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A Monoclonal Antibody That Binds to Cones

Vance Lemmon

A monoclonal antibody that binds to cones has been produced. This antibody, 50-1B11, binds to the outer segments of cones in rhesus monkeys. Immunohistochemical experiments indicate that 50-1B11 binds to a subset of photoreceptors, probably cones, in all vertebrate species tested thus far, including man. In vitro experiments on chicken retina indicate that the antigen is intracellular and associated with the plasma membrane, while electronmicroscopic–immunohistochemical studies demonstrate that the antigen is contained in the lamellae of the outer segments of rhesus cones. Invest Ophthalmol Vis Sci 27:831–836, 1986

The ability to use immunohistochemical techniques to differentiate between different classes of cells has proven to be an invaluable approach in studying both normal cell function and pathological conditions. Over the past several years a number of monoclonal antibodies have been developed that bind to specific classes of cells in the retina of various species.[1–3] Until now, there have been no antibodies produced that bind specifically to vertebrate cones. This, combined with the lack of conventional histochemical techniques to discriminate between rods and cones, has greatly hindered studies of retinal degeneration in both human pathological material and in animal models of photoreceptor degeneration. We have recently produced a monoclonal antibody that binds to cones in monkeys and also binds to cones in formalin fixed human retina. Therefore, we believe that this antibody will greatly facilitate studies of the cell biology and pathology of cones.

Materials and Methods. Mice were obtained from Jackson Labs (Bar Harbor, ME). White leghorn chicken eggs were obtained from S. Sacks and Son; Evans City, PA. All procedures used in this study conform to the ARVO Resolution on the Use of Animals in Research.

To prepare material for immunization of mice, retinas were dissected from E19 chick embryos, and plasma membranes were prepared using discontinuous sucrose density gradients with steps of 0.32 M, 0.8 M, and 1.2 M sucrose.[4] Plasma membranes were collected, solubilized in 0.5% NP-40 in 20 mM TRIS, saline, pH 7.2 and then centrifuged at 100,000×g.av. for 30′. The supernatant was then incubated with Bio-beads (Biorad Laboratories; Richmond, CA) to remove the detergent. The supernatant was used to immunize Balb/C mice using Freund's complete adjuvant. One month later the mice were boosted with antigen in Freund's incomplete adjuvant and then rested for at least another 4 wk. Prior to fusion a mouse was injected with antigen via the tail vein on 3 successive days. All immunizations contained approximately 100 μg of protein.

Hybridomas were prepared following standard procedures using NS-1 cells.[5] Supernatants were first screened using a dot blot immunoassay with the same material that was used to immunize the mice.[6] Wells containing supernatants that were positive on the dot blot were subsequently tested using immunohistological procedures with cryostat sections of paraformaldehyde fixed E19 chick retina. After incubating the sections with hybridoma supernatant the sections were washed with phosphate buffered saline (PBS) and then incubated with a 1:500 dilution of fluorescein labeled goat anti-mouse-IgG (Cappel Labs). Wells containing supernatants that bound to restricted classes of retinal cells were cloned by limiting dilution.

In order to study the specificity of antibody 50-1B11 double label experiments were conducted using techniques developed by de Monasterio et al.[7] Procion yellow (obtained from Dr. de Monasterio) was injected into the vitreous of rhesus monkeys that were being used in neuroanatomical experiments for other pur-

