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CHAPTER 3

Retinal Degenerations of Hereditary, Viral and Autoimmune Origins: Studies on Opsin and IRBP

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1. INTRODUCTION

Blindness is one of the major health problems in the world both in terms of human suffering and loss of work productivity. Along with cancer, it is one of the most feared of human afflictions. Although the eye is a delicately balanced grouping of tissues, it is the retina whose function is most complex and whose dysfunction usually leads to the most severe and the most irreversible consequences.

With the advent of sophisticated molecular biological techniques in the last five years, there have been major advances in our understanding of the causes of some inherited retinal degenerations.

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Even with this, however, we have little information concerning the causes of most of the degenerative retinal disorders. In fact, it is clear that severe retinal pathology can occur when the major problem is not primarily expressed in the retina itself. Gyrate atrophy (Valle *et al.*, 1977; Mitchell *et al.*, 1989) and choroideremia (van den Hurk, 1992) are examples of conditions in which the primary insult is systemic but which quickly lead to retinal dysfunction. In Retinitis Pigmentosa (RP), we have a grouping of diseases that, collectively, diminish retinal function and generally lead to blindness but are genetically and clinically heterogeneous. This family of disorders is the most common inherited form of severe retinal degeneration with a frequency of about 1 in 3000 births. It is the retinal photoreceptor neurons that are primarily affected in RP; molecular genetics studies to date indicate that defects in genes that are photoreceptor-specific (such as opsin) account for these degenerations. Genetically, several forms are seen including autosomal dominant (AD), autosomal recessive (AR) and X-linked (XL) as well as single, isolated cases. It has been estimated that ADRP accounts for about half of all RPs, ARRP for 20%, XLRP for about 8 – 10% with the remaining cases attributed to isolated or unknown causes.

In spite of these advances, it is clear that many of the retinal degenerations cannot be directly attributed to a hereditary defect. This is true, for example, in uveitis where genetic predisposition may be only one of the factors leading to the degeneration. Other factors such as age or viral infection can cause, contribute to or speed up the degenerative process. Does age-related macular degeneration (ARMD), for example, have a true genetic component? Can viruses be harbored in the retina that attack specific cells when active and lead to a slow degenerative condition? These questions have yet to be adequately addressed. Two proteins, however, rhodopsin and interphotoreceptor retinoid-binding protein (IRBP), are leading candidates for being involved in disease processes of the retina whether the disease is of genetic origin or not. The two proteins are uniquely or predominantly synthesized by photoreceptor neurons and play critical roles in retinal physiology. As is well known, opsin is necessary for capture of the photic signal and initiation of ion conductance changes in phototransduction while, as outlined below, IRBP functions both in retinoid transport in the visual cycle and as a 'buffer', protecting both retinoid and photoreceptor membranes from damage in the interphotoreceptor space. Any problem with either of them would be expected to result in retinal dysfunction in one form or another.

This review outlines some of the approaches currently being used to address the problem of retinal degeneration. It focuses on (1) retinal degenerations in the RP family of diseases, (2) experimental autoimmune uveitis (EAU) and (3) degeneration subsequent to viral infection. In most of this, I have tried to use specific examples, usually focusing on opsin (but not the color pigments) and interphotoreceptor retinoid-binding protein (IRBP). In many of the retinal degenerations, there may be primary defects in the polypeptide moiety of these two proteins such that dysfunction is a result. In other cases, these proteins may not be involved in the primary cause of the disease but can be quickly affected in function, concentration, localization, etc. causing secondary yet important sequelae. As such, they can be thought of as important markers for the disease process whose dysfunction themselves can cause severe consequences.

2. RETINOID TRANSPORT IN THE VISUAL CYCLE

Since the Nobel Prize-winning work of Dr George Wald (1935), opsin has been recognized as occupying the central position in the visual cycle. It captures the photic energy and begins the process of visual transduction through a complex series of steps that first produces a chemical (cGMP) signal and then an electrophysiological response. Steps in this process of visual transduction are now known in some detail; for a review, see Baylor (1987). However, many of the steps in the visual cycle that precede and follow the light activation of rhodopsin have been much less well characterized, especially the movement of potentially large amounts of retinoid between

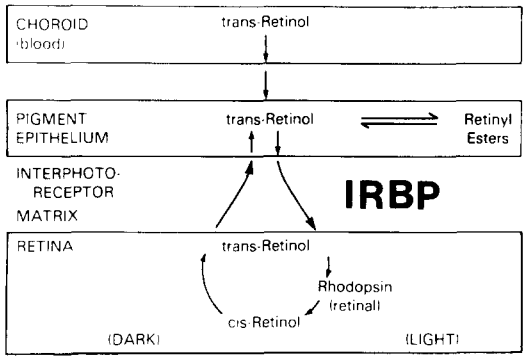

FIG. 1. Movement of retinoid in the visual cycle.

retina and pigment epithelium. For example, the retinoid isomerization reactions have only recently being elucidated by Rando and his colleagues (Bernstein *et al.*, 1987) as have the energetics necessary to drive the isomerization process in the PE cell (Rando, 1991).

Along with opsin, there is another major protein synthesized by the photoreceptor neurons (van Veen *et al.*, 1986) that now appears to be an integral link in the visual cycle. That protein is IRBP, the interphotoreceptor retinoid-binding protein. IRBP is a large lipoglycoprotein that, in contrast to opsin which is inserted into the nascent outer segment membranes, is secreted into the interphotoreceptor space between the retinal photoreceptor outer segments and the microvillous processes of the pigment epithelial (PE) cells (for reviews, see Chader, 1989; Pepperberg *et al.*, 1993).

It has long been known that retinoid moves back and forth across the interphotoreceptor space between the photoreceptor outer segments and the PE cells in cycles of light and dark (Dowling, 1960). This is schematically shown in Fig. 1 with retinoid having to move between different cellular compartments and across a substantial extracellular space. However, three important questions have not been addressed until only very recently. First, what is the form of the retinoid that moves from its stores in the PE cell to the retinal photoreceptor and then back to the PE cell after a light bleach, i.e. is there vectorial transport? Secondly, is this process driven by simple mass action or is it an 'active' process mediated by specific proteins? Thirdly, is there a 'protective' component for the retinoid and/or the cellular membranes? In the following sections, evidence is given implicating the involvement of IRBP in each of these important processes and, as such, indicating the potential importance of IRBP in retinal pathophysiology.

3. OPSIN AND IRPB: PROTEIN STRUCTURE AND FUNCTION

3.1. Opsin Structure and Function

Basic to the study of a hereditary defect in a particular protein that is thought to cause a retinal degeneration or a particular sequence in that protein that could be immunopathogenic is (1) to know the primary sequence of that protein and (2) to uncover the functionally important residues whose presence/mutation will result in a disease phenotype.

The primary sequence of opsin and its insertion into the disc membranes of rod outer segments has been intensely studied for a number of years (Hargrave, 1982; Dratz and Hargrave, 1983) and it is now known in great detail. The opsin molecule is a glycoprotein of approximately 41,000 MW that is an intrinsic membrane protein in the rod outer segment discs. Depending on the type of pigment and the species, it consists of about 348 amino acids (rod pigments and human blue cone pigment) and two oligosaccharide chains that contain 6–8 monosaccharide units each. Functionally, opsin contains a single 'active site' where 11-*cis*-retinal is chemically bound to lysine through a Schiff base. Figure 2 shows a generalized polypeptide chain for visual pigments which is thought to be inserted as seven helical transmembrane domains into the outer segment discs. The amino terminal, with its asparagine-linked carbohydrate chains, extends into the intradiscal space (bottom of figure) while the carboxyl terminal, with its many potential phosphorylation sites on serine and threonine residues, protrudes from the cytoplasmic surface. In bovine opsin, the critical linkage of retinal to form the Schiff base is through lysine 296.

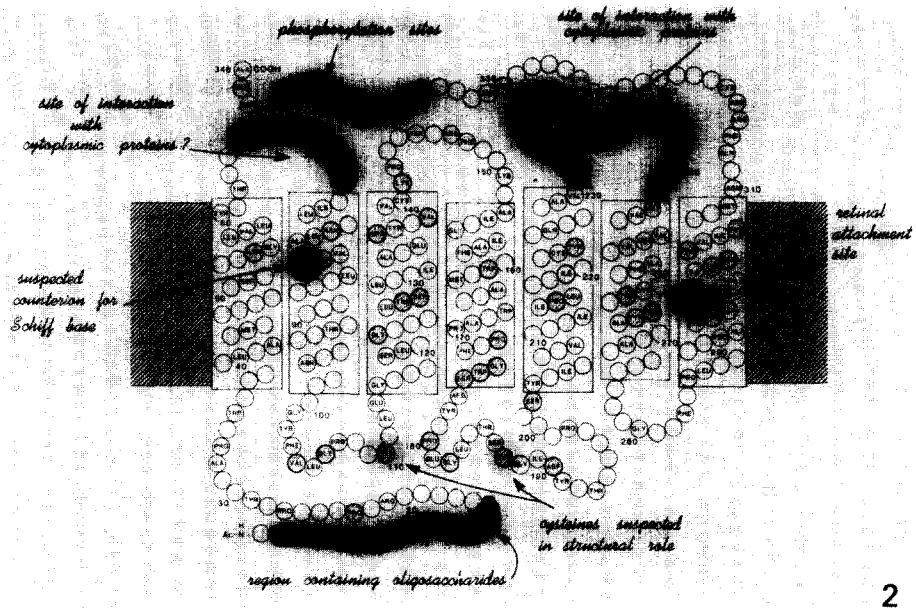

FIG. 2. (A) Model of opsin showing similarities and conserved sequences in different visual pigments. Identified amino acids (3 letter code) are shown when they are identical in 4 or more visual pigments. Heavy circles: invariant amino acids in all pigments. Shaded circles: amino acids homologous in 4 or more visual pigments. Open circles: positions lacking homology in the visual pigments. Taken from Applebury and Hargrave (1986).

Amino acids at many positions in proteins can be changed or mutated with impunity, causing only innocuous polymorphisms and not disease entities. However, even conservative changes in some amino acids can cause loss of function and a hereditary disease phenotype. The functional significance of many of the amino acids in opsin and the other visual pigments have been studied (Fig. 2; see Applebury and Hargrave, 1986 for a review) and many of them have been identified as critical in maintaining proper structure and function in the pigment molecules as they are situated in the membrane. Functional importance also becomes evident from the comparison of pigment sequences from different species where homologues to the vertebrate visual pigment gene can be found through the invertebrates down to the algae *Chlamydomonas* and the archaebacterium *H. halobium* (Martin *et al.*, 1986). In most of these, certain residues can vary widely while others remain invariant, implying their functional importance. These are often linked to specific functions as is the lysine at residue 296 which serves as the retinaldehyde attachment site. Cysteines at positions 110 and 187 form an important disulfide bond that are thought to help to maintain proper structure. Other amino acids such as the proline at position 23 are invariant although their functions have yet to be determined. Finally, many of these residues are also conserved in genes of somewhat different nature within the same supergene family. Obviously, the color pigments show strong sequence homology to opsin. The gene for the β-adrenergic receptor is also part of the same family of membrane receptors and shows many striking similarities to opsin both in amino acid sequence and three-dimensional structure (Dixon *et al.*, 1986).

These earlier observations at the protein level have been confirmed and greatly extended by an excellent series of molecular biological studies culminating in the determination of the complete nucleotide and deduced amino acid sequences of human opsin (Nathans and Hogness, 1984). In parallel, Khorana and his colleagues have developed a system for site-directed mutagenesis of opsin that is designed to probe relationships between protein structure and function (for review, see Khorana, 1992). In this work, a synthetic opsin gene was constructed that allows

for mutagenesis by restriction fragment replacement and thus, site-directed opsin deletions and substitutions can easily be made. Selected single or multiple amino acids can be altered, the opsin protein expressed in COS-1 cells *in vitro* and the effects on rhodopsin regeneration can be assessed (Oprian *et al.*, 1987). Specific amino acid residues thus have been pinpointed that are critical in retinoid binding, maintenance of secondary structure, interactions with other protein components of visual transduction (e.g. transducin) and general functioning of the rhodopsin molecule. Interestingly, some of the mutations have given us unexpected information. For example, Zhukovsky *et al.* (1991) have constructed opsin mutants in which the covalent linkage cannot be formed between the retinoid and protein moieties by substituting alanine or glycine for lysine-296. Yet, when provided with a Schiff base formed from 11-*cis* retinal and an *n*-alkylamine, both mutants can form a visual pigment based on absorption characteristics of the product. Moreover, one of them can activate transducin. Thus, although one would assume such mutants would be dysfunctional (e.g. perhaps causing a retinal degeneration *in vivo*) it seems that the covalent bond is not absolutely necessary for rhodopsin functioning. On the other hand, certain small amino acid deletions in the peptide loops in the intradiscal domain cause structural defects that do seem to lead to functional problems. These mutations prevent the insertion of the protein into membranes, causing it to accumulate in the endoplasmic reticulum. In many ways, these mutants mimic the natural mutations in opsin, many of which are now known to lead to visual dysfunction and photoreceptor cell degeneration in autosomal dominant RP (see Section 4.2).

3.2. IRBP Structure and Function

Much less is known about the IRBP molecule as to precise localization, structure and function. Using a gentle lavage technique, Pfeffer and his colleagues (1983) reported that IRBP was the major, readily-soluble component of the monkey IPM and that it was the only retinoid-binding protein that could be detected. In contrast to opsin whose expression appears to be restricted to the retina at least in primates, IRBP is readily detected in pineal cells albeit at an apparently lower concentration (Rodrigues *et al.*, 1986). This is confirmed by Northern blot analysis of total RNA from retina and pineal which demonstrates an approximate 20-fold difference in IRBP mRNA abundance in the two tissues (Nickerson *et al.*, 1991). As will be discussed later, these results are physiologically confirmed in that both pineal and retina are involved in the pathology of IRBP-induced autoimmune uveitis (see Section 5).

