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Immunochemistry of the Outer Retina

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Summary

The immunochemistry of the outer retina is discussed with particular reference to photoreceptor cells, the retinal pigment epithelium and the interphotoreceptor space. The antigens identified and the techniques utilised are summarised.

The retinal pigment epithelium (RPE) and the photoreceptor cells form a functionally interdependent unit which is responsible for the initial photochemical events in the processes giving rise to vision. As might be expected such specialised cells have a variety of unique proteins to support their specialised functions in addition to elements common to many cell types.

This paper will be concerned with the immunochemistry of the developed outer retina which, for our purposes, shall be defined as the photoreceptor cells, the subretinal or interphotoreceptor space (Fig 1), and the RPE. Müller cells and other elements of the inner retina will not be discussed.

Immunochemistry can be applied to the study of the outer retina in two main areas. Firstly, the identification and localisation of known proteins using antisera or monoclonal antibodies (MAbs). Examples might include the identification of cytoskeletal elements such as actin and tubulin in RPE cells or the localisation of rhodopsin and S-antigen in photoreceptor cells. The second, and possibly more exciting area, is the use of MAbs as dissection tools in the indentification and characterisation of previously unknown proteins. This approach has been elegantly demonstrated by Molday and coworkers[1] in the identification of peripherin, a protein of the rod outer segment.

The two complementary techniques of immunoblotting[2] (sometimes referred to as Western blotting) and immunocytochemistry are particularly useful in the indentification, characterisation and localisation of antigens.

Immunoblotting

Recent advances in biochemical techniques made possible the transfer of a resolved mixture of proteins and other molecules from gels (acrylamide or agarose) onto an immobilising matrix. Nitrocellulose membrane has been the most widely used matrix.[2] This technique has attracted a wide range of applications in basic, clinical and immunologial research. In the latter, antibodies (monoclonal or polyclonal) can react with the antigens bound onto the nitrocellulose membrane (immunoblotting). The reacted antibodies can then be easily identified by autoradiography if they are conjugated to a radioactive ligand (^{125}I) or by the addition of a suitable chromogenic substrate, if the ligand is an enzyme (like peroxidase or alkaline phosphtase). Alternatively, if the first antibody is not linked to a ligand a second antibody (anti-immunoglobulin) conjugated to an appropriate probe (radioactive ligand or an enzyme) could be used in the same manner to visualise antibody antigen reaction onto the nitrocellulose membrane.

Immunoblotting is useful in analysing the immune response in certain retinal diseases, especially those associated with autoimmunity.[3] Its application has also proved invaluable in identifying antigens normally masked by other predominant proteins which co-migrate with

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them on an SDS-polyacrylamide gel.[1] It is becoming evident that immunoblotting is one of the most accurate methods of defining the specificity of an antibody and as a means of identifying common epitopes on related or unrelated molecules (Fig 2).

Immunocytochemistry

As with most microscopical techniques vastly different degrees of resolution are obtainable. At one end of the range, immunofluorescence staining of frozen sections can be useful (Fig 3), while at the other end of the spectrum lies electron microscopy (EM) immunocytochemistry using pre- and post- embedding techniques and a variety of electron dense markers of which colloidal gold is becoming the most popular due to its small size and extreme electron density.[4]

Immunofluorescence or immunoperoxidase staining of frozen sections of lightly fixed materials using methanol and acetone has the

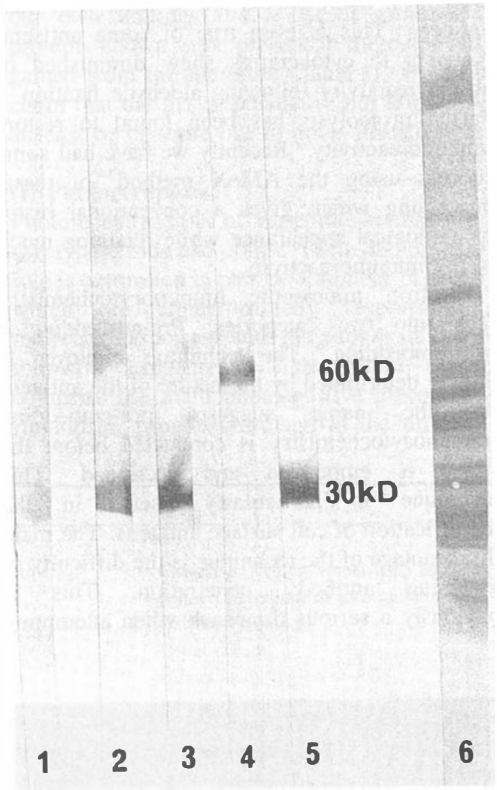

Fig. 2. *Example of an immunoblotting experiment. Lanes 1-5 are blots following gel electrophoresis of purified rod outer segments. Lane 6 is a blot following the electrophoresis of retinal extract. Lanes 1 and 6 have been stained with amido black to show the protein bands present. In lane 1 faint staining of a band corresponding to opsin can be seen. In lane 6 numerous protein bands are present. Lane 2, Blotted Ros proteins immunostained using MAb ROS 4C4. This antibody probably recognises opsin. Faint bands corresponding to the dimer and trimer of opsin are also visible. Lane 3, Blotted ROS proteins immunostained using MAb ROS 4A2. Strong staining of a band at approximately 32kD is visible. EM immunocytochemistry suggests that this MAb recognises peripherin (see Fig. 4). Lane 4, Blotted ROS proteins immunostained using MAb ROS 1F2. Strong staining of a band at approximately 60kD is present. This protein has not been identified as yet. Lane 5. Transferred ROS proteins immunostained using MAb ROS 1F4. Strong staining of a band at approximately 32kD is present. This MAb probably recognises opsin.*

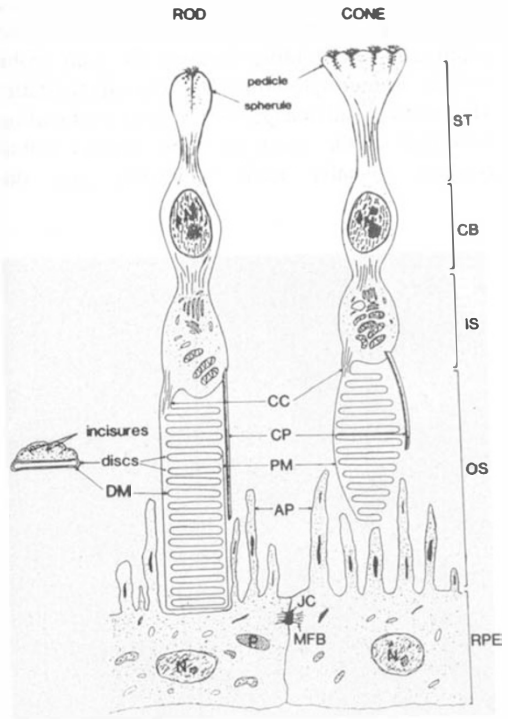

Fig. 1. *Diagrammatic representation of the outer retina showing a rod, cone and the retinal pigment epithelium. AP: apical processes CB: cell body, CC: connecting cilium, CP: calycal processes, DM: disc membrane, IS: inner segment, JC: junctional complex, MFB: microfilament bundle, N: nucleus, OS: outer segment, P: phagosome, PM: plasma membrane, RPE, retina pigment epithelium, ST: synaptic terminal.*

advantage of minimal loss of immunoreactivity. Frequently MAbs will only work on such material. Following conventional fixation with aldehydes, many MAbs fail to react with their antigens. This is even true of some antisera. Antisera to cytokeratins show diminished or absent reactivity following aldehyde fixation. [5] Partial proteolysis has been found to restore immunoreactivity.[6] Recently we have had some success using the AMeX method[7] of tissue processing which gives a conventional histo-phathological appearance while retaining much of the immunoreactivity.