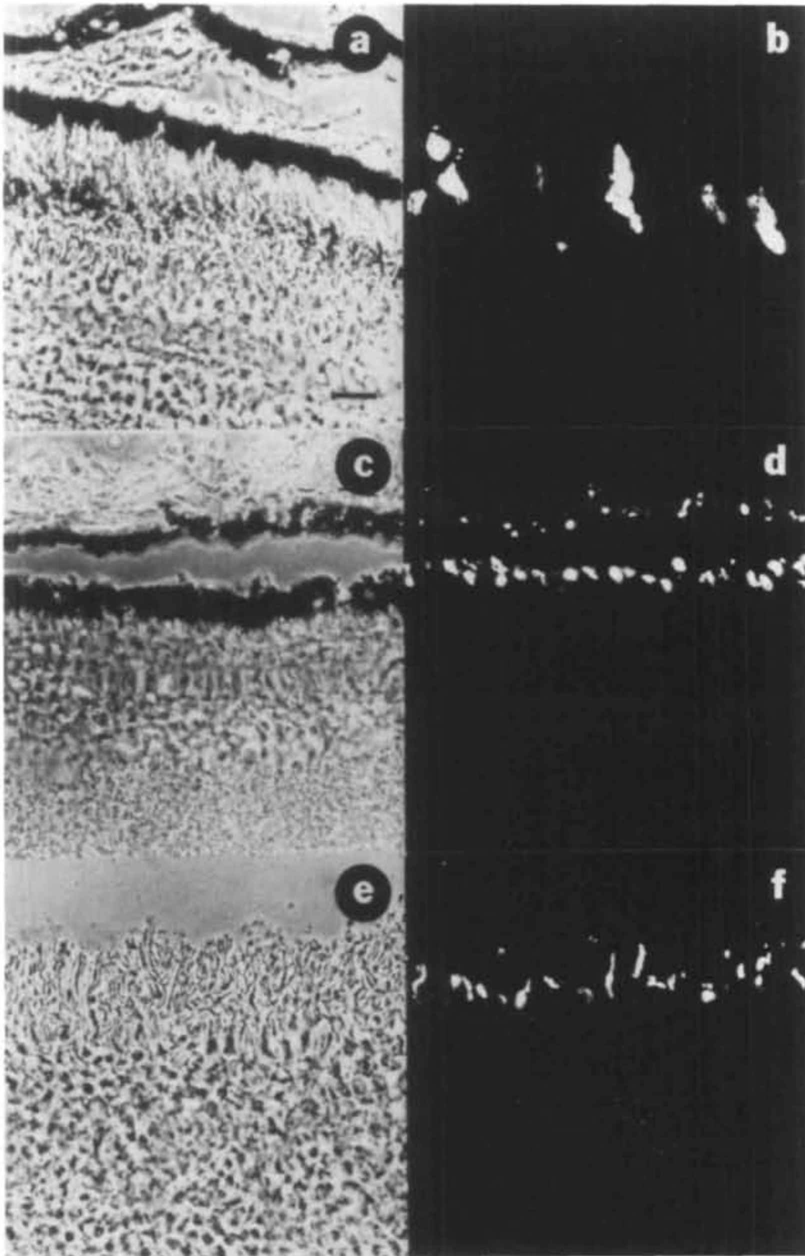

Fig. 1. Immunofluorescence localization of 50-1B11 antigen in different species. **A** and **B** show phase and fluorescence images of adult bullfrog retina. In **B** 50-1B11 immunoreactivity is restricted to a subset of photoreceptors. **C** and **D** illustrate adult chicken retina. In **D** a relatively large percentage of the photoreceptors are stained by 50-1B11. **E** and **F** are of adult human retina. In **F** only some photoreceptors are stained. Original magnification ×250.

poses. The monkeys were anesthetized with ketamine, maintained on a mixture of nitrous oxide, oxygen, and halothane, then deeply anesthetized at the conclusion of the experiment with halothane.[8] After 18–24 hour post-injection survivals the eyes were enucleated and fixed by immersion in 4% paraformaldehyde/0.01% glutaraldehyde. The eyes were cryoprotected and 10-μm sections were cut. After staining with 50-1B11 followed by a rhodamine labeled secondary antibody, the sections were examined with an epifluorescence microscope equipped with FITC and RITC filter cubes.

