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Opsin- and S-Antigen-Like Immunoreactions in Photoreceptors of the Tree Shrew Retina

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In the tree shrew retina individual rod and cone photoreceptors can be readily identified and quantified because their perikarya are arranged in a single layer. This retina is therefore an ideal system for testing the specificity of photoreceptor-directed antibodies. Here we describe the staining properties of polyclonal antibodies against (rhod)opsin and retinal S-antigen in the tree shrew retina. The (rhod)opsin antibody exclusively and completely labelled the rod population. The antibody against S-antigen also labelled all rods and, in addition, a regularly arrayed subpopulation of cones, which we argue to be the blue-sensitive cones. In the context of our findings, the labelling of pinealocytes with these antibodies is discussed. Invest Ophthalmol Vis Sci 30:530–535, 1989

Immunocytochemical studies of the retinae of several mammalian species have shown that antibodies against the rhodopsin apoprotein opsin and S-antigen exclusively label photoreceptors.[1–5] Furthermore, such antibodies have been used to demonstrate that pinealocytes contain photoreceptor-like antigenic molecules and are closely related to retinal photoreceptors.[2–10] For both fields of research, it is of considerable interest to characterize the specific receptor type/s that are labelled by these antibodies. Both opsin and S-antigen immunoreactions have been shown in the vast majority of retinal photoreceptors, but it is possible that not all receptors are labelled.[1–3,10] The mammalian retinae studied so far (eg, rat, mouse, guinea pig, hamster, cat, rabbit, ox, human) are heavily rod-dominated with smaller intermingled populations of cones. Due to these anatomical conditions it remains unclear whether: (1) the antibodies against (rhod)opsin recognize the entire rod population; and (2) the antibodies against S-antigen exclusively label rods,[11,12] or also cones.[1,9]

The tree shrew retina is an ideal system to address these questions. Its photoreceptor layer is cone dominated with rods comprising only approximately 5% of the receptors.[13,14] In contrast to the outer nuclear layer (ONL) of most mammalian retinae, the tree shrew ONL contains only one row of receptor somata (cones), with the few rod somata slightly displaced towards the outer plexiform layer (OPL).[13] Hence, cones and rods can be easily distinguished, quantified and their immunoreactivity observed in vertical or horizontal sections. Here we describe the tree shrew photoreceptor types that are labelled by a polyclonal antibody against bovine (rhod)opsin,[3,15] named CERN-JS 839, and a polyclonal antibody against bovine S-antigen, named SAP.[2] There is good evidence that retinal S-antigen is identical to the "48 kD protein" of the retina,[16] now also named "arrestin,"[17] an enzyme in the phototransduction process.

Materials and Methods

Tissue Preparation

Four eyes from three adult tree shrews (*Tupaia belangeri*) of either sex were used for the present study. Animal care and treatment conformed to the ARVO Resolution on the Use of Animals in Research. The animals were sacrificed by an overdose of pentobarbital given intraperitoneally and the eyes were enucleated immediately. The eyeball was cut open around the equator and the anterior part with lens and vitreous discarded. The posterior eye cup, with the retina attached, was then immersed in fixative (4% formalin in saline or phosphate buffer (0.1 M PB, pH 7.4)). Fixation times ranged from 2 hr to 6 weeks.

Immunohistochemistry was performed on sections prepared by embedding small pieces of retina (3–4 mm^2) in paraffin and serially sectioning the blocks to

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obtain vertical or horizontal sections of 10 or 15 μm thickness. ("Vertical" and "horizontal" refer to planes of section which are orthogonal and parallel to the retinal layers, respectively.) For immunocytochemical staining of whole isolated retinae, adhering parts of the pigment epithelium were brushed off gently, the retina was washed in PB for 2 hr and immersed in 30% sucrose in PB at 4°C overnight. The specimens were then shock-frozen in liquid nitrogen and thawed to improve antibody penetration.