Electron microsopic immunocytochemistry falls into two categories: Pre-embedding[8]or post-embedding.[9] The technique employed is largely determined by the nature of the antigen. As the name suggests pre-embedding immunocytochemistry is conducted before the tissue is embedded and sectioned. This technique is particularly useful in the identification of cell surface antigens. The main disadvantage of the technique is the difficulty of assessing antibody penetration. This is obviously a serious drawback when attempting to dentify intracellular antigens. Cells can be made permeable to antibodies using detergents such as Triton X-100 but this inevitably results in poorer morpholgical preservation. Post-embedding immunocytochemistry attempts to preserve tissue immunoreactivity through all the steps of processing prior to sectioning. The demands of the technique have resulted in the production of specialised resins such as Lowicryl K4M and LR gold which can be polymerised at low temperatures. Following sectioning the antibody solution is incubated with the EM sections on nickel or gold grids. Most commonly, antibody binding is detected using colloidal gold conjugated to immunoglobulins or protein A.[4] For example colloidal gold conjugated with an anti-mouse immunoglobulin would be used to detect the binding of a MAb raised in mouse to the tissue section. It should be noted that in gold conjugates, the binding between the gold probe and the immunoglobulin is purely electrostatic. The main disadvantage of the post embedding technique is the need for some sort of initial fixation (usually mild aldehyde) and the

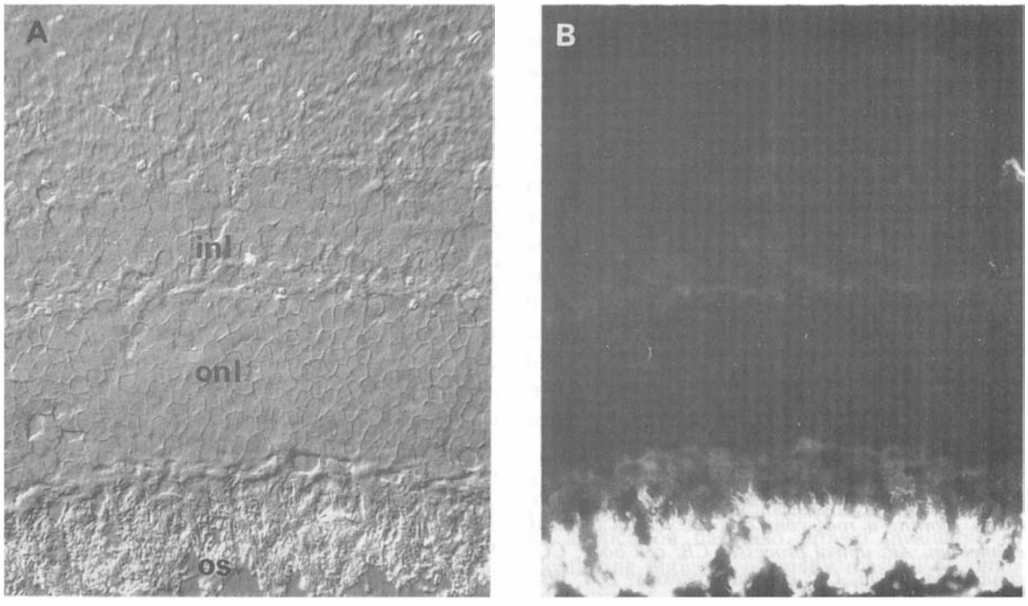

Fig. 3. *(A) Differential interference contrast micrograph of a cryostat section of bovine retina. The outer segments (os), outer nuclear layer (onl), and the inner nuclear layer (inl) are particularly prominant. (B) The same section viewed using epi-fluorescence to demonstrate the intense staining of the outer segments produced using a monoclonal (ROS 1F2) specific for a − 60kD protein of the outer segment. Both x 250.*

subsequent loss of immunoreactivity. Ultrastructural preservation, although often surprisingly good, is not as good as can be achieved by conventional EM techniques.

Applications

Many groups are now producing MAbs to retinal cells as probes of structure, function and development. We are pincipally interested in the interaction between RPE cells and photoreceptors (e.g. phagocytosis) and the identification of RPE cells in cultured and pathological material. In the course of these investigations we have been producing MAbs to photoreceptor and RPE cell antigens. Once such antibodies have been characterised to the degree of knowing molecular weight of the antigen (immunoblotting) and its tissue location (immunocytochemistry) it is of importance to compare these findings with descriptions of known photoreceptor, interphotoreceptor matrix and RPE antigens. This paper presents some of our own findings and a list of some of the proteins known to be present in the outer retina gained from the literature (Table I). We do not claim that the list is exhaustive but hope it will at least be of some use to other investigators.

(I) Photoreceptors

Photoreceptor cells of vertebrate retinas are of two types, rods and cones. Their photosensitive role is controlled in part by a number of proteins which are either directly responsible or indirectly associated with the photolysis cascade following exposure to light. These proteins comprise the main photosenstive glycoprotein, rhodopsin, proteins involved in the process of

Ag	*Ab*	*Ref*	*Spp*	*Tech*	*Localisation*
Photoreceptor					
actin	AS	57	frog	ferritin	Calycal processes, apical RPE and connecting cilium of rods and cones.
actin	AS	58	rat cow monkey man	ferritin/EM	Calycal Processes. Distal cilium.
calmodulin	AS	59	frog	IF	Ciliary body region of ROS.
calmodulin binding protein	AS	59	frog cow	IB	
CNP ase	AS	65	cow	PAP	ROS, elipsoid of RIS.
CNP ase	AS	66	chick	PAP	Photoreceptor plasma membrane. OS and IS of rods and cones.
cone related antigen (1)	MAb COS-1 anti 33kD	62	chick	IB EM	Outer segment of double cones. and some single cones.
cone related antigen (2)	MAb 50−1B11	63	various vertebrates monkey man	IF IP	Lamellae of COS.
cone related antigen (3)	MAb CSA-1 anti-54kd	64	pig	IB IF	COS/CIS cell bodies, axons, Synaptic pedicles.
CSAD	AS α-subunit	71	rat	IP EM	Rod and cones (especially spherules).
G-protein complex	AS α-subunit	47	cow chick	IB	
			cow chick monkey man	IF	Rod photoreceptors.
G-protein complex	MAb α-subunit	48	frog	IF	ROS.
HIOMT	AS	67	rat cow man	PAP IB	Outer segments, inner nuclear layer.
LIMP (ROS 1.2)	AS	51	frog	ferritin/EM	Incisures of ROS discs.
LIMP (ROS 1.2)	AS	52	frog	ferritin/EM	Margins of red and green ROS. Discs and margins of COS lamellae.
LIMP (ROS 1.2)	MAb 3D12 4B2 anti-(ROS 1.2)	53	cow	ferritin/EM	Margins of ROS discs and its exposure on the cytoplasmic surface.

Table 1 continued

Ag	*Ab*	*Ref*	*Spp*	*Tech*	*Localisation*
PDE	AS	39	fish frog rat	immunoprecipitation	
PDE	MAb ROS 1	40	cow man	IB. IF	ROS. COS.
	ROS 1	40	fish frog rat man	immunoadsorption IB	
	ROS 2	40	cow		
PDE	MAb	41	cow	IB	
PDE	AS	42,43	cow	EM	ROS, lumen of connecting cilium. RIS.
peripherin	Mabs 2B6 3B6	1	cow	gold/EM	Rim of discs.
rhodopsin	AS	13	frog	IF	Rod outer segment.
rhodopsin	AS	14	cow mouse	IP/EM	Disc and plasma membrane of ROS, small quantity in the connecting cilium and plasma membrane of the inner segment.
rhodopsin	AS	15	frog	ferritin/EM	Disc and plasma membrane of ROS, RIS, little labelling in the connecting cilium.
rhodopsin	MAb RET-P1	16,17	rat	IF.PAP/EM ferritin/EM	Entire rod photoreceptors except synaptic ending and outer plexiform layer.
rhodopsin	MAb 4D2	18	cow	gold/EM	External surface of rod outer segment.
	4A2	18	cow	gold/EM	External surface of rod outer segment.
	5A3	18	cow	gold/EM	External surface of rod outer segment.
	4A3	18	cow	gold/EM	External surface of rod outer segment.
	4D2	18	frog	gold/EM	Red rod outer segments.
	4A2	18	frog	gold/EM	Light labelling green ROS, COS. & RIS.
	5A3	18	frog	gold/EM	Light labelling green ROS, COS. & RIS.
	4A3	18	frog	gold/EM	Light labelling green ROS, COS. & RIS.
	1D4	18	cow	gold/EM	ROS slightly. RIS & connecting cilium.
	1D4	18	frog	gold/EM	Red and green ROS.
	2C1	18	cow	gold/EM	ROS.
	2C1	18	frog	gold/EM	Red and green ROS.
	3D6	18	cow frog	gold/EM	ROS and COS.
	1C5	18	cow	gold/EM	ROS.
	3A6	18	cow	gold/EM	ROS.
S-antigen	AS	19,25, 29	g.pig	IF	Photoreceptor cells and pineal gland.
S-antigen	AS	54	g.pig rabbit	IF	Photoreceptor cells and pineal gland.
S-antigen	AS	28	rabbit	IF	Outer segments.
S-antigen	AS	30	rat	IF gold/EM	Rod outer segments.
S-antigen	AS	31	rat	PAP/EM	Outer segments and around cell bodies.
S-antigen	AS	29	rabbit light and dark adapted	IP/EM	Photoreceptor discs, plasma membrane, microvilli.
S-antigen	AS	32	man	Gold/EM	Proximal portion of ROS. cones negative.
S-antigen	MAbs S6H8 S8D8,S2D2 S7D6,S8D1 S9E2	34,36	various vertebrate and invertebrates	IF	Photoreceptor cells.
S-antigen	MAbs RSA1/ 83 RSA2/83	33	cow	radio immuno-cytochemical	A1 distal protein of ROS. A2 ROS and RIS.
S-antigen	MAbs A9C6 A1C5	35	vertebrates including human and dev. rat	IF. IP	Photoreceptor cell layer in all species tested. In neuroblastic cell layer – 3 day rat. Entire photoreceptor cell – 7 day rat and all other species.

