Specific Binding of Peanut Lectin to a Class of Retinal Photoreceptor Cells

A Species Comparison

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Although lectins have been used to study surface oligosaccharides of photoreceptor cells in intact retinas and dissociated retinal cells, the specificity of lectin binding to cones versus rods in a variety of species has not been examined closely. The authors previously found that application of fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA), a lectin with high affinity for galactose-galactosamine disaccharide residues, to cryostat sections of unfixed mouse retina results in staining that is confined to synaptic regions and a subpopulation of photoreceptor cells. To further investigate the possibility that PNA binding is specific for cone photoreceptors, the authors extended their studies to include the duplex retinas of fish, rabbit, monkey, and human in addition to the cone-dominant retina of the chick. These studies show that PNA binding is specific for cone inner and outer segments and also is likely to be associated with the large synaptic pedicles of cone photoreceptor cells. In addition, the authors compared PNA binding with that of Ricinus communis agglutinin I (RCA), another lectin that preferentially binds terminal D-galactose moieties. While RCA does bind to cones in the species examined, it also binds to a lesser extent to rod photoreceptor inner segments. The pattern of binding of RCA in other regions of the retina differs markedly from that of PNA. Significantly, RCA serves as a specific marker for retinal vasculature in the human, monkey, and mouse. These results suggest that certain PNA-binding macromolecules may be important in defining the molecular and cellular specificity of cone photoreceptor cells and that PNA may provide a means for the isolation of cones and cone-specific molecules. RCA may prove to be of value in monitoring vascular changes associated with normal development and pathologic conditions. Invest Ophthalmol Vis Sci 25:546-557, 1984

In our previous studies of the binding of a battery of carbohydrate-specific lectins during mouse retinal development, it was observed that peanut agglutinin (PNA) from Arachis hypogaea showed selective binding to discrete patches within the outer synaptic layer and to a subpopulation of photoreceptor cells believed to be cones. This pattern of PNA binding suggested heterogeneity in cellular carbohydrate composition within the photoreceptor layer. The cone-specific binding of PNA suggests that significant differences in glycoconjugate composition of rod and cone photoreceptor cells may exist. In fact, 3H-fucose has been shown to be incorporated into a glycoprotein that is a component of cone but not rod outer segments.

To further investigate the basis of this cellular heterogeneity and its possible relationship to differences between rods and cones, we extended our PNA binding studies to include retinas from a variety of species that differ in their ratios of cone and rod photoreceptor cells. In addition, we compared PNA binding with that of another galactose-specific lectin, Ricinus communis agglutinin I (RCA). We have employed a new embedding method using polyacrylamide (modified from reference 4; Johnson and Blanks, manuscript submitted) to obtain thinner sections and tissue morphology superior to that obtainable with fresh frozen cryostat sectioning techniques.

Materials and Methods

Lectins and Hapten Sugars

Fluorescein-isothiocyanate conjugated (FITC) peanut agglutinin (PNA) and Ricinus communis agglutinin
I (RCA) were obtained from E. Y. Laboratories (San Mateo, CA) and Vector Laboratories (Burlingame, CA). The hapten sugar D (+)-galactose was obtained from Sigma (St. Louis, MO). Hapten-specific inhibition of PNA or RCA binding was evaluated by preincubation of the FITC-lectin (100 μg/ml) in 10 mM sodium phosphate buffered 0.85% saline (PBS, pH 7.2–7.4) containing 25–50 mM D-galactose.

Histochemical Procedures

Experimental animals were treated in conformity with the ARVO Resolution on the Use of Animals in Research. They were killed by cervical dislocation (mice, C57BL/6J), decapitation (fish, Carassius auratus; chickens, Gallus gallus), or an overdose of phe- nobarbital (rabbits, Oryctolagus cuniculus; monkeys, Macaca fasicularis). A total of 50 mouse, 20 chicken, 10 rabbit, 7 fish, 4 monkey, and 3 human eyes were examined in this study. The eyes were enucleated, corneas removed and the eyecups immersed in 4% paraformaldehyde in PBS for 1 hr (unless noted otherwise). The human eyes were fixed longer than the other tissues. One eye was fixed 3 days in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer; the other two were fixed in 4% paraformaldehyde for 24 hr. One of the human eyes came from an enucleation procedure for anterior segment melanoma and the other two eyes came from eye bank donors; the retinas did not appear pathologic. After fixation, the eyecups were rinsed for several hours in PBS and embedded in polyacrylamide according to our modification of the procedure of Hausen and Dreyer4 (Johnson and Blanks, manuscript submitted).