Immunocytochemistry

Sections were dewaxed and treated with antiserum against bovine (rhod)opsin (CERN-JS 839),[3,15] diluted between 1:800 and 1:1600, or with antiserum against bovine S-antigen (SAP),[2] diluted 1:1000. Free-floating whole retinae, after the shock freezing, were washed three times, treated for 3 hr with 0.1 M DL-lysine, 10% normal rabbit serum and 0.3% Triton X-100 made up in PB and then incubated with the primary antiserum at a dilution of 1:200 or 1:1000 without detectable differences. All antisera were diluted in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 and 1% bovine serum albumin and applied for 72 hr at 4°C.

After a 30 min wash in PB, the specimens were incubated with porcine anti-rabbit IgG (Dako, Copenhagen, Denmark), diluted 1:20, for 1 hr at room temperature and rinsed for 30 min. For processing according to the peroxidase-anti-peroxidase technique, the tissues were finally treated with PAP-complex (Dako) diluted 1:80 in PBS, for 1 hr at room temperature,[2] and washed. Peroxidase activity was demonstrated by incubation in 0.025% diaminobenzidine containing 0.003% hydrogen peroxide in PBS. Sections were mounted with Permount (Fisher, Fair Lawn, NJ). Whole retinae were mounted flattened on glass slides, photoreceptor side up, and mounted with glycerol (9 parts glycerol/1 part PB).

To test the specificity of CERN-JS 839 to recognize (rhod)opsin and of SAP to recognize the S-antigen in tree shrew retina, three retinae were used for a Western blot analysis. The retinae were separated from the pigment epithelium, sonicated in ice-cold 0.1 M PB and mixed with sample buffer (2.5 ml 0.25 M Tris-HCl buffer, 4.0 ml 10% SDS, 400 μl 0.25% bromphenol blue, 100 μl 0.25% pyronin, 1 ml mercaptoethanol). The samples were boiled for 3 min, applied to a 1 mm thick, 10% SDS-polyacrylamide gel and then electrophoresed by standard techniques. Proteins were blotted onto nitrocellulose (overnight, 180 mA). The nitrocellulose sheets were incubated overnight with the (rhod)opsin antibody (CERN-JS 839, diluted 1:5000) or the S-antigen antibody (SAP, diluted 1:5000), respectively. Binding of the primary antibody was then visualized by means of the PAP technique essentially as described for the immunocytochemical investigations.

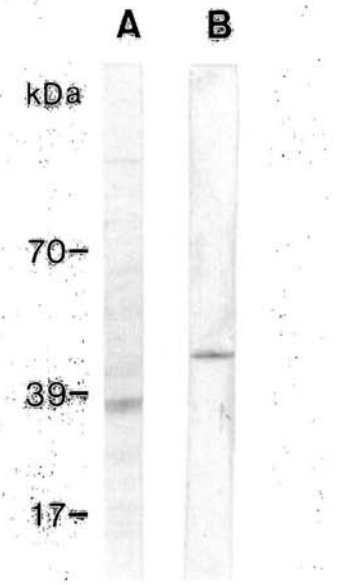

Fig. 1. Immunoblots of 10% SDS-PAGE of proteins in the tree shrew retina. Lane A: Immunoreactive band of approximately 36–38 kD molecular weight detected with the (rhod)opsin antibody CERN-JS 839. Lane B: Immunoreactive band of approximately 48–50 kD molecular weight detected with the S-antigen antibody. Molecular weights were estimated with the use of prestained standards (Biorad, Richmond, CA).

Staining of sections as above but with omission of the primary antibody revealed no labelling in the tree shrew retina.

Some immunocytochemically labelled sections were counterstained as follows: removal of coverslips and permount with xylene, rehydration through ethanol into distilled water, and staining with toluidine blue (0.01%). The sections were then dehydrated, cleared and mounted with Permount.

