Four Cone Types Characterized by Anti-Visual Pigment Antibodies in the Pigeon Retina

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Using three antibodies to visual pigments (monoclonal antibodies COS-1 and OS-2, and a polyclonal anti-opsin serum), four different types of cone cells could be distinguished in the red area (dorsoposterior part with the highest density of cones) of the pigeon retina. Both members of the double cone and the single cone with the red oil droplet were labelled with our monoclonal antibody COS-1 (type I cone). The single cone with the orange oil droplet was positive both with anti-opsin and monoclonal antibody OS-2 (type II cone). The single cone exhibiting a yellowish-green oil droplet, fluorescent in ultraviolet light, also reacted with anti-opsin but lacked the antigenic determinant recognized by OS-2 (type III cone). The thin cone with the small colorless oil droplet was negative with both COS-1 and anti-rhodopsin (type IV cone). We propose that the four immunologically distinguishable cone types correspond to cones expressing visual pigments with different (long-, middle-, short-wavelength and ultraviolet) color sensitivities.

Visual pigments of various color sensitivities have been described in the pigeon retina using early receptor potential, electroretinography, spectrophotometry of extracted pigments, and psychophysical measurements. These investigations showed the pigeon retina to contain shortwave-, middlewave-, longwave-, and, similarly to other birds, an ultraviolet-sensitive photopigment.

In elucidating the neural networks of the retina correct assignment of photopigments to photoreceptor cells is important. The most conspicuous feature of the bird cone cells is the presence of colored oil droplets, a useful morphological marker for recognizing different cone types. Microspectrophotometric (MSP) investigation of dark-adapted outer segments and their correlation with the color (or absorbance) of the oil droplets in the inner segment of the same cell greatly contributed to the understanding of the light sensitivities of morphologically identified cone types. However, this method presents a difficulty in that measurement of very tiny outer segments is at the limit of resolution, and furthermore the oil droplets are of little use for conventional histological specimens since they are dissolved during specimen preparation. A further possibility is to find morphological characteristics for cones earlier identified by other methods and to use them in recognizing the cells in the electron microscope.

A few years ago, we initiated in our laboratory the method of detecting visual pigments immunologically by mono- and polyclonal antibodies. Two monoclonal antibodies (mAbs), OS-2 and COS-1, were used in combination with a polyclonal anti-rhodopsin serum to investigate the chicken and gecko retina, as well as various mammalian retinas. Since the pigeon has been the most widely investigated bird species in visual science and is known to contain cone cells with different color sensitivities, the thorough analysis of the pigeon retina with the available antibodies seemed to be of particular importance.

Materials and Methods

Twenty pigeons (Columba livia) were used and handled according to the ARVO Resolution on the Use of Animals in Research. After decapitation the eyes were enucleated and the retina was separated from the pigment epithelium in fixative. The red area (the dorsoposterior part of the retina with the highest density of cones, mainly with red and orange
oil droplets) was excised; in a few cases, however, the yellow area and the red-yellow transitional zone were also used. The fixative was a 1% glutaraldehyde solution in 0.1 M cacodylate buffer, pH 7.2. Following a 1 hr fixation, the retinal pieces were washed in cacodylate and subsequently in 0.1 M Tris-HCl buffer.

The antibodies used were described previously. Two mAbs, OS-2 and COS-1, were produced by immunizing mice with a crude chicken photoreceptor membrane suspension, selecting the hybridoma clones with immunocytochemistry and finally characterizing the antibodies by immunoblotting. Both mAbs were found to be specific to visual pigments: while mAb OS-2 recognized a common epitope of practically all visual pigments in the chicken, mAb COS-1 identified a single visual pigment with a molecular mass of 33 kD. The third antibody, a polyclonal anti-rhodopsin serum, was produced by excising the opsin band from SDS PAGE gels of bovine photoreceptor membrane proteins and by immunizing rats with the eluted denatured opsin. Therefore in the following we use the term anti-opsin to designate the antibody to the protein component of the rod visual pigment, rhodopsin.

Light microscopic immunocytochemistry was carried out on two kinds of specimens. In the majority of the cases, 0.5–1.0 μm semithin sections of araldite-embedded retinas were used after removing the embedding resin with sodium methoxide. The 0.5 μm sections allowed us to investigate the same photoreceptor cell on adjacent sections with different antibodies. Therefore we frequently used serial semithin sections, the consecutive members of which were incubated with the three antibodies or with a mixture of mAb COS-1 and anti-opsin serum.