Figure 3 shows that IRBP is a biosynthetic product of retinal photoreceptor cells. Using a specific ^{35}S-RNA probe for IRBP, *in situ* studies by van Veen and his colleagues (van Veen *et al.*, 1986) show positive hybridization only in the photoreceptor nuclear layer (Fig. 3a). An HRP-labeled probe shows a similar labeling pattern (Fig. 3c). At a higher magnification, HRP labeling is visualized primarily around rod rather than cone perikarya (Fig. 3d). The synthesized protein, however, appears to be quickly secreted into the interphotoreceptor matrix (IPM). Figure 4 shows IRBP immunofluorescence in the IPM of the monkey retina (Rodrigues *et al.*, 1986). Fluorescence is only seen within the interphotoreceptor space at this light microscope level (Fig. 4a). The intensity of the fluorescence is less in the macular area than in the more peripheral retina (Fig. 4b) and is not seen beyond the ora serrata (Fig. 4c). Immunoelectron microscopic localization of IRBP in the primate retina confirms this general extracellular pattern (Fig. 5), with IRBP predominantly localized in the IPM around rod photoreceptors (Rodrigues *et al.*, 1986, 1987). Recently, Carter-Dawson and her colleagues (1989, 1992) have used silver-enhanced immunogold electron microscopy to show dense labeling around rods in the monkey retina with considerably less staining in matrix regions of the cone sheaths. As compared with the precise positioning of opsin in the photoreceptor membranes, little is known about the localization of IRBP in the interphotoreceptor matrix and its interactions with other extracellular components. However, the IPM is known to have a highly-ordered structure (Hageman and Johnson, 1991) and evidence from Uehara *et al.* (1990) indicates that

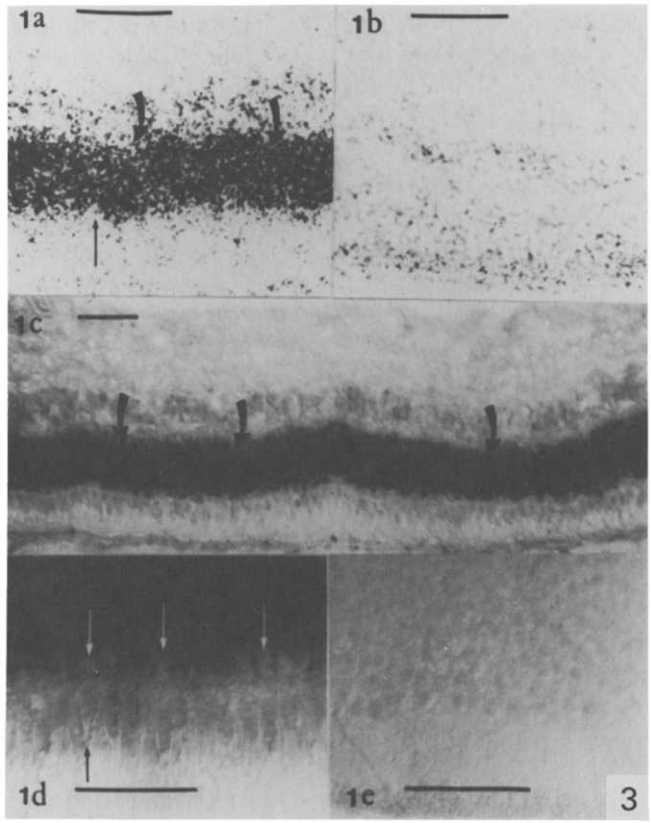

FIG. 3. Nomarski micrographs of *in situ* hybridization of tissue sections of bovine retina. (a) Positive hybridization using a ^{35}S-labeled RNA probe specific for bovine IRBP. Curved arrows point to the positive photoreceptor nuclear layer. Thin arrow points to a less-highly labeled cone perikaryon. (b) Control reaction using sense probe. (c) Positive hybridization with HRP-labeled probe in photoreceptor layer (arrows). (d) Higher magnification showing relative lack of reaction product in area of cone perikarya (white arrows) or outer segments (black arrow). (e) Control reaction using sense probe. Taken from van Veen *et al.* (1986).

IRBP as well as other IPM components may change in position in response to light/dark signals. The latter group found that, in the light, IRBP and other IPM components appear to be found predominantly in bands at the apical surface of the RPE and at the junction of the photoreceptor inner and outer segments. In the dark, though, the concentrations of the components are more homogeneous within the IPM with the interstitial zone more heavily stained than under light-adapted conditions. Importantly, the time courses for the light- and dark-evoked changes in distribution closely match the known rates of retinoid movement between retina and RPE in the Visual Cycle (Dowling, 1960). Light-evoked changes are seen in about 5 min, slightly preceding the movement of retinoid from the PE cell to the photoreceptors; in the dark, distribution changes are much slower (1 – 2 hr) but yet approximate the time frame of retinoid movement in dark adaptation. As postulated by Uehara *et al.* (1990), the apparent high concentration of IRBP at the apical surface of the RPE cell in the light could facilitate retinoid transfer from the photoreceptors to the RPE. Thus, there is the potential for finding IRBP mutants where the mutations not only might directly affect ligand binding but also might affect interactions with other IPM constituents and positioning within the IPM. This would then be loosely analogous to the situation with opsin where, for example, Khorana and his group have found a specific mutation in

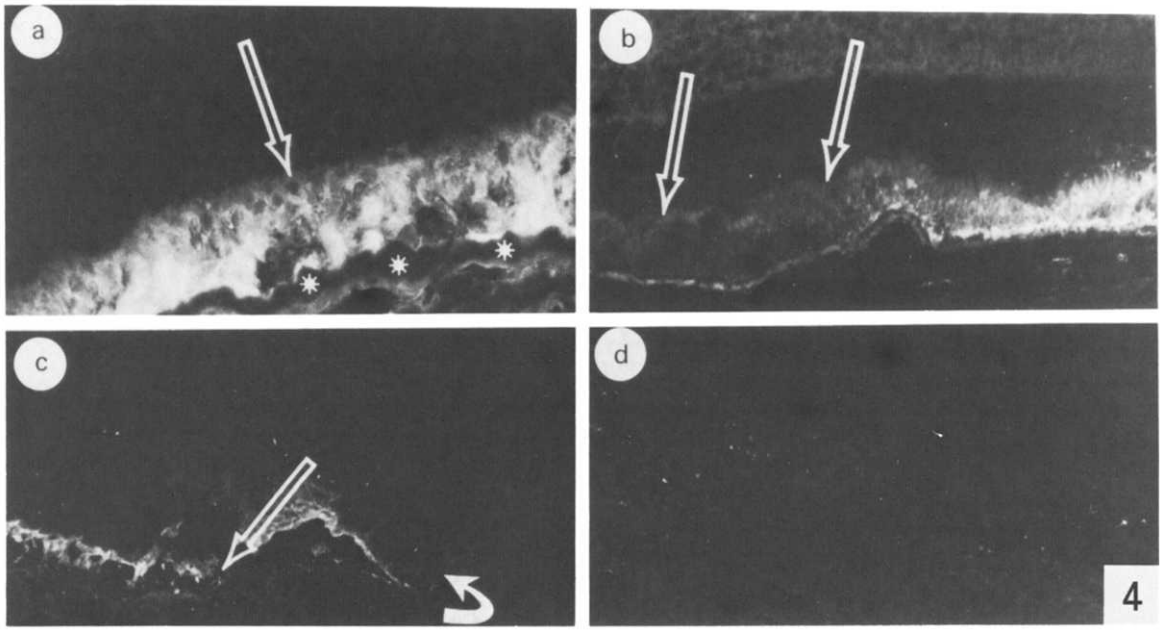

FIG. 4. Immunofluorescent staining for IRBP in the monkey interphotoreceptor matrix using a polyclonal antibody to purified monkey IRBP. (a) Extracellular fluorescence in the IPM; arrow identifies the outer limiting membrane and asterisks identify the pigment epithelium. (b) Decreased immunofluorescence in the macular region (arrows). (c) Fluorescence ends in the transition zone at the ora serrata (arrow). (d) No immunofluorescence is observed if the antibody is preabsorbed with IRBP (×330). Taken from Rodrigues *et al.* (1986).

lysine 248 prevents interaction with transducin and stimulation of GTPase activity (Franke *et al.*, 1988) or other mutations which prevent the insertion of opsin into newly-forming disc membranes.

IRBP is a large lipoglycoprotein that has a molecular size of about 135,000 daltons. The bovine gene codes for 1264 amino acids (Borst *et al.*, 1989), almost 4-fold larger than the opsin polypeptide. Both the protein and the gene demonstrate an interesting 4-fold repeat symmetry, indicating that duplication events were probably a part of the evolutionary history of this protein. The oligosaccharide chains of purified IRBP have been analyzed and have been shown to be somewhat similar to the Asn-linked, hybrid types observed in opsin (Taniguchi *et al.*, 1986). However, Taniguchi and his collaborators found that IRBP is unique in that it contains fucosylated sugar chains. As with opsin, IRBP can be phosphorylated (Wiggert *et al.*, 1988). Interestingly, phosphorylation changes the binding characteristics of IRBP for concanavalin A indicating that phosphorylation *in vivo* could markedly affect its interaction with other IPM components. Adler *et al.* (1987) have examined the shape and hydrodynamic properties of IRBP and have concluded that the molecule has an elongated, rod-like shape. Although much less is known about the structure of IRBP than is known about opsin, Adler and her colleagues have shown IRBP to have a flexible area in the middle that allows for either a straight conformation or a 'hairpin' bend of 60 – 90° that may be determined by the state of retinoid binding. It is interesting to note that, close to the middle of the molecule, there is a proline-rich sequence in both the human protein (residues 697 – 701: Liou *et al.*, 1989) and the bovine protein (residues 690 – 694; Borst *et al.*, 1989). This conserved string of prolines may be involved in the generation of the flexible hairpin loop and thus could be a physiologically and pathophysiologically important region in the molecule.

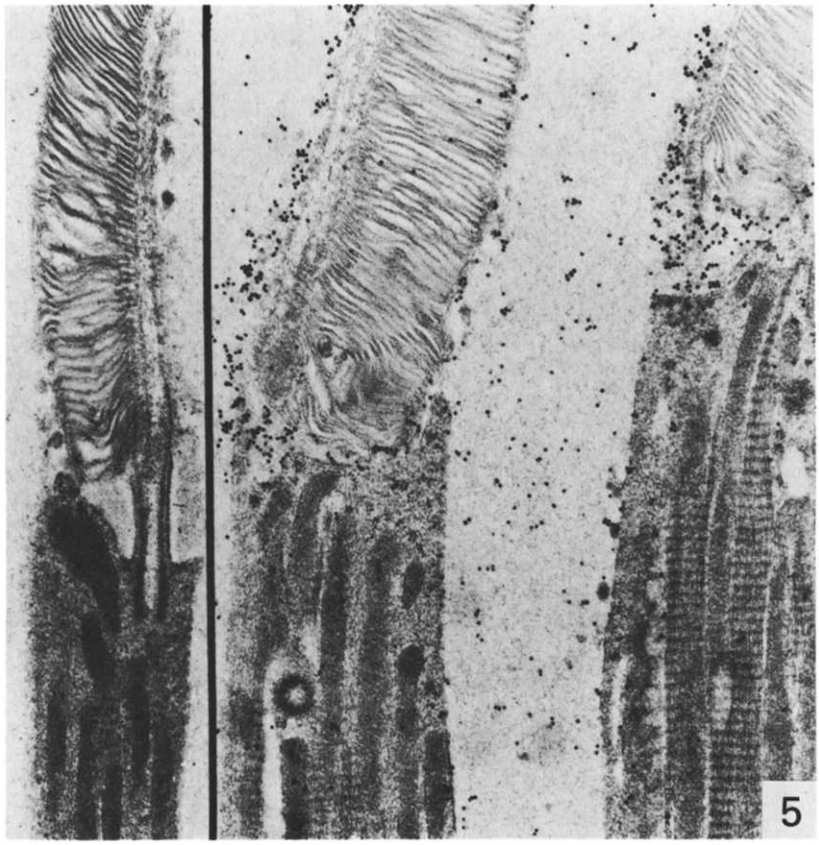

FIG. 5. Immunoelectron microscopic localization of IRBP in the monkey interphotoreceptor matrix. *Left panel:* no immunoreactivity is observed in control sections treated with anti-IRBP that had previously been incubated with purified IRBP. *Right panel:* sections treated with anti-IRBP demonstrate labelling predominantly in the interphotoreceptor space. (×30,000). Courtesy of Dr M. Rodrigues.

IRBP will bind retinoids in a non-covalent, light-dependent manner (Wiggert *et al.*, 1979; Adler and Martin, 1982; Saari *et al.*, 1985; Adler and Spencer, 1991). As with albumin in serum, it will bind a number of hydrophobic ligands at a relatively low affinity. The dissociation constant for all-*trans*-retinol, for example, is only 1.3×10^{-6} M (Adler *et al.*, 1985). This loose binding, however, coupled with the high concentration of IRBP in the IPM would tend to optimize for high capacity, bulk movement of retinoid in the IPM. This would be theoretically necessary to move large amounts of retinoid from the retina to the PE cell after a strong bleach and in the reverse direction for adequate dark adaptation.