Results

Western blot analysis shows that the (rhod)opsin antibody binds to a 36–38 kD protein band (Fig. 1A) and the S-antigen antibody reacts with a 48–50 kD protein band (Fig. 1B). This indicates that they specifically recognize the (rhod)opsin and S-antigen (arrestin) molecule of the tree shrew retina.

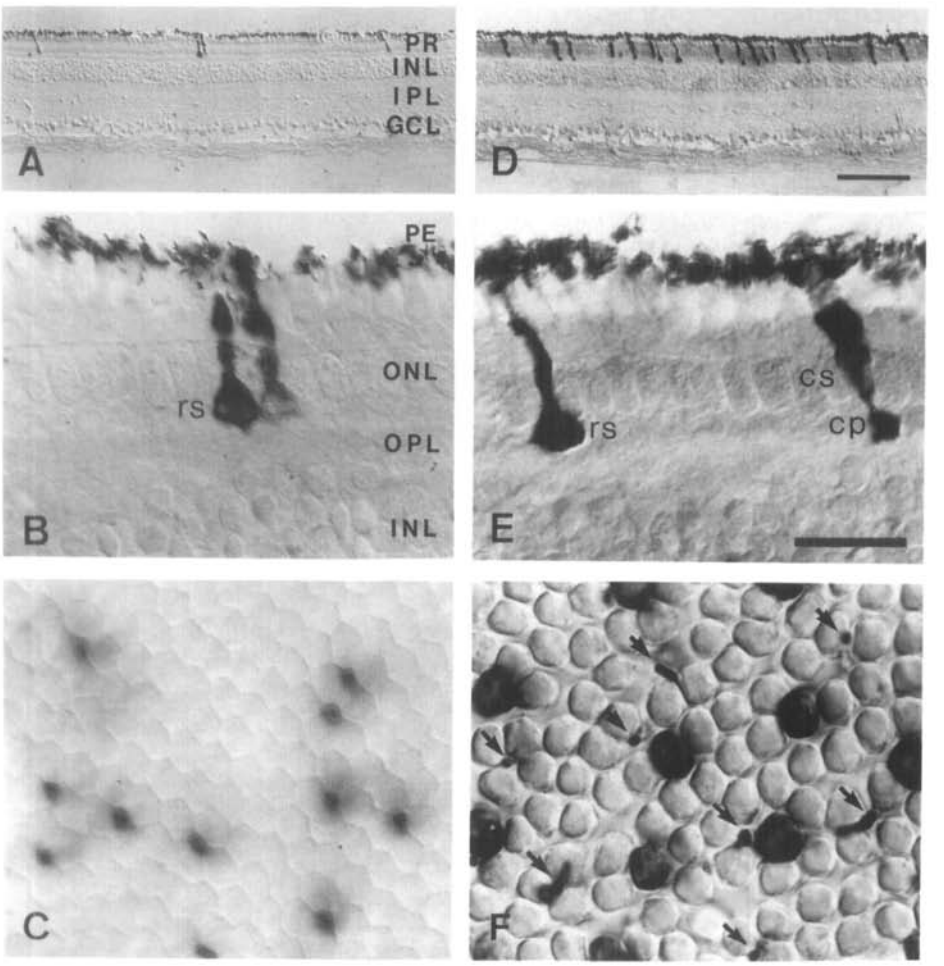

Fig. 2. Opsin-like immunoreactivity (**A–C**) and S-antigen-like immunoreactivity (**D–F**) in the tree shrew retina. (**A, D**) Adjacent vertical paraffin sections at low magnification. Only photoreceptors show immunoreactivity and the antibody against S-antigen labels many more receptors. Pigment epithelium also appears dark. Some retinal layers are indicated. Scale bar in D, 100 μm, applies to A and D. (**B, E**) Details from A and D, respectively, at higher power. In B a pair of rods is labelled, the perikarya are displaced from the row of unlabelled cone somata towards the OPL. In E a rod and a cone are labelled. (**C, F**) Photoreceptor layer seen in whole-mounts at high magnification. C with focus on the cone myoid level. The smaller rod inner segments are immunopositive, but not the larger densely packed cones. F with focus on the cone ellipsoids and rod outer segments. A subpopulation of cones as well as the rods (arrows) are labelled. Scale bar in E equals 20 μm and applies to B, C, E and F. Abbreviations: PE: pigment epithelium; PR: photoreceptor layer; ONL: outer nuclear layer; OPL: outer plexiform layer, INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell and axon layer; rs: rod soma; cs: cone soma; cp: cone pedicle.

Figure 2 shows a comparison of elements labelled by the (rhod)opsin antibody (A–C) and the S-antigen antibody (D–F) in the tree shrew retina. Opsin-like immunoreactivity occurs exclusively in the photoreceptor layer. Only a few photoreceptors are labelled (Fig. 2A). At higher magnification (Fig. 2B), all labelled receptors can be identified as rods by their thin inner and outer segments and the position of their somata. The perikarya, and the inner and outer segments of the rods are labelled. A view of the photoreceptor layer in a whole-mounted retina (Fig. 2C), with cone inner and rod outer segments in focus, shows the sparse and irregular distribution of rods amongst the dense unlabelled cone population, an