Since the colored oil droplets are dissolved during embedding, part of the immunocytochemical work was performed on frozen semithin sections. Small fixed retinal pieces were infused with 2.1 M sucrose 1–2 hr at 4°C, subsequently frozen with Freon 22 on specimen holders and sectioned on an FC4 cryo-ultramicrotome (Reichert, Oberkochen) on Fujicolor 100 or Agfa XR100i color print films; the same method was also used with photography of the frozen semithin sections.

Fluorescence microscopy was carried out with an epifluorescence attachment to a Laborlux K microscope (Leitz, Wetzlar, Germany). The specimens were illuminated through a 340–380 nm ultraviolet filter, while 430 nm was used as a blocking filter. The same retinal areas were photographed subsequently with transmitted light on color print film to identify the color of the oil droplets and with ultraviolet light for fluorescence on a highly sensitive black-and-white film (Fortepan 400). A NPL Fluotar (Leitz) ×100 immersion objective was used for photography.

Results

Morphologically Identifiable Cone Cells

Six different cone types can be identified in the red area of the pigeon retina (Fig. 1). The largest oil droplet can be found in a single cone having a red oil droplet which is situated in the upper (sclerad) level. This cone type contains additional small oil droplets along the plasma membrane of the inner segment. Also in this upper level is the orange oil droplet of the double cone. This droplet is of medium size and has a tendency to form one or two additional small droplets by fragmentation. Another type of orange oil droplet can be found at a deeper (vitread) level and belongs to a single cone. The oil droplet is spherical in shape, somewhat smaller than the red oil droplet and has a deep orange color. At the same level (or slightly deeper), two different types of small oil droplets are situated. One of them belongs to the accessory member of the double cone; this oil droplet is yellow in color and is composed frequently of tiny units forming a group in the apex of the inner segment (Fig. 2). Another typical feature of this complex is that it does not form a diffraction halo while focusing in the microscope. The other small oil droplet at this level is the colorless oil droplet in a thin single cone. In this
Figures 1–4 were originally submitted in color but, due to the high production cost of color figures, they were transformed into black and white. The colors of the oil droplets are marked by letters: r = red, o = orange, do = deep orange, yg = yellowish green, c = colorless. Original color prints are available on request.

Fig. 1. Schematic representation of the morphologically identifiable cone types and their relation to antibody binding. See text for further explanation.

Fig. 2. A double cone extending from the edge of a whole-mount retinal piece. The accessory member of the double cone (arrow) has a small yellow droplet in the apex of the inner segment and additional smaller droplets in the ellipsoid region.

Fig. 3. A pair of micrographs taken from the same area of a whole-mount retina with white (a) and ultraviolet light (b), respectively. Oil droplets at the more vitread level (yellowish-green, orange, and colorless) are in focus. Yellowish-green oil droplets on (a) are fluorescent on (b).

Fig. 4. Immunocytochemical reactions on frozen semithin sections (a, b) and on whole retina (c). If frozen sections were reacted with mAb COS-1 (a), the outer segments of the cone with the large red oil droplet, as well as that of the double cone (the principal member of which is recognizable here by the orange color of its oil droplet in the upper level) show a positive reaction. In contrast, anti-opsin (AO, b) stains cones with a larger deep orange and a smaller yellowish oil droplet, respectively. Red outer segments, also positive with this antibody, are present at the upper margin of the micrograph. The bar on (b) represents 10 μm and applies for all micrographs on this color plate. On a retinal whole-mount reacted with mAb OS-2 and seen from above (c), no positive outer segments can be found belonging to the yellowish-green oil droplets (arrows).

respective observations are in contrast with those of Mariani and Leure-DuPree, who did not distinguish single cones with colorless oil droplets but attributed the colorless droplet to the accessory member of the double cones. The yellowish-green oil droplets are somewhat bigger than the colorless droplets and can be found at the deepest (most vitread) oil droplet level.

This stratification of oil droplets was consistently observed in fresh whole-mount retinas and was preserved also in favorably fixed and embedded specimens. Transverse sections of such retinas made identification of morphologically different cone types in most cases possible. It must be noted, however, that the pigeon retina is very sensitive to fixation and suboptimal fixations can often lead to disordered positions of oil drops and partial loss of outer segments. To increase the reliability of our studies we made parallel observations on frozen semithin sections where the color of the oil droplets could be directly observed. This method has its own limitations, such as loss of oil droplets from their original positions, changes in their color during the immunocytochemical procedure, less intense reaction, and difficulties in achieving favorable orientation of photoreceptor cells. Therefore cone cells could be reliably identified by comparing findings derived from both lines of observations.

