GENERATION OF MONOCLONAL ANTIBODIES TO CELLS OF THE GOLDFISH (*CARASSIUS AURATUS*) RETINA

By

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Dean of the Graduate College

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NOMENCLATURE

GL	ganglionic layer
IN	inner nuclear layer
IP	inner plexiform layer
IS	inner segment
Kd	kilodalton
mAb	monoclonal antibody
mOsm	milli-osmolar
OM	outer limiting membrane
ON	outer nuclear layer
OP	outer plexiform layer
OS	outer segment
rmT	room temperature
ROS	rod outer segment
sp.g.	specific gravity

CHAPTER I

INTRODUCTION

Vision begins when a photon is caught by a photopigment, or visual pigment, of a photoreceptor cell. The absorption of photons bleaches photopigments, which refers to a conformational change in the photopigments (Wald, 1968). Subsequently, this change triggers a series of biochemical and enzymatic reactions which ultimately cause the excitation of the photoreceptor membrane and produces a neural signal (Penn and Hagins, 1969; Stavenga and DeGrip, 1985).

Photopigments are integral membrane proteins. They consist of a chromophore which is derived from vitamin A and an opsin which is an apoprotein of 35-40 K dalton (Kd). Spectrophotometric studies of retinas and individual receptors suggest that there are mainly four different photopigments distributed among the photoreceptors of humans and some vertebrates (i.e., nonhuman primates and many species of fish, that enjoy color vision (Jacobs, 1983; Bowmaker, 1983; Marks, 1965) Rhodopsin, the visual pigment of the rod-shaped photoreceptor is the most abundant photopigment of the retina in most species. It mediates vision in dim light. The other three pigments, that reside

in cone-shaped photoreceptors and mediate color vision, are referred to as cone photopigments. According to their maximum absorption spectrum, cones can be classified into three different subclasses, blue, green, and red cones.

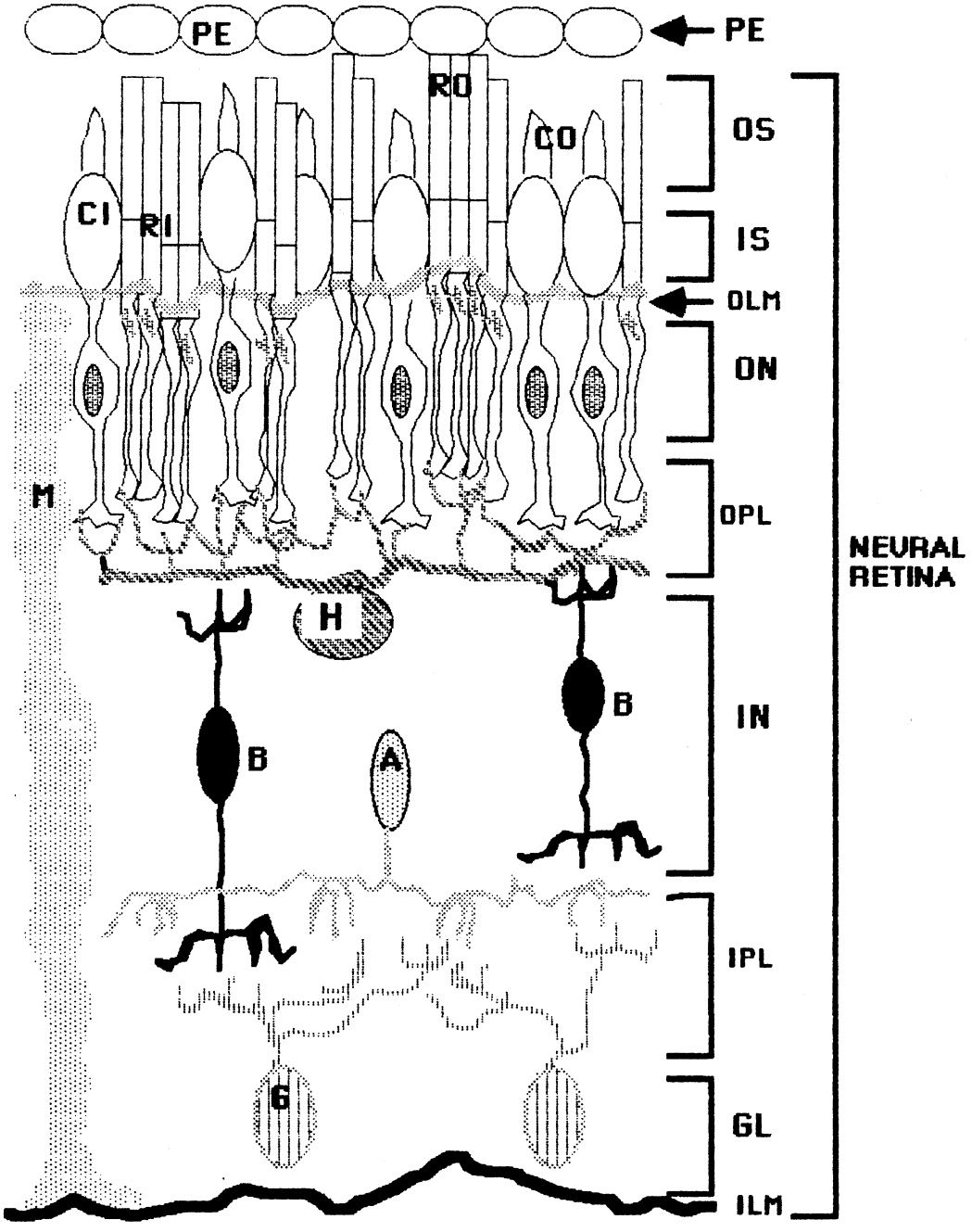
Nature of Vision

Retina

The retina is part of the central nervous system. It is composed of the pigmented epithelium and the neural retina (Fig.1). The pigmented epithelium, which is immediately beneath the choroid of the eye, is a densely pigmented layer. With its light-absorbing pigment granules containing melanin, it absorbs nearly all the light that escapes absorption by photoreceptors (Fein and Szuts,1982). Also, it mediates the delivery of oxygen and nutrients from the choroidal circulation and plays an active role in the continuous turnover of the photoreceptor outer segment (Fein and Szuts, 1982).

The neural retina is a laminated structure composed of several cell layers separated by zones where these cells interact synaptically (Jacobs, 1981). The layers of the retina traditionally have been named with the inner layers referring to those toward the vitreous body, and the outer layers referring to those toward the sclera, the outer coating of the eye (Dunn, 1973). From the outer layer to the inner layer, these structures are as follows:(1) layer

Figure 1. Schematic Drawing of a Generalized Vertebrate Retina. A, amacrine cell; B, bipolar cell; CI, cone inner segment; CO, cone outer segment; G, ganglion cell; GL, ganglion layer; H, horizontal cell; ILM, inner limiting membrane; IN, inner nuclear layer; IPL, inner plexiform layer; IS, inner segment layer; M, Muller cells; OLM, outer limiting membrane; ON, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment layer; PE, pigmented epithelium; RI, rod inner segment; RO, rod outer segment. (Modified from Dowling, 1970).