impression already conveyed by the vertical sections (Fig. 2A).

S-antigen-like immunoreactivity is also restricted to the photoreceptor layer (Fig. 2D). In adjacent sections, S-antigen immunoreactive receptors are more numerous than the opsin-immunoreactive receptors. Inspection at higher magnification (Fig. 2E) reveals that SAP stains the rods and, unlike CERN-JS 839, also certain cones. The latter can be recognized by the thicker inner segment, the position of the soma and the pedicle which is connected to the soma by a thin stalk (see also ref. 13). As with CERN-JS 839, SAP immunolabelling is present in all cellular compartments but the staining is stronger (compare Fig. 2A and D). Immunolabelled whole-mounts further clarify the pattern of labelled receptors. Figure 2F focusses on the cone inner and rod outer segments. The distribution of S-antigen-positive rods (arrows) is similar to the opsin-immunoreactive ones in Figure 2C, but, in addition, a regular subpopulation of cones is labelled. The regular pattern of these cones is particularly striking when a larger area is viewed (Fig. 3). From the whole-mounted retina the S-antigen-immunoreactive cones were quantified as forming 4 to 10% of the entire cone population, depending on retinal location. We presume that the S-antigen immunoreactive cones are the blue-sensitive cones, since their relative density and regular arrangement match those of the blue-sensitive cones described in other species (see *Discussion*).

To determine whether antibodies against (rhod)opsin and S-antigen label the whole rod population, or only a part of it, we counterstained immunocytochemically labelled vertical and horizontal sections with toluidine blue. Figure 4A shows the distribution of opsin immunoreactivity in a horizontal section at high power. The plane of section is through the vitread ONL and ten rod cell bodies are visible. The same field is shown in Figure 4B after counterstaining with toluidine blue, and three additional nuclei of opsin-immunonegative cells at the vitread level of the ONL show up (arrows). Counterstaining of vertical sections reacted with SAP (Fig. 4D) also reveals occasional nuclei of S-antigen-immunonegative cells at the vitread level of the ONL (arrows).