Table 1 continued

Ag	*Ab*	*Ref*	*Spp*	*Tech*	*Localisation*
S-antigen	MAb(rat) S2.4.C5	37	Pig human	gold/EM	Photoreceptor cell.
spectrin	MAb 4B2	61	cow	IB gold/EM	Rims of ROS discs.
tubulin	AS	59	frog	IF	Cilium,ciliary microtubules in ROS.
tubulin	MAb	60	frog	IF	Ciliary microtubules in ROS.
visinin	AS	69	crab, frog, cat, man	IF	Cone cells.
calbindin (visinin)	AS	70	pigeon chick	PAP	Mainly cone cells.
Interphotoreceptor Space					
C-6-S	MAb anti C-6-S	89	monkey	IF	Cone sheath.
IRBP	AS	76	rat cow monkey man	IF. PAP. EM	IPS RPE apical surface. Plasma membrane of CIS and RIS.
IRBP	AS	90	rabbit rat cow man	gold/EM	As above.
IRBP	AS	82	man	IF. IP	Space bounded by RPE apical surface and the external limiting membrane.
IRBP	AS	83	rat	IF	As above.
IRBP	AS	84	developing normal & RCS rat	IF-PAP	P2-P8 space between neuroblastic layer and RPE. P10 = IPS.
			RCS rat	IF-PAP	Staining decreases P18-P45.
IRBP	AS	80	man	IF	Extended fluorescence from the apical border of the RPE to the external limiting membrane.
IRBP	AS	85	frog cow man	ferritin/EM	IPS also ensheathed connecting cilium. None in cytoplasm of cells bordering IPS.
IRBP	AS	86	monkey	IF. IP. EM	IPS. RIS Cytoplasm of rod cells.
IRBP	AS	87	ground squirrel	IP. gold/EM	IPS.
PABP	AS	88	man	IB. IF	IPS.
Retinal Pigment Epithelium					
actin	AS	91	rat IS	IF	Associated with circumferential microfilament bundle.
actin	Ph	91	rat IS	F	Circumferential microfilament bundle.
actin	Ph	92	chick IS	F	Cortical ring.
actin	Ph	93	chick IV	F	Microfilament arrays.
actin	Ph	94	chick IV	F	Variable, dependent on cell morphology.
actin	Ph	95	man IV	F	Microfilament bundles.
actin	As	96	chick IS. IV	IF	Circumferential microfilament bundles.
actin	AS	97	rat IV	IF	In association with phagosomes. Microfilament bundles.
ATP-ase	AS	118	rat IS	IF	α (+) Absent and α present in RPE.
collagen type IV	AS	108	chick IS	IF	RPE basement membrane.
collagen type IV	AS	109	chick IV	IF	Beneath cells.
desmosomal components 82 and 86kD	AS	102	chick IS. IV	IF	Junction complex region. Similar to vinculin.
Fc and C3b		115	dog	rosette	RPE.
Fc and C3b		116	rat	rosette	Absent.
fibronectin	MAb	108	chick IS	IF	Basement membrane area.
fibronectin	AS	109	chick IV	IF	Beneath cells.
fibronectin	AS	110	cow IS	IF	Basement membrane area.
fibronectin	MAb	111	chick IS	IB. IF	Basement membrane area.

Table 1 continued

Ag	*Ab*	*Ref*	*Spp*	*Tech*	*Localisation*
fibronectin	AS	112	monkey IS	IF. EM	Basement membrane area.
fibronectin	AS	113	rat IS	EM	Apical microville Golgi-ER.
HLA-DR	MAb & AS	117	man	IF. IP	Absent in normals. Present in retinitis pigmentosa.
insulin-like growth factor	?	120	man	?	?
integrin	MAb & AS	111	chick IS	IB. IF	Basal – lateral surface.
keratin	AS	100	various, man IS IV	IF IB	Cytoplasmic filaments.
keratin	MAbs AS	106	man IS IV	IF.IP.IGS.IB	Cytoplasmic filaments.
laminin	AS	108	chick IS	IF	Basement membrane.
laminin	AS	109	chick IV	IF	Beneath cells.
laminin	AS	110	cow IS	IV	Basement membrane.
mannose-6-phospate receptors	AS	119	rat IS	gold/EM	Plasma membrane.
myosin	AS	91	rat IS	IF	Circumferential microfilament bundles.
myosin	AS	92	chick IS	If	Cortical ring.
proteoglycans	AS	108	chick IS	IF	Basement membrane.
proteoglycans	AS	109	chick IV	IF	Beneath cells.
proteoglycans	MAbs	114	rat IS	IF IP	Interphotoreceptor space.
retinoid binding proteins	AS	121	rat IS	gold/EM	RPE cytoplasm.
retinoid binding proteins	AS	122	cow rat monkey	IF IP IB	RPE. Interphotoreceptor space.
spectrin	AS	92	chick IS	IF	Cortical ring near membrane.
spectrin	AS	103	chick IV	IF	Cortex of cell.
spectrin	AS	104	chick IV	IF	Surface lamina.
transferrin	?	120	man	?	?
tubulin	AS	98	chick IV	IF	Microtubules.
tubulin	AS	99	rat	IF	Microtubules, normal and dystrophic.
vimentin	AS	94	chick IV	IF IB	Cytoplasmic filaments.
vimentin	AS	102	chick IS IV	IF IB	Cytoplasmic filaments.
vimentin	AS	92	chick IS	IF	Contact rings adjacent to membrane.
vimentin	AS	98	chick IV	IF	Focal contacts.
vimentin	AS	102	chick IS IV	IF	Associated with cell junctions.
vimentin	AS	103	chick IV	IF	Areas of adhesion cortical band of adhearing junction.
vimentin	AS	105	chick IV	IF	Focal contacts.
Others					
?	9 MAbs	123	chick	IF	RPE.
72kD protein	MAbs C6,C8	124	bovine	IB	Membrane.
42kD protein	MAb	125	frog rat cow chick monkey man	IP	RPE.
?	MAbs RET-PE1 MAbs RET-PE2	126	rat	IF	RPE.

Table I

This table summarises the antigens of the outer retina identified using immunocytochemical methods. The abbreviations used are: Ab: antibody, Ag: antigen, AS: antiserum. EM: electron microscopy, F: fluorescence, IB: immunoblotting, IF: immunofluorescence, IGS: immunogold silver, IP: immunoperoxidase, IS: in situ, IV: in vitro, MAb: monoclonal antibody, PAP: immunoperoxidase using peroxidase-anti-peroxidase conjugate, Ph: fluorescent phallotoxin, Ref: reference, Spp: species, Tech: Technique.

phosphorylation and signal transduction and a number of contractile or filamentous proteins. Other retinal proteins may assume a structural or supportive function. A great deal of our understanding of the roles of these retinal proteins stems from their localisation. Immunochemical and cytochemical techniques played a vital role in unravelling the location and chemical structure of these proteins. These immunological findings are briefly discussed.