In comparing color pictures with pictures made from the identical area in the fluorescence microscope (Fig. 3a, b), fluorescent oil droplets can be easily identified as the yellowish-green oil droplets. Characteristic of this fluorescence is that at the beginning of ultraviolet irradiation it is almost zero and gradually increases in intensity to reach a maximum at 20–40 sec. The fluorescence fades after 1 min and is abolished after about 2 min. Along with these changes in fluorescence intensity, the oil droplets gradually lose their color and become completely colorless. Other oil droplets retain their color during this period and only the very small red droplets along the plasma membrane of the inner segment with the large oil droplet become clear.

Cone Types Distinguished with Immunocytochemistry

Immunocytochemical investigations of semithin sections showed cone cells to bind antibodies as summarized on Figure 1. According to visual pigment antigenicity we designate cones which are labelled with mAb COS-1 as type I cones. These were principal and accessory members of the double cone and the single cone with red oil droplet. The identification of the COS-1 positive cones according to their oil droplet colors was easily possible on frozen semithin sections (Fig. 4a), while the double cone could be recognized according to its characteristic morphology even on semithin sections of araldite-embedded retinas (Fig. 5). When using higher (1:20,000, 1:40,000) dilutions of this antibody, the staining pattern remained the same, with the intensity of the binding decreasing gradually in all three cone outer segments. In contrast, a lower (1:5,000) dilution of COS-1 resulted in weak or moderate staining of additional photoreceptor outer segments (rods, type II and III cones).

In addition to rods, our anti-opsin serum recognized two different cones with oil droplets of medium and somewhat larger size located at the lower level of
The six morphologically identifiable cone types of the pigeon could be grouped into four classes according to the antigenicity of their visual pigments (Fig. 1). One cone type (type I) represents a group consisting of three morphologically different cones while three represent one morphological type each. The question can be asked if cones in the type I group express the same visual pigment protein or homologous pigments with identical or similar epitopes which are recognized by mAb COS-1. Although the latter possibility cannot be ruled out and further monoclonal antibodies may differentiate between them, there are a few data favoring the idea that the three cones in type I group all contain the same visual pigment. (1) Members of a protein family may have closely related epitopes with different affinities to a certain mAb; in such cases diluting the antibody can differentiate between them. With type I cones of the pigeon this was, however, not the case: decreasing the antibody concentration resulted in equal and gradual decrease of the labeling intensity in all three cones. This observation indicates that the antigenic determinant in the visual pigment of these cones is identical, although it does not necessarily preclude that the determinant belongs to different visual pigments. (2) Microspectrophotometric data have shown that the outer segments of these cones contain a visual pigment having the same absorption maximum in the pigeon. Similar results were reported on chicken and turtle retinas.

The classification of cones into four immunologically types was intended to make discussion of the results simpler and to avoid repetitions of lengthy morphological definitions. According to this classification (Fig. 1) the four cone types are: type I, the COS-1 positive cones; type II, cones labeled both with anti-opsin and OS-2; type III, cones labeled with anti-opsin only; and, finally, type IV, cones that are negative with both COS-1 and anti-opsin, but positive for OS-2. If we compare this immunocytochemical labeling pattern with morphologically identifiable cone
types, the conclusion can be drawn that both members of double cones and the single cone with the large red oil droplet belong to type I, the single cone with the orange oil droplet represents type II, the single cone having a yellowish-green droplet is identical with type III and the thin cone with the small colorless droplet can be identified as type IV.

The situation becomes more complex if we want to assign color sensitivities to the four immunologically identifiable cone types. Data about visual pigments in the whole retina and especially in different visual cells of the pigeon are not completely unequivocal and further microspectrophotometric and electrophysiological investigations are needed until a clear-cut idea about the color sensitivity of individual photoreceptor cells can be obtained. Nevertheless, in the following we speculate as to the color-perceiving properties of the four cone types distinguished by our antibodies. What seems to be well established is that both members of the double cones and the single cone with the red oil droplet contain a long wavelength-sensitive visual pigment, the absorption maximum of which is between 560 and 570 nm. The yellowish-green oil droplet of the cone having a yellowish-green droplet is identical with the type I cones recognized by mAb COS-1 which can therefore be regarded as specific to the long wavelength-sensitive pigment.

It is most likely that the single cone with the orange oil droplet containing a $514\,\text{nm}$ green-sensitive visual pigment (type C of Bowmaker) corresponds to our type II cone which reacts with both anti-opsin and mAb OS-2. Whether this pigment represents a separate green-sensitive cone pigment or is identical with the $507\,\text{nm}$ pigment thought to be similar or identical with rhodopsin remains to be established.