3.2.1. TRANSPORT FUNCTION OF IRPB IN THE IPM

Even though the basic bleaching/regeneration reactions of opsin have been known for decades, only recently has information emerged as to the type of retinoid transported in the IPM, the vectorial nature of the movement and the apparently active role of IRBP in the process. For example, Saari *et al.* (1985) have shown that different retinoids are associated with IRBP in the IPM in dark- and light-adapted cow eyes. More recently, Adler and Spencer (1991) have reported that the major ligand carried by IRBP that had been partially purified from dark-adapted cow eyes is 11-*cis*-retinal while, in light-adapted eyes, it is all-*trans*-retinol.

In a series of experiments, Okajima *et al.* (1989, 1990) have used a simple toad eye-cup preparation to study retinoid flow in which the natural components of the visual cycle are all present and can be manipulated independently. For example, in an 'RPE-Choroid' eye cup from which the neural retina is removed, ^{3}H-retinaol can be incubated within the eyecup with or without proteins such as IRBP or albumin. In this way, rates of ^{3}H-retinyl ester accumulation in the pigment epithelium *in situ* can be determined and the effect of the presence or absence of APO-IRBP can be assessed. Lower amounts of added APO-IRBP ($1-10$ μM) were found to greatly facilitate ester formation, well beyond that of buffer alone or added serum RBP or albumin. Addition of higher amounts of IRBP ($20-30$ μM) retarded ester accumulation (Okajima, 1989) as previously reported by Ho *et al.* (1989) in studies with liposomes. However, retinoid movement *in vivo* is not a rapid event (Dowling, 1960) and, based on the apparent concentration gradients of IRBP in the IPM seen by Uehara *et al.* (1990), IRBP could both facilitate retinoid transport and 'buffer' its activity in the IPM within the timeframe of bleaching and dark-adaptation.

Most importantly though, IRBP appears to facilitate the release of 11-*cis*-retinal from pigment epithelial cells and to promote the regeneration of rhodopsin under dark-adapted conditions. Using the toad eye-cup system, Okajima and her colleagues (1990) demonstrated that no retinoid was released from the pigment epithelium if IRBP were not present and that, with the addition of apo-IRBP, only the aldehyde in the *cis* form was withdrawn. Albumin is devoid of activity in this regard. With the addition of rod outer segments into the eye cup, the presence of IRBP was found to greatly facilitate the regeneration of rhodopsin with the withdrawn retinoid over a 3 hr incubation period. Jones *et al.* (1989) have previously shown that full regeneration of rhodopsin in isolated rod photoreceptors of the tiger salamander can be achieved in the presence of IRBP and that these recovery times are close to those seen in the intact eye. These studies differ in a major way from previous studies designed to examine the effects of IRBP on retinoid transfer (e.g. Ho *et al.*, 1989) using synthetic liposomes. In particular, the eye-cup preparation used by Okajima and coworkers has built-in, physiological 'traps' at both ends of the visual cycle, i.e. retinyl ester formation in the RPE and rhodopsin synthesis in retinal ROS. As discussed in detail in Pepperberg *et al.* (1993), this allows for the 'directed flow' of retinoid as postulated by Saari (1990).

Similarly, Carlson and Bok (1992) have completed an elegant series of experiments on retinoid uptake into and release from cultured RPE cells. In this work, ^{3}H-all-*trans*-retinol is delivered in a physiological manner in a complex with serum retinol-binding protein (RBP) to the basal surface of the RPE cell. With apo-IRBP present in the apical medium, ^{3}H-11-*cis*-retinal is specifically released at this surface. When the cells were incubated with another retinoid-binding protein, cellular retinaldehyde-binding protein (CRALBP) under similar conditions, the level of extracted ^{3}H-retinal is markedly reduced rather than increased. This constitutes an important control experiment, again demonstrating the selective ability of IRBP to extract 11-*cis*-retinal from RPE cells. Carlson and Bok conclude that "...IRBP is actively involved in the apical release of ^{3}H-11-*cis*-retinal, perhaps via a receptor in the RPE apical membrane." Although such a receptor has yet to be identified in RPE cells, Politi *et al.* (1989) have presented evidence suggesting such a 'receptor' could be present in rod photoreceptors. In this work, IRBP immunoreactivity was detected on the surfaces of non-permeated, rat photoreceptor neurons in culture. This immunoreactivity is restricted to the inner segment region and could be due to binding sites that could facilitate retinoid transport or uptake. In spite of the question of the presence or absence of IRBP 'receptors' on RPE or photoreceptor cells, the results cited above indicate that (1) with light bleaching, IRBP facilitates the movement of all-*trans*-retinol from retina to RPE along with ester formation in the RPE cells and (2) with dark adaptation, it mediates the release of 11-*cis*-retinol from RPE to the retinal outer segments and subsequent regeneration of functional visual pigment. Thus, as with opsin, IRBP should be considered an integral link in the visual cycle and, also as with opsin, problems in its functioning due

to genetic or other reasons could be expected to lead to retinal degeneration.

3.2.2. PROTECTIVE FUNCTION OF IRPB IN THE IPM

Retinoids are fairly labile and seem to be generally protected in the body by interaction with specific binding proteins (e.g. serum retinol binding protein, RBP). On the other side of the coin, retinoids themselves are well known to be toxic *in vivo* at slightly above physiological levels and, in the theoretical amounts that can be generated after a strong, light bleach, could be damaging to delicate membranes of the cells surrounding the IPM. As previously postulated (Chader, 1989), one clear result of a defect in IRBP concentration and/or function could be an impairment of retinoid transport. Not only would this mean that the proper retinoid would be unavailable for opsin regeneration and dysfunction of the visual process but, as a corollary, there would be an inappropriate build-up of retinoid somewhere in the retina, IPM and/or RPE. Similarly, an opsin defect that led to its inability to use retinoid for visual pigment regeneration could lead to dramatic increases in the concentration of free retinoid and possible subsequent membrane damage. Although there is yet no direct evidence demonstrating a deleterious effect of increased free retinoid on photoreceptor cell integrity due to either opsin or IRBP defects, there is ample evidence of potentially deleterious effects of retinoids on specific aspects of cell functioning, e.g. retinal lysosome lability (Dewar *et al.*, 1975) and permeability effects on subcellular organelles (Stillwell and Ricketts, 1980). In the last study cited, relatively low levels of all-*trans*-retinol were shown to cause adverse effects on liposome membrane permeability that, *in vivo*, would result in the uncoupling of oxidative phosphorylation. Similarly, Meeks *et al.* (1981) have shown that retinoids at the micromolar level induce membrane 'destabilization', i.e. a reduction in microviscosity, and that the rank order of retinoids that cause this effect corresponds to vitamin A toxicity observed in bioassays.

Finally, Jones *et al.* (1989) have shown that IRBP can alleviate a 'toxic' effect of retinol build-up on recovery of rod photoreceptor sensitivity. Using an electrophysiological technique to record membrane currents from individual photoreceptor cells (Fig. 6), they demonstrated that 11-*cis*-retinol causes a marked loss in sensitivity that is reversed by the addition of IRBP to the bathing medium. Thus, IRBP can sequester retinoids that are intrinsically 'toxic' at lower levels or ones that could be potentially membranolytic at higher levels, protecting photoreceptor functional and structural integrity.

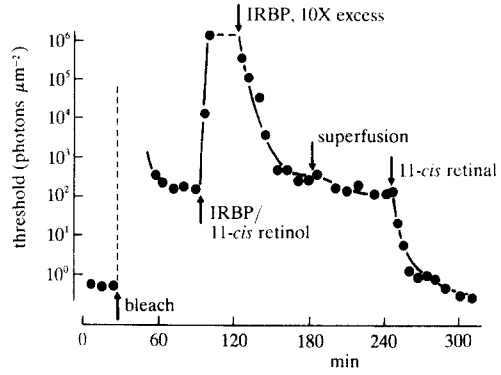

FIG. 6. Membrane current responses in isolated photoreceptor cells of *A. tigrinum* using a suction electrode demonstrate the reversal of toxic effects of 11-*cis*-retinol on dark current generation. After initial bleaching, IRBP or retinoids were added as indicated and threshold values were measured. The dashed line indicates a period when the circulating dark current was apparently zero. Taken from Jones *et al.* (1989).

Recent work also leaves little doubt that IRBP can exert a major protective influence on retinoid integrity. In *in vitro* studies, Crouch *et al.* (1992) have shown that, due to its allylic nature, all-*trans*-retinol is a highly labile compound in aqueous solution or even if it is incorporated into phospholipid vesicles. Considerable degradation is seen within 30 min under these conditions; one of the major products observed is retinal. Thus, both auto-oxidation and isomerization could be significant problems in the highly oxygenated and reactive environment of the IPM. When added at an equimolar concentration, IRBP fully protects retinol for at least 50 min in a buffered aqueous solution and is much more effective than albumin. Importantly, Crouch and her colleagues found that IRBP does not interfere with rhodopsin regeneration in rod outer segment preparations or

the restoration of full sensitivity in isolated rods. Thus, the physiological importance of IRBP in the IPM seems clear as (1) an active facilitator of retinoid transport, (2) a 'buffer', protecting membranes against 'toxic' retinoids and (3) protecting the oxidative and isomeric integrity of the retinoids traveling in both directions between retina and RPE. The pathological ramifications of problems with these aspects of IRBP action seem clear.

4. OPSIN AND IRBP: MOLECULAR PATHOLOGY

4.1. General Approaches to Molecular Genetics

In starting to study any disease process, the basic questions are: (1) What is already known about the disease? and (2) is the disease accessible to a molecular biological approach for understanding its etiology and/or its treatment? Often a direct hereditary component is known as in many of the RPs but the underlying genetic problem could be complex. This could range from simple nucleotide base pair changes (point mutations) as now known to occur in many of the RPs to large chromosomal deletions, insertions, rearrangements or instabilities. In this section, two of the major methods for identification of disease genes are briefly described along with some of the basic molecular biological concepts used in these analyses (e.g. RFLP usage).

The simplest and most straight forward method involves a 'candidate gene approach'. This is used when biological or biochemical information is available about the disease such that an educated guess can be made as to the defective gene. The second method is called 'reverse genetics', 'linkage analysis' or 'positional cloning' (Collins, 1992) when the chromosomal locus of the disease is first determined and then the actual abnormal gene at that site is identified. This is a much more time consuming, costly process but has been used very effectively in uncovering gene mutations in a number of cases as outlined below.

4.1.1. RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLPs)

The human genome is huge and complex. It consists of about 3×10^9 base pairs with over 2×10^5 functional genes, controlling elements, pseudogenes, etc. as well as a large amount of intervening sequence of unknown function. Much variability (polymorphism) in single or multiple base pairs is seen within the genomic nucleotide sequences between individuals of a given species. Also, within the genomic intervening sequences are large numbers of repetitive elements that, in eukaryotes, can compose up to 40% of the genome (Hardman, 1986). These also can be highly variable between individuals. A well known example of these repetitive elements is the *Alu* repeat family which is a grouping of short, similar sequences which are reiterated over and over again and interspersed throughout the genome. Other examples include the LINE (L1) elements that are longer in sequence, VNTRs (variable number of tandem repeats) and STRs (short tandem repeats). The term 'microsatellites' usually refers to the situation when there is di- and tri-nucleotide variability in copy number. Of great importance in gene mapping and linkage analysis is the polymorphic nature of these repeats and of the other small differences in DNA base sequence between individuals within the rest of the genome. In fragile X syndrome, for example, the fragile site in the FMR-1 gene is actually composed of repeat sequences, p(CCG) (Yu *et al.*, 1991; Oberle *et al.*, 1991). Similarly, the myotonic dystrophy (MD) gene of affected individuals has a large and variable increase in the nucleotide repeat p(AGC)n/pCTG) (Harley *et al.*, 1992). It is thus clear that these repeat sequences can not only be used to tag and follow a disease locus but can themselves be directly involved in the primary genetic defect as 'heritable unstable DNA sequences' (Richards and Sutherland 1992).

Polymorphisms were first demonstrated in the β-globin gene in 1978 and have subsequently been used to help in pinpointing the chromosomal loci of a large number of abnormal genes. These DNA base variations can be detected by using specialized enzymes called restriction endonucleases. Each of these enzymes recognizes a specific, short nucleotide sequence (restriction site) in DNA from any species and cleaves the DNA strands in a reproducible pattern. If, in a particular individual's DNA, a mutation does occur which alters the nucleotide sequence, a

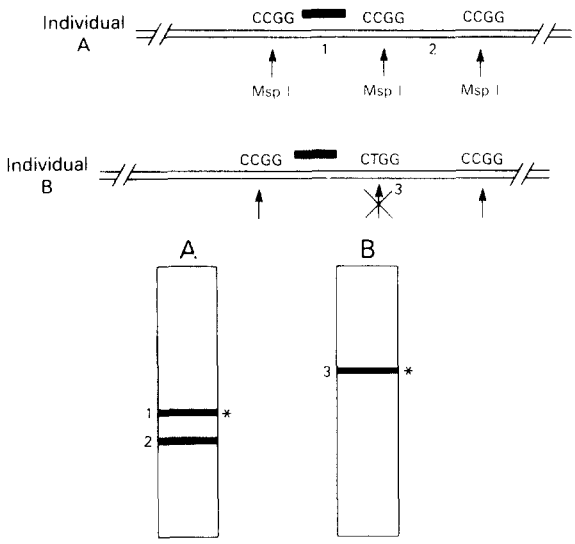

FIG. 7. Model for restriction fragment length polymorphism (RFLP) analysis. The restriction enzyme Msp 1 cuts the DNA samples at different points in the two individuals (top) producing different fragment lengths of DNA as depicted in the schematic electrophoretic separation (bottom). The solid bar on the DNA strands represents a labelled probe that recognizes and binds to the DNA sequence and can be followed in migrational position on the electrophoretic gel (asterisk).

restriction site may be destroyed or, perhaps, a new one created. This is shown in Fig. 7 where the sequence CCGG in individual A has mutated to CTGG in individual B. The restriction enzyme, Msp I, which recognizes the CCGG sequence and cuts the DNA of individual A in three places as seen in the figure, no longer recognizes and cuts the middle site in the B mutant. This produces DNA restriction fragments of different sizes due to the Restriction Fragment Length Polymorphism (RFLP). If one had a specific probe (e.g. radioactive, fluorescent, etc.) that would recognize and bind to the DNA area of the two individuals as shown by the bar between the first two restriction sites in individual A, it would bind to and highlight DNA fragments of different lengths in the two individuals after electrophoretic separation (Southern blotting) as shown by the asterisks (lower part of Fig. 5).