PE
PE
RO
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RI
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OLM
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M
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NEURAL
RETINA
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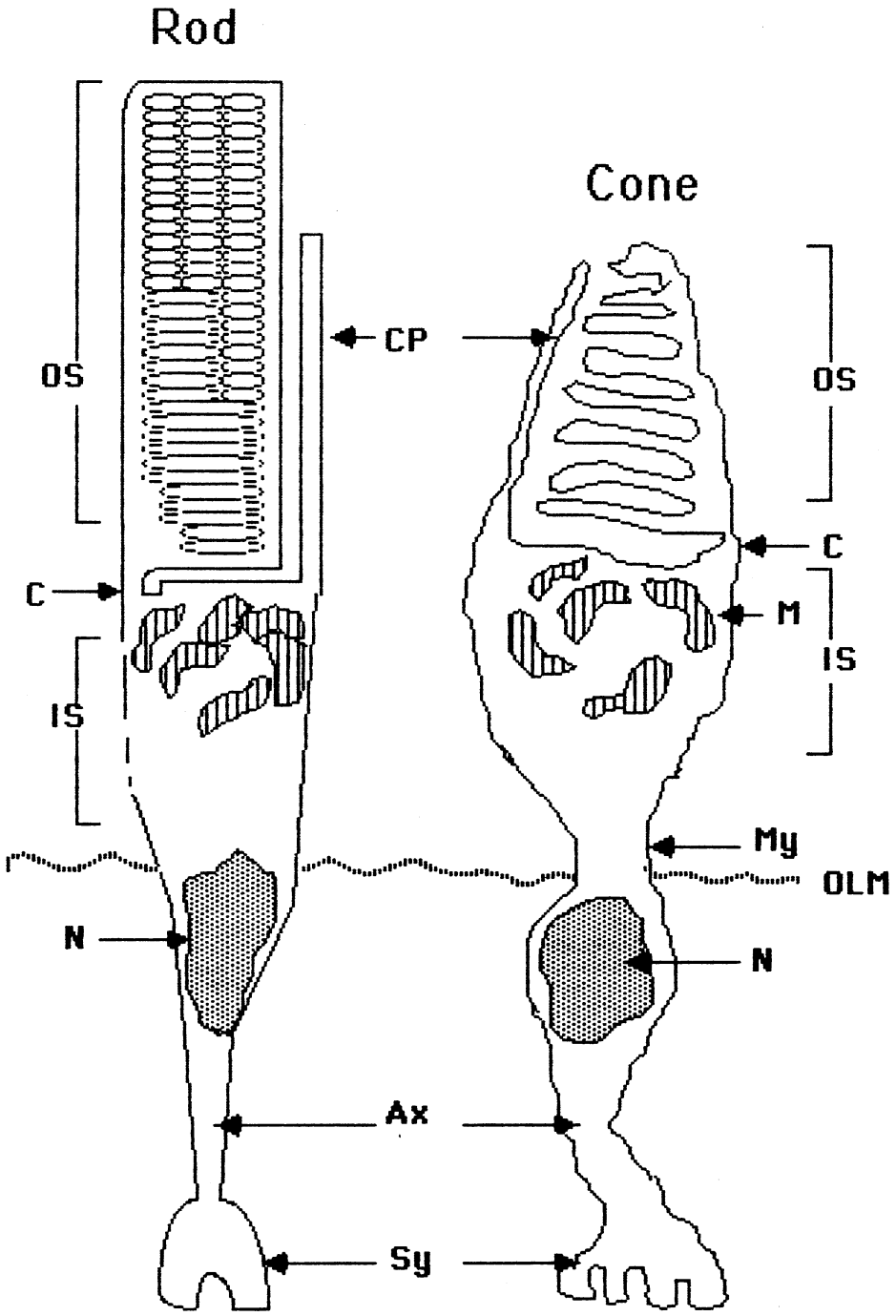
of rods and cones, (2) outer limiting membrane, (3) outer nuclear layer, (4) outer plexiform layer, (5) inner nuclear layer, (6) inner plexiform layer, (7) ganglionic layer, (8) layer of optic nerve fibers, and (9) inner limiting membrane (Guyton, 1976). The layer of rods and cones are the distal processes of photoreceptors, which are abutting into the pigmented epithelium. These cells will be discussed in greater detail later. Rather than a true membrane, the outer limiting membrane (OM) is a region of tight junctions between receptors and Muller cells which are not neurons but cells contributing to the framework of a retina. The cell bodies of the photoreceptors are arranged as a layer called the outer nuclear layer (ON). The outer plexiform layer (OP) is a dense synaptic layer where the processes of the photoreceptors, bipolar cells and horizontal cells all interact. The cell bodies of bipolar cells and other neurons are located in a zone called the inner nuclear layer (IN). The inner plexiform layer (IP) is a second zone of complex synaptic interactions, in this case between bipolar cells, ganglion cells and amacrine cells. The cell bodies located closest to the vitreal surface belong to the ganglion cells, which is called the ganglionic layer (GL). Their axonal processes constitute the optic nerve fibers which pass over the retinal surface and are gathered together at the optic nerve (Jacobs, 1981).

Photoreceptors

Vertebrate photoreceptors can be classified as either rods or cones (Fig.2). Both of them are composed of a membrane-infolded outer segment (OS), an inner segment (IS) which connects to the outer segment through a slender ciliary bridge (Sjostrand, 1953), and a nucleus-containing cell body with a synaptic terminal (Fein and Szuts, 1982; Dunn, 1973). The outer segment is a highly specialized region composed of laminated membrane infolding in an orientation perpendicular to the longitudinal axis of cells. The membrane, occupying about 50% of the outer segment total volume, is where the photopigments reside (Fein and Szuts, 1982). From the distal to the proximal end, the inner segment consists of 3 regions: ellipsoid, paraboloid, and myoid. They are characterized by special organelles and inclusions in their regions of cytoplasm (Sjostrand, 1953; Dunn, 1966) . The synaptic terminal is the site of information transfer to the higher-order retinal neurons.

The classical distinction made between rods and cones was based on observations with the light microscope, which were initiated by Max Schultze, an early nineteenth-centry biologist (Underwood, 1968). Basically the rod is cylindrical with the inner and outer segment having roughly the same diameter, whereas the cone outer segment tapers and has an inner segment of greater diameter than the outer segment (Jacobs, 1981). Nevertheless, the morphological

Figure 2. Rod and Cone Structure. Ax. axon; C, cillary connection; CP, calycal process; IS, inner segment; M, mitochondria; My, myoid; N, nucleus; OLM, outer limiting membrane; OS, outer segment; Sy, synaptic terminal. (After Nilsson, 1964.)


Rod
Cone
OS
CP
C
IS
OS
C
M
IS
My
OLM
N
N
Ax
Sy

characteristics of cones described above is not so clear in some species or in some regions of the retina (Underwood, 1968). Currently, the structure of photoreceptor outer segments provides perhaps the best single criterion for separating rods from cones. As seen in the electron microscope, the rod outer segment appears as a stack of membrane-limited discs, which are isolated from one another and from the cell membrane. On the other hand, the membrane discs of cones are typically continuous with the outer cell membrane. Because of the difference in disc construction, the cone outer segment, is open to extracellular space whereas the rod outer segment discs are not (Jacobs, 1981).

