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Immunohistochemical Detection of Retinal Cones in Monkey Retina: Light and Electron Microscopic Study

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Purpose: Retinal cones have wave-length-specific visual pigments. To identify subclasses of cones, opsin-specific antibodies were previously established for the immunohistochemical studies of frozen sections. In this study, we produced retinal cone antibodies and examined (1) the specificity of these antibodies with Western blot analysis, (2) the application of these antibodies to paraffin-embedded monkey retinal sections, and (3) the use of these antibodies in light and electron microscopic immunohistochemical analyses of the localization of retinal cones.

Methods: The N-terminal peptide of blue opsin, and the C-terminal peptide of green/red opsin were used as immunogens in New Zealand White rabbits. Immunohistochemical staining was performed using the ABC method and immunogold method. As antigen retrieval treatment, paraffin-embedded cynomolgus monkey retinas were subjected to enzyme and microwave treatment.

Results: Both anti-red/green and anti-blue cone opsin antibodies detected 40 kDa native cone opsins in crude retina extract. The red/green and blue cone opsin immunostaining after trypsin treatment revealed that a positive signal was observed in the cone outer segment. Immunogold labeling also showed that gold particles were concentrated on the cone outer segment.

Conclusion: In the antigen retrieval method, trypsin treatment is the appropriate method to obtain optimal staining of paraffin sections of retina. Using this method, retinal cone in conserved old paraffin sections can be identified immunohistochemically. **Jpn J Ophthalmol 2000;44:503–510**

Key Words: Cone, immunohistochemistry, opsin photoreceptor, retina.

Introduction

The human retina contains two classes of photoreceptor cells: rods and cones. Rods are responsible for dim-light vision, while cones are responsible for bright-light and color vision.[1,2] Human color vision results from the absorption of light by three classes of cone photoreceptors: long-wave-sensitive (red), middle-wave-sensitive (green), and short-wave-sensitive (blue) cones. The three types of cones show maximal spectroscopic absorption at different wave lengths. The ratios among the quantal catches by the three classes of photoreceptors is interpreted by the brain as a color along the spectrum. Using these three classes of photoreceptors, humans, Old World monkeys, and some species of New World monkeys have trichromatic color vision.[3,4]

The outer segments of cones contain visual pigment, which catches the light of the retinal image. Visual pigment consists of molecules composed of two portions: a protein portion, opsin, and a light-catching portion, which is the cis-retinal chromophore. Variations in the protein portion of the molecule determine its sensitivity to light of different

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regions of the visible spectrum. Each primate photoreceptor contains only one type of visual pigment molecule. Proteins of this visual pigment family have structures composed of the hepta-helical transmembrane bundles, within which the chromophore is held.[5]

The absorption of a single photon of light causes the isomerization of the retinal chromophore of photopigments from the 11-cis to the all-trans retinal chromophore configuration. This isomerization results in the formation of an activated photopigment, which triggers a signal amplification cascade.[6–8] The first step in this cascade involves activation of transducin, which in turn activates a cyclic guanosine monophosphate (cGMP) phosphodiesterase. The resulting decrease in the cGMP level triggers closure of cGMP-gated membrane cation channels and hyperpolarization of the photoreceptor cell.

Cones and rods are histologically distinguishable components of the retinal photoreceptor mosaic.[9,10] To identify the subclasses of cone, anti-cone opsin-specific antibodies have been obtained and used for immunochemical studies by several researchers.[11–13] However, these antibodies were applied to frozen sections, not paraffin sections. Generally, paraffin sections can be manipulated more easily and conserve tissue structure, such as lamination of retina more tightly than frozen sections.

In this study, we obtained anti-cone opsin antibodies, and examined as follows: (1) the specificity of these antibodies against monkey retina using an immunoblotting method; (2) the reactivity of anti-red/green and anti blue cone opsin polyclonal antibodies with paraffin-embedded *Macaca* monkey retina using an antigen retrieval method, and (3) the ultrastructural localization of cone opsin using a postembedding immuno-electron microscopic method.