Many of the base changes (mutations) that result in RFLPs are silent in that they do not result in amino acid changes in the translated protein. Other mutations do result in amino acid substitution but are 'silent' in that they do not result in protein dysfunction. Yet other base mutations code for changes in critical amino acids that can lead to a disease state. Since these changes are inherited in a Mendelian manner, however, they are all important genetic markers that can be used to 'tag' and follow a disease gene in a family pedigree.

4.1.2. LINKAGE ANALYSIS

Linkage analysis involves a mathematical assessment of the frequency of coinheritance of two traits within a family. The closer the two DNA sequences encoding the two traits are to each other in the genome, the greater the tendency to be coinherited with less and less crossover or recombination. Technically, one can calculate the 'lod score' from the linkage data to give an odds ratio for the cosegregation. This 'log of the odds' is a logarithmic value that, when exceeding 3.0, indicates the assignment of the disease gene to a particular locus when the chromosomal position of the marker is known. This approach has been successful in determining the chromosomal localization of retinoblastoma which is linked to Esterase D on chromosome 13 (Sparks, 1980) and with cystic fibrosis which is linked to an anonymous marker (DOCRI-917) (Tsui *et al.*, 1985). These principles are more thoroughly covered in a number of excellent textbooks including that by Gelehrter and Collins (1990) and review articles by Caskey (1987) and Musarella (1992).

4.1.3. CANDIDATE GENE ANALYSIS

In contrast to linkage analysis (positional cloning), a candidate gene approach to uncovering a disease gene presupposes that some knowledge of the disease process is available. Using this information, the investigator can make an educated guess as to the involvement of a particular gene. In gyrate atrophy, for example, Takki (1974) identified hyperornithinemia as a major feature of the disease such that the enzyme

ornithine aminotransferase became an obvious candidate for the gene lesion. Although this is an example of a situation where a systemic gene defect rapidly affects retinal function, one would usually focus on unique enzymes or proteins within the retina – PE complex in assessing their possible involvement in a retinal degeneration. Thus, specialized proteins like opsin, IRBP, transducin, etc, with strong functional roles within the visual process are the most likely candidates for study. In this approach, genes are cloned and sequenced from affected individuals and the sequences are compared with those from normal controls. Abnormalities can thus be determined as in the examples with opsin given below.

4.2. Opsin: Molecular Pathology

It has been known for years now that genes for the red and green visual pigments map to the X chromosome, while blue cone sensitivity segregates in an autosomal manner. In 1986, Nathans and his colleagues cloned the genes for the human green, red and blue color pigments (Nathans *et al.*, 1986a) and also demonstrated that defects in these specific genes directly resulted in color blindness (Nathans *et al.*, 1986b; Piantanida, 1988). Color blindness, however, is usually a static condition that does not progressively lead to photoreceptor degeneration and blindness. At about this time though the *ninaE* gene was found to be responsible for a hereditary retinal degeneration in *Drosophila*, and that this gene encoded a sequence homologous in structure and function to vertebrate opsin (O'Tousa *et al.*, 1985). Thus, it was apparent that, at least in one species, a defect in the opsin visual pigment could cause a 'typical' hereditary retinal degeneration. Only in 1986, however, was the chromosomal assignment for human opsin made on a particular autosome. Both through the use of somatic cell hybrids (Nathans, 1986b; Sparks *et al.*, 1986a) and *in situ* hybridization (Sparks *et al.*, 1986b), the gene was localized to the q21 – q24 region of chromosome 3. A connection between the defect in the opsin gene and a specific hereditary retinal degeneration in the human, however, could not yet be made.

In pioneering work, the first linkage study demonstrating the locus for an RP-related gene was reported by Bhattacharya *et al.* in 1984. This was localized to the proximal part of the X chromosome near the probe L1.28. Using a sophisticated series of multilocus homogeneity analyses with a number of available probes, Ott *et al.* (1990) have now shown that there are at least two and probably three *XLRP* genes in this region. It was not until 1989, though, that linkage studies on a family with *ADRP* made it apparent that a candidate gene approach to the study of the RP family of diseases was warranted. Humphries and his collaborators, as recently reviewed in this series (Humphries *et al.*, 1993) had been working on a large Irish pedigree with *ADRP* for some time (Farrar *et al.*, 1989; Daiger *et al.*, 1989) and found that the anonymous DNA marker D3S47 tightly segregated with the disease phenotype in this family (McWilliams *et al.*, 1989). The lod score calculated for the coinheritance of D3S47 and the *ADRP* disease gene was 14.7, an exceedingly high value which gave strong evidence for the locus of the disease on the long arm of chromosome 3 (i.e. 3q). Farrar *et al.* (1990) then used a microsatellite marker (see Section 4.1.1) in the first intron of the opsin gene to link it to the *ADRP* and to confirm that opsin was, in fact, a likely candidate gene for the disease.

Dryja and his coworkers (1990a) were the first to fully utilize this information and to demonstrate a nucleotide sequence abnormality in opsin in a grouping of RP patients in the United States. Specifically, they found a C to A nucleotide transversion in codon 23 that leads to a proline to histidine substitution in the opsin polypeptide sequence in about 12% of 148 RP patients but in none of 102 normal controls tested. In another study, they also demonstrated other ADRP mutations at codon 58 and 347 (Dryja *et al.*, 1990b). As can be seen in Fig. 8, the proline in normal opsin at codon 347 (coded by the CCG triplet in normal subject N 87) is changed to either a leucine (CTG triplet in patient AD 71) or to a serine (triplet TCG in patient AD 92).

Interestingly, this region contains the CCGG sequence specific for Msp I digestion and thus the

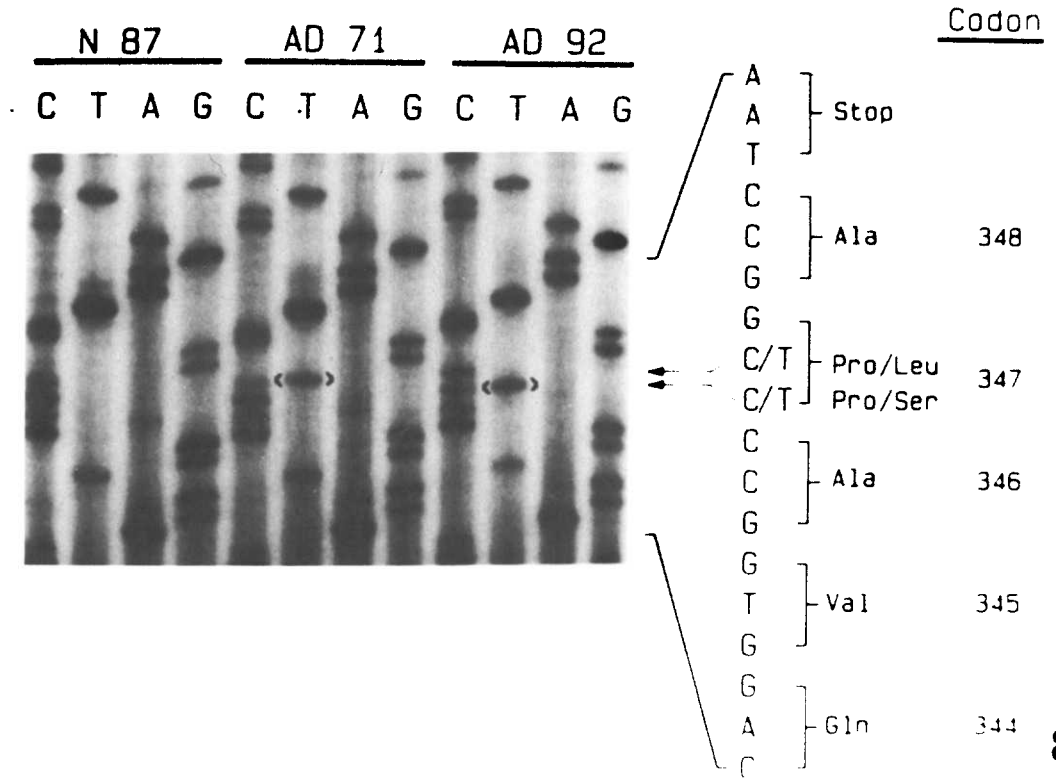

FIG. 8. Nucleotide sequence analyses of mutations in the opsin gene. DNA samples were from a normal control (n 87) and RP patients (AD 71 and AD 92). On the right, nucleotide mutations and deduced amino acids are given that correspond to the mutated bases shown in brackets on the gel. Note the CCGG MspI restriction site. Taken from Dryja *et al.* (1990).

patients can be identified by their altered Msp I RFLP pattern as outlined in Fig. 9. This figure shows the transmission of the Pro-347 – Leu mutation in a pedigree containing patient AD 71. In this family, DNA from RP-affected individuals with the mutated opsin (shown in the solid circles and squares) have a 221 bp Msp I restriction fragment. This restriction fragment is not seen in non-affected members of the family (open circles and squares). Affected members of the family are heterozygous for the mutant 221 bp fragment. Non-affected members of the family only show smaller restriction fragments after Msp I digestion. Most importantly, the mutated sequences 'segregated perfectly with the disease' phenotypes within the individual pedigrees. This is an excellent example of the use of RFLPs in 'tagging' a mutant sequence, in this instance, where the restriction site is the actual site of mutation.

The functional or structural significance of these mutations is not readily apparent. The mutations in prolines at codons 23 and 347 are not only at different ends of the molecule but in distinctly different microenvironments; Pro23 is intradiscal and Pro347 is at the cytoplasmic interface. It is known, though, that Pro23 is one of the invariant amino acids and Pro347 one of the conserved amino acids discussed in Section 3.1. Prolines, because of their unique chemical structure among the amino acids are thought to often change the structural domain of the protein area in which they reside, forming 'kinks' or 'bridge regions' between adjoining structures. Thus, these prolines are probably important in maintaining proper opsin structure intrinsically and/or for its interactions with other proteins in visual transduction.

In spite of the obvious co – segregation of the Pro – His change with some of the ADRP pedigrees in the USA, Farrar *et al.* (1990) were unable to find this particular conversion in a number of European pedigrees indicating the

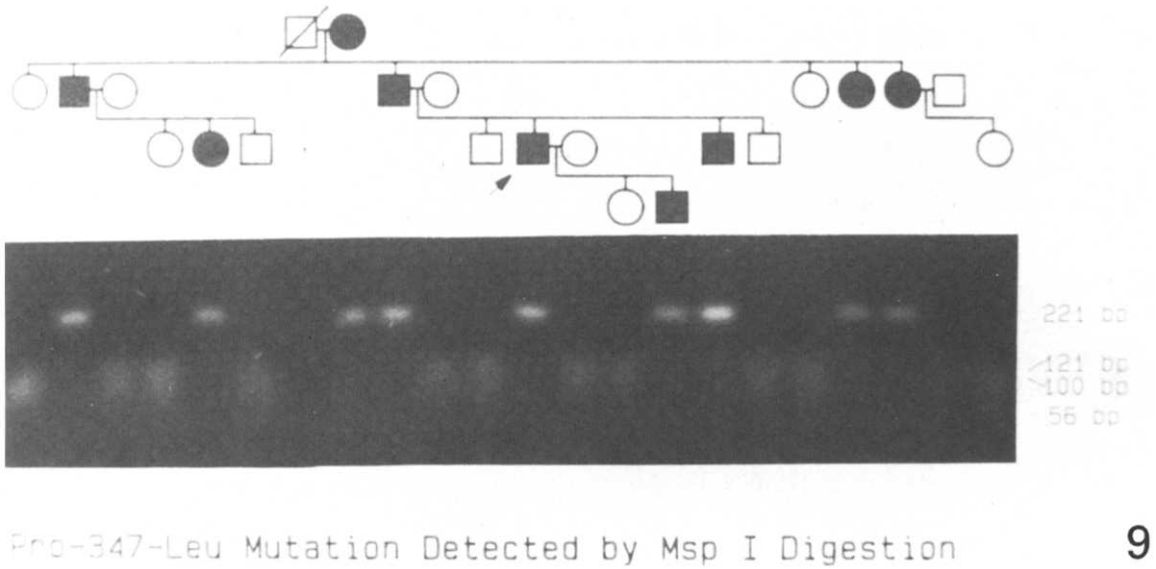

FIG. 9. Gel electrophoretic pattern of ethidium bromide-stained DNA fragments digested with MspI from an ADRP pedigree with a Pro-347 – Leu mutation. Solid symbols: affected members; open symbols; non-affected members. Arrow: patient AD 71 as given in Fig. 8. Note that the 221 bp restriction fragment co-segregates with the affected members of the family. Taken from Dryja *et al.* (1990).

diversity of the mutations underlying the clinical RP phenotypes. Heckenlively *et al.* (1991) have studied two other *ADRP* families with codon 23 mutations in opsin and found the clinical phenotype was that of sectoral RP. As with the report by Farrar *et al.* (1990), all patients with sectoral RP did not demonstrate the Pro to His substitution. Sectoral RP is an RP variant in which degeneration is restricted to the inferior nasal quadrant of the retina rather than throughout the retina as in the more typical RPs (Massof and Finkelstein, 1981). Interestingly, several of the patients reported by Heckenlively *et al.* (1991) had a history of potential light damage to the retina from intense sunlight, reflection from water, welder's arc light, etc. They have thus hypothesized that photic toxicity, coupled with an instability in the opsin molecule due to the Pro mutation, could lead to the retinal degeneration. This would mean that at least some forms of RP could be multifactorial in that 'two hits' would be necessary to express the disease phenotype. The first would be a genetic mutation (e.g. a Pro – His transversion) leading to a structural abnormality in the opsin protein which, based on whether or not a second hit (e.g. photic toxicity) occurred, may or may not be translated into an overt retinal degeneration. This possibility is substantiated by results from Berson *et al.* (1991) who have seen extensive heterogeneity in RP disease characteristics and severity in patients with the same opsin point mutations. They suggest that 'risk factors' other than the actual gene abnormalities may play a significant role in the progression of the retinal degeneration, complicating our understanding of the disease etiology but leaving the door open to alternate routes of control and therapy.