Flat mounts of retina were prepared by fixing eyes by immersion in 4% paraformaldehyde and 0.01% glutaraldehyde for 1 hr at 4°C as were the following steps. The retinas were then removed from the eyes and the pigment epithelia separated from the retinas. The retinas were post-fixed in the same fixative overnight. The next day the retinas were treated with phosphate buffered saline (PBS) with 0.05% Triton X-100 for 10′ followed by PBS with 10% horse serum for 30′. The retinas were incubated with 50-1B11 overnight, washed extensively with PBS, and then reacted with the Vectastain secondary antibody and avidin-biotin-HRP complex (Vector Labs; Burlingame, CA) for one hour each. The retinas were processed with an intensified diaminobenzidine procedure.[9] Flat mounts were examined

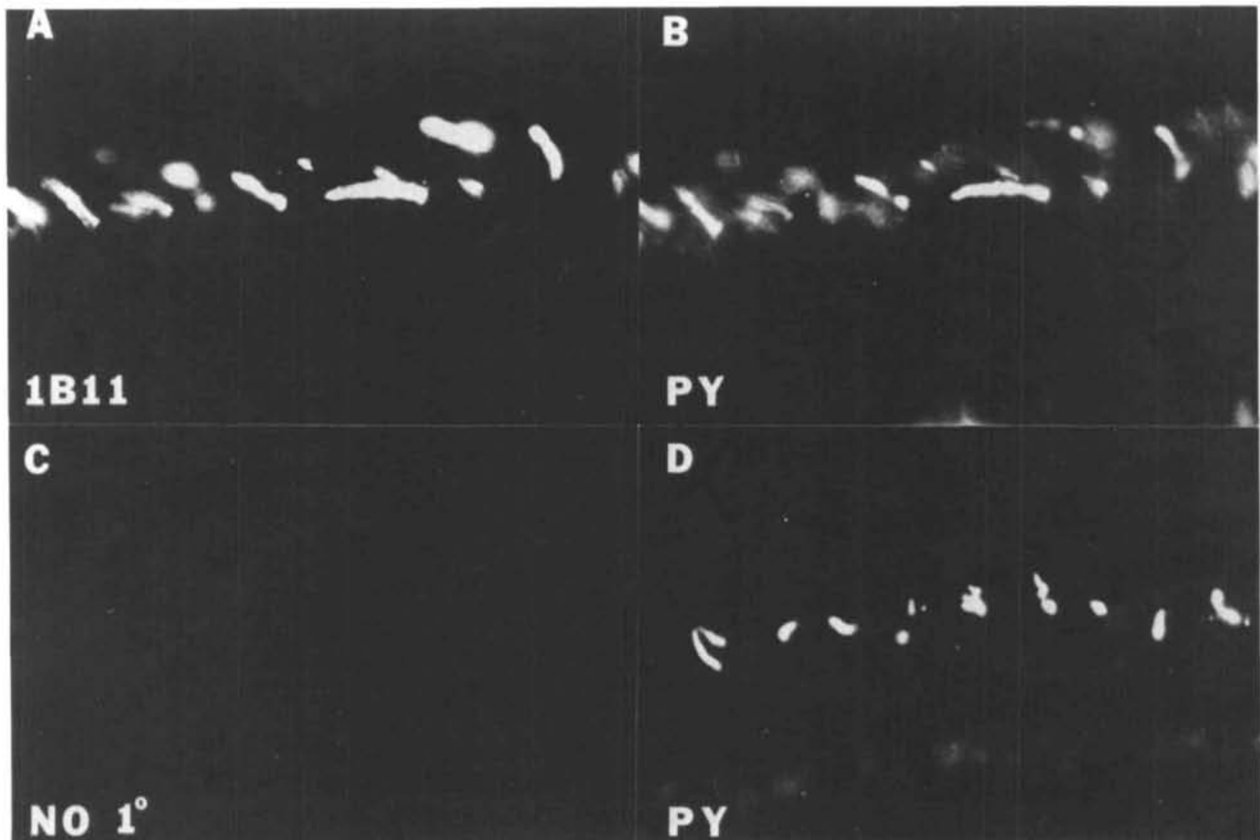

Fig. 2. Double label experiment in rhesus monkey using procion yellow and 50-1B11. A and B show the same region of retina. In A the 50-1B11 positive outer segments are seen while in B the procion labeled is seen. C and D show a control section from the same procion injected eye as in A and B. The section was stained with the rhodamine labeled secondary antibody but not the 50-1B11 primary antibody. C shows that the secondary antibody did not bind to the procion filled cells seen in D. Original magnification ×500.

with a phase microscope. Some pieces of the flat mounts were embedded in plastic and sections cut perpendicular to the plane of the retina for examination in both the light microscope and electron microscope.