Materials and Methods

Animals

Four eyes of cynomolgus monkeys (*Macaca fascicularis*) were obtained from Tsukuba Primate Center (Tsukuba). All use of animals was in accordance with the guidelines established in the ARVO statement for the Use of animals in Ophthalmic and Vision Research.

Production of Anti-Cone Opsin Antibody

The amino-terminal peptide of blue opsin, including an extra cysteine added at the N-terminus (C-MRKMSEEEFYLFKNISSV, residues 1–18) and the carboxy-terminal peptide of green/red opsin (LQLF-GKKVDDGSELSSAS, residues 334–351) were synthesized, and conjugated to bovine serum albumin (BSA) using *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS; Toray Research Center, Ohtsu).

Three New Zealand White rabbits (obtained from Clea, Japan, Tokyo) were injected intradermally at multiple sites on the back with an emulsion of 600 μg of the conjugate and an equal volume of Freund's complete adjuvant (DIFCO Lab, Detroit, MI, USA). Rabbits were given a booster of 100 μg peptide-BSA conjugate in Freund's incomplete adjuvant at approximately 1-week intervals. The antiserum, along with pre-immune serum, was tested for antibody activity using a BIA core instrument (Pharmacia, Uppsala, Sweden).

Polyclonal Antibody Titer Assay with BIA Core

Approximately 100 μg of retinal cone opsin peptide was coupled to a dextran-loaded biosensor chip. The presence of opsin-specific immunoglobulin in the purified serum was studied by application of antibody at 25 μg/mL to the biosensor. The increase in resonance units (RU) at equilibrium was considered to be a measure of antigen–antibody interaction, and was used to calculate an apparent association constant.

Purification of Anti-Opsin Antibody

The antiserum was purified using two steps, including BSA-sepharose and protein A-sepharose affinity chromatography. The affinity adsorbents were prepared by coupling BSA and protein-A to CNBr-activated Sepharose 4B (Pharmacia Biotech, Piscataway, NJ, USA) by the method of Cuatrecasas.[14] Immunoglobulin fractions were prepared from the rabbit anti-opsin antiserum by precipitation with 40% saturated ammonium sulfate. The fractions were passed through the BSA affinity column, and nonadsorbent fractions were then applied onto the protein-A affinity column. After successive washings with 5 M NaOH (pH 8.9) containing 3 M NaCl and 1.5 M glycine, the bound antibodies were eluted with citrate buffer (0.1 M, pH 3). Protein was determined by Bradford's method with BSA as a standard.

Western Blot Analysis

Monkey eyes were enucleated in the Tsukuba primate center and chilled immediately in a light-proof icebox. All procedures were performed at 4°C unless otherwise stated. Monkey retinas were dissected from eyes and stored at −80°C until use. Retinas were homogenized in ice-cold phosphate-buffered saline (PBS) and centrifuged at 15,000 *g* for 20 minutes. After centrifugation, the supernatants were collected,

and electrophoresed in 12.5% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE), and electrophoretically transferred to nitrocellulose membranes using a semi-dry transfer system. After transfer, membranes were incubated in PBS containing 3% skim milk for 30 minutes, and then overnight in the same buffer containing diluted anti-red/green (1:2000) or anti-blue opsin (1:1000) antibodies. Controls included pretreatment of these antibodies with 10 μg of red/green or blue opsin peptides. After several washes in PBS, the membranes were incubated with biotinylated goat anti-rabbit immunoglobulin coupled to horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) for 1 hour. After several washes in PBS, the membranes were incubated in avidin-biotin complex for 1 hour, and immunoreactive bands were visualized using diaminobenzidine.

Immunohistochemical Studies

Sample preparation. Four enucleated eyes from two cynomolgus monkeys were fixed in 4% paraformaldehyde/PBS (pH 7.4) at 4°C overnight. Samples were dehydrated in absolute methanol, cleared in xylene, and routinely embedded in paraffin. Sections were cut 4-μm thick and mounted on silanized slides.

Antigen retrieval method. Sections were deparaffinized in xylene, rehydrated in a series of ethanol, and then rinsed in PBS (0.01 M, pH 7.4). Various antigen retrieval methods were tested (Table 1).