Once the candidate gene approach was shown to be successful in pinpointing the first opsin mutation in *ADRP*, intensive work from a number of groups subsequently identified a very large number of mutations in the opsin molecule that segregate with retinal degeneration phenotypes in well-characterized pedigrees. Dryja *et al.* (1990b, 1991) and Sung *et al.* (1991) have uncovered numerous mutations affecting a large percentage of the amino acids of the polypeptide. Changes are now documented on both cytoplasmic and intradiscal surfaces of the opsin molecule and within the transmembrane region (see Fig. 2). Most of these changes result from point mutations with a high frequency of *CpG* mutations. A

deletion mutation in opsin resulting in ADRP, however, has been reported. Inglehearn *et al.* (1991) has demonstrated a three base pair deletion in the fourth exon of an RP family that is in frame and results in the deletion of one of two isoleucine residues at codons 255/256. The authors speculate that the mechanism by which the mutation is generated appears to be similar to that which creates new microsatellite variants. Such deletion mutations demonstrate the diversity of mutational events causing ADRP as well as the large number of sites at risk in the opsin molecule.

Much less is known about autosomal recessive and X-linked (ARRP) forms of Retinitis Pigmentosa. Linkage analysis is difficult with recessive diseases due to the smaller number of patients within defined pedigrees. The candidate gene approach has been successful in one case, however, since Rosenfeld *et al.* (1992) have reported that a 'null' mutation in the opsin gene can be detected in an ARRP patient. In this case, the mutation (GAG to TAG) at codon 249 probably results in the deletion of the fourth and fifth transmembrane domains of the polypeptide. Even though the first genetic linkage to any form of RP was to the short arm of the X-chromosome (Xp) in 1984 by Bhattacharya *et al.* the actual genes involved have not been identified because of the complex nature of the problem (Ott *et al.*, 1990).

To date, only relatively simple mutations have been uncovered that lead to changes or deletions in amino acid sequence within the opsin polypeptide. Other mutations, however, can be envisioned that are more subtle and do not code for actual mutations in the polypeptide sequence but which yet could lead to retinal degeneration. Lem *et al.* (1991), for example, have examined the tissue specificity and developmental regulation of opsin chimeric genes in transgenic mice. They found a 500 bp fragment that specifies expression in photoreceptor cells as well as other upstream regions that are critical to the uniform spatial expression of the gene. A mutation in either of these regions might be expected to result in inappropriate temporal and/or spatial expression of the gene with possible resultant tissue degeneration. Opsin mRNA levels are also known to be regulated by light and by a circadian oscillator at least in some species (Korenbrot and Fernald, 1989). Mutations which lead to inappropriate light-driven or circadian expression of the opsin gene also might result in photoreceptor degeneration due to faulty control of the rod disc renewal process. In this scenario, one could envision genes specific to the pineal melatonin system as well as regulatory genes in the retina as playing a possible role in the abnormal expression. Finally, the opsin gene is only one of the genes that will ultimately be shown to be mutated in autosomal dominant and other forms of RP. The gene responsible for the retinal degeneration seen in early development in the *rds* mouse has recently been cloned and identified as peripherin (Travis *et al.*, 1989; Connell *et al.*, 1991). Peripherin is a protein that has features in common with opsin in that it is a membrane protein that seems to be structurally important to the rod outer segment. Point mutations and deletions in the human homolog of this gene have now been identified as cosegregating with patients in ADRP pedigrees (Farrar *et al.*, 1991; Kajiwara *et al.*, 1991). The cloning of *ROM-1*, a new member of the peripherin gene family that is localized in the photoreceptor disc rim (Bascom *et al.*, 1992), offers an obvious new candidate gene for genetic studies as does the gene for the β-subunit of the ROS phosphodiesterase enzyme known to be mutated in the retinal degeneration seen in the *rd* mouse (Bowes *et al.*, 1990). Daiger and his coworkers (Blanton *et al.*, 1991) have used linkage analysis to excellent advantage in demonstrating cosegregation of the *ADRP1* gene with a number of DNA markers on chromosome 8 and mapping of this disease gene to the pericentric region of the chromosome. The identity of this gene has yet to be determined.

4.3. IRBP: Molecular Pathology

To date, IRBP has not been shown to be the locus of any of the hereditary retinal degenerations. As pointed out above though, it is a likely candidate based on both structural and functional grounds. Its large size, however, makes it more difficult to screen RP pedigrees, do direct sequencing, etc. Are there any specific hereditary retinal

degenerations which could be 'candidate diseases' in which IRBP might likely be involved?

One possibility is Fundus Albipunctatus (FA). FA is a night blindness, inherited in an autosomally recessive manner, first described by Lauber (1910). This congenital condition is relatively benign and stationary. Marmor (1990) has followed two patients for over a decade and has found that the dark adaptation times and ERG signals remained 'remarkably stable' over this period although he noted an evolution of the fundus lesions from 'flecks in childhood' to subsequent 'relatively permanent punctate dots'. These dots seem to be at the level of the retinal pigment epithelium and, interestingly, are similar to the yellow dots observed in some vitamin A deficiencies (Hing, 1965). Electrophysiologically, Carr *et al.* (1974) used dark adaptometry to demonstrate that the signature defect in this condition is an abnormally slow dark adaptation time. They found that regeneration after a strong bleach could take up to 3 hr or more but that, importantly, full recovery was achieved, comparable to the level seen in normal subjects. Fundus reflectometry is another non-invasive technique for determining rhodopsin levels in small areas of the retina *in vivo*. This technique also demonstrates that, although visual pigment regeneration is slow, regeneration eventually becomes complete. This indicates that, although visual pigment is affected in the disease process, opsin itself is probably not defective along with proteins involved in subsequent steps within the visual transduction pathway. Rather, the evidence points to proteins and enzymes interacting with rhodopsin and to processes controlling vitamin A metabolism and movement in the RPE and/or IPM as more likely candidates (Ripps, 1982). This yet gives a wide range of possibilities including: (1) proteins directly involved in opsin regeneration such as kinases, phosphatases, etc. in the photoreceptor cell, (2) proteins involved in retinoid storage, isomerization and mobilization in the RPE such as the cellular retinaldehyde-binding protein (CARLBP) or cellular retinol-binding protein (CRBP) and (3) soluble and membrane-bound proteins/receptors involved in retinoid transport across the IPM and uptake into the retina. Unfortunately, very little is known about most of these proteins/processes, making it impossible to study them as candidates in an FA pedigree. In contrast, not only is IRBP a likely candidate but proper cDNA probes are readily available.

Saperstein *et al.* (1990, 1991) have used the molecular biological data available on IRBP to study a small FA pedigree using a combination of the candidate gene approach and linkage analysis. Basic to the study is the fact that a number of polymorphisms have been determined for IRBP such that an adequate RFLP analysis could be performed. Polymorphisms are usually identified and followed in a pedigree using Southern blotting. Saperstein and Nickerson (1991), however, have shown that RFLPs can be easily detected in DNA fragments after amplification by PCR if gene sequence information is available for the construction of proper primers (which is the case for IRBP). In this way, the more laborious, costly and time-consuming Southern blotting can be circumvented by the use of only a small fraction of the total sample DNA conventionally used. Figure 10 demonstrates the usefulness of the technique in identifying Sty I and Msp I RFLPs, originally described by Chin *et al.* (1988) in a pedigree consisting of two parents, two daughters and a son in which the two daughters are affected by FA. First, genomic data on *IRBP* was used to construct primers from the first intron of the gene to amplify a 1196 bp product which contained both restriction sites. These were then used with genomic DNA from each of the family members in the PCR amplifications, and digested with the restriction enzymes and the products separated by electrophoresis for identification of possible polymorphisms.

In this family, the restriction pattern shows both polymorphic bands as well as non-polymorphic bands (Fig. 10). The Sty I alleles are seen as bands at 280 bp and 390 bp (allele 1) or 670 bp (allele 2) while the Msp I alleles are visualized by bands at 400 bp and 610 (allele 1) and 1010 bp (allele 2). Non-polymorphic bands are seen at 430 and 110 bp with Sty I and at 190 bp with Msp I. The father and one daughter are heterozygous for both the Sty I and Msp I RFLPs (1,2) while the other three members of the family (mother, son, other daughter) are homozygous (2,2) for both.

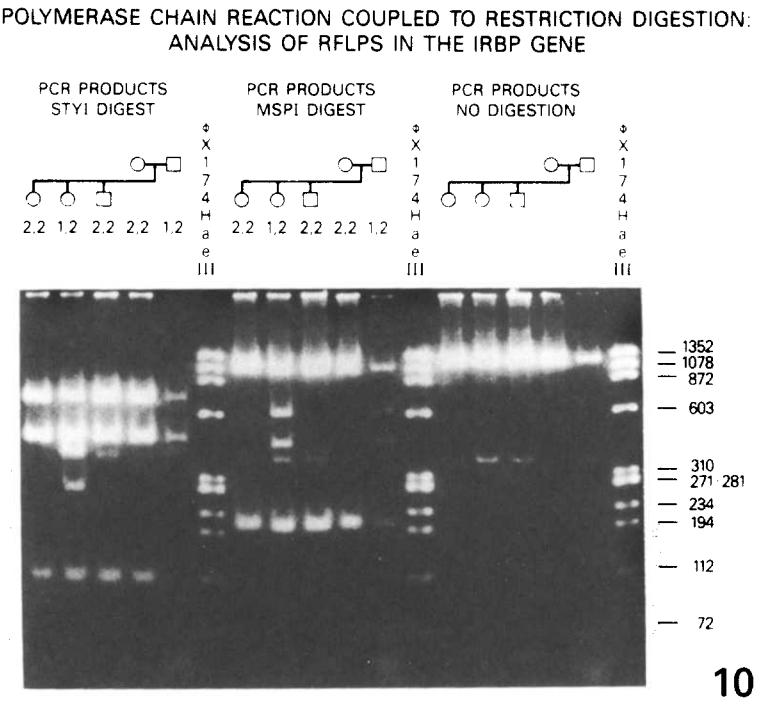

FIG. 10. RFLP analysis of a pedigree with Fundus Albipunctatus using PCR coupled to restriction digests. Specific PCR products from the intron A/exon 1 region of the IRBP gene were digested with Msp I, Sty I or neither. The gel electrophoretic pattern demonstrates the allele patterns (1,2) for the family members. In this case, the two daughters are affected. Taken from Saperstein and Nickerson (1991).

As analyzed by Saperstein *et al.* (1990) in more detail, the inheritance pattern indicates that the father is a double heterozygote for both FA and for IRBP and that, in one daughter, allele (2) cosegregates with the FA gene while, in the other daughter, it is allele (1) that cosegregates with the disease. The IRBP and FA loci are therefore not linked indicating that they are separate and distinct, at least in this family grouping.

Even though this study excludes IRBP from further consideration as the FA locus in this family, it yet may be involved in other FA pedigrees and in other types of retinal degenerations. What clues are currently available? As delineated above, the high concentration of IRBP in the IPM, its potentially protective use and its functional role in rhodopsin regeneration would mitigate for such a role. Similarly, the very early expression of IRBP in embryonic development also suggests a role for the protein in normal retinal development. In all species studied to date, IRBP appears well before opsin or even somewhat before the elongation of outer segments. For example, Johnson *et al.* (1985) found a correlation between IRBP production and the beginnings of disc formation in nascent ROS in the embryonic human retina. In the cow, Hauswirth *et al.* (1992) have reported significant IRBP levels in IPM in the fifth embryonic month, well before outer segments or opsin begins to accumulate. In rodents, outer segment development is delayed until after birth beginning at about postnatal day 7. Carter-Dawson *et al.* (1986), however, detected IRBP at embryonic day 17 when even rod inner segments are at an early stage of development. Gonzalez-Fernandez and Healy (1990) have found an "unexpected early expression of the mRNA for IRBP" in the rat and suggest that it could help in the transport of 'nutrients and morphogens' to the developing retina. Interestingly, van Veen *et al.* (1988) found that most of the IRBP immunoreactivity in the normal mouse retina remained within the photoreceptor cell until approximately postnatal day 7 (P7). Only after this is the bulk of the immunoreactivity found in the IPM with little

TABLE 1. *Immunoreaction of Photoreceptor-Specific Proteins in Retinae of Control Cats and Abyssinian Cats with Progressive Retinal Atrophy*

Antibody raised against	IRBP	S-antigen	Opsin	Transducin-α
Control 12 w	+ + +	+ + +	+ + +	+ + +
Control 2 year	+ + +	+ + +	+ + +	+ + +
Stage 0	+ + +	+ + +	+ + +	+ + +
Stage 1	+ +	+ + +	+ + +	+ + +
Stage 2	+	+ + +	+ +	+ +
Stage 3	(+)	+ + +	+ +*	+ +*
Stage 4	0	+ + +	+ +*	+ +*

*Intracellular localization. (Taken from Narfstrom *et al.*, 1989.)

if any detectable within the photoreceptor perikaryon. In a number of mouse mutants of retinal degeneration (*rd,rds*), however, substantial intracellular IRBP is detected throughout the degenerative process. This indicates that the normal secretory mechanism is aborted as a result of the primary gene defect(s). Despite a secondary event, van Veen and his colleagues have postulated that such an accumulation of IRBP and possibly other secretory products could lead to "a degenerated and less differentiated phenotype". Thus, a delay in expression of IRBP, a problem with its secretion, as well as more classical mutations as now are known for the opsin gene, could all cause or contribute to loss of photoreceptor function and, ultimately, to retinal degeneration.