Rhodopsin

The outer segment of rods contains stacks of flattened discs formed by lipid bilayers which provide a matrix for the protein molecules. These discs are not continuous with the plasma membrane of rod outer photoreceptors, but are connected to it by filamentous structures.[10-11] Cone photoreceptors differ from rods in that discs generally maintain continuity with the plasma membrane.[11] The visual pigment rhodopsin is the major protein of the rod outer segment (ROS). Rhodopsin is an intrinsic glycoprotein which spans the disc membrane thickness, crossing the membrance interface several times.[12] Immunocytochemical techniques, on the light microscopic level, have helped in the localisation of rhodopsin on both the discs and plasma membrane. This observation was made almost two decades ago when Dewey et al.[13] used polyclonal anti-rhodposin antiserum to localise rhodopsin on the frog ROS. Jan and Revel[14] have confirmed the localisation of rhodopsin on both sides of discs and plasma membrane of mice and bovine ROS using the immunoperoxidase technique for analysis by transmission electron microscopy. Rhodopsin was also visualised in small quantities along the connecting cilium and the plasma membrane of the rod inner segment (RIS). Using biotinylated sheep anti-opsin antiserum and avidin ferritin markers, Papermaster and co-workers[15] reported similar localisation of rhodopsin in frog photoreceptors. Recent advances in immunocytochemistry have made possible the use of monoclonal antibodies in the localisation of epitopes on thin sections. A monoclonal antibody to rhodopsin (RET-P1) was used in an indirect immunofluorescent labelling of rat photoreceptor cells. It showed intense labelling of the entire photoreceptors with the exception of the synaptic ending in the outer plexiform layer.[16,17] Closer examination of the rat retinal sections showed reaction in the cell bodies, outer and inner segments of rods but not cones. This antibody also reacted with a group of photoreceptors, in retinal sections, from the tiger salamander. Another monoclonal anti-opsin antibody (RHO-C7), whose epitope is in the N-terminal region of the rhodopsin molecule has been found to label only the outer segment of rat retina.[17]

However, different epitopes of the rhodopsin molecule have different immunocytochemical localisation patterns. This could be clearly verified by ultrastructural examination using immunoelectron microscopy. This was well demonstrated when RET-P1 antibody (using PAP staining technique) showed dense labelling of the plasma membrane of both outer and inner segments of rod photoreceptor.[17] A more diffuse reaction was also evident near the edges of the photoreceptor discs and in the peripheral cytoplasm of the inner segment. Staining over the external face of the plasma membranes of the outer and the inner segments was also seen when a ferritin-conjugated marker was used.[17] In contrast, RHO-C7 antibody was found to label only the external face of the plasma membrane of photoreceptor outer segment and occasionally the surface of the inner segments.

A more detailed ultrastructural study was described by Molday and co-workers, using a panel of monoclonal antibodies to rhodopsin. Various degrees of labelling of the external surfaces of the rods plasma membrane was noted when monoclonal anti-opsin antibodies, specific to the N-terminal region were used. Visualisation by an immunogold-dextran marker, on gluteraldehyde fixed bovine retinas, revealed that only one monoclonal antibody (4D2; specific to fragment 1 at the N-terminus of rhodopsin) exhibited dense labelling of the external surface of the ROS plasma membrance.[18] Another monoclonal antibody (4A2) which recognised the same F1 fragment showed a marked reduction in its labelling of the external surfaces of the plasma membrane. In contrast, three other N-terminus-specific monoclonal antibodies (4A3, 3D3 and 5A3) exhibited no labelling of the rod outer or inner segments. Treatment of fixed retinas with ethanol followed by rehydration, or treatment with detergent (saponin) and sodium borohydride, seemed to increase the accessiblity of antigen sites. All but one (3D3) of the above

five monoclonal antibodies reacted more intensely.[18] Gold-dextran particles were observed along the external surface of bovine ROS when 5A3 and 4A3 were used. An increase in the intensity of labelling was found when 4D2 or 4A2 monoclonal antibodies were employed. Antibody 3D3 again failed to react with ROS under any of the above conditions. On a thin section of frog ROS, 4D2 showed intense labelling of the red ROS. The other monoclonal antibodies exhibited significantly lower reaction. Green ROS, cone outer segments (COS) and rod inner segments (RIS) all reacted slightly with all five monoclonal antibodies.[18]

On the other hand, a C-terminus-specific monoclonal antibody, 1C5, did not show any labelling of the external surface of ROS under any of the above conditions. Using antibodies on Lowicryl-embedded thin sections of the bovine retina, C-terminus-specific (1D4), or N-terminus specific (4D2 and 5A3) monoclonal antibodies, strongly labelled the ROS and slightly the inner segment and the connecting cilium. Monoclonal antibodies 1D4 and 2C1 (C-terminus-specific) reacted with both red, and more intensely, the green frog ROS on thin sections.[18] Other C-terminus specific anti-bovine monoclonal antibodies, 3A6 and 1C5, exhibited no cross reactivity with frog ROS, COS and RIS. Only one C-terminus-specific antibody, 3D6, showed strong labelling of the bovine and frog COS as well as ROS. No reaction was observed in the inner segments.[18]

This account demonstrated the power of using specific monoclonal antibodies in association with different pre- and post- embedding treatment strategies.

S-Antigen

S-Antigen, a soluble retinal protein, was isolated and characterised for the first time by Wacker et al.[19] The protein has an apparent M. wt. of 50kD on SDS-polyacrylamide gels [20] and has stimulated a great deal of interest due to its ability to induce experimental autoimmune uveoretinitis (EAU) in various animal species (for review see Faure et al.[21] Gery et al.[22]) Recently, Pfister et al.[23] identified S-Ag as the 48kD protein which binds specifically to photo-excited and phosphorylated rhodopsin and inhibits cGMP hydrolysis. This makes it an important part of the phototransduction process of the photoreceptor cells.

The localisation of S-Ag by immunocytochemistry has been demonstrated in both the photoreceptor cells and the pineal glands.[24-26] Using indirect immunofluorescent staining and an antiserum to bovine S-Ag, Wacker and co-workers[19] found the protein in the entire photoreceptor cells and the staining pattern suggested an association with the plasma membrane.[27] No specific fluorescence was detected in the RPE or choroid. Yajima et al.[28] obtained similar results by using direct immuno-peroxidase staining of rabbit retinas. At the electron microscopic level they found immuno-reaction on both sides of the disc membranes of the outer segment with particles distributed in a pattern symmetrical to that of the membrane. Positive reaction was also shown in the plasma membrane of the outer segment. Some labelling was found in the tips of the outer segments before phagocytosis but not after. However, studies on 30h light adapted rabbit retinas showed abundant reaction products in the cytoplasm of the RPE cells and also in Bruch's membrane and the endothelium of choriocapillaris.[29] The outer segments of the photoreceptor cells showed swelling and destruction of the disc membranes and contained only a little reaction product. On the other hand, Broekhuyse et al.[30] found, at the EM level and using 24h light adapted rat retinas, that the immungo-gold particles were seen in the ROS over the areas containing stacked discs. The plasma membranes were only slightly labelled.

Using an antiserum to S-Ag and the peroxidase antiperoxidase immunohistochemical technique on fixed rat retina, Uusitalo et al.[31] found, at the light microscopic level, intense reaction in the outer segment of the photoreceptor layer with immunostaining around the cell bodies of the outer nuclear layer. Their EM studies showed immunoreactivity mainly in the inner surfaces of disc membranes of the rods and its plasma membranes. Plasma membranes of outer nuclear layer also showed a positive reaction. However, studies on the localisation of S-Ag in human retinas, McKechnie et al.[32] showed no specific staining of the ROS plasma membrane. They used fixed sections embedded in Lowicryl K4M, cut and treated with anti-bovine S antisera and protein A/gold complex. Specific labelling was observed in the proximal portion of the ROS which diminished towards the top of the ROS.

Positive staining was distributed uniformly showing preferential deposition on the ROS disc membranes. The connecting cilium was also labelled but no reaction was seen in rod spherule, outer nuclear layer or the inner segment.