Immunohistochemical study by light microscopy. After antigen retrieval treatment, sections were again rinsed with PBS and quenched in 0.3% hydrogen peroxidase in methanol for 30 minutes. Sections were rinsed in PBS, blocked with 1.5% normal goat serum in PBS and incubated in a humidified chamber at 4°C overnight with anti-cone opsin antibodies: anti-red/green opsin antibody, 1:2000; anti-blue opsin antibody, 1:1000. After three rinses in PBS, sections were treated with biotin-conjugated goat anti-rabbit secondary antibody and then with avidin and biotinylated horseradish peroxidase complex (Vector) for 1 hour each. Slides were again rinsed in PBS, and the horseradish peroxidase reaction was developed with diaminobenzidine. Controls included omission of these antibodies and preabsorption of the antibodies with 10 μg of red/green or blue opsin peptides.

Table 1. Antigen Retrieval Procedures

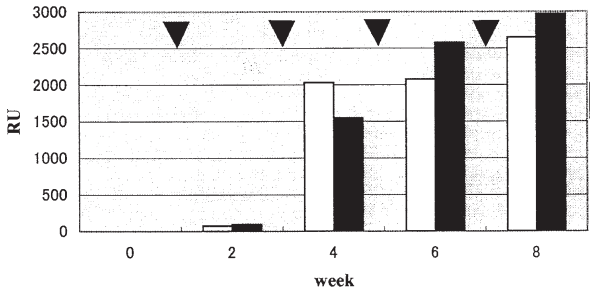
Antigen retrieval procedures	Temp./Time
Enzyme treatment	
0.1% Trypsin	37°C/30 min
Proteinase K (10 mg/mL)	Room temperature/10 min
Microwave treatment (MWF-2, Nisshin-EM) 0.01 M citrate buffer (pH 6.0)	95°C/25 min

Immunohistochemical study by electron microscopy. For electron microscopic immunocytochemical analysis, retinas were further dissected into small pieces after fixation in 2% paraformaldehyde/0.1% glutaraldehyde. Fixed retinas were then dehydrated with an ascending series of ethanol up to absolute, incubated in a 1:1 mixture of LR white resin (London Resin, London, England), and then incubated overnight in pure LR white resin mixture at 4°C. Retinas embedded in the fresh resin were cured under ultraviolet light for 1 week. Ultrathin sections were mounted on nickel grids. The sections were blocked with PBS containing 1.5% goat serum for 30 minutes, incubated with anti-red/green (1:100) or anti-blue (1:50) opsin antibody overnight, rinsed three times with PBS, and then incubated on 10 nm gold-conjugated goat anti-rabbit IgG (1:20, Amersham, Tokyo) overnight. After rinsing, the sections were post-stained with 1% uranyl acetate for 5 minutes.

Results

Antibody Titer Assay

The resonance units to each opsin were gradually increased by each booster immunization, as shown in Figure 1. After the fourth booster immunization, all rabbits were sacrificed and all of their blood was collected.



RU: resonance unit

Figure 1. Antibody titer assay with BIA core. Binding response increased during course of successive immunizations. Booster immunization is indicated by ▼. □ Red/green antibody; ■ Blue antibody.

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Properties of Purified Anti-Red/Green and Anti-Blue Opsin Polyclonal Antibodies

The results of the purification procedures are summarized in Table 2. With four steps of purification, the anti-red/green opsin antibody was purified 11-fold, and the anti-blue opsin antibody was purified 11-fold.

Western Blot Analysis

Immunoblotting of the monkey retina with either anti-red/green or anti-blue opsin polyclonal antibody revealed prominent bands of 40 kDa. When the immunostaining with the anti-cone photo-pigment antibodies was performed in the presence of red/green or blue opsin peptide (10 μg/mL), none of the immunostained proteins were detected (Figure 2).

The Effect of Antigen Retrieval Treatment

Table 3 shows the results of antigen retrieval treatment. Without any treatment, no signal was detected in retinal sections. Proteinase K treatment destroyed retinal morphology. Microwave treatment caused high background staining in the inner and outer nuclear layers. Overall, optimal staining was achieved with trypsin treatment.