The nuclei of these immunonegative cells morphologically differ from rod nuclei in their Nissl staining.[14] They are smaller and of triangular or irregular shape. Their chromatin granules are fairly numerous and more evenly distributed than the clustered chromatin of rod nuclei. Immunonegative cell nuclei therefore appear smoother and sometimes more translucent and their nuclear membrane can be easily distinguished. Applying an enzyme histochemical method specific for microglial cells[18] to the tree shrew retina, we have demonstrated a population of microglial cells which by their soma position and cell density fully account for the immunonegative cell population.[14] Microglial cells at the inner margin of the outer nuclear layer are also found in other mammals.[19]

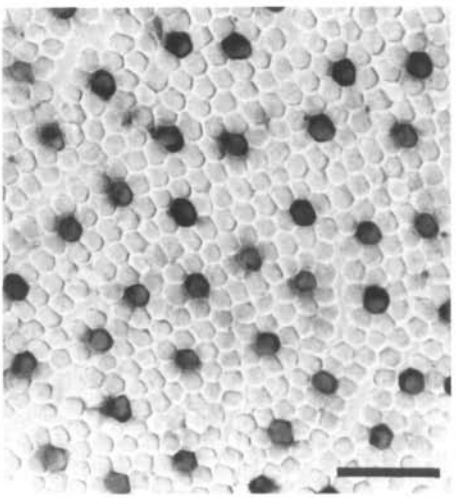

Fig. 3. Larger field from a whole-mounted retina stained with the S-antigen antibody. The focus is on the cone inner segments. The regular distribution of immunopositive, presumed blue-sensitive, cones is very prominent. Scale bar equals 30 μm.

Thus, the analysis of counterstained sections reveals that the entire rod population of tree shrew retina displays opsin and S-antigen immunoreactivity.

Discussion

The current study characterizes the photoreceptor cell types which bind polyclonal antibodies against purified bovine rhodopsin (CERN-JS 839) and retinal S-antigen (SAP) in tree shrew retina. With both antibodies, immunoreactivity is found in the perikarya, inner and outer segments and pedicles of retinal photoreceptor cells. Previous studies using these antibodies have already established that staining is not confined to the outer segments.[1-3,5,9] This is in contrast to the immunoreactivity obtained with another antiserum against rhodopsin, which is restricted to the photoreceptor outer segments.[20] As discussed by Foster and colleagues,[5] these differences can be attributed to different antigenic sites recognized by the antibodies, eg, expressed by opsin in the process of biosynthesis or transport.

The special composition and arrangement of photoreceptor types in the tree shrew retina enabled us to