Recent developments in MAb technology has allowed the isolation of Abs to S-Ag by various groups.[33-37] MAbs allow the study of different epitopes of the protein, their location by immunocytochemistry, and phylogenic distribution. Faure and co-workers isolated six mouse MAbs to S-Ag., four of which stained ocular tissues from various classes of vertebrate and invertebrates.[36] They labelled the entire photoreceptor cell, excluding the nucleus. This indicates the presence of distinct epitopes even in distant species. A similar staining pattern of the photoreceptors was shown by Donoso and co-workers.[35] They reported two MAbs to S-Ag characterised by the ELISA and immunoblotting techniques. However, only one of them, MAb A9-C6, reacted on fixed embedded tissue sections of vertebrate retinas including human, bovine, guinea pig, mouse and the developing rat. Fluorescent and immunoperoxidase staining techniques were used.

At the EM level, using two mouse MAbs and indirect autoradigoraphy, Das et al[33] reported the localisation of S-Ag in fixed bovine explants. The protein was shown in the apical portion of the ROS with the use of MAb RSA 1/83. The second MAb RSA 2/83 showed the Ag both in the inner and outer segment of the rod cells.

More recently, a rat monoclonal antibody (S 2.4CS) produced against bovine S-Ag and characterised by immunoblotting, was shown, by EM localisation, to uniformly label the ROS disc membranes of human and pig photoreceptors.[37] Labelling was also seen in the perinuclear area and over the connecting cilium. Newly phagocytosed ROS membrane in the apical region of the cell was also labelled. Those in deeper areas of the cell showed less label. It was suggested that the antigenicity of the protein decreased as the digestion of the RPE proceeded.

cGMP-PDE

Hydrolysis of cGMP by cGMP-phosphodiestrase (cGMP-PDE) is one of the early events that occur in retinal rod cells, following illumination.[38] The frog cGMP-PDE has a molecular weight of 240 kD while the mammalian enzyme has an apparent M.wt. of 170-185 kD and is composed of three subunits; two catalytic, α and β, of 88 and 84 kD and the inhibitory γ−subunit of approximate M.wt. of 10-13 kD.

Specific rabbit antisera to bovine PDE and its inhibitory γ-subunit were used to determine their effect on enzyme activity.[39] The antisera also reacted with immmunologically-related polypeptides in pig, rat, frog and fish using the immunoprecipitation technique. Two monoclonal antibodies were produced against bovine retinal cGMP-PDE (ROS-1 and ROS-2). Both ROS-1 and ROS-2 reacted with one polypeptide (corresponding to the PDE) by immunoblotting.[40] Using the indirect immunofluorescent technique and ROS-1 antibody, it was possible to localise the enzyme's antigenic determinant to both rod and cone outer segments of the bovine retina. Immuno-adsorption technique, using ROS-1 monoclonal antibody, demonstrated the presence of polypeptides of similar mobility to bovine cGMP-PDE in gold fish, mammals (human and rat) and frog retinas. ROS-1 and ROS-2 absorbed all the three (α, β and γ) polypeptides of bovine PDE. Indirect immunofluorescent staining with ROS-1 antibody on frozen sections of human retina revealed labelling in both rod and cone photoreceptor outer segments. The same pattern was seen in other vertebrate retinas with no reaction being evident in the inner segments.[40] In the primate retina, the long slender outer segments of the foveal cones were also stained with ROS-1.[40] Another monoclonal antibody to frog PDE failed to block light-activated guanine nucleotide binding nor did it inhibit the PDE activity. This suggested that the antibody did not bind the functional moiety of the enzyme.[41]

Ultrastructural localisation of cGMP-PDE using affinity purified anti-bovine PDE antiserum, on Lowicryl K4M embedded sections, indicated the presence of the enzyme, specifically in rods and mainly in the outer segment region. Some labelling, in some sections, was also observed in the lumen of the connecting cilium and RIS at the Golgi apparatus.[42,43] The biotin-avidin immunoferritin labelling was absent from cone photoreceptor cells.

G-Protein (Transducin "T") complex.[42]

Several retinal proteins bind strongly to the visual pigment rhodopsin in the disc membrane,

following illumination (reviewed by Kuhn.[44]) Amongst the proteins that bind to rhodopsin in a light-dependent manner is the G-protein complex. It consists of three polypeptide chains, α, β and γ of apparent molecular weights of 39-40 kD, 36-37 kD and 6-8.5 kD respectively.[45,46]

Rabbit antiserum (GI-2) to bovine retinal α-subunit was tested for its reactivity with retinal proteins, using the immunoblotting technique.[47] The antiserum reacted specifically with the α-subunit of transducin and no cross reactivity was noted with other bovine retinal proteins. It also reacted with a protein band from chicken retina that had the same electrophoretic mobility as α-transducin from bovine retina. Using the indirect immunofluorescent technique, the antiserum labelled only the photoreceptor cells layer of frozen sections of chicken, bovine, monkey and human retinas.[47] It was also found that rods but not cones were heavily labelled with this antibody. This may suggest that the α-subunit structure of cones differ from that of the rods. It is also possible that rod and cone cells utilise different phototransduction mechanisms.

Eleven monoclonal antibodies to frog G-protein complex were found, by immunoblotting, to recognise the α-subunit (40 kD) only and not the β or γ-subunits.[48] Two of these antibodies, using the indirect immunofluorescent technique on isolated frog ROS (fixed, either in light or in dark, with paraformaldehyde), showed uniform fluorescence along the whole isolated rod outer segment. Ultrastructural localisation with other monoclonal antibodies to the G-protein complex, has not yet been presented.

LIMP (ROS 1.2)

Although disc membranes of rod photoreceptor cells are densely packed with rhodopsin, another large intrinsic membrane protein (LIMP), of apparent molecular weight (on SDS-PAGE) of 220-240 kD in cattle and human and 290 kD in frog, was found to be continuously (and may be synchronously with rhodopsin) synthesised and assembled into disc membrane.[49-50] This large glycoprotein was specifically localised by EM immunocytochemistry (using retina embedded in gluteraldehyde cross-linked BSA and indirectly visualised by ferritin-conjugated antibody) to the incisures of frog ROS discs and to the disc margins.[51] The incisures divide the disc into lobes (Figure 1). Recent ultrastructural localisation, using the same purified antiserum on frog outer segments, showed that the immuno-labelling was on the short incisures and margins of red and green ROS and the margins of COS lamellae.[52] Labelling of the inter-incisures surface of all photoreceptor classes was also noted by using higher antibody concentration.

Monoclonal antibodies to bovine LIMP were also used for its ultrastructural localisation.[53] Immunoferritin labelling with these antobodies (3D12 and 4B2) and transmission EM, confirmed the location of the protein in the margins of bovine ROS discs and its exposure on the cytoplasmic disc surface. Both monoclonal antibodies recognised different sites of the protein.

Peripherin

The use of monoclonal antibodies and the immunoblotting technique helped in unveiling the presence of yet another membrane protein (M. wt. 33 kD) that is usually masked under the thick abundant band of rhodopsin when bovine ROS are analysed by SDS-PAGE[1] (see Figure 2).

Because this protein was localised by immunocytochemistry on the rim of ROS discs (Fig 4) it was termed "peripherin" by Molday and co-workers. Immunogold labelling of morphologically intact isolated discs, prepared by mild trypsinisation of the ROS fraction, confirmed the localisation of this protein at the rim region of discs. Immunogold dextran labelling of ROS sections embedded in Lowicryl resin indicated that this protein is located around the periphery of ROS organelle where the discs come in close contact to the ROS plasma membrane[1] (see Figure 4).