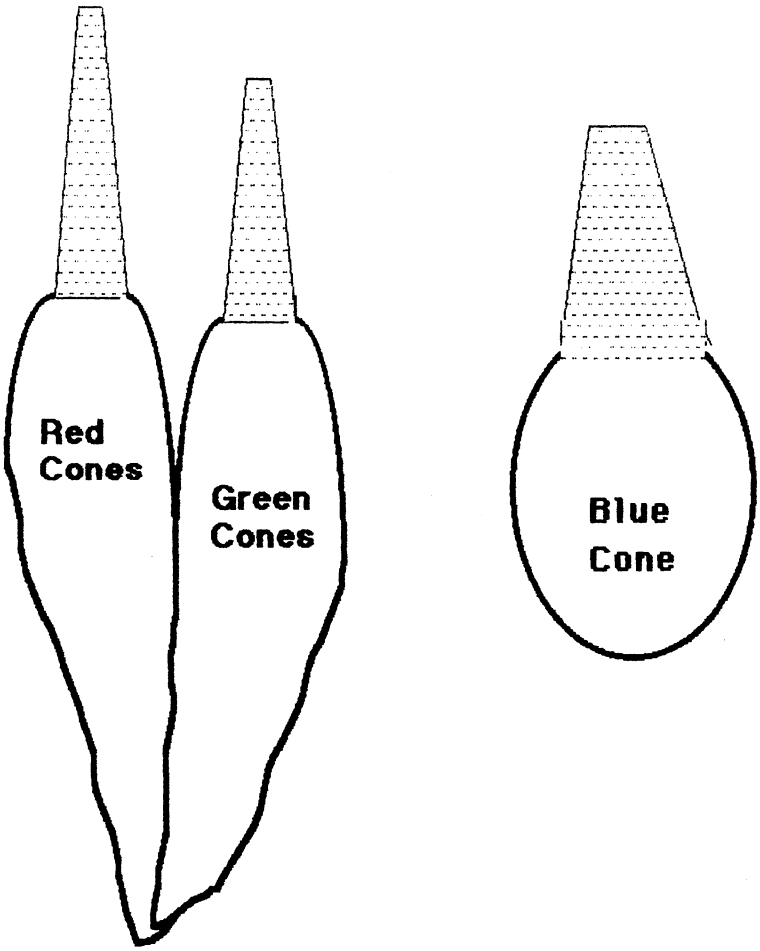
According to microspectrophotometric measurements, which can be used to measure the absorbance spectrum of the photopigments in an single cell, there are cone subgroups existing in retinas of those species which enjoy color vision (Jacobs, 1983; Harosi and MacNichol, 1974; Liebman and Granda,1971; Bowmaker, 1983). One of the most extensive studies of color vision has been made on the common goldfish (Jacobs, 1983; Stell and Harosi, 1976; Marks, 1965; Marc and Sperling, 1976; Harosi and MacNichol, 1974; Harosi, 1976). One type of rod and three types of cones exist in the goldfish retina. There are rods which give a spectrum with maximum absorbance at 522 nm, short-wave cones with maximum absorbance at 455$\pm$5 nm (so-called blue cones), middle-wave cones with maximum absorbance at 530$\pm$5 nm (so-called green cones), and long-wave cones with maximum absorbance at

625±5 nm (so-called red cones) (Marks, 1965; Harosi, 1976; Harosi and MacNichol, 1974). It has been shown that each goldfish cone contains one of three color specific photopigments (Tomita et al., 1967). Using microspectrophotometric measurements and a reduction assay of nitro-blue tetrazolium chloride, accompanied by microscopic identification, it was revealed that a strong correlation exists between structure (cone type) and function (visual pigment) in goldfish (Fig. 3; Stell and Harosi, 1976; Marc and Sperling, 1976). The blue cones appear as a short-fat single cone (SS) which has a short, broad OS and ellipsoids and virtually no myoid, the green cones appear as the short member of the double cone (SD), while the red cones are the long member of double cone (LD). Both green and red cones also exist as single cones and as such are indistinguishable from one another. The total chromatic composion of the goldfish retina was found to be 45% red cones, 35% green cones and 20% blue cones (Marc and Sperling, 1976).

Photopigment

Photopigments are integral membrane glycoproteins. Each photopigment molecule consists of a prosthetic group known as a chromophore and a large apoprotein known as an opsin, which are covalently bound to each other with a one to one stoichiometry (Wald, 1968). In all the species that have been studied, the chromophore is either retinal or

Figure 3. Morphology of Cone Subclasses of Goldfish Retina.

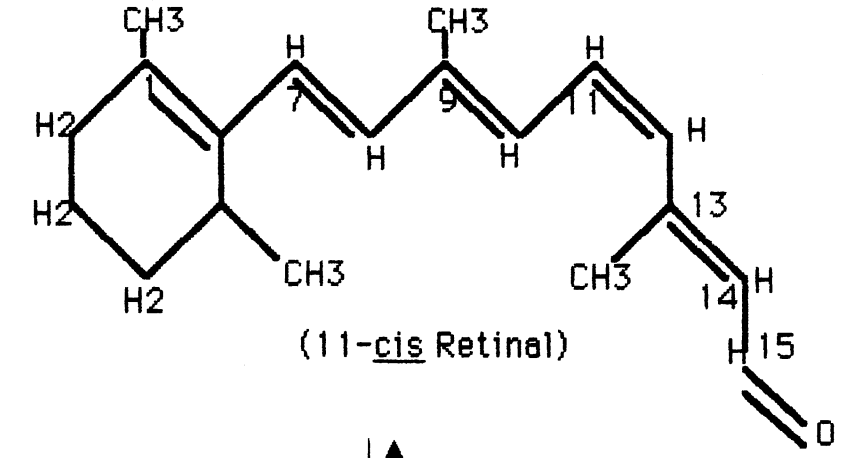

Red
Cones
Green
Cones
Blue
Cone

3-dehydroretinal, whereas the opsin's structure appears to be less conserved (Fein and Szuts, 1982). Therefore, it is generally agreed that the physical, biochemical, and spectral differences among photopigments are the consequence of variation in the opsins.

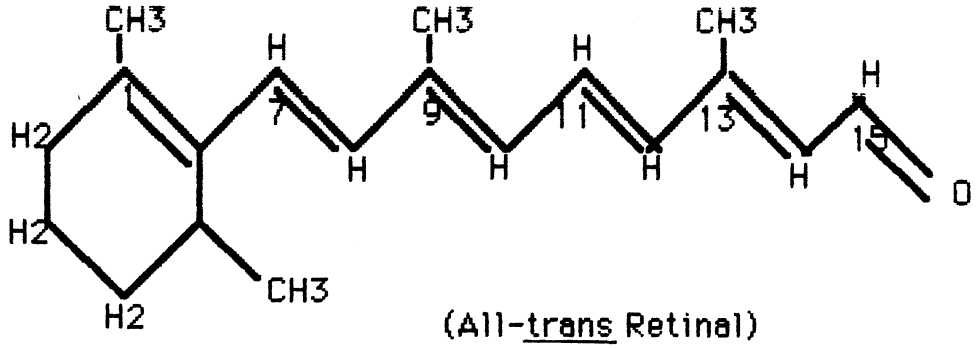
Most of the knowledge about photopigments today has been obtained from biochemical studies on rhodopsin, the photopigment of rods. The bovine retina is the most popular source of rhodopsin since it is of large size and, more essentially, its rhodopsin-riched rod outer segments can be easily isolated (Fein and Szuts, 1982).