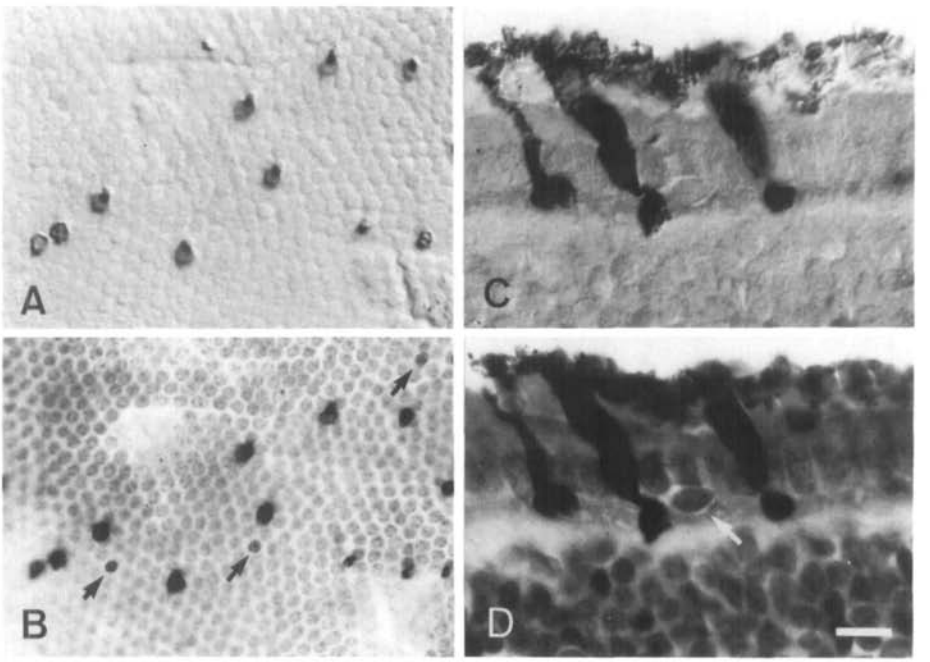

Fig. 4. Counterstaining of antibody-treated sections. **(A, B)** Horizontal section at the level of rod perikarya, which are labelled with the (rhod)opsin antibody; (A) before and (B) after counterstaining. Three immunonegative perikarya (arrows) appear in B, they are microglia (for details see text). Slight shrinkage occurred during the counterstaining procedure. **(C, D)** Vertical section, labelled with the S-antigen antibody, (C) before and (D) after counterstaining. One rod (left) and two cones are immunopositive, whereas a microglial cell (arrow) at the rod nuclear level is immunonegative. Scale bar in D equals 20 μm for A and B, and 10 μm for C and D.

demonstrate that the (rhod)opsin antibody labels the whole rod population and no other retinal elements. The S-antigen antibody also labels the entire rod population and, in addition, a regularly distributed subpopulation of cones which resembles the blue-sensitive cone population in other mammalian species.

Blue-sensitive cones have been described or postulated on the basis of staining differences. In the primate retina (baboon) Marc and Sperling[21] histochemically demonstrated the distribution of three types of cones by the light-stimulated reduction of nitroblue tetrazolium chloride. These authors described the blue-sensitive cones as a regularly distributed population with a low relative density (3–20%). Cone subpopulations with similar properties have been found to preferentially accumulate procion dyes or to stain prominently with toluidine blue in several species.[22–27]

The S-antigen-positive cone population in tree shrew retina also has a low relative density (4–10%) and regular distribution, and therefore we suggest that they are the blue-sensitive cones. This is corroborated by the fact that a cone subpopulation with the same quantitative properties appears darker in toluidine blue-stained horizontal sections.[14]

Long and Aguirre[28] briefly reported S-antigen immunoreactivity in ground squirrel photoreceptors. They found immunoreactivity in rods (which make up less than 10% of photoreceptors in this species) and in a subpopulation of cones, also thought to be blue-sensitive. This suggests an intriguing similarity in the pattern of S-antigen immunoreactivity in the two species.

Western blot analysis shows that the SAP antibody specifically recognizes the S-antigen (48 kD protein) of the tree shrew retina. The high degree of monospecificity and cross-reactivity of S-antigen antiserum in many species[1,4,10] makes it tempting to speculate that this antibody might recognize the blue-sensitive cone population in all mammalian retinae. It has now to be explained why the SAP antibody is selective for the presumed blue-sensitive cones. Is blue cone S-antigen particularly similar to rod S-antigen? Do the other cones not contain the S-antigen or do their corresponding molecules exhibit different epitopes?

Our results with the tree shrew retina may help to

clarify the relationship between rods and cones, on the one hand, and pinealocytes, on the other. Pinealocytes of mammals may contain immunoreactive S-antigen[2,10,29] and (rhod)opsin,[3,6] depending upon the species (see ref. 30 for a review). Generally, S-antigen-immunoreactive pinealocytes are more numerous than opsin-immunoreactive cells.[3,30] These findings, together with the evidence of the current study, suggest the existence of at least two types of pinealocytes, one displaying "rod-like" characteristics (ie, S-antigen and opsin immunoreactivity) and the other displaying "blue cone-like" features (ie, S-antigen immunoreactivity, but not opsin immunoreactivity).

In summary, the tree shrew retina was used to clarify the labelling specificity of polyclonal antibodies against (rhod)opsin and S-antigen. This retina, with its relatively simple arrangement of photoreceptors, may well also prove to be a useful system for testing other poly- or monoclonal antibodies directed against phylogenetically old epitopes of photoreceptor-specific proteins.

Key words: opsin immunoreactivity, S-antigen immunoreactivity, cone photoreceptors, rod photoreceptors, pineal organ

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