Staining Procedure

The staining procedure with FITC-conjugated lectins was based on that of Hatten et al.5 Staining solutions were prepared by dilution of lectin to 100–200 μg/ml with PBS containing 500 μg/ml bovine serum albumin (PBS-BSA) followed by centrifugation. In brief, sections were preincubated for 10 min in PBS-BSA followed by application of 25–50 μl of lectin solution and incubation in a humid chamber for 15–30 min. Sections were then rinsed in PBS and mounted in PBS-BSA containing 1 mg/ml p-phenylenediamine to inhibit quenching of the fluorochrome.6 Photographs were made on a Zeiss Photomicroscope III (New York, NY) equipped for epifluorescence.

Whole Mounts

Whole-mount preparations of rabbit retina were obtained by dissection of the retina from eyecups fixed for 2 hr in 4% paraformaldehyde as described above. Retinas were rinsed overnight in PBS and then incubated in the presence of 200 μg/ml FITC-PNA in PBS containing 1 mg/ml BSA for 30 min at room temperature. The whole retina then was rinsed in three changes of PBS over a 90-min period. Four to six small peripheral incisions were made to allow the retina to be flat-mounted on a microscope slide. The flattened retina was covered with a mounting medium of PBS containing 1 mg/ml BSA and 1 mg/ml p-phenylenediamine. A coverslip was floated on the mounting medium and medium was slowly withdrawn so the coverslip contacted but did not crush the retina. Observations were made by epifluorescence microscopy using an Olympus BH-S microscope (New Hyde Park, NY) equipped with narrow band blue excitation filters.

Results

In all retinas, except that of the cone-dominant chick eye, heterogeneity of PNA and RCA binding within the photoreceptor layers was observed. Lectin binding was not detectable in the nuclear layers of the retina but, rather, was specific for the synaptic layers and the inner segment (IS) and outer segment (OS) regions of certain photoreceptor cells. More detailed descriptions of FITC-PNA and FITC-RCA binding patterns in the individual species examined are as follows:

Human

In the paramacular region of the human eyes, the cone inner segments, and, to some extent, the outer segments showed PNA binding (Fig. 1A). Rods, apparent as negative images (r, Fig. 1A), did not show PNA binding. PNA binding to the outer synaptic layer (OSL) was not observed but lectin binding in OSL typically is not detectable in tissues subjected to prolonged fixation, such as the human tissue had been. Tangential sections of the human retina in the photoreceptor layer showed rings of fluorescence corresponding to cone inner segments cut in cross-section (arrowheads, Fig. 1B). In some areas the negative images of rods were detected (r, Fig. 1B).

FITC-RCA binding to human retina showed a different pattern of binding compared with that of PNA. Photoreceptor outer segments of both rods and cones were fluorescent (Fig. 1C), but it also was possible to identify the large inner segments of the cones (single arrowheads, Fig. 1C). A band of fluorescence representing the outer limiting membrane (OLM) was evident. Spots of intense fluorescence were visible in the IS-OS junctional region (double arrowheads, Fig. 1C). Patchy areas in the OSL and the entire ILM were fluorescent. Retinal blood vessels (BL) were intensely fluorescent.
Monkey

These animals were perfused with the same dialdehyde fixative described above for one of the human eyes or fixed by immersion for 1 hr in 4% paraformaldehyde after enucleation. Application of FITC-PNA resulted in intense fluorescence only in the IS and OS of cones (Figs. 2A–C). Often the pattern of PNA bind-
ing in the OSL appeared patchy (Fig. 2A). The inner synaptic layer (ISL) was barely fluorescent with PNA. The pattern of binding of FITC-RCA was quite different in the monkey retina (Fig. 3) since both rod and cone photoreceptor outer segments were lightly stained with this galactose-specific lectin. A higher magnification of the photoreceptor region (Fig. 3C) shows that the staining pattern with FITC-RCA appeared to “cap” the region of the cone ellipsoid. Diffuse fluorescence is visible on the rod outer segments, being so intense at the region dividing the outer and inner segments that spots of fluorescence were seen (double arrowheads, Fig. 3C). Fluorescence also was observed in the OSL with FITC-RCA (Fig. 3C). Blood vessels (BL, Fig. 3A) were intensely stained with RCA.