Chromophore. Retinal, or 3-dehydroretinal, is an aldehyde derivative of vitamin A with a molecular weight of 285 dalton. In bovine rhodopsin, the chromophore attaches to the Lysine at position 296 of the opsin with a Schiff base linkage (Ovchinnikov, 1982). Retinal is a beta-ionone ring possessing a side chain with four carbon-to-carbon double bonds that terminates in an aldehyde group (Fein and Szuts, 1982). Each double bond of the side chain might potentially exist in either cis- or trans-configuration. All the photopigments that have been analysed possess as chromophore 11-cis retinal (Fig.4), or 11-cis dehydroretinal (Wald, 1968). When, however, a visual pigment is bleached by light, the Schiff base linkage is broken and a series of short-lived intermediates is formed. The final product of this visual cascade is the all-trans form of the chromophore

Figure 4. Structure of 11-cis Retinal and All-trans Retinal. (After Wald, 1968).



(11-cis Retinal)



(All-trans Retinal)

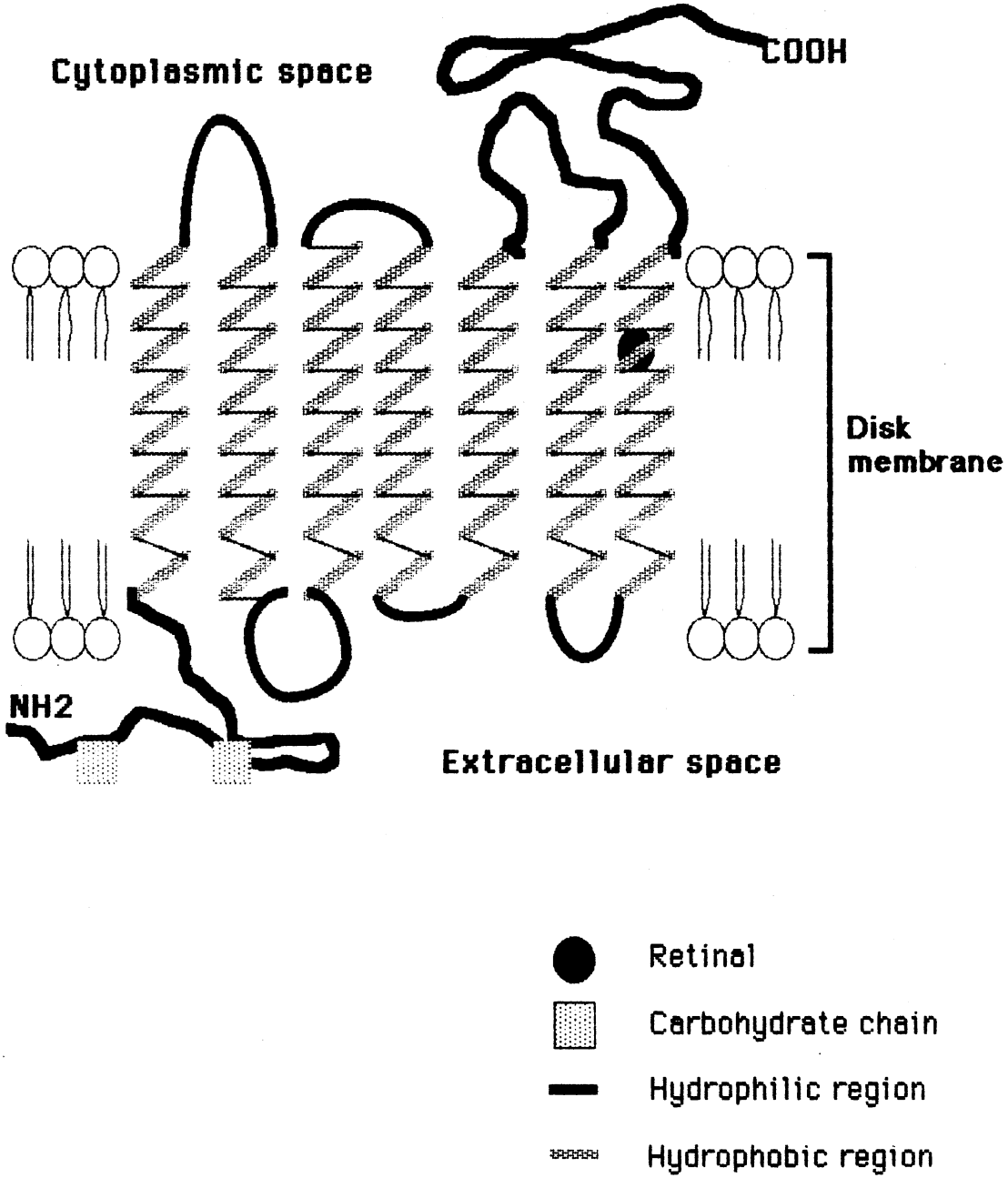
which is the most stable and prevalent form (Wald, 1968). The latter may remain in the photoreceptor outer segment and be recycled through further light-dark sequences or be reduced to retinol and removed back to the pigmented epithelium for storage (Chader, 1982). It was showed by Hubbard and Kroph (1958) that this photoisomerization of retinal is the only action of light in vision. The isomerization of the chromophore is proposed to trigger a series of conformational changes in the attached opsin which creates or unveils an enzymatic site on its cytosolic face. Through a number of enzymatic processes including the activation of phosphodiesterase (Bennett, 1982; Yoshizawa and Fukada, 1983), and the change in the cyclic GMP concentration (Farber et al., 1977), the ion transport systems of the membrane are actived. This results in the eventual hyperpolarization of the photoreceptor membrane and produces a neural signal (Jacobs, 1981). The steps between absorption and changes in membrane permeability are currently under intensive investigation (Abrahamson and Ostry, 1981; Farber, et al., 1977; Liebman and Pugh Jr., 1979; Ortez, et al., 1983).

Rhodopsin. Rhodopsin is a single polypeptide chain to which two short oligosaccharide segments are connected (Hargrave, 1977). In a rewiew article by DeGrip (1985), some current knowledge and studies of rhodopsin have been described. In purified washed rod outer segment (ROS)

membranes, rhodopsin contributes over 95% of the membrane protein content (DeGrip, 1985). The primary structure of bovine rhodopsin has been elucidated by protein analysis (Ovchinnikov, 1982 ; Hargrave,et al., 1983) and gene cloning (Nathans and Hogness, 1984). Hargrave et al. (1983) reported that bovine rhodopsin consists of a single polypeptide chain of 348 amino acids with a molecular weight (MW) of 39 Kd. The sequence shows a characteristic alternation of hydrophilic and hydrophobic stretches. These hydrophobic stretches have a high probability for alpha-helical organization (Hargrave, et al., 1983).