Tissue culture experiments were conducted on embryonic day 6 (E6) chick retinas that were dissociated and cultured for 6 days.[10] Cells were fixed with 4% paraformaldehyde/0.01% glutaraldehyde in 0.1 M phosphate buffer (PB), treated with 0.1% Triton X-100/PB, and then stained with antibodies.

For both light microscopic studies and electron microscopic studies, controls were always performed that included incubating sections without the primary antibody but with the secondary antibody. This allowed us to determine if the secondary antibody bound to the tissue. We found no binding of either fluorescein or HRP labeled antibodies in sections incubated only with secondary antibodies (data not shown).

Results. Antibody 50-1B11 was found to stain a subset of photoreceptors in the E19 chick retina. Examination of bull frog, chicken, and human retinas demonstrated that 50-1B11 had wide species cross-reactivity while maintaining specificity for a subset of photoreceptors (Fig. 1). The 50-1B11 antibody also bound to some photoreceptors in rat retina (data not shown).

Since the distance between stained photoreceptors in the rhesus retinas appeared to correspond to that described for cones, double label experiments were performed using procion yellow to allow the localization of cones. After injections of procion yellow into the vitreous, the outer segments of cones were stained. The staining was concentrated in the outer segments but was also found in the inner segments, somas, and dendrites of some cells. When these same cells were examined for staining with 50-1B11 they were found to also be labeled, but the 50-1B11 staining was restricted to the outer segments (Figs. 2a, 2b). Examination of numerous sections from four different injected eyes indicated that there was excellent correspondence between the procion label and the 50-1B11 staining. As a control, sections from procion injected eyes were stained with the RITC-secondary antibody but not the 50-1B11 antibody. In these sections there was no RITC stain of the outer segments of the procion