Immunohistochemical Localization of Red/Green and Blue Opsin in Monkey Retina

Light microscopy. Photomicrographs of retina sections stained with anti-red/green opsin or anti-blue opsin antibody are shown in Figure 3. Positive signals for retinal cone opsin immunohistochemistry were observed only in the photoreceptor layer. Neither antibody labeled the rods. The population of anti-red/green cone opsin-positive cells was larger than that of blue cone opsin-positive cells throughout the whole retina. Especially, photoreceptors in the fovea were dominantly labeled by anti-red/green opsin antibody. In addition, immunocytochemical labeling of cone cells by each antibody was blocked by competition with a 10- to 100-fold molar excess of the appropriate peptide (*data not shown*).

Electron microscopy. Electron micrographs showing preparations of retina stained for red/green (Figure 4) and blue opsin (Figure 5) by the postembedding immunogold method. Gold particles were consistently found on the plasma membranes of the outer segment. Staining with control serum showed no immunoreactivity (*data not shown*).

Discussion

In this study, we used antibodies against peptides derived from human retinal cone photoreceptors. Anti-peptide antibodies are especially useful because antigenic epitopes can be designed to maximize the specificity of the antibodies so that they can distinguish even closely related molecules. As shown in Figure 1, both anti-red/green and anti-blue cone polyclonal antibodies immunoreacted with single bands with molecular weights of about 40 kDa in whole monkey retina homogenate, which agrees approximately with the molecular weights of red/green and blue cone opsins predicted from the respective cone opsin amino acid sequences.[15] Thus, our anti-cone antibodies appeared to detect native retinal cone opsins.

In general, paraffin-embedded tissues are commonly available, but are not always suitable for immunohistochemistry, because membrane proteins like cone opsin are inactivated during the preparation. However, using trypsin treatment, we could detect red/green and blue cone opsins in monkey retinas. Trypsin might permit antigen retrieval by unmasking retinal cone opsin antigens by increasing the tissue and cell permeability and breaking the cross-links re-

Table 2. Partial Purification of Anti-Cone Opsin Polyclonal Antibody from Rabbit Serum

	Protein (mg/mL)	RU* (/mL)	Specific Activity	Purification (-Fold)
Anti-red/green cone opsin antibody				
Purification step				
Crude extraction	8.20×10^4	7.25×10^5	8.8	1
Protein-A affinity	5.03×10^2	4.85×10^4	96.4	11
Anti-blue cone opsin antibody				
Purification step				
Crude extraction	4.20×10^4	5.7×10^5	13.5	1
Protein-A affinity	7.1×10^2	1.13×10^4	158.5	11

*RU: Resonance unit.