According to these studies a putative model for the organization of the rhodopsin polypeptide chain through the disc membrane was developed (Fig.5; Ovchinnikov, 1982, Hargrave, et al., 1983). There are seven hydrophobic alpha-helical stretches which pierce the membrane, and are interconnected by six hydrophilic stretches, which reside on the membrane-cytoplasm interface and in the intradiscal aqueous space. The largest hydrophilic stretches are present in the C- and N-terminal region of the opsin. The C-terminus is located on the cytoplasmic side of the disc and contains 25-35 amino acid residues (Hargrave and Fung, 1977). The N-terminus is located on the intradiscal side of the membrane and contains 30-35 amino acids whose properties are probably strongly influenced by the presence of two oligosaccharide units (Hargrave,1982). Such an organization of hydrophobic and hydrophilic segments in the cell membrane

Figure 5. Putative Model of Bovine Rhodopsin in Cytoplasimc Membrane. (Modified from Hargrave et al. 1980.)


Cytoplasmic space
COOH
Disk membrane
NH2
Extracellular space
Retinal
Carbohydrate chain
Hydrophilic region
Hydrophobic region

gives the rhodopsin structure a typical amphipathic character.

Cone photopigments. In contrast to rhodopsin, our knowledge of cone photopigments is very limited since it has been impossible to obtain purified color-specific photoreceptors (cones) or their respective visual pigments. Besides the fact that the amount of color-specific photopigments is much less than that of rhodopsin, the main problem arises from the fact that the opsins are so physically/chemically similar that separation from one another by classical biochemical procedures has proven impossible (Fein and Szuts, 1982). For a long time almost all of the knowedge about color vision was based on investigations that employed either photometric techniques or electrophysiology and psychophysics to measure the in situ properties of visual pigments (Jacobs, 1983).

In 1986 Nathans et al.(1986,a.b.) described the isolation of the genes that specify the opsins of the three different color-sensitive pigments in the human eye. Their work confirmed the trichromacy theory of human color vision which was first proposed by Young in 1802 and proved that there are indeed separate genes for each of the color specific opsins (Botstein, 1986). According to the genes they cloned, Nathans et al. deduced amino acid sequences of the three color photopigments, which showed 41±1 % identity with that of rhodopsin. The red and green pigments showed

96% mutual identity but only 43% identity with the blue pigment.

Although the amino acid sequence has been revealed, extraction and purification of cone photopigments is still a big challenge to vision scientists. Further understanding of cone photopigments, for example the kinetics of bleaching and regeneration, and the special configurations which cause the unique spectra, still requires the availability of purified color photopigment.

The purpose of this study was to develop monoclonal antibodies (mAbs) to components of goldfish retina that would be useful in the purification of color-specific photoreceptors or their respective color-specific photopigments.

Hybridoma Technology And Vision Study

In 1975 Kohler and Milstein demonstrated that normal antibody-secreting cells could be immortalized by fusion with myeloma cells. The myeloma is a malignant tumour of transformed antibody-secreting cells. For the purpose of mAb production, a non-secreting myeloma cell line that is defective in hypoxanthine guanine phosphoribosyl transferase (HGPRT) is usually used. When cells are treated with Sendai virus or polyethylene glycol (PEG), their membranes fuse and multinucleate cells called heterokaryons are formed . At the next cell division, the nuclei of heterokaryons fuse, and the daugher cells possess a more or less equal share of

the genetic material of orginal cells (Goding, 1981). In a selective medium containing hypoxanthine, aminopterin and thymidine (HAT), only hybrids of lymphocytes and myelomas, called hybridomas, which possesses HGPRT inherited from the lymphocyte, servive. With proper screening and cloning procedures, the individual, or monoclonal hybridoma can be isolated and expanded. Using this technique, virtually unlimited quantities of homogenous antibodies can be produced.

The development of hybridoma technology not only solves the major problems of traditional polyclonal antiserum, namely specificity and reproducibility, but also provides a powerful tool to study antigens which are unavailable in purified form (Goding, 1980). For example, Burger et al. (1981) developed a mAb specific to guinea pig Ia antigen by immunizing mice with an Ia-positive B cell line and Molday and Mackenzie (1983) developed a mAb against the C-terminal region of rhodopsin by immunizing mice with bovine ROS disc membranes.

Monoclonal antibodies against photoreceptors and photopigment

Barnstable (1980) elicited mAbs against various retinal cell types by immunizing mice with a membrane pellet of rat retina. Seven antibodies interacting with rat retinas were characterized. Three of these antibodies, P1, P2, and P3, all more or less interacted with rod inner and outer

segments. None of them appeared to be directed against rhodopsin as tested by competitive binding with partially purified rhodopsin and immuno-electroblotting. Another three antibodies, G1, G2, and G3, all labelled Muller cells. The remaining antibody recognized an antigen on neurons.

In 1982, Mackenzie and Molday developed monoclonal antibodies by immunizing mice with illuminated purified bovine ROS membrane. Six antibodies were characterized by radioimmune labeling of ROS membrane proteins separated by sodium dodecyl sulfate (SDS) gel electrophoresis (MacKenzie and Molday, 1982; Molday and MacKenzie, 1983). Two of those, 3D12 and 4B2, recognized a large membrane protein of the photoreceptor (MW 220,000), while the other four, 1C4, 3D6, 4A2, and 4B4, recognized the opsin. Limited proteolysis experiments of ROS membranes indicated that 1C4 and 3D6 bound to antigenic sites close to the C-terminus, 4A2 bound to an antigenic site at the N-terminus, and 4B4 bound to an internal region of the cytoplasmic surface.

In 1984, Takahashi et al. developed five mAbs which bound to opsin on Western blots of frog ROS (Takahashi et al., 1984). When tested with the chicken retina these mAbs appeared to label selectively outer segments of rods but not cones. In rat, all five mAbs labeled OS with weaker staining in the IS and ON layer. Western blot analysis showed these mAbs bound to rhodopsin monomer and oligomer as well as to two bands at 80 and 85 Kd. Further experiments showed that these mAbs, developed against frog rhodopsin,

recognized antigenic determinant common to both photoreceptors and Muller cells in rats.

Lemmon (1986) developed a mAb by immunizing mice with retinas of chicken embryo. This mAb, 5O-1B11, bound to OS of cones in rhesus monkey. In vitro experiments on chicken retina indicated that the antigen was intracellular and associated with the plasma membrane. Electronmicroscopic-immunohistochemical studies demonstrated that the antigen was contained in the lamellae of the OS of rhesus cones.

It has been suggested by DeGrip (1985) that the mAb is the most useful immunological tool for the functional studies (photolytic rearrangement, interaction of activated rhodopsin with the G-protein or opsin kinase, and regeneration of rhodopsin) of photopigments since polyclonal sera have multi-site interactions.