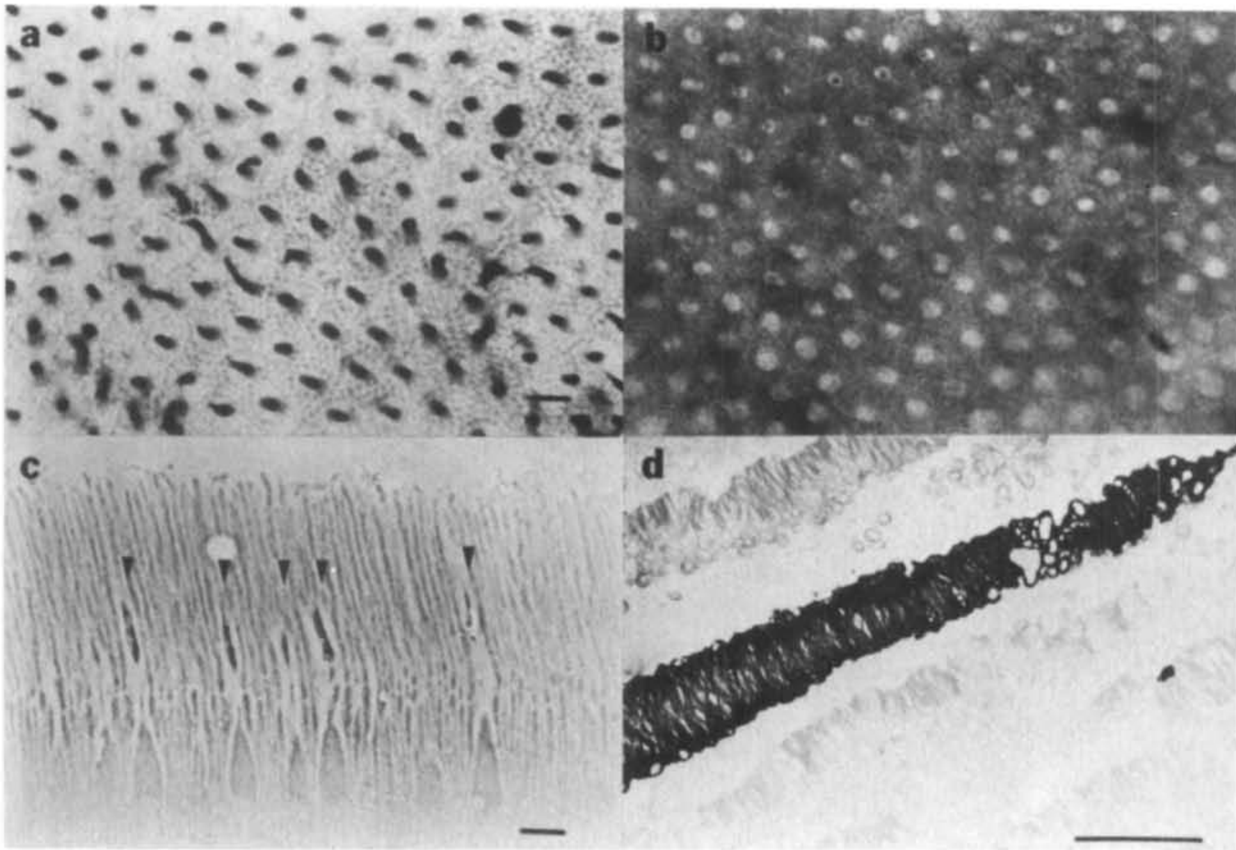

Fig. 3. 50-1B11 staining of rhesus monkey retina. **A** and **B** show a flat mount of HRP stained retina. In **A** the outer segments are seen, whereas in **B** the plane of focus is on the inner segments. The pattern of the stained outer segments in **A** corresponds to the pattern of cone ellipsoids in B. In **C** a 1-μm plastic section shows five stained outer segments (indicatd by arrowheads) that are located above cone ellipsoids. In **D** an electronmicrograph shows 50-1B11 immunoreactivity in the lamellae of a cone outer segment, as judged by its connection to an ellipsoid out of the field of view. Scale bar in A = 14 μm, in C = 7 μm, in D = 0.7 μm.

yellow positive cells (Figs. 2c, 2d). This indicates that (1) our filter combinations were appropriate to discriminate between RITC and procion yellow and (2) that the secondary antibody did not bind to procion stained cells.

In a second series of experiments, flat mounts of retina were examined for a correspondence between the pattern of stained outer segments and the pattern of cone ellipsoids. Figure 3a shows 50-1B11 positive outer segments from a rhesus retina and 3b shows the same field focused at the level of the inner segments. In Figure 3a the outer segments of rods are unstained but visible due to the use of phase optics. The pattern of the cone ellipsoids corresponds perfectly to that of the 50-1B11 positive cells. This indicates that all cones in the monkey retina contain the 50-1B11 antigen. Figure 3c shows the pattern of 50-1B11 staining seen in a 1-μm thick plastic section perpendicular to the plane of a rhesus retina stained as a flat mount. The outer segments of the cones are stained while the rods are not stained. Figure 3d shows a thin section taken adjacent to the thick section shown in 3c. It demonstrates that the 50-1B11 antigen is associated with the lamellae of the outer segment of a cone and that nearby rod outer segments are unstained. In order to obtain staining in the flat mounts 0.05% Triton X-100 treatment was required. Untreated tissue or tissue treated with 1.0% saponin did not react with 50-1B11.