In this regard, knowledge is now accumulating as to the elements involved in function and expression of IRBP. Functionally, little is yet known about the retinoid binding site(s) as compared with the precise knowledge of the retinoid pocket within the opsin molecule. More information has recently been shown for fatty acid binding in that Putilina and her colleagues (1993) have used fluorescent probes to demonstrate that IRBP contains one high-affinity binding site for fatty acid analogs that could function *in vivo* to bind and transport a number of natural fatty acids. Mutations in any of the fatty acid and retinoid binding sites could lead to lipid/retinoid imbalances and initiate photoreceptor cell death. The five adjacent proline residues in the middle of the molecule constitute another logical site for beginning to study structure – functional relationships and for looking for IRBP mutants that might cause retinal degenerations. At a higher level of control, Liou *et al.* (1991) have examined the promoter region of the IRBP gene for sequences involved in expression. They have pinpointed a stretch of 212 base pairs in the 5′-promoter region as being critical for normal basal expression and tissue-specific expression. Beyond this, Albini *et al.* (1990) have studied the methylation patterns of the IRBP gene and found that hypomethylation of a CpG-rich island in the promoter region (−1578 to −1108) and sites in the first exon are linked to expression of the gene. Such demethylation was only found in a cell line known to express IRBP (i.e. Y-79 retinoblastoma) and not in other, non-expressing cell types. All of these are areas in which small changes could result in retinal dysfunction. The chromosomal localization for IRBP has been reported to be chromosome 10p11.2 – q11.2 by Liou *et al.* (1987) although Ngo *et al.* (1993) have recently made the assignment to 10q21.1. Although no RP is yet known to map to this chromosome, it is possible that one of the yet uncharacterized types of RP will be mapped to this region of the genome.

Clues from animal models of RP have also implicated IRBP in the degenerative process. As just mentioned, abnormalities in IRBP secretion are present in combinations of *rd* and *rds* mouse mutants (van Veen *et al.*, 1988). Narfstrom *et al.* (1989) have shown a reduced level of IRBP in a cat model of hereditary retinal degeneration. In these studies, a number of photoreceptor proteins were studied by immunocytochemistry in the slowly degenerating retinas of Abyssinian cats; only IRBP was found to be markedly reduced at an early stage before the onset of cell death. As seen in Table 1, levels of S-antigen, opsin and

transducin-α remained fairly stable well past the time of IRBP disappearance. IRBP should thus be considered as a possible candidate for the locus in this disease or at least as an early lesion that can lead to important secondary complications. Finally, Duncan *et al.* (1992) have demonstrated that there is a cognate of IRBP in *Drosophila melanogaster* heads, apparently within the retinas. This glycoprotein carries endogenous retinoid and fatty acids and has many of the same physicochemical characteristics as mammalian IRBP. Since so many visual system mutants for the fruitfly are available, it will be interesting to see if this 'fly gene' maps to one of these mutant loci such that the IRBP gene can be pinpointed as the mutated gene. If this occurs, the characteristics of the 'fly disease' should give valuable clues as to which type (or types) of human RPs to specifically examine for IRBP mutations.

5. EXPERIMENTAL AUTOIMMUNE UVEITIS

5.1. General Considerations

As with Retinitis Pigmentosa, Uveitis is a grouping of diseases that usually leads to retinal degeneration. Although there is thought to be a hereditary component of susceptibility, no direct genetic inheritance has been detected as in many of the RPs. Although the causes of many of the different forms of Uveitis are yet unknown, it is most often considered to be an autoimmune condition that primarily affects the eye. Some of the disease entities, however, are manifest as a generalized systemic syndrome. In man, Vogt – Koyanagi – Harada syndrome, Sarcoidosis and Behcet's disease are examples of generalized, immune-mediated conditions, while Sympathetic Ophthalmia and Birdshot Retinochoroidopathy are conditions that are thought to be restricted to the eye.

Several animal models have now been developed in which immunization with specific proteins of retinal origin induces Experimental Autoimmune Uveitis (EAU). Much of this early work has been reviewed by Gery *et al.* (1986a) in this review series. Opsin is one of the antigens that has been reported to induce a mild form of EAU in guinea pigs (Marak *et al.*, 1980; Myers-Eliott *et al.*, 1983) and, more recently, in rats (Schalken *et al.*, 1988) and monkeys (Broekhuyse *et al.*, 1988). Interestingly, rhodopsin is more immunopathogenic than is opsin in inducing EAU (Schalken *et al.*, 1988). In contrast, the S-antigen is a soluble uveitopathogenic antigen characterized by Wacker and his colleagues (Wacker *et al.*, 1977) that invariably causes an intense ocular inflammation and subsequent retinal degeneration when injected into test animals. Pinealitis is another manifestation of the disease process indicating the presence of S-Antigen in this photosensitive gland. S-antigen is also called '48 k protein' and 'arrestin' and is a relatively abundant, soluble protein of the photoreceptor cell. Its binding to photoreceptor membranes is light-dependent and it has been postulated to participate in visual transduction (Kuhn *et al.*, 1984). S-antigen genes have now been well characterized in several species and form a family grouping of similar proteins (Shinohara *et al.*, 1992). S-antigen-induced EAU in rats is rapid and devastating; specific peptides of the protein are also capable of inducing inflammation and subsequent retinal degeneration. Interestingly, Shinohara and his coworkers (Shinohara *et al.*, 1990; Singh *et al.*, 1992) have found that a number of peptides from a variety of sources (yeast histone, hepatitis virus, leukemia virus, etc.) have sequences similar to those in S-Antigen and can induce EAU in test animals.

5.2. IRBP-Induced EAU

In 1986, Gery and his coworkers (Gery *et al.*, 1986b) first reported that IRBP could induce EAU in the rat. As with S-antigen, both uveoretinitis and pinealitis were observed. Significant differences between the characteristics of the diseases induced by IRBP and S-antigen were found, however. First, IRBP-induced EAU has a markedly shorter time course than the S-antigen-induced disease. Secondly, it is produced at a much lower dose of antigen. Thirdly, immunization with higher doses of IRBP produces a less intense uveitic response, primarily affecting

FIG. 11. Section of the retina of a Lewis rat 27 days after immunization with IRBP. Note the loss of photoreceptor cells and lack of evidence of inflammation.

photoreceptor cells, and does not produce as marked a panuveitic effect as does S-antigen. The pathology of the IRBP-induced disease has been well documented in rat (Gery *et al.*, 1986b), mouse (Chan *et al.*, 1990) and monkey (Hirose *et al.*, 1987; Sanui *et al.*, 1990) and seems to be limited to the eye and pineal. In Fig. 11, a section of a rat retina is shown 27 days after immunization with IRBP. As is evident, photoreceptor cells are degenerated or missing but much of the rest of the retina remains intact. This is, as such, the end-stage of the disease with no signs of the initial inflammation and little to distinguish it from a hereditary photoreceptor degeneration. In mouse, many of the characteristics of the disease process are similar to those seen in the human, i.e. a relapsing course of the disease and the presence of patches of infiltrating macrophages (Dalen–Fuchs nodules). Most importantly, the disease characteristics in monkey are also close to those seen in human, making it clear that, indeed, these are all true animal 'models' of human uveitis (Hirose *et al.*, 1987; Sanui *et al.*, 1990).

5.3. Immunopathogenic Sequences in IRBP

With this relative specificity of effect of the IRBP protein, it was especially interesting to identify the amino acid sequence(s) of the molecule that causes EAU. Redmond *et al.* (1988) first demonstrated that cyanogen bromide could be used to cleave IRBP and, after separation by HPLC, some of the fragments were found to be uveitogenic when administered to rats. In particular, a fragment comprising amino acids 1169 through 1191 was found by Sanui *et al.* (1989) to be the major immunopathogenic site in the IRBP molecule. In rats, the dose of peptide 1169–1191 needed to produce EAU was

TABLE 2. *Analogs of Peptide 1181 – 1191 with Alanine Substitutions**

Peptide	Sequence
1181 – 1191	S W E G V G V V P D V
A (1182)	S A E G V G V V P D V
A (1183)	S W A G V G V V P D V
A (1184)	S W E A V G V V P D V
A (1185)	S W E G A G V V P D V
A (1186)	S W E G V A V V P D V
A (1187)	S W E G V G A V P D V
A (1188)	S W E G V G V A P D V
A (1189)	S W E G V G V V A D V
A (1190)	S W E G V G V V P A V

*Peptide 1181 – 1191 is the natural uveitogenic peptide sequence found in IRBP. Peptides A(1182) through A(1190) were synthesized with alanine (underlined) substituted for the natural amino acid. (Taken from Kotake *et al.*, 1991.)

remarkably low and the disease resembled that produced by the intact protein in its kinetics and histopathology. This peptide also constituted the immunodominant epitope of the IRBP molecule. Truncation of 1169 – 1191 from the carboxyl terminal resulted in no loss of ability to induce EAU after removal of the valine at position 1191 but complete loss of activity with deletion of the aspartic acid at 1190. Truncation from the amino-terminal end of the peptide yielded a series of active peptides as short as the decapeptide 1182 – 1191. Removal of the tryptophan at 1182, as with the aspartate at 1190, destroyed all activity, demonstrating their critical immunological role. Together, these data pinpoint the nonapeptide 1182 – 1190 with the sequence W – E – G – V – G – V – V – P – D as the least common denominator for both the immunological and immunopathological site.

As Khorana and his colleagues (Khorana, 1992) have studied the mutagenesis of the opsin protein to determine structure – function relationships as discussed in Section 3.1, Kotake *et al.* (1991) investigated whether or not each of the amino acids in the nonapeptide was necessary in provoking EAU and an antigenic response in test animals. For this purpose, alanine, a small, relatively neutral amino acid, was substituted for each of the residues in synthetic peptide 1181 – 1191 (Table 2). When rats were injected with the intact 1181 – 1191 at the low dose of 200 pmol/rat, EAU was produced. Table 3 outlines the results obtained with the analogs as to the incidence, day of onset and severity of the disease produced with each alanine substitution. Alanine substitution for either tryptophan at position 1182 (analog A1182) or aspartic acid at position 1190 (analog A1190), abolished or drastically reduced uveitogenicity. These data confirm the truncation studies and underscore the pivotal role of these residues in the disease. Residues at 1183, 1185 and 1189 (glutamic acid, valine and proline, respectively) were found to be important but not critical in that alanine substitution of these residues led to a low but detectable uveitogenic response. Substitutions of the other amino acids were found to be relatively innocuous in that the uveitogenic activities of the alanine-substituted peptides were not markedly altered. Thus, a fairly good map of the uveitogenic site on the IRBP molecule is available.

Interestingly, Kotake *et al.* (1992) have recently found that there are two completely different antigenic sites within the 1182 – 1191 active peptide fragment. The first site is delineated by the entire sequence while the second site becomes detectable only when tryptophan at 1182 is removed. Importantly, responses at both sites are restricted to the same major histocompatibility complex product (MHC) (I-A) and not to two different products. It thus may be that peptides for the two sites can change configuration and interact differently with the MHC molecule on antigen-presenting cells (APC) to produce the distinct antigenicities. Complex as they are, these data should be valuable in ultimately allowing for a molecular biological and peptide approach to the control of uveitis. The specificity of the IRBP peptide causing uveitis and its interaction with the MHC and APC may make it possible to find a method of peptide-mediated immunotherapy. In autoimmune encephalomyelitis, for example, peptide analogs of areas on the myelin basic protein inhibit the induction of disease in mice by the native peptide (Wraith *et al.*, 1989). Thus, modulation of the ocular immune response through alterations of IRBP peptides, molecular biological intervention directly at the MHC/APC sites, etc. may ultimately make it possible to control the different human forms of uveitis.