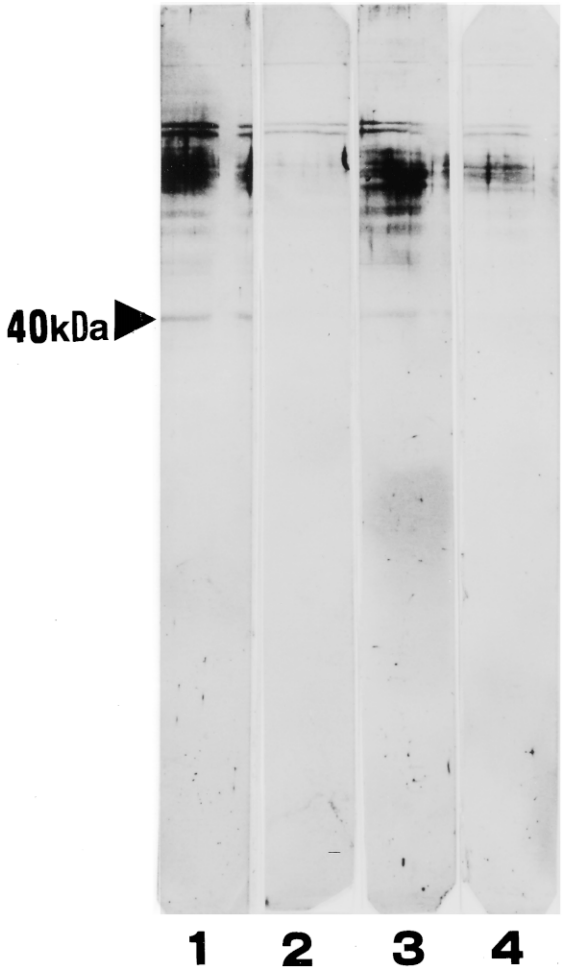

Figure 2. Western blots of red/green and blue opsin in monkey retina (10 μg/lane). Blots in lanes 1 and 2 were probed with anti-red/green opsin polyclonal antibody. Blots in lanes 3 and 4 were probed with anti-blue opsin polyclonal antibody. Monomers migrate with apparent molecular weight of approximately 40 kDa. Higher bands represent oligomers, which are artifacts of sodium dodecylsulfate solubilization. No immunoreactive signals were detected with preadsorbed anti-cone opsin antibodies. Lane 1; red/green opsin; lane 2; pre-adsorption with red/green opsin peptide; lane 3; blue opsin; lane 4; pre-adsorption with blue opsin peptide.

sulting from formaldehyde fixation.[16] By trypsin treatment of preserved paraffin-embedded surgical eye specimens, we could also examine retinal cone opsin expression in preserved surgical specimens of retinoblastoma (*data not shown*). This fact reveals conservation of antigenicity of retinal cone opsins in routinely formalin-fixed, paraffin-embedded specimens. Therefore, opsin expression in other retinal diseases can be examined retrospectively. In the near future, using this antigen retrieval method, newly obtained antibodies against phototransduction-associated proteins might be applicable not only in frozen sections but also in paraffin sections.

Table 3. Effects of Antigen Retrieval Methods

Treatment	Sensitivity*	Tissue Damage	Background*
No treatment	ND	No damage	ND
Trypsin	Detected	Moderate	Low
Proteinase K	Detected	Severe	Low
Microwave	Detected	Moderate	High

*ND: not detected.

Red/green and blue opsin were observed predominantly in the outer segments of cones by light microscopic immunostaining. This observation is in agreement with previous studies.[11–13] Ultrastructural immunogold labeling for electron microscopy also showed that gold particles were concentrated in the plasma membranes of outer segments. However, in previous immuno-electron microscopic studies in the human retina,[17,18] anti-cone opsin antibodies raised against the same peptide epitopes of the cone opsins as those used in the present study could stain red/green cones but not blue cones in LR-white resin-embedded sections. This discrepancy may have one or more of the following explanations. First, there may have been differences between the antibodies used in the present study and in previous studies. The titers of our antibodies were monitored using a biosensor. The biosensor monitors protein-to-protein interactions in real time, using an optical detection principle based on surface plasmon resonance.[19,20] In these measurements, the red/green and blue cone opsin peptides were immobilized on the surface of the sensor chip, while antibody-containing serum fractions were injected in a continuous flow over the surface. The surface plasmon resonance response reflects a change in the refractive index, and hence a change in mass, at the detector surface as the antibody binds. In short, the higher the value of the RU, the stronger the antigen–antibody interaction. Since we quantified antibody binding strength by biosensor, the quality of our cone opsin antibodies would be higher than that of the previously established antibodies. Second, reduction or destruction of opsin antigenicity might have occurred in previous studies. The times required for sampling and preparation of specimens for electron microscopy after the postmortem were longer in the previous studies than in this study. Because of the smaller population of blue cones in the retina, the blue cone opsin antigenicity might have been more obviously affected by postmortem changes. Third, there may be a lower

