by this assay were rescreened, using an immunofluorescence staining technique.

ABC-Immunoperoxidase and Immunofluorescence Staining. Retinas from carp, white perch (Roccus americana), or goldfish (Carassius auratus) were fixed for 1-1.5 hr at 4°C in 0.1% glutaraldehyde/25% paraformaldehyde/0.13% picric acid in 0.08 M sodium phosphate buffer, pH 7.4. Cryostat sections (10-12 μm) were cut and dried onto gelatin-coated slides, preincubated with 15% normal goat serum in phosphate-buffered saline for 1 hr at 4°C, and incubated with the primary antibody overnight at 4°C. After washing (1 hr), the slides were incubated with either biotin-conjugated goat anti-mouse immunoglobulins (Cappel Laboratories, Cochranville, PA) overnight at 4°C for immunoperoxidase staining or with rhodamine or fluorescein-conjugated goat anti-mouse immunoglobulins (Cappel) for 1 hr at 4°C for immunofluorescence staining. The slides were again washed; those prepared for immunoperoxidase staining were incubated with avidin-biotinylated peroxidase complex (Vectorstain, prepared according to the manufacturer's instruction) for 2.5 hr at 4°C, washed, and underwent reaction with diaminobenzidine (Polyscience, Warrington, PA); those prepared for immunofluorescence staining were mounted with 50% (vol/vol) glycerol in 0.08 M phosphate buffer.

Establishing Horizontal Cell Culture. Aliquots of isolated horizontal cells were distributed onto sterile polylysine-coated coverslips inside Petri dishes. The preparations were used either immediately after washing, or were incubated in modified L-15 medium (16) in humidified chambers at RT. For immunofluorescence staining, the coverslips were fixed in 0.1% glutaraldehyde for 5 min at RT, blocked with 15% nor-

Abbreviations: CH1, CH2, and CH3, cone horizontal cells types 1, 2, and 3, respectively; RH, rod horizontal cells.

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nal goat serum for 15 min at RT, and incubated with horizontal cell-specific monoclonal antibodies for 1 hr at 4°C. They were rinsed and incubated in fluorescein-conjugated goat anti-mouse immunoglobulins (Cappel). After incubation for 30 min at RT, coverslips were washed and mounted on slides with 50% (vol/vol) glycerol in 0.08 M phosphate buffer. Slides were viewed with phase contrast and fluorescence optics.

For cell-adhesion experiments, coverslips were coated with antibody HC-II.7 (ammonium sulfate-precipitated ascites, 0.2 mg/ml), polylysine (25 μg/ml), or an unrelated IgG1 for 2 hr at 37°C

Horizontal cell isolation. Plasma membrane preparations were isolated from enriched horizontal cell fractions using the two-phase dextran-polyethylene glycol method of Brunette and Till (18) with some modifications adapted from Yeh et al. (19). The membrane proteins were solubilized with 1% Triton X-100 and iodinated using the method of Bolton and Hunter (20) (125I-labeled Bolton–Hunter reagent; Amersham). Iodinated plasma membrane proteins were incubated with horiztonal cell-specific antibody (ascites precipitated with ammonium sulfate) or preimmune serum (precipitated with ammonium sulfate) diluted in the immunoprecipitation buffer (IP, 150 mM Tris-HCl/0.15 M NaCl/1 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride, pH 7.4) with 2% bovine serum albumin and 1% Triton X-100 at 4°C on a shaker. After incubating for 1 hr, Immunobead (Bio-Rad, previously equilibrated in the IP buffer/1% Triton) was added into each tube and allowed to incubate for another hour. Tubes were spun in a Microfuge for 5 min at 4°C and supernatants were discarded. Pellets were washed 3 times with ice-cold IP buffer/1% Triton, and bound iodinated proteins were eluted by boiling in 5% NaDodSO4/1% 2-mercaptoethanol for 2 min. Supernatants were subjected to 10% polyacrylamide gel electrophoresis by using the procedure of Laemmli (21). Iodinated proteins were visualized by autoradiography.

