Selective Destruction of Photoreceptor Cells by Anti-Opsin Antibodies

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This article reports that cultured photoreceptor cells are selectively vulnerable to complement-mediated lysis by antiopsin antisera. The study has been carried out using a culture system that permits the growth of embryonic retinal neurons and photoreceptor cells in the absence of glial, pigment epithelial, connective, or endothelial cell contamination. Exposure of these cultures to an antiopsin antiserum in the presence of complement results in the lysis of 85% of the photoreceptor cells, without extensive loss of neurons. Photoreceptor lysis by the antibody is dose-dependent and specific, as demonstrated by the ability to block photoreceptor lysis by preincubation of the antiopsin antiserum with purified rhodopsin. Photoreceptor sensitivity to lysis by these antibodies develops in vitro in parallel with the appearance of immunocytochemically detectable opsin. Multipolar neurons (which do not contain opsin) are not affected by the treatment, as shown by microscopic analysis and determination of neuronal "markers" such as choline acetyltransferase and GABA high affinity uptake. These results show that antiopsin antibodies can cause selective photoreceptor degeneration. Moreover, this in vitro system appears useful as a bioassay to test a variety of possible mechanisms of retinal cell destruction which may be important in vivo. The results suggest also a mechanism for the generation of enriched retinal neuronal cultures by selective lysis of photoreceptor cells. Invest Ophthalmol Vis Sci 28:118-125, 1987

Autoimmune responses to retinal antigens might be involved in diseases such as uveitis, pars planitis, retinal vasculitis and retinitis pigmentosa, but their precise role in the pathophysiology of these ocular lesions remains to be established. Many different but complementary questions must be answered to verify this hypothesis, including the identity of the relevant retinal antigens, the nature of the immune responses that they trigger, the role of the blood-retinal barrier in the immunization process, and the differential susceptibility of each retinal cell type to destruction by specific cellular or humoral immune mechanisms. Evidence derived from animal studies indicates that several soluble and particulate proteins of the outer segment can act as antigens and induce immune-mediated retinal lesions. One of the suspected antigens is the visual pigment, rhodopsin, which is selectively concentrated in rod outer segments. In laboratory animals, retinal damage resulting from experimental immunization with rhodopsin is very complex, since degenerative changes are not always restricted to photoreceptors, and are frequently accompanied by retinal infiltration with macrophages and other inflammatory cells. It is not clear whether opsin-containing photoreceptors and opsin-free neurons are destroyed by similar mechanisms. Furthermore, whether photoreceptor destruction is caused by cellular or humoral immune mechanisms has not been elucidated. The complexity of the intact animal poses great difficulty for the investigation of some of these questions in vivo.

Recently, conditions have been described in this laboratory that allow the growth of embryonic retinal neurons and photoreceptors in the absence of glial, pigment epithelial, connective, or endothelial cell contamination. Neurons and photoreceptors can be studied with accurate analytic techniques and identified by objective criteria. Immunocytochemical studies have shown that opsin immunoreactive materials are not detectable in cultured retinal neurons, but are present on the surface of the photoreceptors. These observations suggested that the culture system might be of use in the analysis of selective cellular responses to antiopsin immune mechanisms.

We report here that cultured photoreceptors are se-
lectively vulnerable to complement-mediated lysis by antiopsin antisera. Specificity was shown by the absence of neuronal degeneration in treated cultures, as well as by the ability to block photoreceptor lysis by preincubating the antiopsin antisera with purified rhodopsin. These results show that antiopsin antibodies can cause selective photoreceptor degeneration even in the absence of inflammatory cells. Our results suggest a mechanism for the generation of enriched retinal neuronal cultures by selective lysis of photoreceptor cells, and provide an in vitro system to test a variety of possible mechanisms of retinal cell destruction which may be important in vivo.