Study objectives

To solve the problem of purification of color-specific photopigments, a long-tern project was proposed by our laboratory. We proposed to develop mAbs to be used as specific binding ligands to affinity purify photopigments from a homogenate of whole retina. As a first step in the generation of photopigment-specific antibodies, it may be necessary to obtain purified color-specific cones. To accomplish this, mAbs specific to color-specific cones could be developed and used to recover blue, green, or red cones from suspension of retinal cells by panning procedures

(Ortez, 1982). In this project the goldfish retina was used because their blue, green and red cones can be morphologically distinguished (Marc and Sperling, 1976; Marc, 1982).

To accomplish these long-tern goals three major works were undertaken in the current study. The first was to develop a procedure for purification of cone-enriched antigen without fixation. The second was to develop a reproducible, high yielding hybridoma technology in our laboratory. The third was to develop monoclonal antibodies specific to individual components of the cone-enriched antigen preparation.

CHAPTER II

MATERIALS AND METHODS

Animals

Goldfish (Carassius auratus) of 15 to 25 gm were used in this project. They were obtained from a local pet shop as 1-2 gm fish, and raised in laboratory aquaria equipped with an air supply and water filtering system until they reached a usable size. Purina catfish food was fed daily.

BALB/c mice of 8 to 12 weeks were used as recipients of the antigen and donors of spleen cells and feeder cells. They were obtained from colonies maintained either in the Jackson Laboratory (Bar Harbor, Maine) or the Oklahoma State University laboratory animal care facilities . All the mice used in this project had been adapted in the animal room of the university for at least one week before use.

Chemicals and Reagents

Buffer and water

Water used in the preparation of all buffer and media was obtained from a Branstead NANOpure system. Phosphate-buffered saline (PBS, pH 7.4) was widely used in this project. Each liter of PBS contains 154 mM NaCl, 8 mM

Na_2HPO_4, and 2.6 mM NaH_2PO_4.

Reagents for cone preparation

Each liter of Hickman's Teleost Saline (HTS, pH 7.7) (Hoar and Hickman, 1967) contained 110 mM NaCl, 2 mM KCl, 1 mM $NaHCO_3$, 5 mM NaH_2PO_4, 5 mM EGTA, and 0.25% gelatin (60 bloom, Sigma). The osmolarity of HTS was adjusted with sucrose.

The fixtive used for retina preparation was composed of 6.0 mM sodium cacodylate, 2.5% glutaraldehyde, 1.0% paraformaldehyde, and 3.0% sucrose, pH 7.4.

The solution used for siliconizing glassware was composed of 5% dimethyldichlorosilane in chloroform.

Tissue culture medium

Each liter of RPMI-1640 contained one pack of dehydrated RPMI-1640 (Gibco), 2.0 gm $NaHCO_3$, 2.5 mg Amphotericin B (Sigma), 2.05 mg deoxycholate (Sigma), 58.0 mg (1720 IU/mg) penicillin, and 135 mg (740 mg/g drug material) streptomycin.

Each liter of enriched RPMI-1640 (Enr-RPMI, pH 7.4) consisted of RPMI-1640 as described above to which was added 110.0 mg sodium pyruvate (Gibco), 292.0 mg L-glutamine (Gibco), 20 ml MEM amino acids, 50x (Gibco), 10 ml MEM non-essential amino acids, 100x (Gibco), 4.0 ml MEM vitamins (Gibco), and 1.0 ml 0.35% 2-mercaptoethanol stock solution.

Hypoxanthine thymidine (HT) medium consisted of Enr-

RPMI, to which was added 10% by volume of fetal calf serum (FCS, Gibco) and 1% by volume of an HT stock solution. The HT stock contained hypoxanthine 10 mM, and thymidine 1.6 mM and was sterilized by filtration through a membrane filter of 0.45 μm pore size.

Hypoxanthine/aminopterin/thymidine (HAT) medium consisted of Enr-RPMI/10% FCS, hypoxanthine 200 μM, aminopterin 0.8 μM and thymidine 32 μM. Aminopterin was prepared as a 0.8 mM (100x) stock solution in 0.1 N NaHCO3 and sterilized by membrane filtration. The hypoxanthine and thymidine were introduced into HAT by adding 2% by volume of the HT stock solution described above.

The cloning medium was composed of 45% Enr-RPMI, 45% conditioned medium, 10% FCS, and 2% HT stock by volume. The conditioned medium was obtained from logarithmically growing cultures of 653 myeloma cells.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Each liter of gel buffer (pH 8.9) was composed of 22 mM TRIS (Bio-Rad), 200 mM glycine, 1.0 gm SDS and 0.59 gm EDTA.

The 8% resolving gel (18ml) was made by dissolving 1.4 gm acrylamide (Bio-Rad), 43 mg Bis-acrylamide (Bio-Rad), and 36 mg ammonium sulfate (Bio-Rad) in 18 ml gel buffer. TEMED (Bio-Rad) 25 μl was added right before use.

The 3.5% stacking gel (9 ml) was composed of 300 mg acrylamide, 7 mg Bis-acrylamide, 18 mg ammonium persulfate,

1% SDS and 0.75 ml of 1.5 M TRIS (pH 6.8). TEMED (12.5 µl) was added immediately before use.

Gel mix (5x) was composed of 10% SDS, 10% 2-mercaptoethanol, 50% glycerol, 0.1% bromophenol blue and 120 mM TRIS.

Western blot assay

Each liter of transfer buffer (pH 8.4) was composed of 25 mM TRIS, 192 mM glycine, and 20% (v/v) methanol.

Each liter of TRIS-buffered saline (pH 7.4) consisted of 50 mM TRIS and 17 mM Nacl.

Each liter of PBS-Tween (pH 7.4) contained 140 mM NaCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4.7H2O, 2.7 mM KCl and 0.5 ml Tween 20 (Sigma).

Sterilization

Serum bottles, dissecting instruments, pipettes, PEG-1000 (polyethylene glycol, MW 1000, Sigma), and other autoclavable supplies were autoclaved at 121°C, 15 pounds of pressure, for at least 15 min.

Tissue culture media were sterilized by filtration through millipore memberane filter with pore size of 0.45 µm. The sterility of tissue culture media and reagents was tested by adding aliquots into 5 ml of BHI (Bacto-Brain-Heart Infusion, Difco) and/or Bacto-Sarbouraud liquid medium (Difco). Sterility tests were conducted at 37°C for at least one week.

Preparation of Color-specific Photoreceptor Cells (CSP)

Color-specific photoreceptors (cones) were prepared from retinas of dark-adapted goldfish (Ortez et al., 1983). Dark-adaptation for at least 2 h facilitates separation of the neural retina from the pigmented epithelium. The fish were anesthetized with MS-222 (ethyl m-aminobenzoate, 0.2 mg/ml, Sigma) and sacrificed by cervical dislocation. Retinas were removed from the eyes, and collected in a small volume of ice cold Hickman's teleost saline (HTS). The retinas were fixed for 15 min in tissue fixative. Then, the fixative was removed and the fixed retinas were minced into small fragments with scissors. The photoreceptor cells were released by gently disrupting these fragments in a siliconized glass tissue grinder. Three types of tissue grinders were used, Thomas No. AA 85, Kontes No. 22, and Kontes No. Duall 20. They will be described in more detail later. Additional HTS was added into the preparation as needed. Large fragments were removed by passing the cell suspension through a 1 cm deep loosely packed glass wool column in a 5 cc syringe barrel. The filtrate was the crude retina preparation, which contains both rod and cone cells.