TABLE 3. *Immunopathogenic Properties of the Alanine-Substituted Analogs**

Peptide injected	Dose (nmol/rat)	EAU incidence	Onset day (mean)	Severity (mean)
1181 – 1191	200	4/4	10.0	1.5
	20	4/4	10.5	1.8
	2	3/3	11.0	1.7
	0.2	3/3	13.7	1.3
A(1182)	200	0/4		
A(1183)	200	4/4	11.5	1.0
	20	0/4		
A(1184)	200	4/4	9.5	1.8
	20	4/4	10.8	1.3
	2	3/3	11.0	1.7
	0.2	2/3	16.0	1.0
A(1185)	200	4/4	12.0	1.0
	20	0/4		
A(1186)	200	4/4	10.5	1.0
	20	4/4	11.5	1.0
	2	2/3	12.0	1.0
	0.2	2/3	15.5	1.0
A(1187)	200	4/4	10.8	1.0
	20	3/4	13.3	1.0
	2	0/3		
A(1188)	200	4/4	10.3	1.0
	20	4/4	12.0	1.0
	2	3/3	12.7	1.3
	0.2	1/3	16.0	1.0
A(1189)	200	4/4	11.8	1.0
	20	1/4	14.0	1.0
A(1190)	200	1/4	15.0	1.0
	20	0/4		

*Lewis rats were injected with the indicated dose of peptide. Peptide 1181 – 1191 is the natural uveitogenic peptide. Peptides A(1182) through A(1190) have alanine substitutions as given in Table 1. (Taken from Kotake *et al.*, 1991.)

5.4. Genetic Considerations in Uveitis and EAU

Even though the various uveitic conditions are not inherited in a direct Mendelian fashion, there does appear to be a genetic component in phenotypic expression of the disease (Rose *et al.*, 1978). In the human, for example, Nussenblatt *et al.* (1982) found an association between birdshot retinochoriodopathy, expression of the HLA-A29 antigen and immune responsiveness to S-antigen. In rats, it is well known that there are strain differences in susceptibility to uveitis. It is the Lewis rat strain that seems to be particularly susceptible to the induction of uveitis and is usually used as a test animal in this regard (Gery *et al.*, 1986a). Table 4 (see Fox *et al.*, 1987) shows that various rat strains immunized with IRBP or S-antigen fall into three general categories: (1) high responders (Lewis, Buffalo, F344 and ACI) with the Lewis rat most susceptible, (2) intermediate and variable responders (BN) where there is a broad range of severity depending on the antigen and (3) non-responders to IRBP (WF, RCS-rdy$^+$) even though rats in these strains are susceptible to S-Ag-induced EAU. Differences in IRBP-susceptibility between the Lewis rat and the WF and RCS strains are particularly striking. At least in the WF strain, its poor responsiveness may be due to genetically-regulated differences in their cellular immune responses.

As also seen in Table 4, the susceptibility to uveitis in the different rat strains is generally parallel to susceptibility to experimental allergic encephalomyelitis (EAE) induced by myelin-basic protein (MBP). Again, these similarities may be due to genetically-regulated responses of the animal's immune systems to the particular protein epitopes or to other genetically-determined factors

TABLE 4. *Susceptibility of Rats of Different Strains to EAU Induced by IRBP or S-AG and to EAE**

Rat strain	IRBP – EAU Diseased/ total	Onset day	Severity†	S – Ag – EAU Diseased/ total	Onset day	Severity	EAE Diseased/ total	Onset day	Severity
Lewis	7/7	8.9	2.4	16/16	10.0	3.5	5/5	8.9	4.0
Buffalo	3/3	10.7	1.7	5/5	11.6	4.0	4/5	11.5	2.0
F344	4/5	10.5	1.5	5/5	12.6	3.2	5/5	10.2	4.0
ACI	5.5	12.0	1.0	5/5	11.0	3.0	4/5	11.0	2.8
BN	8/8	9.1	2.4	18/24	14.7	3.3	1/4	14	2
WF	2/12	—	0.5	9/9	16.3	3.3	9/10	12.1	2.0
RCS-rdy†	0/6	—	—	6/6	17.4	2.8	4/4	16.0	1.5

*Rats were immunized with IRBP, S – Ag or myelin basic protein (EAE) at 40 μg/rat.

†Severity was determined on a scale from 0 to 4. In WF rats, IRBP-EAU was detected by histological sectioning. (Data are adapted from Fox *et al.*, 1987.)

such as numbers of choroidal mast cells (Mochizuki *et al.*, 1984). The Lewis rat is also susceptible to a severe form of arthritis that is induced by a streptococcal wall antigen (Sternberg *et al.*, 1989). Recent work on this animal model indicates that the high immunosusceptibility probably involves a generalized problem in biosynthesis/secretion of corticotrophin-releasing hormone (CRH) in hypothalamic CRH neurons (Calogero *et al.*, 1992). These investigators hypothesize that the defect may involve 'alteration(s) in transduction mechanisms' in CRH neurons or to direct genetic problems with 'the regulatory region of the CRH gene' in these rats. It would be very interesting to determine if this or a similar genetic susceptibility were also the basis of IRBP- or S-Ag-provoked EAU. If so, molecular biological techniques could then be used for disease prevention and/or intervention.

Finally, Caspi and her coworkers (Caspi *et al.*, 1992) have studied the genetic component(s) that define the susceptibility of mice to EAU induced by IRBP. Figure 12 shows the uveitic responses of mice with most of the known independent H-2 haplotypes. Ocular pathology was seen only in those strains exhibiting the H-2^k, H-2^r, H-2^b and H-2^d haplotypes and could further be mapped to the I-A subregion of H-2^k. Interestingly, Caspi and her coworkers found that expression of the I-E^k gene product had a marked ameliorating effect on the disease process, reducing EAU induction in the I-E-positive strains. The situation is not simple, though, since the severity and even the incidence of the disease differs in strains exhibiting the H-2 haplotype when they are on different genetic backgrounds. The expression of EAU in these animals is therefore not only dependent on the I-A subregion of H-2 but also partly determined by non-MHC genes. Thus, susceptibility to EAU and other autoimmune diseases is multigenic and intervention could be envisioned at any of a number of yet-to-be-determined gene loci.

6. VIRAL-INDUCED RETINAL DEGENERATIONS

6.1. General Considerations

As pointed out in Section 1, many of the forms of retinal degeneration are 'sporadic' and cannot be ascribed to a specific inherited cause. It is now thought that at least some of these are of viral etiology. To date, the best characterized of these entities is Acute Retinal Necrosis (ARN), a relatively rapid form of retinal degeneration. In man, ARN was first described by Urayama *et al.* (1971) and is now known to be a blinding syndrome involving retinitis, arteritis and retinal detachment. Although it usually presents little or no other systemic manifestations (Gartry *et al.*, 1991), it is increasingly being recognized in patients with Acquired Immunodeficiency Syndrome (Forster *et al.*, 1990). A mild form has also been described in which many of the rapid, devastating effects on the retina are not seen (Matsuo *et al.*, 1988).

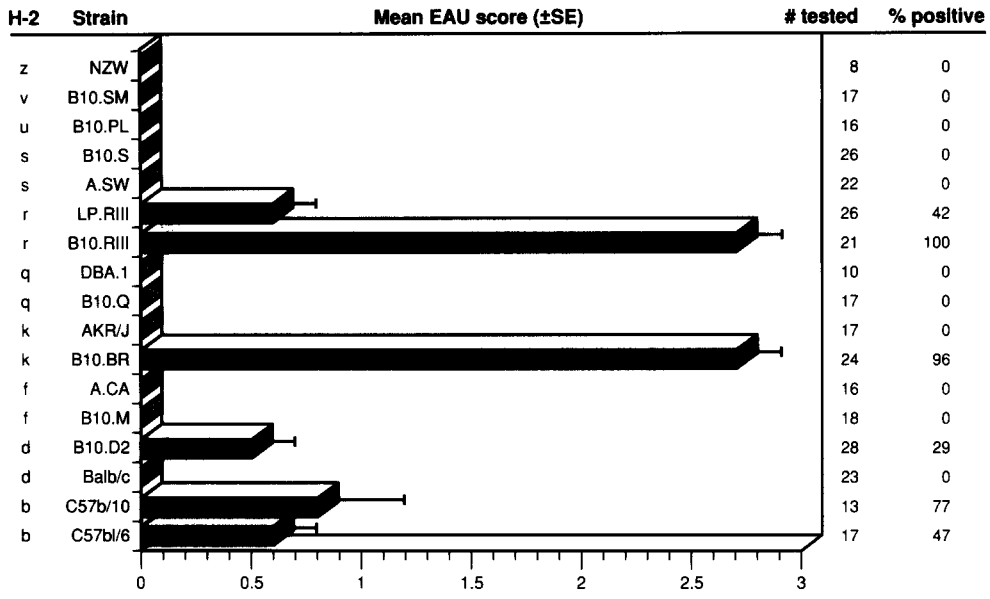

FIG. 12. EAU induced by IRBP in mice carrying different H-2 haplotypes. Taken from Caspi *et al.* (1992).

6.2. Viral Involvement in Retinal Degeneration

More than a decade after its first description in the literature, Culbertson *et al.* (1982) implicated the herpes grouping of viruses in the etiology of ARN. It is now known to be mainly associated with reactivation of latent herpes simplex (HSV) or varicella-zoster virus, although Culbertson *et al.* (1991) have shown a mild form of ARN to be associated with primary infections of varicella-zoster (chickenpox). Importantly, Holland *et al.* (1989) have demonstrated a link between ARN and HLA-DQw7 in Caucasians; Ichikawa *et al.* (1989) have similarly shown an association with Aw33, B44 and DRw6 in a Japanese grouping of patients. Matsuo and Matsuo (1991) have found HLA-DR9 to be high in patients with fulminating ARN compared with patients with a milder form of the disease. Thus, as with the various uveitic conditions outlined in Section 5, it is probable that immunogenetic predisposition plays a role in ARN.

Many viral infections, however, are of slow time course and, importantly, the clinical signs manifest well after the acute viral infection subsides. In this way, the diseases mimic hereditary degenerative conditions since the putative viral causes become cryptic before the overt signs of the disease become apparent. Several diseases of the CNS are well known to have this type of course including kuru and Creutzfeld – Jacob syndrome in humans and scrapie in sheep. Ocular models of some of these diseases have been developed in animals. These include Creutzfeld – Jacob disease (CJD) (Hogan *et al.*, 1983) and scrapie (Buyukmihci *et al.*, 1980). The CJD model in mice is particularly interesting since in many ways it mimics some of the aspects of human Retinitis Pigmentosa with a progressive retinal degeneration observed after a long 'incubation' period. In both of these entities, there is a marked and preferential degeneration of the photoreceptor inner and outer segments while the inner nuclear and pigment epithelial layers remain normal in appearance. Hogan and his coworkers also comment that the morphological damage induced by the CJD agent is similar, in some respects, to that in retinitis punctata albicans and senile macular degeneration. Nothing is known, however, as to the biochemical or molecular

TABLE 5. *Viral Detection, Pathology and IRBP Levels after Coronavirus Infection*

Day	Detection of viral antigen*	Infectious virus	Ocular pathology	IRBP (% of control)
0	0/5	0/5	0/1	100
3	2/2	3/3	1/4	57
6	2/3	3/3	3/4	61
7–11	1/3	0/3	1/2	ND
21	0/2	ND	2/3	ND
23	ND	ND	ND	46
84–98	ND	ND	4/4	ND

*Values for detection of viral antigen, infectious virus and pathology are number of positive eyes/total eyes examined. IRBP levels are given a % of control at time of innoculation (day 0).

ND = not determined. Adapted from data in Robbins *et al.* (1991, 1992).

biological pathogenesis of the CJD-induced retinal degeneration.

Animal models also have been developed for studying ARN, although as in animals with IRBP- or S-antigen-induced EAU, all of the disease manifestations in animal and man are not the same. These models do, however, underscore that severe retinal degeneration can be caused by viruses under several different conditions. Seventy years ago, von Szily (1924) described a retinitis in rabbits induced by herpes simplex virus. Whittum *et al.* (1984) have injected HSV into the anterior chambers of mice with resultant inflammation and degeneration of the contralateral retina but, oddly, sparing the ipsilateral retina. Foster *et al.* (1991) have studied the genetics of viral-induced retinal degeneration in the mouse and, not surprisingly, have found a distinct genetic component where certain strains of mice are susceptible to contralateral retinitis (e.g. BALB/c) and others are resistant (e.g. CB17). They indicate that the uveitis produced is controlled by gene products from the Igh-1 locus on chromosome 12 and that CD8 and CD4 lymphocytes play a role in protecting the contralateral retina from damage.