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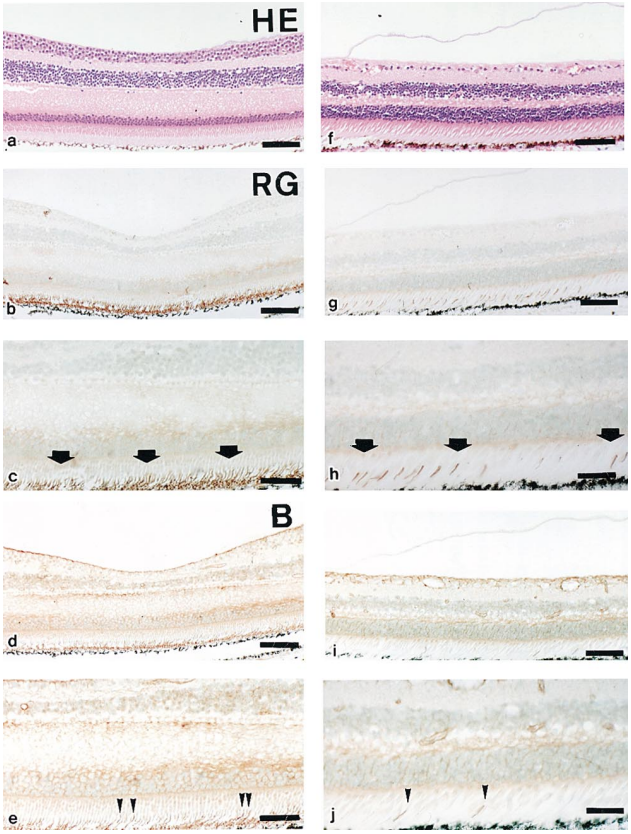

Figure 3. Photomicrographs showing labeling of monkey retina with anti-red/green or anti-blue cone opsin antibodies using trypsin treatment. Figures (**a**) and (**f**) were hematoxyline-eosin staining for histological interpretation. Bar = 200 μm. In low magnification, both retinal cone opsin antibodies labeled dominantly in photoreceptor layer throughout retina [red/green: (**b**) and (**d**), blue: (**g**) and (**i**), Bar = 200 μm]. In high magnification, positive signal was observed in outer segment, and in part of inner segment of cone photoreceptor [red/green: (**c**) and (**e**), blue: (**h**) and (**j**), Bar = 100 μm]. Arrows: red/green cones; Arrowheads: blue cones. (**a**)–(**e**): macula. (**f**)–(**j**): peripheral retina. HE: hematoxyline eosin staining; RG: red/green cones, B: blue cones.

expression of blue cone opsin than of red/green opsin in individual cone cells. In a postembedding immunogold electron microscopic study, it was shown that the immunogold intensity could be used as a quantitative measure of the antigen in the tissue.[21] In preliminary observations in the present study, the immunogold intensity of blue cones was lower than that of red/green cones. Therefore, the opsin expression in the blue cones themselves might be lower than that of the red/green cones. Cone opsins have been isolated from retinal tissues of cone-cell-rich animals, for example, chickens[22] and lizards,[23–25] but not of higher vertebrates, because of their relatively low expression compared, for example, to rhodop-

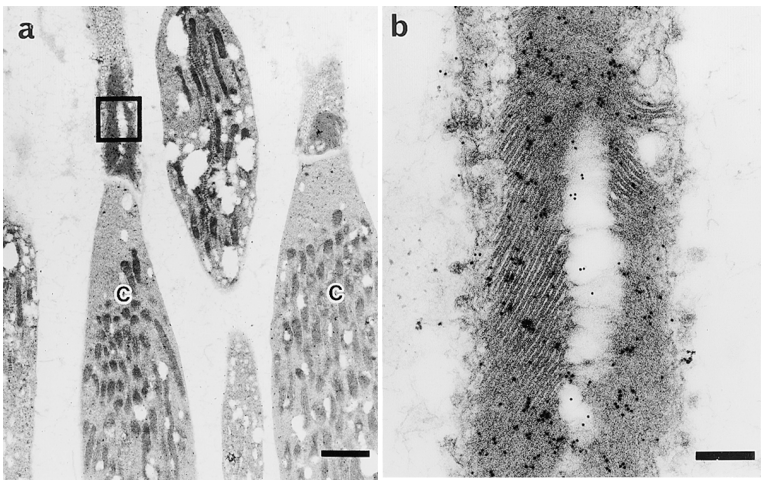

Figure 4. Electron micrograph of red/green cones in foveal retina (labeling with anti-red/green opsin antibody). In fovea, red/green cones had long, tapering inner segments (**a**). Many long, ellipsoid mitochondria were observed. Gold particles from immuno-gold labeling were concentrated on plasma membrane of cones (**b**). Magnification (**a**) ×7500, (**b**) ×45,000. Bar (**a**):0.2 μm, (**b**):0.02 μm. C: cone.