The cone cells were enriched by layering this crude retina preparation on a two step discontinuous Ficoll/Hypaque gradient with specific gravities (sp.g.) of 1.16 and 1.26 gm/ml. The gradient was centrifuged at 500

xg, 15 min, room temperature (rmT). Cone cells were collected from the interface between the 1.16 and 1.26 gm/ml sp.g. layers. The Ficoll/Hypaque solution was washed from the cone preparation with HTS by centrifugation at 650 xg, 15 min, rmT. The pelleted cone cells were resuspended with HTS and the number of cells was determined with a hemacytometer. Through this cone-enriching procedure, the majority of rods had been removed, however, there were still some rods as well as red blood cells and other unidentified components remaining in this preparation.

Hybridoma Technology

Immunization

BALB/c mice of 8 to 12 weeks were used as donors of spleen cells in the cell fusion. The animal was given two subcutaneous (s.c.) injections of $4\text{-}6\text{x}10^5$ cone cells two weeks apart. For the first injection, the immunogen was in 0.3-0.6 ml of 50% (v/v) Freund's complete adjuvant (FCA). For the second injection, the immunogen was in 50% Freund's incomplete adjuvant (FIA). Three to four days before the fusion, a third injection containing a comparable number of cone cells in 0.2-0.4 ml HTS was given intraperitoneally (i.p.). The duration between the second injection and the final injection was 1 to 8 weeks. The immune responses of immunized mice were investigated by screening their sera, which were obtained from the brachial vein at the time of

sacrifice.

Preparation of cells

Myeloma cell. The mouse myeloma line X63-Ag8.653 (653 cell) was chosen for the cell fusion. It was kindly provided by Dr. Mark Sanborn. The 653 cells were cultured in enriched RPMI-1640 containing 10% fetal calf serum and incubated at 37°C in a humidified 3% carbon dioxide enviroment.

The cell line was maintained either by 1 to 10 (v/v) cut with Enr-RPMI/10% FCS weekly or frozen in freezing medium composed of 10% dimethyl sulphoxide (DMSO) in FCS (Goding, 1983; DeSt. Groth and Scheidegger, 1980). To achieve exponential growth, 653 cells were cut in 1 to 2 (v/v) dilution daily for at least 3 days prior to the fusion.

Immediately prior to fusion, 653 cells were collected by centrifugation at 500 xg, 5 min, rmT and were resuspended in serum-free RPMI-1640. The number and the viability of the 653 cells were estimated using trypan blue (0.1% in PBS) dye exclusion.

Spleen cell. The immunized mice were sacrificed with chloroform, followed by cervical dislocation and then disinfection in 70% isopropyl alcohol. The spleen was removed from the animal aseptically and rinsed with 70% alcohol once and serum-free RPMI-1640 twice. The spleen was

minced into small fragments with scissors and then gently dissociated in a Duall No.22 (Kontes) glass tissue grinder. Approximately 10 ml of RPMI-1640 were added during this process. To remove the large tissue fragments, the spleen cell suspension was layered on a gradient containing 3 ml of ice-cold FCS and allowed to set for 10 min. Spleen cells were collected from the RPMI layer and washed with 30-40 ml of serum free RPMI-1640. These cells were centrifuged at 500 xg, 5 min, rmT and resuspended in 10 ml of RPMI-1640 containing 5% FCS. The number and viability of these spleen cells were estimated with the trypan blue dye exclusion method.

Feeder cell. Peritoneal exudate cells of BALB/c mice were used as feeder cells. The animal was sacrificed and disinfected with 70% alcohol. The fur of the animal in the abdominal area was dissected aside and 5-6 ml of ice-cold RPMI-1640 was injected intraperitoneally. The abdomen was massaged for approximately 3 min and then the peritoneal cells were collected with sterile Pasteur pipets. Cells were washed with 20 ml RPMI-1640 and centrifuged at 500 xg, 8 min, 15°C, and resuspended in Enr-RPMI/10% FCS. The number of viable feeder cells was determined by the trypan blue dye exclusion method.

Fusion protocol

The fusion protocol was modified from the method described by Galfre and Milstein (1981). In general, approximately $5x10^7$ spleen cells were mixed with $1x10^7$ 653 cells in a 50 ml plastic conical tube (Falcon). The cell mixture was brought to 50 ml with serum-free RPMI and spun at 550 xg, rmT for 8 min. The supernatant was decanted thoroughly and the cell pellet was loosened by gently tapping the tube. For fusing cells, 1 ml of fusogen (35% PEG-1000 and 7.5% DMSO in RPMI-1640 at 37°C unless otherwise stated) was added dropwise over a 1 min duration. Then the fusogen was slowly diluted with 10 ml of RPMI-1640 containing 5% FCS (37°C) over 10 minutes. It was further diluted by adding another 10 ml of the same medium. The cell suspension was centrifuged at 550 xg, rmT, for 8 min and the supernatant was removed. The cell pellet was gently resuspended with Enr-RPMI/10% FCS containing HAT (hypoxanthine 200 μM, aminopterin 0.8 μM,and thymidine 32 μM) to a density of $5x10^5$ cells/ml.

Feeder cells were added into the culture at $1x10^4$ cell/ml. The cell suspension was distributed to 23 wells of 24-well tissue culture clusters (Costar) in 1 ml aliquot. One remaining well of each cluster was seeded with approximately $2x10^5$ 653 cells and was treated as the other wells to confirm the selective effect of the HAT. The culture was incubated at 37°C with 3% CO2. The cells were

fed by replacing approximately one half of the culture supernantant with HAT medium 24 h after the cell fusion.

The cells were fed 6-7 days later with HAT medium and incubated for another week. Thereafter 2x HT medium was fed as needed. The HT was kept in the cultures for at least 4 weeks to allow for the recovery of the main synthetic pathway of the fused cells (Goding, 1983).

Screening

An indirect fluorescein-labeled immunocytochemistry assay (FLICA) was used as the screening procedure in this project. Either cone cells immobilized on glass slides or cryostat sections of retina were used as antigen.

Antigen preparation

Aliquots of cone preparation containing approxmately 3000 cones were centrifuged onto gelatin-coated microscope slides at 220 xg, 5 min, and air dried at rmT. The gelatin-coated slides were prepared by coating a microscopic slide with a warm (40°C) solution composed of 0.5 gm gelatin (300 bloom, Sigma) and 0.05 gm chromium potassium sulfate per 100 ml. Immediately prior to each assay the cone-fixed slides were hydrated in PBS for 10 min.

Cryostat sections of retina were prepared using the method described by Ortez et al. (1983). Retinas were obtained from dark-adapted goldfish and fixed as before. The tissues were frozen in blocks of Optimal Cutting

Temperature (OCT) compound (Fisher Scientific) in a methanol/dry ice bath. Eight micron sections were prepared using a cryostat microtome. The sections were mounted to gelatin-coated slides and air dried at rmT. The OCT compound was washed away by PBS (10 min for 3 times) prior to use in the FLICA.