Cytoskeleton and structural proteins

Immunolocalisation techniques have played a vital role in understanding the distribution of structural and membrane associated proteins of the outer retina. Localisation of a number of these filamentous or supportive proteins was made possible by using specific antibodies. These techniques are particularly powerful when the protein(s) are present in small amounts. The following is a brief account of the majority of identified components:

(1) *Actin*: It is now accepted that actin is present in muscle as well as in non-muscle cells.[55] Actin appears to have a cytoskeletal or struc-

tural function, in addition to its contractile role. In the ROS of the retina, actin bundles are thought to play a vital role in the contractile process of rods and cones in addition to its role in the process of shedding and phagocytosis by pigment epithelial cells.[56]

Immunocytochemical localisation of actin in frog retina, fixed and embedded in aldehyde cross-linked serum albumin, showed heavy labelling in the apical pigment epithelial cell processes and in the calycal processes of photoreceptor cells, which are the microvillus-like projections that arise from the inner segment and extend alongside the basal outer segment.[57] Actin was also found, in large quantities, in the connecting cilium (distal area at the site of outer segment disc formation) of both rods and cones using affinity purified rabbit antiserum to chicken gizzard actin, visualised by biotin-avidin-ferritin marker on albumin embedded retinal sections. Labelling with anti-actin was also observed below the new discs in rods while no reaction was noted in the plasma membrane and the interior of the connecting cilium or the remainder of outer segment. Further EM localisation of actin in mammalian retinas, aldehyde fixed and embedded in Lowicryl K4M, was recently described. Thin retinal sections were allowed to react with anti-actin antiserum by the indirect immunoferritin method.[58] Again actin was localised in the distal cilium of rat, cow, monkey and human photoreceptors. This suggested that an actin-mediated contractile mechanism may also regulate outer segment morphogenesis in vertebrate photoreceptor cells. It was also noted that the anti-actin, labelled the bundle of filaments within photoreceptor calycal processes extending into the inner segment subjacent to the plasma membrane.[58]

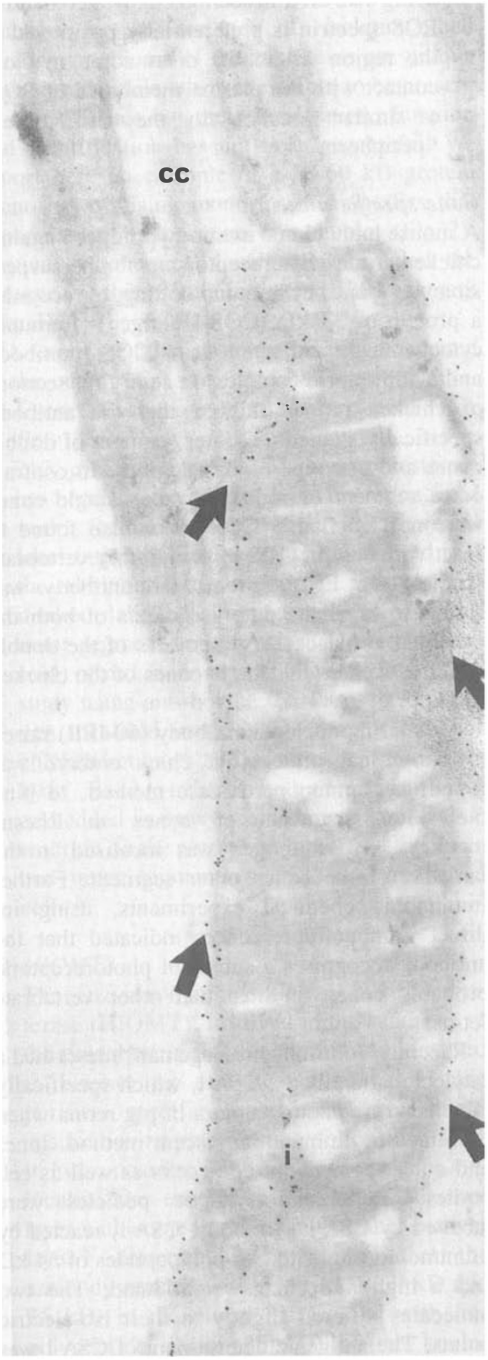

Fig. 4. *Post embedding EM immunocytochemistry using Lowicryl K4M embedded bovine retina. The section has been incubated with a monoclonal antibody (ROS 4A2), which on immunoblotting recognises a polypeptide with a molecular weight of approximately 32kD. Binding of the antibody has been demonstrated using 10mm diameter colloidal gold conjugated with goat anti-mouse immunoglobulin. The antigen appears to be localised at the margins of the outer segment of the photoreceptor (arrows). The staining at (i) is probably following the line of an incisure. (cc) connecting cilium. The localisation at the margins of the outer segment and the apparent molocular weight of the antigen suggest that this antibody recognises peripherin (x30,000).*

(2) *Tubulin*: Tubulin is another major cytoskeletal protein found primarily in the cilium of retinal photoreceptors. Roof and Applebury[59] used anti-tubulin for the immunolocalisation of the protein in isolated frog outer segments, using the indirect immunofluorescent staining method. It was found that the labelled ciliary microtubules can reach distally to the point near the end of the outer segment. Recently, Kaplan et al[60] used a monoclonal anti α-tubulin antibody followed by a Texas Red fluorophore second antibody to demonstrate the extent of microtubules in isolated frog ROS (fixed with paraformaldehyde). It was possible, by fluorescent microscopy, to visualise the labelling which extended to slightly over half the length of the ROS on diurnal light cycle. Although frog ROS kept in a constant darkness for 3-4 weeks are longer than those maintained on cyclic lighting, the distribution of fractional lengths of anti-tubulin labelling of ciliary microtubules was the same. In fewer than 15% of rods the immunolabelling extended into the distal region of the outer segment where disc membrane shedding occurs.[60]

(3) *Calmodulin and Calmodulin binding protein*: Calmodulin is present in vertebrate ROS in very low quantities. However, it was possible to localise this protein by immunofluorescence using anti-calmodulin antiserum. The labelling was restricted to a stripe in the ciliary body of ROS.[59]

A number of polypeptides were found to bind calmodulin in a Ca^{2+}-dependent manner. One of these proteins of 240 kD, was found in cattle and toad retinas. Using antiserum to the mouse brain fodrin (a spectrin like actin- and calmodulin-binding protein) it was possible to show, by immunoblotting, that this antibody cross reacted with the 249 kD-calmodulin binding protein. In toads, the fodrin-like 240 kD polypeptide was found to be distinct from the "large intrinsic membrane protein (ROS 1.2)".[59]

(4) *Spectrin-like protein*. Wong and Molday[61] reported the presence, in bovine photoreceptor cells, of a 240 kD polypeptide related to the α-subunit of red blood cell spectrin. Its properties and molecular weight were confirmed by immunoblotting, using a monoclonal antibody (4B2). Biochemical studies showed that it was not an integral membrane protein but a tightly membrane-associated one. Ultrastructural localisation, using 4B2 on thin sections, indicated that the ROS spectrin is preferentially positioned in the region where the discs come in close contact with the plasma membrane of ROS, a similar location to the rim protein "peripherin".[61]

Cone specific proteins

A mouse monoclonal antibody produced against chicken crude photoreceptor membrane suspension was found, by immunoblotting, to recognise a protein of 33 kD (COS-1 antigen). Immunocytochemical localisation using COS-1 antibody and avidin-biotin complex on semi-thin sections of chicken retina, showed that the antibody specifically stained the outer segment of double cones and one type of single cones. In contrast outer segment of rods and other single cones were not labelled.[62] COS-1 was also found to react with certain COS of many other vertebrate species. On EM sections, the antibody was shown to label the outer segments of both the principal and accessory members of the double cones as well as the single cones of the chicken retina.[62]

Another monoclonal antibody (50-1B11) raised to the retinal proteins of chick embryo was found, by immunoperoxidase method, to bind the outer segments of cones in Rhesus monkeys.[63] The antigen was localised in the lamellae of the cones outer segment. Further immunohistochemical experiments, using indirect immunofluorescence, indicated that the antibody recognises a subset of photoreceptors, probably cones, in man and other vertebrate retinas.

Recently, Johnson and Hageman[64] described a monoclonal antibody, CSA-1, which specifically labelled cone photoreceptors in pig retina when examined by immunofluorescent method. Inner and outer segments of cone cells as well as cell bodies, axons and synaptic pedicles were labelled by CSA-1. Antibody, CSA-1, reacted by immunoblotting with two polypeptides of 54 kD and a high molecular weight band. The two molecules differed slightly in their iso-electric points. The antigenic determinant of CSA-1 was found to be a galactose-containing carbohydrate

moiety as judged by enzymatic digestion.[64]

Miscellaneous

A number of other proteins are found in the retina. Their characterisation is not well achieved, partly due to the lack of a known function or a method of isolation and purification. Some of these proteins are unique to the retina while others are known to be present in other tissues but their localisation, function and identification has not been successful. We report here an example of a 58-60 kD protein identified by using monoclonal antibodies that recognise its epitopes on immunoblot (Fig 2). Immunocytochemical localisation revealed the presence of this protein in the rod outer segment (Fig 3).