RESULTS
Staining Patterns Produced by Horizontal Cell-Specific Antibodies. Sixteen hybridomas were identified that secrete antibodies specific to horizontal cells. The hybridomas were classified into three groups with respect to the staining of horizontal cells produced by their light microscopic level using standard indirect immunofluorescent and ABC-immunoperoxidase staining techniques. The three cone horizontal cell subtypes and one rod horizontal cell subtype found in the carp retina are located in well-defined layers (Fig. 1a). The type I cone horizontal cells (CH1) are located most distally in the inner nuclear layer, while the type 2 and type 3 cone horizontal cells (CH2 and CH3) and rod horizontal cells (RH) are located proximal to the CH1 horizontal cells. One of the hybridomas was classified in group I—i.e., staining by its antibody appeared to be associated with all horizontal cells. Fourteen of the hybridomas were classified in group II—i.e., staining by these antibodies was associated only with the distally positioned CH1 horizontal cells. One hybridoma was in group III—i.e., staining by its antibody was associated with a proximal non-CH1 type of horizontal cell. Fig. 1b–d shows immunoperoxidase staining patterns elicited by the three different groups of antibodies; Fig. 1e shows staining of most, if not all, horizontal cell perikarya with an antibody from a group I hybridoma; Fig. 1c shows staining of the perikarya of the most distal horizontal cells, the CH1 cells, with an antibody from a group II hybridoma; Fig. 1d shows staining of horizontal cell perikarya proximal to the CH1 cells by an antibody from a group III hybridoma.

In all three micrographs of Fig. 1, some staining deeper in the inner nuclear layer can be seen, which corresponds to the location of the axon-like processes that extend from the cell perikarya and terminate close to the inner plexiform layer. The staining of these processes is shown particularly clearly in Fig. 1c and d (arrows). In cyprinids, only cone horizontal cells appear to extend axon-like processes (7, 8, 10), suggesting that the cells stained in Fig. 1d are either CH2 or CH3 horizontal cells, or both.

CH1-Specific Antibodies. Group II hybridomas were most commonly isolated probably because the majority (~65%) of the horizontal cells in the cyprinid retina are of the CH1 type (7). Of the CH1-specific antibodies, a number appeared to recognize different antigens or antigenic determinants on the CH1 cell because they demonstrated different staining patterns. Since there is available a variety of group II antibodies, we have so far focused much of our work on these, particularly on one antibody HC-II.7, which was determined to be an IgG1 monoclonal antibody. Fig. 2a and b shows staining by antibody HC-II.7, using an indirect immunofluorescence staining technique. The staining extends all along the CH1 cell body layer and, in addition, there is some fainter staining deeper in the inner nuclear layer (arrowheads)—i.e., this antibody appears to stain both the cell perikaryon and

Fig. 1. (a) Schematic diagram indicating the location of the subtypes of horizontal cells in the inner nuclear layer of carp retina. The type I cone horizontal cells (H1) are located most distally and their cell bodies form a distinct band. Type II and type III cone horizontal cells (H2 and H3) and the rod horizontal cells (RH) are located proximal to the CH1 cells and their cell bodies are less distinctly layered. Arrows indicate axonal processes that extend from the CH1 cells toward the inner plexiform layer (IPL). ONL, outer nuclear layer. (b–d) ABC-immunoperoxidase staining by three different antibodies. (b) Staining of all subtypes of horizontal cells. (c) Staining of CH1 cells only. (d) Staining of non-CH1 horizontal cells, which are located proximally to a clearly unstained CH1 layer. Axonal processes located deeper in the inner nuclear layer are indicated by arrows. (Bar = 10 μm.)
by antibody HC-II.7. Staining in all cases appeared to be confined to the cell surface. This is shown particularly clearly in the lower part of Fig. 3b and the axon terminal in Fig. 3c, in which the micrograph focus was approximately at the cell equator. We have also observed that living cells show similar immunofluorescence staining, again indicating that the specific antigens are localized on the cell surface. Not all group II antibodies stained all parts of the cultured CHI horizontal cells; four (such as HC-II.2 and HC-II.3) reacted only with the cell perikarya and did not stain the axonal processes.

Further evidence that the antigens recognized by antibodies HC-II.7 and HC-II.3 are plasma membrane proteins and also different molecules was provided by a biochemical characterization of the antigens. Plasma membranes from enriched horizontal cell mixtures were isolated, and the membrane proteins were iodinated using a mild iodination procedure to ensure minimal loss of immunological activity (20). After reaction with antibody, the proteins were precipitated and run on a polyacrylamide gel. With antibody HC-II.7, as shown in Fig. 4, a single band of 48–50 kDa appeared on the resulting autoradiogram. With antibody HC-II-3, using a similar approach, a plasma membrane molecule of 40–45 kDa was identified.

We have also tested the cell specificity of antibody HC-II.7 by examining its reactivity with other parts of the carp central nervous system. Staining was found to occur in only

For the axon process. At higher magnification (Fig. 2b), it can be seen that in the CHI cell body layer, staining is confined to cell contours, suggesting that the specific antigen is on the cell surface. The immunoperoxidase staining produced by this particular antibody is shown in Fig. 1c.