Materials and Methods

Materials

The following supplies were used: Costar tissue culture plastic, 16-mm wells (Costar Plastics; Cambridge, MA); Falcon tissue culture plastic, 35-mm dishes (Falcon; Oxnard, CA); trypan blue (GIBCO; Grand Island, NY); medium 199, penicillin, trypsin (Irvine); fetal calf serum (Hyclone); polyornithine, bovine serum albumin, pyruvic acid, transferrin, progesterone, linoleic acid/bovine serum albumin, glutaraldehyde, glutamine, tetrathynyl boron, butylethylketone, EDTA, choline chloride, acetylcholine chloride Tris hydrochloride (Sigma Chemical; St. Louis, MO); NVP, guinea pig complement (Calbiochem; San Diego, CA); Ammonyx-LO® (Onyx Chemical Co); Nuclear Track Emulsion NTB2, NaCl, Dextol, Fixer (Kodak; Rochester, NY); Hematicolor® (American Scientific; McGraw Park, IL). Isotopes used were: 3H-GABA [amino butyric acid, gamma-2- 3H-(N)], specific activity 84.7 Ci/mmol (New England Nuclear; Boston, MA); acetylecoenzyme A [1-14C], specific activity 58 mCi/mm (Amersham; Arlington Heights, IL). Purified bovine rhodopsin was a gift from Dr. P. O'Brien (NEI, NIH). Sheep antirhodopsin antisemur was a gift from Dr. D. Papermaster (Yale University).

Cell Cultures

Methods to generate low density monolayers of neurons and photoreceptors from 8-day white Leghorn chick retinal embryos have been described in detail elsewhere. Briefly, neural retinas were dissected from other eye tissues and dissociated mechanically after mild trypsinization. The cells were suspended in 199 medium supplemented with 10% heat-inactivated fetal calf serum, 110 μg/ml linoleic acid BSA complex, 2 mM glutamine, and 10,000 U/L penicillin, and seeded in polyornithine-coated 35-mm plastic dishes or 16-mm plastic wells at a ratio of 8 X 10^5 or 1.5 X 10^5 cells per dish or well, respectively. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

Antibody Treatment

All the experiments (except when indicated) were carried out using 6-day in vitro cultures. The cultures were rinsed twice in serum-free medium 199 containing pyruvate (3.8 X 10^{-3} M), insulin (8.3 X 10^{-3} M), transferrin (6.3 X 10^{-3} M), progesterone (2.0 X 10^{-5} M), linoleic acid (4.0 X 10^{-3} M), bovine serum albumin (1.7 X 10^{-3} M), glutamine (2 X 10^{-3} M), and penicillin (10,000 U/L) as described by Lindsey and Adler, and incubated for 1 hr in the same medium. They were then incubated at room temperature for 15 min with different dilutions of antiopsin antiserum in this same chemically defined medium. At that time, the cultures received complement at a final concentration of 1.5% (V:V), and were further incubated for 1 hr at 37°C. Control cultures received either complement alone or antisemur alone.

Quantitative Analysis of Cell Survival

Total number of cells was determined with an AR-TEK image analysis system (Artek Systems Co., Farmingdale, NY). The relative distribution of different cell types was established by phase contrast microscopic analysis of 200 cells chosen at random. Cells with two or more neurites were classified as multipolar neurons. Photoreceptors were identified by their elongated shape, the presence of a single, short neurite, and a conspicuous inner segment containing a lipid droplet. The identity of these cells has been corroborated by scanning and transmission electron microscopy, by the cytochemical demonstration of peanut lectin receptors, and by immunocytochemical detection of the visual pigment protein opsin. Cell viability was confirmed by dye exclusion using trypan blue. Lysed photoreceptor cells could be easily recognized as swollen, usually circular, phase dull “ghosts,” which appeared empty except for the presence of the nucleus and lipid droplet. These cells were stained by trypan blue in the dye exclusion test. The data are averages of at least two separate experiments run in triplicate.