A few of the other proteins and enzymes found in the outer retina are listed below:

(1) *Cyclic nucleotide phosphodiesterase (CN Pase)*

2',3'-cyclic nucleotide 3'-phosphodiesterase has been localised in fresh bovine retina fixed with formaldehyde. Immunocytochemical localisation by light microscopy showed that the rabbit antiserum used in the indirect PAP method labelled the photoreceptors in the outer segment and an external region (ellipsoid) of rod inner segment layer.[65] In another study using anti-bovine CN Pase, the antigen was localised in the photoreceptor layer of chicken retina.[66] EM sections of gluteraldehyde fixed, treated with antiserum and embedded in epoxy resin showed strong PAP reaction on the plasma membrane of the inner and outer segments of both rod and cone cells.[66]

(2) *HIOMT*

The enzyme hydroxyindole-O-methy transferase (HIOMT), involved in the synthesis of the pineal hormone, melatonin was localised in bovine, rat and human retinas. Using purified specific rabbit antiserum raised against bovine pineal extract followed by PAP, it was possible, by light microscopy, to localise the enzyme in the outer segment layer with some labelling in the inner nuclear layer.[67] Immunoblotting showed that the antibody reacted with two bands (39 kD and 25 kD) in the pineal extract and with one polypeptide chain (25 kD) of the bovine retinal extract.

(3) *Visinin and Calbindin*

Visinin (M wt 24 kD) was first isolated from chick retina in 1983.[68] Antiserum against visinin of the chick retina also labelled the photoreceptor cells of human, cat, frog and crab retinas.[69] Staining of cone cells was clearly evident in the photoreceptor layer while rods showed barely detectable labelling in the direct immunofluorescent microscopy. Recently, Pasteels et al,[70] presented evidence based on immunoblotting cross reactivity between visinin and calbindin, a 27 kD vit-D-dependent calcium binding protein, that these two proteins are the same, though the latter occurs in brain and other tissues at a slightly higher molecular weight. Antiserum to calbindin again stained cone cells in pigeon and chick retinas, using the PAP method and light microscopy. Other retinal cells were also labelled but to a lesser extent.[70]

(4) *CSAD*

The taurine synthesising enzyme, cysteine sulphinic acid decarboxylase (CSAD) was localised in the retina using thin sections of gluteraldehyde fixed, antiserum-treated and embedded in low gelling-temperature agarose. Peroxidase labelling, visualised by EM, was found in rat retinal neuronal types and their processes including the photoreceptor rod and cone cells.[71] The staining was clearly evident in certain cone pedicles and rod spherules. Other cells (like ganglion cells) also showed heavy staining.

(II) Interphotoreceptor Matrix

The IPM occupies the area between the neural retina and the RPE. This matrix lacks collagen and fibronectin and contains proteins and glycosaminoglycans (GAGs). Proteins constitute 98% of the soluble fraction of the IPM compared to the 2% composed of GAGs.[82-83]

The IPM has a strategic location and could have various physiological roles including the mediation of the transport of nutrients and vitamins such as vitamin A and vitamin E.

Most of the soluble IPM proteins appear to be synthesised by either the RPE or photoreceptor cells.[73] The major protein of the IPM was found to be the interphotoreceptor retinoid binding protein (IRBP).[74]

IRBP

Interphotoreceptor (or interstitial) retinoid binding protein (IRBP), is a large glycolipoprotein of the interphotoreceptor matrix. It has an apparent M. wt. of 140 kD as assessed by SDS-polyacrylamide gels. This protein has been shown to be the only retinoid-binding protein in the IPM[72,75-76] and could be involved in the transport of vitamin A between the RPE and retinal photoreceptors.[77-78] Accumulating evidence indicates that the rod photoreceptor cells are the site of its biosynthesis.[79-81] This IRBP has been well characterised, and localised mainly to the IPM. Using light and electron microscopy, Bunt-Milam and Saari[76] found IRBP in the space surrounding photoreceptor outer segment and the apical surfaces of inner segments, with the heaviest labelling in the line corresponding to the RPE apical surface. This was resolved on the electron microscopic level to the IRBP coating the RPE microvilli. The label was also found around rod and cone inner segment and in the intralamellar spaces of the COS. The Müller cell microvilli were also heavily stained. Fong et al[82] using LM and fluorescent or peroxidase staining on fixed human tissues embedded in gelling agarose, demonstrated the presence of IRBP in the space surrounded by the epical surfaces of the RPE cells and in the region of the external limiting membrane (ELM). No IRBP was detected in the inner segments of photoreceptor cells. Similar results were found in a rat retina.[83-84] Recently, Schneider et al[85] localised IRBP, by EM microscopy in bovine, human and frog retinas, using affinity purified rabbit and bovine IRBP and visualised by avidin-ferritin or biotinyl-ferritin markers. Tissues were fixed then embedded in Lowicryl K4M. The protein was found, unevenly distributed, in the IPM. The highest labelling was in areas immediately adjacent to photoreceptor plasma membranes or PE processes. Cytoplasm of rods and cones was unlabelled if cells were intact. This is in contrast to the result of Bunt-Milam and Saari[76] who found labelling of IRBP in the COS. Schneider et al[85] also found labelling surrounding the connecting cilium. No label was detectable within photoreceptor IS, pigment epithelium or Müller cells. However, Rodrigues et al[86] found immunostaining of IRBP at the EM level in monkey rod cell cytoplasm and inner segments. No immunoreactivity was found in Müller cells or the fovea where there was no staining of the CIS or COS. Anderson et al[87] found IRBP in the cone-dominant ground squirrel retina. Hollyfield et al[80] also found immunoreactivity in the IPM of the fovea of human retina.

Peanut agglutinin-binding protein (PABP):

This large glycoprotein of unknown function was identified and purified from human retina using its lectin-binding property. Rabbit antiserum to the affinity purified protein was used to confirm its molecular weight (135 kD on SDS-PAGE) by immunoblotting and its distinction from IRBP which binds Con A lectin.[88] Fluorescent microscopic localisation of the antigen in human retina indicated that it is an interphotoreceptor matrix component apparently around both rod and cone cells.[88]

Antigen specific to the Cones' Sheath

Using a panel of monocolonal antibodies to various unsaturated glycosaminoglycans, it was possible to identify chondroitin-6-sulphate (C-6-S) as the specific component of primate cone matrix sheath. This was demonstrated by indirect immunofluorescent technique on sections of primate retina.[89]

(III) Retinal Pigment Epithelium

Immunochemical studies have been conducted on RPE cells both *in situ* and with cultured cells. A large proportion of this work concerns the cytoskeletal elements of chick RPE. In culture embryonic chick RPE continues to differentiate to produce cells of very similar morphology to those found *in vivo*. Usually the cells appear as colonies with the cells in the centre having a morphology similar to the *in vivo* appearance. Toward the edge of the colony the cells express an undifferentiated, well spread morphology and develop large areas of cell-substrate adhesion.