Staining by another group II antibody, HC-II.2, is shown in Fig. 2c. Although prominent staining is again seen associated with CHI cell perikarya, it is distributed in a patchy fashion. The patchy staining also appears to be confined to the CHI cell surface. Another clearly distinguishable staining pattern was observed with antibody HC-II.3. Although its staining of CHI cell perikarya appeared to be similar to that of antibody HC-II.7, no staining was found deeper in the inner nuclear layer—i.e., the antigen recognized by this antibody is apparently not present on the axon-like processes. With antibody HC-II-2 also, no staining was observed deeper in the inner nuclear layer.

To analyze further the location of the horizontal cell-specific antigens, and to identify directly staining of the so-called axon processes of the cone horizontal cells, we tested several group II antibodies on isolated horizontal cells in freshly prepared or in 24-hr-old cell cultures of carp retina. Fig. 3 shows immunofluorescence labeling of fixed isolated horizontal cell perikarya (a and b) and an axon terminal (c)
one other type of cell, which was in the optic tectum. With regard to species crossreactivity, staining in the closely related goldfish retina with antibody HC-II.7 was identical to that in the carp retina, but there was no staining in the white perch retina. The absence of staining in the perch retina suggests that the antigen recognized by antibody HC-II.7 is not a common horizontal cell antigen shared by all teleosts. On the other hand, with antibody HC-III.1 (shown in Fig. 1d), staining of horizontal cells was observed in both goldfish and white perch retinas.

**Cell Culture Enhancement Provided by Antibody HC-II.7.**

Adult carp horizontal cells ordinarily do not survive more than 7–10 days in cultures, even when grown on polysylisine-coated coverslips. After 3–4 days, vacuoles appear in the cytoplasm of many cells and the number of adhering cells decreases rapidly thereafter. After 7 days in culture, the majority of those horizontal cells still attached to the coverslip are rounded, and processes extending from the cells are only rarely seen.

Horizontal cells maintained on HC-II.7 antibody-coated coverslips, on the other hand, were more numerous, had more processes, and survived longer than cells grown on control or polysylisine-coated coverslips (Fig. 5). For example, immediately after settling (3 hr), there was a 20- to 25-fold increase in the number of horizontal cells attached to HC-II.7-coated coverslips as compared to control coverslips coated with an unrelated IgG monoclonal antibody, and a 4- to 5-fold increase as compared to polysylisine-coated coverslips. Furthermore, at least 85% of the horizontal cells observed in the HC-II.7 antibody cultures had extensive processes after 3 hr, whereas in control cultures, only 50% showed such processes. Horizontal cells on antibody HC-II.7-coated coverslips also survived longer, with a large fraction living >2 weeks. At least half of the cells retained processes, which were usually observed only for the first few days of cultures grown on polysylisine-coated coverslips, and only for the first few hours on cells grown on control coverslips. The processes on the cells showed qualitative differences as well; horizontal cells in HC-II.7 cultures tended to have a complex network of processes (Fig. 5), in contrast to cells maintained on polysylisine-treated coverslips, which typically had only 1–3 processes. The majority of horizontal cells grown on antibody HC-II.7 did not develop vacuoles until after day 15. Table 1 provides a detailed comparison of horizontal cell survival in culture when maintained on control, polysylisine-, or antibody HC-II.7-coated coverslips over the course of 21 days. Finally, horizontal cells maintained on antibody HC-II.7-coated coverslips were tested electrophysiologically, and they showed normal resting potentials (~60 to ~80 mV) and vigorous responses to L-glutamate and its analogue kainate (16).

**DISCUSSION**

In undertaking these experiments, we had three questions in mind. First, is it possible to raise monoclonal antibodies to a specific type of neuron—namely, the retinal horizontal cell; second, is it possible to raise antibodies that will react only with the surfaces of these cells; and third, is it possible to raise antibodies that will react only with horizontal cell subtypes? Our experiments indicate positive answers to all three of these questions, and they have provided other interesting observations as well. We have raised monoclonal antibodies that appear to recognize (i) all horizontal cells in the retina but no other type of retinal neuron, (ii) specific subtypes of horizontal cells, and (iii) discrete regions of one subtype of horizontal cell. Furthermore, of the seven hybridomas that have been studied most extensively, all appear to secrete antibodies specific to the horizontal cell surface. This was the case whether the antibodies reacted with cells fixed in the intact retina, with cells isolated and fixed, or with isolated cells unfixed and living. These data thus suggest that there are (i) surface antigens common to all horizontal cells but not to other retinal neurons, (ii) surface antigens specific to subtypes of horizontal cells, and (iii) surface antigens found only on certain parts of horizontal cells.