High Affinity Uptake of 3H-GABA

These experiments were carried out using protocols already described in detail. The cultures were rinsed twice with a Tris-Hepes buffered medium (40 mM Tris-Hepes, 1 mM MgCl2; 5 mM KCl and 140 mM NaCl at pH 7.4) and incubated for 15 min at 37°C with a 5 X 10^{-3} M solution of tritiated GABA in the same buffer. After two rinses in isotope-free buffer, the cells were either lysed with distilled water for determination of intracellular radioactivity by scintillation counting, or fixed with 2% glutaraldehyde for 1 hr, dehydrated in an increasing ethanol series, and coated with Kodak

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treated cultures as compared with the corresponding contrast, multipolar neurons appear unchanged in nucleus and lipid droplet usually remained visible. In dish appeared as swollen, phase-dull cells with a circular outline and an "empty" appearance, although the nucleus and lipid droplet usually remained visible. In contrast, multipolar neurons appear unchanged in treated cultures as compared with the corresponding controls. No photoreceptor lysis could be seen in cultures treated separately with either complement alone or antiserum alone.

Dose-Response Curves

To determine the optimal antiserum concentration necessary for maximal photoreceptor lysis, 6-day cultures were incubated for 1 hr with different antiserum dilutions in the presence of a constant amount of complement. Photoreceptor lysis occurred in a concentration-dependent manner (Fig. 2). Some photoreceptor losses were seen with a 1:400 dilution, and a maximum lysis of 85% of the photoreceptors was reached with a 1:30 dilution. Less than 10% of the neurons were lost from cultures treated with these different concentrations of antibody (Fig. 2). Higher numbers of photoreceptors could be lysed at antibody dilutions lower than 1:50, but some evidence of neuronal damage, such as neurite loss or fragmentation, became evident at the higher concentrations (data not shown).

Specificity of the Antipsin Antibody

The observations described above indicate that an antibody present in the antipsin antiserum could cause photoreceptor lysis in the presence of complement. Although antipsin antibodies are known to be present in this antiserum, further data appeared necessary to elucidate whether they were actually responsible for photoreceptor lysis. To evaluate this point, experiments were carried out to determine whether purified rhodopsin could prevent the lysis of photoreceptors exposed to the antiserum in the presence of complement.

The experiments were somewhat complicated by the toxicity of the rhodopsin solution used which contained 0.55 mg/mL rhodopsin, 0.3% (V/V) Ammonyx-LO®, detergent, and 0.01% sodium azide. For this reason, "blocking" experiments were designed as follows. A 50-µl aliquot of antipsin antiserum was incubated for 3 hr at 4°C with 150 µl of the untreated rhodopsin solution. Another antiserum aliquot was incubated under the same conditions with 150 µl of a solution containing 0.3% Ammonyx-LO® detergent and 0.01% azide in Tris-HCl buffer, pH 8.0 (ie, all the substances present in the opsin solution, except opsin itself). At the end of the 3-hr incubation, both samples were dialyzed against 2500 ml of serum-free culture medium using a constant flow microdialysis system. The samples were then tested in the cytotoxicity assay at a 1:50 final dilution in the presence of complement. Untreated antiserum was also tested as a "positive control." Two separate experiments showed the same results: there was no photoreceptor lysis in cultures exposed to antipsin antiserum preadsorbed with rhodopsin, while antiserum treated only with detergent

Choline Acetyltransferase (CAT) Assay

CAT activity was investigated in retinal cultures following with minor modifications the method of Fonnum.17 14C-acetyloenzyme A (AcCoA) was used as radioactive acetylcholine precursor. Cultured cells were harvested after treatment of the dishes for 3–5 min with 0.05% trypsin and 0.02% EDTA in Ca++-Mg++. Free buffer, washed twice by centrifugation, and homogenized by sonication in 5 µl of 0.5% Triton X-100 solution. Homogenates were incubated with an assay mixture containing 14C-labeled AcCoA at 37°C for 15 min and the reaction was terminated with a solution containing 35.7 mg/L acetylcholine chloride in 0.01 M Na2HPO4, pH 7. Boiled samples, run in parallel, were used as controls. The enzymatic activity was completely abolished by 4 mM NVP (hydroxyethyl-4-naphthylvinylpyridium), a selective CAT inhibitor. After separation of radioactive acetylcholine from the unutilized 14C-AcCoA, radioactivity of the samples was measured by liquid scintillation counting. Results are expressed as fmoles acetylcholine/min/35-mm dish.