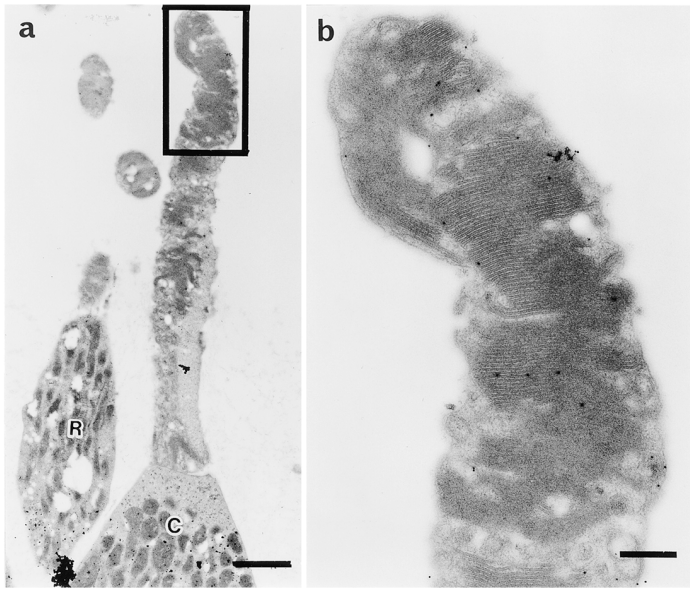

Figure 5. Electron micrograph of blue cone in peripheral retina (labeling with anti-blue cone opsin antibody). Gold particles were observed predominantly on plasma membrane of outer segment. However, immunogold density of blue cones was lower than that of red/green cones. Magnification (**a**) ×5000, (**b**) ×20,000. Bar (**a**) 2 μm, (**b**) 0.5 μm. C: cone; R: rod.

sin, and because of their instability.[22] As a result, biochemical quantification of retinal cone opsins in retinas is difficult and requires further study.

Topographical studies of photoreceptors using whole-mount immunohistochemistry showed that the adult *Macaca* retina had an average of 3.1 million cones, with the fovea containing the highest cone density, which averaged 200,000/mm^2.[26,27] Outside the fovea, the cone density decreased sharply in the periphery, but a higher density was maintained along the nasal horizontal meridian, the "cone streak."[28] Rods were very sparse in the *Macaca* fovea.[27] The rod density increased rapidly outside the fovea, and peaked in the rod ring at the eccentricity of the optic disc. Outside the rod ring, the rod density decreased uniformly as the periphery was approached. The average rod: cone ratio of the *Macaca* retina was 15:1.[29] Regarding the distribution of cone subclasses, red/green cones were found throughout the fovea, while blue cones were found only around the foveola.[31] The immunolabeling pattern in the present study was in agreement with these findings. However, a disadvantage of the retinal whole-mount technique is that cone outer segment loss occurs because of the large number of cones and the length of the cone outer segment in the fovea, and the difficulty of removing the retinal pigment epithelium from the neural retina.[28] In the present study, the anterior segment removed from the eyeball was embedded in paraffin. Concerning loss of the cone outer segment by detachment of the neural retina from the pigment epithelium, the foveal region in our specimens was relatively intact. By examining serial retinal paraffin sections, structural features in the retina, especially between the fovea and cone, can be determined in detail, and our ultrastructural studies are continuing.

In conclusion, we studied (1) the specificity of our retinal cone opsin antibodies by immunoblotting, (2) the application of these antibodies to paraffin-embedded sections using trypsin treatment, (3) the conservation of cone opsin antigenicity with the formalin fixation and paraffin-embedding procedure, and (4) the identification of red/green and blue cones by light and electron microscopy. An unresolved problem is discrimination between red and green cones. Based on microspectrophotometric studies,[30] phototransmittance imaging studies of primate mosaic samples,[31,32] and quantitative studies of red and green cone opsin mRNA expression,[33] it has been suggested that the distribution of red and green cones is random, and that they should be at a ratio of 1:1–1:4. Because of the high degree of homology between red and green cone opsin amino acid sequences,[15] it is very difficult to produce immunochemical probes specific for only red or only green cones. This problem must be addressed in a future study.

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