6.3. Murine Coronavirus Model of Retinal Degeneration

Hooks and his collaborators (Robbins *et al.*, 1990) have established a new mouse model for retinal degeneration using murine coronavirus as the infecting agent. In contrast to previous work, however, they have also begun to examine the morphological and biochemical sequelae of the infection to begin to sort out the mechanism by which damage actually occurs and cell death is effected (Robbins *et al.*, 1991, 1992). In this model, a mild anterior uveitis is observed 3–6 days after infection; focal retinal lesions, mainly in the photoreceptor layer, appear at this time. By day 10 postinjection, much of the retina is involved with some degenerating photoreceptor nuclei apparent. By day 21, widespread retinal atrophy is observed although the anterior chamber appears to be normal. Of interest is that the infectious virus cannot be detected in retinas of animals after about day 10 postinjection (Table 5). Figure 13 shows the pattern of immunoperoxidase staining in the retina of virus nucleocapsid protein as detected by a specific monoclonal antibody. At day 3 postinjection (Fig. 13c), virus is mainly visualized in strands of Müller fibers and their adjacent photoreceptor cells, although some ganglion and RPE cell involvement is observed. By day 6 (Fig. 13d), the pattern is yet focal although more extensive staining of Müller, photoreceptor and ganglion cells is apparent. Concomitantly, there is an early effect on the concentration and distribution pattern of IRBP. Table 5 shows that, even at 3 days postinjection, retinal IRBP levels are significantly decreased. This effect persists at least through day 23. Unexpectedly, staining of retinas using a specific IRBP antibody showed that the viral infection resulted in not only a decrease in IRBP

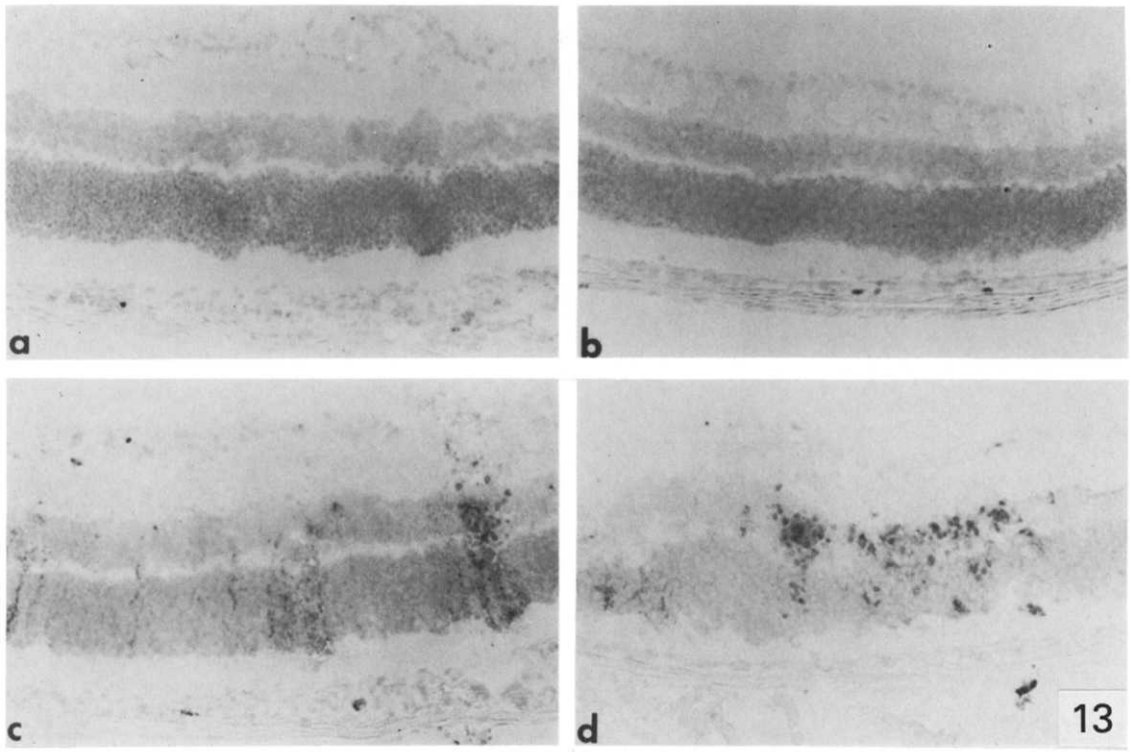

FIG. 13. Pattern of distribution of nucleocapsid antigen of coronavirus in retinas of virus-injected eyes as detected by immunoperoxidase staining. (a) Control retina harvested 3 days after injection with virus and incubated with normal mouse IgG. (b) Control retina harvested 6 days after mock injected and incubated with nucleocapsid antibody. (c) Retina harvested 3 days after injection of virus and stained with nucleocapsid antibody. (d) Retina harvested on day 6 after injection of virus and stained with nucleocapsid antibody (50×). Taken from Robbins *et al.* (1992).

concentration but a major redistribution of the location of IRBP within the retina. By day 6 postinjection, IRBP, which is normally restricted to the interphotoreceptor matrix, diffuses into the retina in patches (Fig. 14b) that closely correspond to areas that are positive for the viral nucleocapsid protein (Fig. 14d). This seems to indicate that an important result of viral infection is disruption of the outer limiting membrane, i.e. the tight junctions between Müller and photoreceptor cells. One would assume that there would be a subsequent redistribution of many IPM components along the Muller fibers along with the intraretinal diffusion of IRBP. A loss of IRBP in particular from its normal position between photoreceptor outer segments and RPE cells could lead to a block in retinoid transit between the two compartments and a buildup of toxic amounts of retinoid at the level of the photoreceptor cell. Certainly, other factors such as disruption of nutrient flow, etc. would also be expected to play secondary yet important roles in the retinal degenerative process as well.

Along with the evidence showing the first biochemical changes induced by viral infection of the retina, the work of Hooks and his collaborators is important since it demonstrates the rapidity with which the virus becomes cryptic after the initial infection. Within two weeks, no sign of the infectious virus or viral antigen can be detected and, if one examines the animals several weeks later, it is not obvious that the retinal lesions are due to viral infection. It should be remembered that several diseases such as scrapie and Creutzfeld – Jacob disease were originally thought to be genetic in nature. The disappearance of virus as in the coronavirus model (Robbins *et al.*, 1992), the long incubation time observed in the Creutzfeldt – Jacob model (Hogan *et al.*, 1983) as well as in human kuru and Creutzfeldt – Jacob

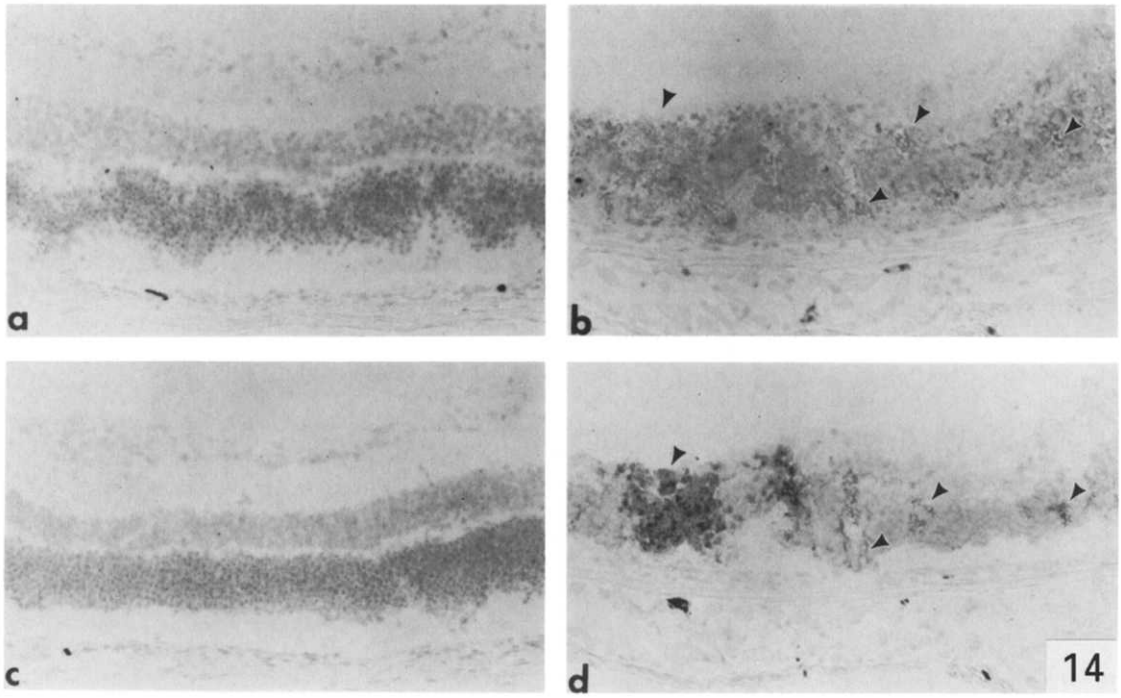

FIG. 14. Pattern of distribution of IRBP and viral coronavirus nucleocapsid staining 6 days after virus injection. (a) Control retina incubated with goat IgG. (b) Retina incubated with IRBP antibody showing patches of IRBP diffused into the retina. (c) Control retina incubated with biotinylated normal mouse IgG. (d) Retina incubated with nucleocapsid antibody showing patches that correspond to the areas of IRBP diffusion. Arrowheads in panels (B) and (D) point to corresponding areas of the retina (50×). Taken from Robbins *et al.* (1992).

disease all are temporally similar to a true inherited condition. Could some forms of 'hereditary' RP in the human result from early virus infection? Techniques of modern molecular biology now allow for testing for the presence of virus in very small tissue samples. Amplification by Polymerase Chain Reaction (PCR), for example, has recently been used to positively identify varicella-zoster infection in small intraocular samples from patients with Acute Retinal Necrosis (Nishi *et al.* 1992). It thus will be possible to test samples of retina in young 'RP' patients if they become available to see if viral 'footprints' can be detected.

7. TOWARDS AN UNDERSTANDING OF PHOTORECEPTOR CELL DEATH

It is clear that many of the mutations causing hereditary retinal degeneration in the human and in animal models are now being uncovered and, within a few years, the genetic causes of many of these conditions should be understood. Similarly, uveitis/EAU and the involvement of viruses in retinal degenerations should be more fully defined. We can now begin to think beyond just determining the molecular lesions of the different retinal degenerations and to think about their control or reversal. For example, knowing the molecular lesion in ornithine amino transferase in gyrate atrophy, Kaiser-Kupfer *et al.* (1980) have taken a direct approach in trying to control systemic ornithine levels in their patients. They have shown that restriction of arginine in the diet can lead to lowered plasma ornithine and a marked improvement in visual function as seen in improvements in the electroretinogram, dark-adaptation and color vision. In uveitis, induction of 'tolerance' by feeding or injection of specific peptides deduced from molecular biological data

may be successful in controlling ocular autoimmune diseases. Likewise, advances in antiviral agents may ultimately lead to the control of ARN and similar virally-caused retinal degenerations. With the plethora of causes of the different forms of RP as seen to date, however, it may be difficult to address these entities by specific gene therapy in each individual case. One might alternatively ask: Is there a more 'generic' way to intervene in these diseases? As an approach to this, the corollary question could be: Is there a common mechanism of cell death and is there a way to, at least, halt or delay this process, until adequate forms of gene therapy or transplantation become available?

What actually kills the photoreceptor or other retinal cell in RP or other retinal degeneration? At least in ADRP, it has been postulated that defects in opsin could lead to an inability of the protein to be transported to the photoreceptor outer segment and be incorporated into the nascent discs (Dryja *et al.*, 1991). This would lead to a build-up of defective opsin in the inner segments, disruption of normal metabolic function with the possibility of the cell, as such, suffocating in its own garbage. In this regard, inhibition of glycosylation, as might be mimicked by a mutation at the asparagine carbohydrate-binding site in opsin, has been shown to lead to a photoreceptor degenerative condition due to the inability of the cell to form normal outer segments (Fliesler *et al.*, 1984, 1985). At present, though, it is difficult to see how many of the other mutations, that seem almost innocuous, can all lead to cell death. Future studies on the correlation of clinical findings (e.g. age of onset, severity, etc.) with particular mutations should shed light on this question.

At present though, one attractive possibility to consider, is that a number of different types of cellular insult all may result in the initiation of programmed cell death or 'apoptosis' in the retinal photoreceptor cell. Apoptosis (Fesus *et al.*, 1991) is a process of cell suicide in many cell types which is characterized morphologically by chromatin condensation, DNA fragmentation amd the dispersion of the dying cell into small cytoplasmic bodies (apoptotic bodies; Kerr *et al.*, 1972). Mechanistically, a series of genes such as transglutaminase and TRPM-2 (also known as clusterin) are activated. Although the roles of these gene products are poorly understood, their appearance is closely linked to cell dissolution.

Apoptosis is extremely important in normal development of many tissues when an overabundance of cells needs to be quickly removed (Schwartz, 1991). Apoptosis has recently been reported in chick embryo retina at the time of marked ganglion cell death (Ilschner and Waring, 1992). It is also operative in remodeling of hormone-dependent tissues such as the prostate and mammary gland where active cell death is induced by hormone ablation (Tenniswood *et al.*, 1992) and in the immune system where there is a particularly high cell turnover rate even under normal conditions (Cohen, 1991). Recent evidence indicates that a large number of insults such as drugs, radiation and toxins as well as the presence or absence of certain hormones, neurotransmitters, etc. can initiate apoptosis. Glutamate toxicity, for example, induces apoptotic DNA cleavage in cultured neurons (Kure *et al.*, 1991). At least in early development, retinal cells exhibit apoptosis as noted above; it is thus possible that RP, uveitis, viruses, etc. afford an insult to the retina that activates the latent 'death program' in the adult. This indeed may be the case in RP since Jones *et al.* (1992) have examined gene expression in normal human retinas and retinas from RP patients and have found a marked increase in expression of the clusterin (TRPM-2) gene in diseased retinas. Although only a small number of retinal specimens were examined and no tissue from younger patients was available, this study does indicate that genes intimately linked to apoptosis can be activated in adult retinas and that RP increases the expression of at least one of the putative 'death genes'.

In general then, apoptosis may be a mechanism for cell regulation and/or removal in many tissues, including the retina, both in normal development and in terminal disease processes. Intervention in this program through activation/repression of specific genes involved in the apoptotic cascade may therefore offer a unified mechanism by which a large variety of degenerative insults can be controlled. This possibility has become more appealing since specific oncogenes (e.g. *myc*, Evan *et al.*, 1991) and tumor-suppressor genes (e.g. *p53*, Yonish-

Rousch *et al.*, 1991) have been found to activate apoptosis while activation of other genes (e.g. *bcl-2* oncogene) has been shown to block the death program. Interestingly, Yonish-Rouarch *et al.* (1991) have found that interleukin-6 inhibits the p53-induced apoptosis of myeloid leukemic cells. Because growth factors are intimately involved in the regulation of many of these genes and because the retina – PE complex seems to be a rich source as well as a target for a number of growth factors, cellular as well as molecular biological intervention may be practical in at least slowing retinal cell death.

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