The cone with the yellowish-green oil droplet, corresponding to our type III cone, binds anti-opsin less intensely and is negative with mAb OS-2, indicating that its visual pigment is different antigenically from the other pigments. This cone was described by microspectrophotometry to be blue-sensitive with a pigment of $\lambda_{\text{max}} 460\,\text{nm}$ (type A$_8$ of Bowmaker). A similar blue-sensitivity ($\lambda_{\text{max}} 467\,\text{nm}$) was also reported on the basis of selective bleaching and early receptor potential. The yellowish-green oil droplet of this cone is fluorescent and in this respect is similar to the larger variety of colorless oil droplets in the turtle. The analogy with the turtle is even more evident after ultraviolet irradiation of the pigeon retina, because the yellowish-green oil droplet becomes entirely colorless in contrast to the other droplets. The turtle cone with the fluorescent colorless oil droplet was described as blue-sensitive, although another view on the color sensitivity of this cone was also published.

The thin cone with the colorless oil droplet representing type IV in our classification deserves special attention. It certainly expresses a visual pigment which is different from the others since neither rhodopsin nor COS-1 (at the optimal 1:10,000 dilution) was bound to it. This pigment was only recognized by mAb OS-2; higher dilutions of OS-2 showed this cone outer segment to bind antibody stronger than other photoreceptor outer segments. The color sensitivity of the visual pigment in this type IV cone is unclear at present. Bowmaker described it as a red-sensitive cone with $\lambda_{\text{max}} 567\,\text{nm}$, similar to the one present in the double cones and in the single cone with the large red oil droplet (type I of our classification). A similar single cone with a small, colorless oil droplet can be found also in the turtle, where data are contradictory as to whether it contains a red- or a blue-sensitive photopigment. Certainly, this pigment in the pigeon retina cannot be identical with the one in type I cones because it is not recognized by mAb COS-1. Another photoreceptor protein with the same longwave sensitivity would be unusual from the evolutionary point of view and is rather unlikely. In contrast, no photoreceptor cell was described to which ultraviolet sensitivity, common in certain vertebrates and shown to be present also in the pigeon could be assigned. The possibility that the cone with the colorless oil droplet is an ultraviolet receptor is supported by its oil droplet being highly transmittent for ultraviolet light, which can therefore freely reach the receptor molecule in the outer segment. Similar considerations prompted Chen et al. and Kolb and Jones to assume the cone with the colorless oil droplet to be responsible for ultraviolet light perception in birds and turtle.

In addition to the ultraviolet-sensitive pigment, a short wavelength (violet)-sensitive visual pigment was also described by several independent methods to be present in the pigeon and chicken. No microspectrophotometric or electrophysiological data are available as to which cone type this visual pigment ($\lambda_{\text{max}}$, between 410 and 420 nm) could be assigned. Since colored oil droplets absorb light shorter than 430 nm, the only candidate would be the cone with the colorless oil droplet. The question of how the violet-sensitive pigment is related to the ultraviolet-sensitive photopigment and whether it is expressed by a subclass of the cones with the colorless droplet (type IV in this study) remains to be solved.

It is interesting to compare the binding properties of the antibodies to visual pigments in other species. As was shown in this paper, mAb COS-1 recognized the long wavelength-sensitive visual pigment in the pigeon. Similar results were obtained in the chicken as well as in the turtle (unpublished). In the gecko retina, which contains green- and blue-sensitive photoreceptors only, this antibody labeled the green (middle wavelength)-sensitive cells. The antibody
showed a uniform binding pattern in mammals, where it bound to the middle wavelength (green)-, or middle-to-long wavelength (green and red)-specific visual cells. Consequently, this mAb seems to recognize an epitope on visual pigments sensitive to the middle-to-long wavelength light from reptiles to man. In addition to rods, our polyclonal antisera to bovine rhodopsin recognized two morphologically distinct cone types. Similar observations were made also in the chicken. Our study shows that the two morphologically different cone types are also immunologically different (types II and III of our classification), since mAb OS-2 could distinguish between them. It must be noted, furthermore, that in the gecko as in the pigeon it was the blue-sensitive cone (accessory member of type C double cone) which showed a specific labeling by anti-opsin. Our mAb OS-2 recognized all photoreceptor outer segments with the exception of one, containing most likely the blue-sensitivite pigment. It is therefore surprising that in mammals the same antibody binds to the blue-sensitive photopigment and leaves outer segments of all other visual cells unstained. This complementary behavior of mAb OS-2 in lower vertebrates on the one hand and in mammals on the other is very interesting and merits further clarification.

Key words: pigeon cones, visual pigments, antibodies, immunocytochemistry, oil droplets

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References