**Rabbit**

Intense binding of PNA was observed in the cone IS (single arrowhead) and OS (double arrowhead) regions, in addition to the OSL (Fig. 4A). There also appeared to be slight binding of FITC-PNA to the cone cell bodies and their axonal extensions (curved arrow, Fig. 4A), leading to the stained patches in the OSL (arrowheads in OSL, Fig. 4A).

In contrast, FITC-RCA intensely stained the inner segments of apparently all photoreceptor cells in the rabbit retina (IS, Fig. 4B). Cone—but apparently not rod—outer segments also were labeled with FITC-RCA (double arrowheads, Fig. 4B). The OSL, ISL, and the inner limiting membrane (ILM) also were intensely stained with FITC-RCA. The regions surrounding the nuclei of the photoreceptor cells stained with FITC-RCA (ONL, Fig. 4B).

In an attempt to further localize FITC–PNA binding, a whole mount of the rabbit retina was prepared (Fig. 5). Since the retina was dissected away from the retinal pigment epithelium, it was possible to see the tips of the outer segments—a view that was not possible in sectioned tissue. The tips of the cone outer segments were intensely fluorescent, as was the plasma membrane of the cone inner and outer segments. The optic disk area also stained with FITC-PNA (arrow, Fig. 5).

The negative images of unlabeled rod photoreceptor cells (r, insert, Fig. 5) are adjacent to fluorescently labeled cones (c, insert, Fig. 5).

**Mouse**

PNA staining again was observed to be selective for a subpopulation of photoreceptor cells. PNA binding was intense in cone IS and OS (Fig. 6A); punctate staining was observed in the OSL. The ISL displayed uniform diffuse staining.

In contrast, FITC-RCA stained the region of the OLM and photoreceptor ISs and the ILM, in addition to a diffuse staining in the OSL (Fig. 6B). As reported previously, retinal blood vessels stained intensely with RCA.

**Chicken**

The chicken retina showed the most intense staining in the photoreceptor layer with FITC-PNA (Fig. 7A); virtually all photoreceptor cells (both IS and OS) appeared to bind this lectin. Faint staining was observed in the OSL and several bands with varying fluorescent intensities were present in the ISL (arrowheads, Fig. 7A). These bands were not seen in the other animals, except when FITC-PNA was injected intraocularly in the mouse (unpublished observations). The nerve fiber layer (NFL, Fig. 7) also was intensely fluorescent.

FITC-RCA stained fewer photoreceptor outer segments (double arrowheads, Fig. 7C) and inner segments (single arrowheads, Fig. 7C) in addition to the OSL (Fig. 7C). The staining in the OSL appeared beaded. The NFL also showed binding to FITC-RCA.

As a control for the specificity of lectin binding, sections of adult chick retina were exposed to FITC-PNA or FITC-RCA preincubated with 50 mM D-galactose. Incubation in the presence of this hapten sugar dramatically decreased PNA (Fig. 7B) and RCA (Fig. 7D) binding. Preincubation with the nonhapten sugar glucose had no effect on the pattern of fluorescent staining by either lectin (data not shown). Similar controls were performed on the other tissues used in this study; similar results were obtained (data not shown).

**Fish**

In the fish, PNA showed binding to cone inner and outer segments (Fig. 8A). Higher magnification shows intense PNA binding to the region connecting the OS and IS (insert, Fig. 8A). The rest of the retina was only faintly fluorescent. This species posed the largest problem in obtaining optimum fixation for ease of cryostat sectioning and consistent lectin binding results.

FITC-RCA showed intense binding in the OSL, ISL, and ILM (Fig. 8B). The rest of the retina was only faintly fluorescent.