The main elements of the cytoskeleton, actin[91-97] tubulin[93,98,99,103,104] and intermediate filaments[94,98,100] have all been shown to be present. *In situ* there is considerable cytoskeletal specialisation associated with the zonula adherens region of the junctional complex (JC), the circumferential actin bundles.[91,96] The microfilament bundles are present in chick RPE *in situ* and in culture in the differentiated central region of cell colonies. Circumferential microfilament bundles (CMB) have also been demonstrated in rat RPE *in situ* and are seen by

electron microscopy in the *in situ* RPE of all mammalian species studied. In primary culture, adult mammalian RPE adopts a polygonal appearance, similar to its *in vivo* appearance, and circumferential microfilament bundles do appear to be present.[101] However, this morphology is usually lost on passage of the cells. A number of cytoskeletal associated proteins are localised in the region of the circumferential microfilament bundle. Vinculin, a Mwt 130 kD protein is closely associated with the membrane. It may be attached to a membrane lipid or to an integral membrane protein.[92,102,103] Actinin (Mwt 190 k) and spectinin also localise to this area[91,92,103,104]). All these proteins are thought to be involved in the attachement of actin microfilaments to the membrane. Myosin has also been detected in association with the CMBs.[91] In the well spread cultured chick RPE cells these proteins are associated with regions of focal cell adhesion and microfilament bundles.[98,103,105]

In the case of intermediate filaments the RPE might be expected to express cytokeratins as a reflection of its epithelial character. However, the nature of the intermediate filaments possessed by the RPE appears, at present, to be species dependent. It is known that chick RPE lacks cytokeratins and expresses vimentin.[102] Human RPE and that of other mammalian species appears to show co-expression of vimentin and cytokeratins both in *in situ* and *in vivo,* [100,106] In human RPE the cytokeratins detectable are characteristic of simple or glandular epithelia (Fig. 5). Following the numerical classification system of Moll et al 1982[107] numbers 5, 6, 7, 8, 18 and 19 have been identified by immunoblotting and immunocytochemistry.[106] Antibodies to some desmosomal components, (the 92 and 86 kD proteins) have been shown to react with the chick RPE but true desmosomes appear to be absent.[102] The situation with regard to mammalian RPE possessing desmosomes is unclear, particularly in light of the cytokeratin immunoreactivity of human but not chick RPE.[100,106]

The RPE cells are attached to Bruch's membrane by their basal surface and number of basement membrane protein, and basement membrane associated proteins, have been identified in this area. The basement membrane components, type IV collagen[108,109] and laminin[108,109,110] have been shown to be present. Fibronectin, a protein possessing specific high-affinity binding sites for the cell surface,

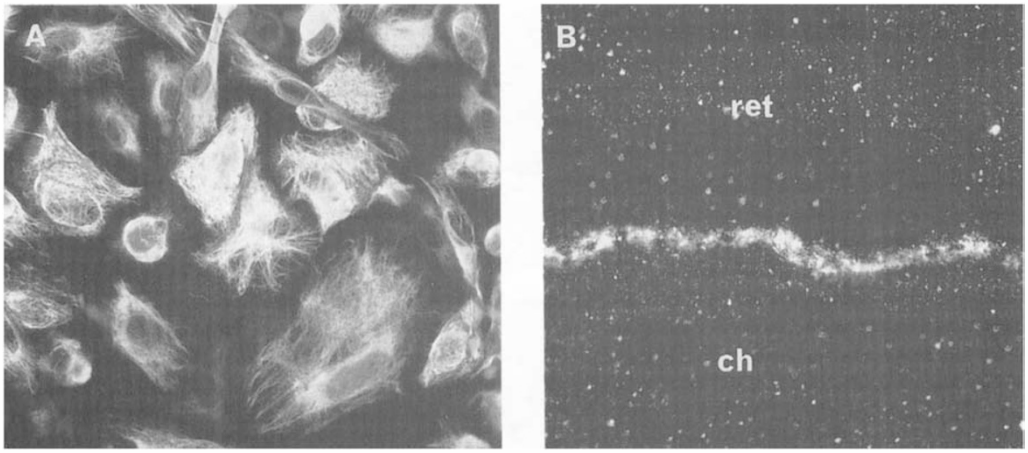

Fig. 5. *(A) Immunofluorescence staining of cultured human RPE cells (obtained from Dr Boulton) using a commercially available monoclonal specific for cytokeratins 5 & 8 (RCK 102). (B) Immuno gold/silver staining of a cryostat section of human retina (ret) and choroid (ch) using the same monoclonal and detected using the immunogold method followed by silver enhancement. When viewed using epi-polarisation the metallic reaction product is clearly visible. This technique avoids the problems due to autofluorescence and pigmentation of the RPE which tend to interfere with other techniques. The RPE is intensely positive. Both x 250.*

collagen, fibrin and sulphated proteoglycans has been localised to the basal aspect of the RPE cell *in situ*[108,110-112] and in culture.[109] One report[113] has also localised fibronectin to the apical microvilli and the Golgi endoplasmic reticulum system of rat RPE. Integrin, a trans membrane protein involved in the linkage between fibronectin and actin has been localised to the basal aspect of chick and rat RPE[111]. Various proteoglycans have been localised to the Inter-photoreceptor space[114] and to the basal aspect of the RPE cell.[108,109]

Several authors have attempted to identify Fc and C3b receptors on the retinal pigment epithelium of dogs and rats.[115,116] However, Eckhert and Hafeman in a recent study were unable to identify Fc or C3b receptors in the RPE cells of black eyed RCS rats or normal congenic (rdy +) rats. In the case of human RPE, HLA-DR antigens have been shown to be present on RPE cells obtained from retinitis pigmentosa patients but were absent from normal controls.[117]

A variety of other membrane proteins and receptors have been shown to be present in RPE cells. Sodium potassium ATPase, which comprises of two subunits of 95kD and 40kD, has been detected by immunofluorescence. Preparations of Na,K-ATPase from the brain and the retina have two biochemically distinct isozymes of the catalytic (95kD) subunit called α and α (+). The α but not the α (+) isozyme appears to be present in the retinal pigment epithelium of the rat.[118] Receptors for mannose-6-phosphate,[119] transferrin[120] and insulin-like growth factor [120] have been demonstrated to be present. Proteins associated with the transport of retinol/retinal, cellular-retinol binding protein (CRPB)[121] and 11-cis retinaldehyde binding (CRALBP)[122] have been detected in the retinal pigment epithelium by immunocytochemical techniques.

Several abstracts outlining the production and preliminary characterisation of MAbs to specific RPE antigens have been published but to the best of our knowledge no detailed papers have appeared.[123-126] Our own efforts in this direction have yielded two groups of MAbs: one showing considerable reactivity with other epithelia and the other showing striking specificity for the RPE *in situ* but failing to react with proliferating RPE in pathological material or cultured RPE cells (Fig. 6). Rather than finding useful markers

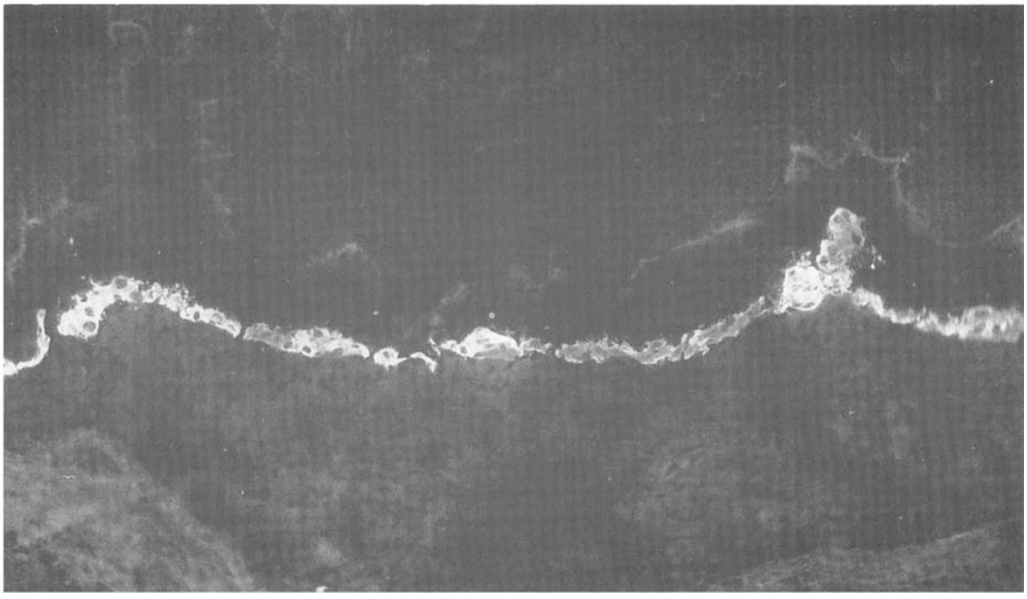

Fig. 6. *Cryostat section of cat retina and choroid (tapetal region). The section has been immunostained with a monoclonal antibody (RPE 42D3) which was obtained from a fusion where the mouse had been immunised with whole bovine RPE cells. The RPE is intensely fluorescent. This antibody also recognises non-ocular simple and glandular epithelial cells (x 160).*

of RPE cells in culture and pathology as initially intended these antibodies may prove useful in the identification of proteins specific to functional RPE.

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