Results

The Retinal Cultures

At the seeding densities used in these experiments, 6-day cultures from 8-day neural retina contain about 9.7 X 10⁴ cells per 35-mm dish and 1.4 X 10⁴ cells per 16-mm well. The cultures showed three easily recognizable cell populations: multipolar neurons (68%), photoreceptors (9%), and neurite-free round cells (23%). The phase microscopic, ultrastructural, and cytochemical criteria used to identify these cells have been presented in Materials and Methods. A representative field of these cultures is shown in Figure 1A.

Cultures Treated With Antipsin Antiserum in the Presence of Complement: Qualitative Description

Fig. 1B shows a culture treated for 1 hr with antipsin antiserum in the presence of complement. Selective lysis of the majority of the photoreceptor cells could be observed (see quantitative analysis below). Those lysed photoreceptors which remained attached to the dish appeared as swollen, phase-dull cells with a circular outline and an "empty" appearance, although the nucleus and lipid droplet usually remained visible. In contrast, multipolar neurons appear unchanged in treated cultures as compared with the corresponding controls. No photoreceptor lysis could be seen in cultures treated separately with either complement alone or antiserum alone.

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Fig. 1. Monolayer cultures from 8-day chick embryo neural retina grown for 6 days in vitro. A, Untreated culture. Note the presence of multipolar neurons (short arrow) and photoreceptor cells (long arrow). The identity of these photoreceptor cells has been corroborated by scanning and transmission electron microscopy, lectin cytochemistry, and by the immunocytochemical demonstration of opsin immunoreactive materials. B, Similar culture treated with a 1:50 dilution of antiopsin antiserum in the presence of complement. Multipolar neurons appear unaffected. Note the presence of photoreceptor "ghosts" (arrows). C, Higher magnification phase contrast photomicrographs of photoreceptor cells in a control culture (C1), and in a culture treated with antiopsin antibody plus complement (C2). Note that the lysed photoreceptor in C2 appears phase-dull and swollen. The nucleus (N) and lipid droplet (L) can still be recognized in this cell (A, B: X375; C1, C2: X685).

and azide (in the absence of opsin) caused the complement-mediated lysis of approximately 85% of the photoreceptors. Thus, the experiments supported the contention that antiopsin antibodies are specifically responsible for complement-mediated photoreceptor lysis.

Development of Sensitivity to Antiopsin Antiserum

Opsin antigens are not detectable in photoreceptor cells at the beginning of the culture period, but develop as the cells mature in vitro.11,13 Experiments were carried out to determine whether the appearance of photoreceptor sensitivity to antiopsin antiserum followed a similar developmental timetable. Two-, 4-, 5-, and 6-day cultures were incubated for 1 hr with a 1:50 antiserum dilution in the presence of complement. As shown in Figure 3, no losses of either neurons or photoreceptors were detectable in 2-day cultures. Some photoreceptor sensitivity to the antiopsin antiserum was already present by 4 days in vitro, when about 50% of photoreceptors were destroyed by the treatment. A plateau was reached by 5 days in vitro, when a maximal destruction of 80–85% of the photoreceptors was ob-
A MULTIPOLAR NEURONS • PHOTORECEPTOR CELLS

Fig. 2. Concentration dependence of the complement-mediated photoreceptor lysis by antiopsin antisera. Replicate cultures were incubated for 1 hr in the presence of a constant amount of complement and different antibody dilutions. The results are the average of triplicate determinations from two separate experiments.