**Discussion**

This paper details a comparative study of peanut agglutinin and *Ricinus communis* agglutinin, two lectins with binding affinities for galactose-containing oligosaccharides, to retinal sections from a variety of vertebrate species. In general, the method involves fixing...
Fig. 2. Light micrographs showing retinal binding of FITC-PNA in monkey. A, Fluorescence micrograph of the macular region with intensely fluorescent cone OS and cone IS (C). Punctate fluorescence in OSL may be due to cone pedicles. Faint fluorescence is present in the ISL (x375). B, Phase micrograph of same section as in A. Cone IS (C); PE, pigment epithelium; OLM, outer limiting membrane; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer (x375). C, A retinal section from the paramacular region of a monkey retina simultaneously illuminated for fluorescence and phase-contrast. The cone IS (C, single arrowhead) are visible directly above the OLM. Several cone OS are evident (double arrowheads). The negative images of rods (R) are visible between the intensely fluorescent cones (x500).
Fig. 3. Binding of FITC-RCA to peripheral region of monkey retina. A, Fluorescence micrograph showing cone IS and OS stained by FITC-RCA. Intensely fluorescent regions are evident at what appears to be the rod IS-OS junction. Patchy fluorescence in OSL may be due to cone pedicles. Blood vessels (BL) stain intensely with RCA (x430). B, Phase contrast micrograph of same area of monkey retina shown in A. Cone IS (arrowhead, C) can be clearly differentiated from adjacent rod photoreceptor cells. Arrows depict the same blood vessels seen in A (x430). C, Higher magnification of outer retina showing FITC-RCA staining of cone IS (C). Rod OS are more fluorescent than the IS. Intense fluorescent areas (double arrowheads) are present at the OS-IS junction of the rods. Intense, somewhat patchy fluorescence is present in the OSL (x740).
Fig. 4. Fluorescence micrographs showing binding of FITC-PNA (A) and FITC-RCA (B) to rabbit retina. A, Cone OS (double arrowheads), cone IS (single arrowheads) and patches within the OSL (arrowheads) stain intensely with FITC-PNA. The cell bodies of the cones are lightly fluorescent as are, in some cases, their narrow axonal extensions (curved arrow) to the OSL (×600). B, FITC-RCA diffusely stains the region of the OLM and photoreceptor IS. Cone IS and OS (double arrowheads) also are stained with RCA, as are the ILM, ISL, and OSL. In addition, the paranuclear regions of photoreceptor cells in the ONL are stained by RCA (×475).
tissue in 4% paraformaldehyde for a brief time, embedding in acrylamide, followed by freezing and cryostat sectioning, and, finally, application of the lectin to the tissue sections. The tissue is fixed prior to application of the lectin to preserve tissue morphology and to prevent lectin-induced rearrangement of binding sites. In addition, this treatment allows lectins access to intracellular and extracellular components; the observed staining patterns, thus, are likely to be the result of binding to cytoplasmic as well as cell membrane-associated and extracellular matrix materials. The results obtained confirm and extend our previous observations on the binding of these two lectins to frozen, unfixed mouse retina.1

PNA is shown to bind specifically to photoreceptor cells morphologically identifiable as cones in a variety of species, including human, monkey, rabbit, mouse, chicken, and fish. In these species, PNA binds intensely to cone inner and outer segments and often shows a patchy distribution of binding in the outer synaptic layer. When present, the size of the PNA-binding patches in the OSL is comparable with that of cone pedicles. The staining in the OSL appears to be especially sensitive to prolonged fixation. In some cases, the cone photoreceptor cell bodies and their narrow axonal extensions leading to the OSL were lightly fluorescent (Fig. 4A). In addition, PNA binding in the inner synaptic layer of the chick retina exhibits a stratified pattern. To a lesser extent, the retinal vasculature and the inner limiting membrane also show PNA binding specificity.

Our results with FITC-PNA do not agree with the binding in isolated photoreceptor preparations in frog. Bridges and Fong7 found that FITC-PNA bound to the OS and not to the IS of freshly isolated ROS preparations, some of which had the IS attached, and Bridges8 reported similar results using isolated preparations of frog retinas. The latter paper also demonstrates FITC-PNA binding to accessory cones in frog. It seems probable that these discrepancies are due to species differences. It is interesting to note that PNA has been shown to bind preferentially to photoreceptor cell precursors in the eye disc of ommatidia in the Drosophila eye.9