We have been successful in obtaining monoclonal antibodies specific to the surfaces of horizontal cells and their subtypes probably because we used fractions enriched in intact horizontal cells for both immunizing and screening purposes. This clearly differs from the commonly used shotgun approach of using heterogeneous cell or membrane preparations to generate antibodies against neural cells. Thus, by preparing specific cell preparations for immunization and

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**Table 1.** Relative cell-adhesion enhancement provided by antibody HC-II.7 and polysylisine

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<th>Process</th>
<th>Relative number of horizontal cells/percentage with processes</th>
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<tr>
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<td>Control</td>
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<td>Polysylisine</td>
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<td>HC-II.7</td>
<td>20–25X/85</td>
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Enriched horizontal cell fractions were plated on coverslips coated with antibody HC-II.7, polysylisine, or an unrelated IgG (control). X was assumed to represent the number of horizontal cells counted in a field of fixed size from control (3 hr) cultures, and the number of horizontal cells counted in antibody HC-II.7 and polysylisine cultures were calculated and recorded relative to control. The number after the slash gives the percentage of cells that had processes.
screening, it was possible to generate monoclonal antibodies of surprising specificity. For example, antibody HC-II.7 reacts with carp CH1 horizontal cells but no other retinal cell and none of the other horizontal cell subtypes. Furthermore, when tested in other parts of the carp central nervous system, staining was found in only one other type of cell, which was, interestingly, in the optic tectum. This finding may be significant in that the optic tectum is also part of the central nervous system involved in visual processing. If identical antigens are being recognized, it will be important to identify the functional role of this molecule. Finally, the nonreactivity of antibody HC-II.7 with perch retina suggests that certain cell-specific antigens may be species specific.

The different staining patterns observed with the variety of group II antibodies, along with data on the biochemical characterization of the CH1-specific antigens, indicate that at least 3, and perhaps as many as 6, of the 14 CH1 hybridomas secrete antibodies that recognize different antigens or antigenic determinants. These results suggest that a single cell subtype, in this case the CH1 horizontal cells, carries a number of different specific antigenic markers.

An obvious question is whether other neurons have this antigenic diversity associated with their surfaces. Evidence from a number of laboratories and from a variety of preparations indicates that this is likely to be the case (22–32). For example, monoclonal antibodies have recently been described that recognize the surface of all or part of rat photoreceptors (22), subsets of leech neurons (23), and a variety of types of vertebrate and invertebrate central nervous system neurons (24–32). The present paper provides evidence that the surfaces of structurally and functionally distinct subtypes of a particular class of vertebrate central nervous system neuron can be distinguished antigenically.

MacLeish et al. (33), using salamander neurons, showed that certain antibodies can enhance mature neural cell growth in culture. Their findings have been confirmed for mammalian cells (34). With antibody HC-II.7, we have shown that a cell-subtype-specific antibody can enhance selectively the appearance of horizontal cells in culture and prolong their survival. Horizontal cells maintained on antibody HC-II.7-coated coverslips have been tested electrophysiologically, and they showed normal resting potentials and strong responses to drugs such as t-glutamate and its analogue kainate. How the antibody exerts these effects is not clear. The enhancement of horizontal cell survival in culture does not appear to be a nonspecific effect of mouse immunoglobulins, as no improvement was found when preimmune serum or an unrelated IgG1 monoclonal antibody was used to coat coverslips. As was pointed out by MacLeish et al. (33), it will be important to determine whether the antibody is simply serving as a better substrate for the cells (i.e., provides better cell adhesion) or is doing something more specific to the isolated neurons.

Finally, it should be noted that the monoclonal antibodies we raised may be useful for preparing homogeneous fractions containing single subtypes of horizontal cells for biochemical and physiological studies. Furthermore, antibodies that recognize surface molecules restricted to cell subtypes and to regions within a single cell subtype, may lead to the identification of cell surface molecules that mediate functions specific to these cells, thus extending our understanding of the molecular basis of neuronal cell structure and function. In this regard, we have tested the effects of several group II antibodies on cyclic AMP production in dispersed retinal cell preparations. As yet, however, we have not observed significant accumulations of cyclic AMP induced by any of the antibodies tested, nor do they seem capable of blocking the accumulation of cyclic AMP induced by dopamine (15).

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