MULTIPOLAR NEURONS • PHOTORECEPTORS

Fig. 3. Development of photoreceptor sensitivity to complement-mediated lysis by antiopsin antisera. Retinal cultures grown for 2, 4, 5, or 6 days in vitro were treated with antiopsin antiserum in the presence of complement, as described in Methods. No photoreceptor lysis could be seen after 2 days in vitro. There was some lysis in 4-day cultures and a maximum response by days 5–6. This behavior correlates well with our previous report that immunocytochemically detectable opsin antigens do not appear in these cultured cells before 4 days in vitro.11,13

Fig. 4. Neuronal viability after culture treatment with either complement alone (open bars) or complement plus a 1:50 dilution of antiopsin antiserum (solid bars). At the end of the treatment, the cultures were returned to antibody-free, complement-free, chemically defined medium, and allowed to develop for a further 24-hr period. Neuronal survival was practically unaffected by the antibody treatment. The results are the average of triplicate determinations in two separate experiments.

Neuronal Viability After Treatment With Antiopsin Antiserum Plus Complement

Long-term neuronal survival: Six-day cultures, treated for 1 hr with either antiserum plus complement or complement alone, were rinsed in and returned to antiserum-free, complement-free, serum-free culture medium, and allowed to develop for a further 24-hr period. As seen in Figure 4, the number of neurons surviving in cultures treated with antiserum plus complement was only slightly lower than that seen in control cultures. In contrast, only 17% of the control number of photoreceptors could be seen in cultures treated with antiserum plus complement.

High affinity GABA uptake: Some metabolic activities known to occur in neurons but not in photore-
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cceptor cells were investigated in treated and control cultures. One of them was the high affinity uptake mechanism for GABA.\textsuperscript{14} Autoradiography shows that in untreated cultures the uptake is present in many of the neurons and process-free round cells, but is not detectable in the photoreceptors (Fig. 5). On the other hand, analysis by scintillation counting showed that control cultures had an uptake of $58932 \pm 6308$ disintegrations per minute (dpm)/dish, and that cultures treated with antiopsin antiserum plus complement retained about 97% of the control level (Table 1).

**Choline acetyltransferase activity:** Treated and control cultures were also compared with respect to the activity of choline acetyltransferase (CAT), the enzyme responsible for acetylcholine synthesis. Given that this enzyme seems to be selectively restricted to some populations of retinal neurons,\textsuperscript{18} no extensive changes should occur in cultures depleted only of photoreceptor cells. As shown in Table 1, this was indeed the case, since cultures treated with antiopsin antibodies plus complement showed 96% of the CAT activity measured in control cultures.

**Discussion**

The experiments reported in this article show that antiopsin antibodies can cause complement-mediated photoreceptor lysis in cultures in which lymphocytes, macrophages, or other cells from the immune system are not present. The specificity of this effect is demonstrated by the following facts: (1) photoreceptor lysis is not accompanied by extensive neuronal death, as determined by quantitative microscopic analysis and by the measurement of neuronal markers such as CAT activity and high affinity GABA uptake; (2) it can be blocked by preincubation of the antiopsin antiserum with purified rhodopsin; and (3) it is not observed when either antiserum or complement are tested individually. Antibody effects are concentration-dependent, and photoreceptor sensitivity to lysis by these antibodies develops in vitro in parallel with the appearance of immunocytochemically detectable opsin antigens.

It has been well established that animals injected with purified rhodopsin in the presence of Freund's adjuvant develop an immune reaction that affects the retina and leads eventually to its degeneration.\textsuperscript{4,9,19} It is less clear, however, whether these degenerative disorders are mediated by humoral or cellular mechanisms.\textsuperscript{4,8} It is also unclear whether photoreceptors are directly attacked by these immune mechanisms, or whether their degeneration is an indirect consequence of other ocular alterations. The studies reported here are relevant in this context insofar as they offer direct evidence that, at least in an in vitro bioassay, photoreceptor cells can be selectively lysed as a direct con-