Tissue culture experiments were conducted using chick retina cells in order to determine the cellular location of the 50-1B11 antigen. Adler and associates have demonstrated that live photoreceptors can be stained with peanut lectin under similar tissue culture conditions.[10] We have also obtained several monoclonal antibodies that can be used to stain the surface of photoreceptors in culture (Lemmon, unpublished). When live cells were incubated with antibody 50-1B11 there was no detectable staining of cells in culture. However, when the cells were fixed and solubilized with Triton X-100 a subset of cells could be stained with 50-1B11 (Fig. 4). The staining appeared to be associated with the plasma membrane since the cells had a characteristic "ring fluorescence," with the edges of the cells appearing brighter than the center of the cells. There

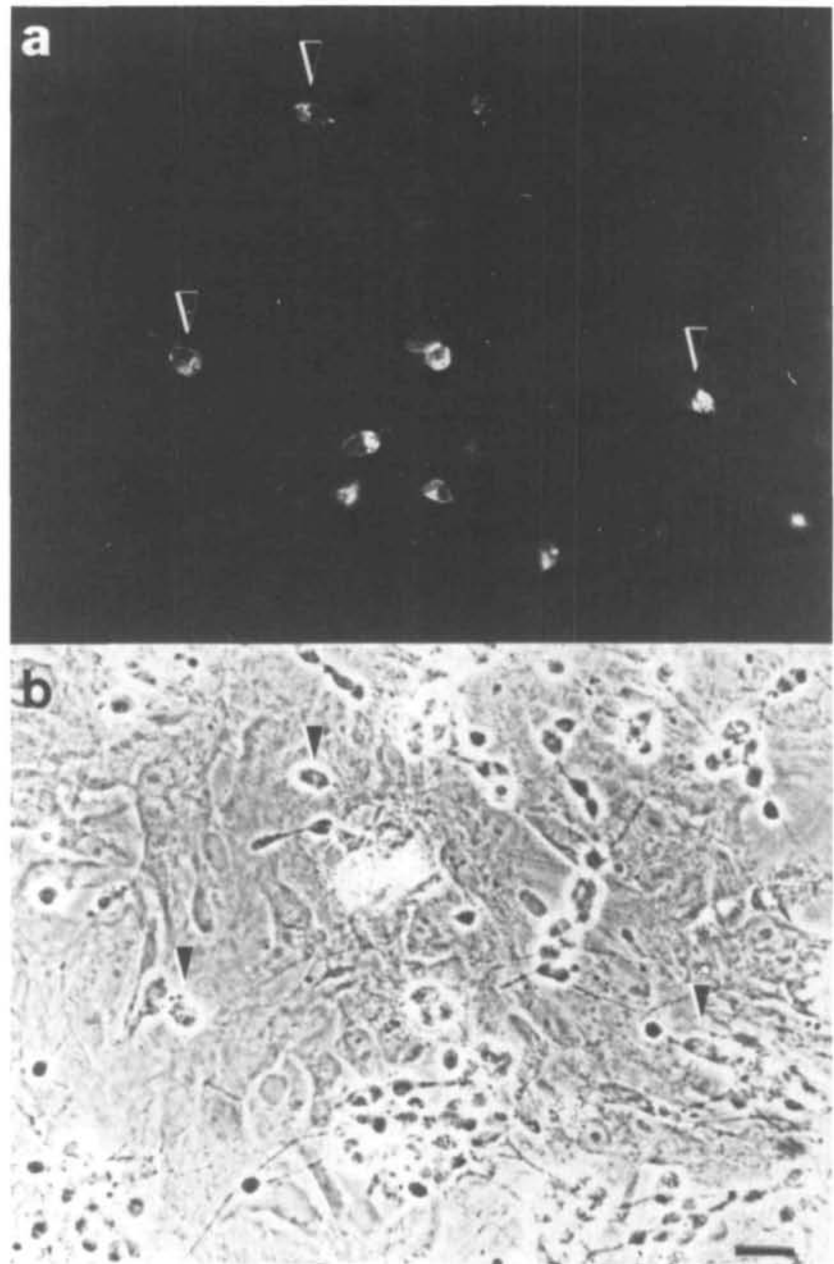

Fig. 4. 50-1B11 staining of E6 chick retina cells in culture for 6 days. A shows immunofluorescence of 50-1B11 positive cells. **B** indicates that relatively few of the cells in culture contain 50-1B11 immunoreactivity. Three of the 50-1B11 positive cells are indicated with arrowheads. Scale bar = 10 μm.