Although the binding of RCA is similar to PNA binding in retina, it is not identical. The major difference is that PNA binds cones exclusively in all species studied while RCA binds rods to some extent in addition to cones. In the primates studied, FITC-RCA showed patchy fluorescence of the outer segments,
Fig. 6. Fluorescence micrographs of mouse retina showing binding of FITC-PNA (A) and FITC-RCA (B). A, Intense fluorescence of selected IS (single arrowhead) and OS (double arrowheads). There is a punctate pattern of heavy fluorescence in the OSL, while bright fluorescence is apparent in the ISL (×510). B, The OLM-IS region and the OSL are barely fluorescent. Blood vessels (BL) are brightly fluorescent. The ILM is also fluorescent (×550).

being more intense at the region connecting outer and inner segments and much weaker in the inner segments. These fluorescent patches were reported first by Uehara et al\textsuperscript{10} on sections of fixed paraffin embedded monkey retina. It is possible that these patches (see Figs. 1C and 3C) correspond to the region of the connecting cilium in rods.

Our results in human and monkey with RCA confirm earlier studies using ferritin-RCA by Nir and Hall,\textsuperscript{11} Bridges,\textsuperscript{3} Bridges and Fong\textsuperscript{7} in the frog, and by Uehara et al\textsuperscript{10} in monkey in which it was shown that RCA binds preferentially to rod outer segment (ROS) plasma membranes. McLaughlin and Wood\textsuperscript{12} also showed that horseradish peroxidase conjugated RCA binds to ROS in the rat. In rabbit, RCA appeared to bind to the OLM, the IS region and to a few photoreceptor outer segments believed to be cones (Fig. 4B). In mouse, the OLM and to a lesser extent the IS region were faintly fluorescent with FITC-RCA (Fig. 6B). In chick, FITC-RCA binding produced a beaded appearance of fluorescence in the OSL and appeared to stain some photoreceptors (Fig. 7B). However, their identification remains unclear. And finally, in the fish retina FITC-RCA did not bind to either the OS or IS region but only to the synaptic layers and the ILM (Fig. 8B). We conclude that there is considerable species variation in RCA binding to retina.

Our results of RCA binding to blood vessels confirms our earlier observation in the mouse retina.\textsuperscript{1} Although this study was confined to the retina, Raedler et al\textsuperscript{13} have reported that RCA (referred to as RCL in their study) binds to the vasculature of the central nervous system in general. Other researchers\textsuperscript{14} have used another lectin, \textit{Ulex europaeus} (UEA), as a marker for vascular endothelium in human tissues such as kidney, liver, pancreas, and cerebellum.

The differences in photoreceptor binding of these two galactose-binding lectins probably is best explained...
by the fact that PNA has its highest affinity for the disaccharide sequence D-Gal-β(1 → 3)D-Gal NAc, \(^{15}\) while RCA has a specificity for β-linked D-galactose residues. \(^{16}\) Our observation that PNA binds to cones preferentially, while RCA binds to some extent to both rods and cones suggests that oligosaccharides present...
Fig. 8. Binding of FITC-PNA (A) and FITC-RCA (B) to a goldfish retina fixed by cardiac perfusion with 4% paraformaldehyde. A, Fluorescent micrograph showing connecting region of cone OS intensely fluorescent due to FITC-PNA binding; lighter binding of ellipsoid region of the IS (×480). Insert: There appears to be a ring of fluorescence (arrows) separating the cone OS and IS (×1,010). B, Fluorescent micrograph showing intense fluorescence of the OSL, ISL, and ILM with FITC-RCA. There appears to be more fluorescence in the region of the ISL directly above the GCL layer (×430).

on rod photoreceptor IS and OS lack the disaccharide linkage noted above or the linkage is inaccessible to lectins in sectioned material.

The preferential binding of FITC-PNA to cones in a variety of species suggests a major biochemical difference between rods and cones that may be related...
to their different visual pigments. The marked appearance of PNA binding in the species presented here suggests that it may provide a means of separating cone from rod photoreceptors. Electron microscopic investigations are underway to further characterize PNA-positive photoreceptor cells and specific regions of the synaptic layers, as are biochemical characterizations of PNA-binding molecules in the retina.

**Key words:** peanut agglutinin (PNA), *Ricinus communis* agglutinin I (RCA), retina, photoreceptors, cones

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**References**