Fig. 5. Photomicrograph of an autoradiogram prepared with a culture exposed to $^3$H-GABA under conditions which allow detection of high affinity uptake mechanisms. The same field is shown photographed with phase contrast microscopy (A) and bright field optics (B). Note extensive labeling of many of the multipolar neurons. Photoreceptor cells do not express this uptake mechanism (arrows) (original magnification, $\times 215$).

sequence of the action of antiopsin antibodies and complement. It does not appear unreasonable to speculate that a similar mechanism could occur in vivo in animals or patients which have circulating antiopsin antibodies and whose blood-retinal barrier is defective.\textsuperscript{20-23}

The culture system used in this article offers a suitable bioassay for the investigation of the presence of

<table>
<thead>
<tr>
<th>Culture treatment</th>
<th>Choline acetyltransferase (CAT) $\text{dpm/dish}$</th>
<th>GABA uptake $\text{dpm/dish}$</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>460.8 ± 162</td>
<td>58932 ± 6308</td>
</tr>
<tr>
<td>Complement alone</td>
<td>456.6 ± 126</td>
<td>64898 ± 23300</td>
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<tr>
<td>Antiopsin +</td>
<td>444.0 ± 19</td>
<td>57512 ± 17809</td>
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Results are the average from triplicate determinations in two separate experiments, plus or minus standard deviation. Treatment of the cultures and biochemical determinations were carried out as described in Methods.

* CAT activity is expressed in fmol acetylcholine/minute/dish.
sensitive micromethods for various molecules important in retinal function, this approach should be useful for neurons (by kainic acid and /3-bungarotoxin treatment), biochemically with populations depleted from either neurons and photoreceptors can now be compared as choline acetyltransferase activity, or the high-affinity uptake mechanism for GABA. Cultures containing receptor cells. It is noteworthy that neurons are not antibodies, on the other hand, yields a population of by complement-mediated treatment with antiopsin neurons largely devoid of contamination with photoreceptors is desirable for the biochemical analysis of photoreceptor cells with those of other neurons present in the cultures. The unavailability of adequate in vitro preparations of mammalian photoreceptors makes it necessary to use a chick system to study antibodies against mammalian antigens. Fortunately, there seems to be enough interspecific homology in molecules such as rhodopsin to cause extensive immunologic cross-reactivity. The culture bioassay described here is now being used to investigate the presence of antiphotoreceptor antibodies in sera from retinitis pigmentosa patients, as well as from animals affected by different retinal degenerative disorders of genetic origin. An in vitro bioassay using retinoblastoma cells has already been used in another laboratory to investigate cellular immune mechanisms in patients affected by retinal degenerations.

The studies reported in this article are also relevant as a method for cell purification in vitro.25-27 The cultures of retinal neurons and photoreceptors used in these studies are already devoid of contamination with glial, pigmented epithelial, fibroblastic, or vascular cells.12,28 However, further separation of neurons and photoreceptors is desirable for the biochemical analysis of these two cell types. We recently reported a method by which some 80% of the neurons present in the cultures can be eliminated by treatment with selective neurotoxins such as kainic acid or /3-bungarotoxin.16 This method allows the generation of highly enriched populations of photoreceptor cells. Photoreceptor lysis by complement-mediated treatment with antiospin antibodies, on the other hand, yields a population of neurons largely devoid of contamination with photoreceptor cells. It is noteworthy that neurons are not extensively affected by the treatment, either in terms of overall survival, or in the activity of markers such as choline acetyltransferase activity, or the high-affinity uptake mechanism for GABA. Cultures containing both neurons and photoreceptors can now be compared biochemically with populations depleted from either neurons (by kainic acid and /3-bungarotoxin treatment), or photoreceptor cells (by complement-mediated lysis by antiospin antibodies). Given the availability of sensitive micromethods for various molecules important in retinal function, this approach should be useful for the study of the chemical differentiation of retinal cells.

Key words: antiopsin antibodies, photoreceptor degenerations, retinal degeneration, retinal cultures, cell separation

Acknowledgments

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