was also usually a large concentration of staining at one end of the cells suggesting that the 50-1B11 antigen is localized in a specialized region of the cells in culture. This is similar to the polarization seen with peanut lectin staining of photoreceptors in culture.[10]

Discussion. These results demonstrate that antibody 50-1B11 binds to an antigen present in a subset of photoreceptors in most vertebrate classes, including amphibians, birds, and mammals. The fact that the antibody binds to outer segments of photoreceptors in the monkey retina suggests that the antigen is probably associated with some aspect of the light transduction mechanism. The double label experiments using procion yellow[7] and the flat mount studies indicate that the 50-1B11 antigen is restricted to cones in monkey retinas. Based on the distribution of the 50-1B11 positive cells in other species it is likely they are also cones.

Three different pieces of evidence suggest that the 50-1B11 antigen is associated with the plasma membrane. The first is that plasma membrane associated material was used both to immunize the mice and as a target antigen in the initial screening step. The second is that immunohistochemical studies of cells in culture gave "ring fluorescence," a pattern typical of plasma membrane antigens. The fact that only fixed, permeabilized cells could be stained suggests that the antigen may be associated with the intracellular aspect of the plasma membrane. The last is that electronmicro-

scopic–immunohistochemical studies demonstrate that the 50-1B11 antigen is associated with the lamellae of the cone outer segments in the monkey. Further experiments will be required to determine what kind of association the antigen has with the plasma membrane. For example, is it an integral or peripheral membrane protein?

Although the 50-1B11 antigen has not yet been characterized biochemically, it is clear that this antibody will be a useful reagent for studying the distribution of a unique class of cells in the retina. It should also permit in vitro and transplantation studies on factors that influence photoreceptor differentiation and survival. Finally, this antibody should be of value in studies on differences between rods and cones in various pathological conditions.

Key words: monoclonal antibodies, cones, human retina, chick retina

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Immunocytochemical Localization of Opsin in the Inner Segment and Ciliary Plasma Membrane of Photoreceptors in Retinas of rds *Mutant Mice*

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Homozygous 020/A mutant mice bearing the rds gene for slow inherited retinal degeneration have been observed to develop normal photoreceptor inner segments connecting cilia and synaptic contacts but fail to form outer segments. Their retinas are responsive to light, however. In order to assess the sources of these physiological responses we investigated the distribution of opsin in photoreceptors by means of immunoelectron microscopy. Opsin was detected in the inner segment plasma membrane and the distal ciliary plasma membrane. Antibody also bound to lamellar and vesicular membranes in the interphotoreceptor space and, in a small fraction of the photoreceptors, to membranes projecting from the distal cilium. These membranes may represent abortive formation of rod discs in this form of retinal degeneration. Failure to form an organized outer segment may contribute to the persistence of opsin in the inner segment plasma membranes of adult mutant mice. Invest Ophthalmol Vis Sci 27: 836–840, 1986

In the developing retinas of newborn 020/A mutant mice bearing the rds/rds genotype, outer segments fail to differentiate while the development of other retinal layers is normal. By 3 wk of age, the photoreceptors consist of normal appearing inner segments and cilia extending to their maximum length. Subsequently, photoreceptors die at a slow rate and may be removed by macrophages in the subretinal space.[1–4] The retinas of these mice are responsive to light despite the absence of outer segments. Reduced ERG responses and a limited light-evoked decline in cyclic nucleotide levels have been noted.[3,4] Although opsin was not detectable spectroscopically,[3] it was detectable by use of a sensitive immunoassay with antiopsin antibodies.[5] Its distribution in photoreceptors has not yet been reported. The possibility that opsin was present in the photore-