Screening procedures

To block non-specific binding sites, the antigen-fixed slides were incubated with 1:20 diluted normal rabbit serum for 30 min, rmT, in a humid chamber. The slides were washed with PBS for 3 min, then an aliquot of the primary antibody was applied and incubated at 4°C, overnight. The primary antibody solution was either the culture supernatant or sera to be tested. PBS and 1:100 diluted normal mouse sera were used as negative controls. A polyclonal mouse anti-retina serum was used as the positive control. This serum was obtained from mice immunized with the cone preparation. The incubation with primary antibodies was terminated by washing the slides with PBS, 3 min for 3 times. Non-specific binding was blocked again as described above. Thereafter, an aliquot of the secondary antibody solution was applied to the cell spot and incubated at 4°C for 1 h. In this project, a fluorescein-conjugated IgG fraction of goat-anti-mouse immunoglobulins (IgA, IgG and IgM) antiserum (Cooper Biomedical) was used as the secondary antibody. The dilution factor of the secondary antibody was determined

with a FLICA whenever a new batch of fluorescein-conjugated antiserum was used. Usually a 1:40 to 1:80 diluted antiserum was optimal. After being washed with PBS 3 min for 3 times, each slide was covered with 15 µl of 50% glycerol in PBS and a coverslip. The results were observed using a Leitz Dialux microscope with UV light source and fluorescein filter cube.

Soft Agar Cloning

The soft agar cloning technique was used for isolation of individual hybridoma clones. The protocol was modified from the method described by Coffino et al. (1972). Briefly, bacto-agar (Difco) was prepared as a 5% stock in water and autoclaved. It was diluted in warm (45°C) cloning medium to a concentration of 0.5%. A cell suspension of 0.1-1.0 ml, which contained an appropriate number of cells based on visual observation, was brought to 4.0 ml with cloning medium in a 100 mm tissue culture dish (Corning). To this, 8.0 ml of 0.5% agar in cloning medium was added and swirled, bringing the final concentration of agar to 0.33%. The cloning culture was incubated at 37°C and 3% CO2. Two weeks later, the visible clones were picked up with Pasteur pipettes and placed in 0.5 ml of cloning medium. The cultures were fed with Enr-RPMI/10% FCS/HT as needed. When the cells had grown to sufficient numbers, the supernatants were tested for antibody production with FLICA. Positive wells were recloned to confirm the monoclonality of each

hybridoma .

Western Blot Assay

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Whole goldfish retinas were vigorously homogenized in HTS. Aliquots of this protein solution were separated by SDS-PAGE as follows. A slab gel (12 cm x 14 cm, 0.75 mm in thickness) with 3.5% stacking gel of 2 cm in length and 8.0% resolving gel of 9 cm in length was used. Thirteen 0.6 cm wide wells were set in the top of the gel. For each well, 10 μl of protein sample was mixed with 2.5 μl of 5x gel mix and heated at 100°C for 3 min before loading onto the gel. The electrophoresis was conducted with a 20 mA current. A protein standard solution containing myosin (MW 220,000), Phosphorylase A (MW 95,000), bovine serum albumin (BSA, MW 68,000), ovine albumin (OVA, MW 43,000), and RNAse (MW 12,000) was used to estimate the molecular weight of unknown proteins.

Western Blot assay

Nitrocellulose paper of 0.45 μm pore size (BA 85, Schleicher and Schuell) was soaked in transfer buffer 30 min before blotting. The gel was removed from the mold and soaked in transfer buffer for 5-10 min. The nitrocellulose paper and the gel were placed in a Genie Electrophoretic

Blotter (Idea Scientific Company, Corvallis Oregon) and blotted at 12 volt for 1.5 h. The nitrocellulose paper was rinsed with Tris-buffered saline (TBS) after blotting and cut into strips. One strip containing the protein standard was stained in diluted water soluble India Ink (1 µl/ml water) overnight and destained with water (Hancock and Tsang, 1983). The remaining strips were immersed in a solution containing 0.5% non-fat dry milk (Carnation) in TBS for 30 min to block non-specific binding sites. Then the nitrocelluse strips were washed with PBS-Tween once and introduced into individual solutions containing the supernatants of mAb producing clones in 0.5% non-fat milk at 4°C overnight. In this step, a 1:50 diluted mouse anti-retina antiserum in 0.5% non-fat milk solution was used as positive control and a conditioned medium obtained from culture of 653 cells was used as negative control. The next day these strips were washed with PBS-Tween 5 times for 3 min each. and then the non-specific sites were blocked in a non-fat milk solution as before. After one wash, the strips were transfered to a solution which was either 1:2000 diluted peroxidase-conjugated sheep anti-mouse IgG antiserum or 1:1000 diluted goat anti-mouse IgG, IgM, and IgA antiserum (Cappel Laboratories) in blocking solution and incubated at 37°C for 1 h. After being washed 3 min for 5 times, these strips were transferred into a substrate solution which contained 0.05% diaminobenzidine tetrahydro-chloride, and 0.01% hydrogen peroxide in 0.5 M Tris buffer

(pH 7.6)(Ortez, et al., 1983). A red-brown color appears in a positive reaction indicating the presence of an antibody against the proteins in that band.

CHAPTER III

RESULTS

Antigen Preparation

In earlier experiments of this project cones were prepared from fixed goldfish retinas since in the absence of fixation most of the cones would become fragmented. It would be valuable to be able to prepare those cones from unfixed tissue and have them remain intact. The percentage of cones to which the outer segment remained attached was used as a measure of cone integrity. In attempt to develop a procedure for preparing unfixed cone-enriched antigen, two features of the antigen preparation protocol were evaluated (i.e. the osmolarity of the buffer and the mechanics of dissociation).

Osmolarity

In the first experiment, cones were prepared in HTS of either 325 mOsm or 410 mOsm. Sucrose was used to adjust the osmolarity. For each goldfish, one eye was fixed as before and the other was not. All other aspects of the cone cell preparation were performed as described earlier. The data in Table I (Exp I) show that the HTS at 325 mOsm gave

approximately 40% more intact cones than that at 410 mOsm when retinas had not been fixed. Therefore, increasing the osmolarity to 410 mOsm did not improve the yield of intact cones. On the other hand, fixation gave twice as many more intact cones as unfixed tissue irrespective of osmolarity in keeping with previous observations.

In the second experiment, cones were prepared from one eye in HTS at 325 mOsm and the other eye in HTS at 280 mOsm. No retinas were fixed in this experiment. The data show that HTS at 280 mOsm gave half as many intact cones as that of the higher osmolarity. Therefore, reducing the osmolarity of the HTS did not improve the yield of intact cones. It was concluded from these experiments that the cone integrity is best preserved when the osmolarity of the HTS is 325 mOsm and that we could not achieve the number obtained with fixation by altering the buffer osmolarity. All subsequent experiments were performed with cone preparations made using fixed retinas and HTS of 325±5 mOsm.

Mechanics of retina dissociation

The grinding instrument used to dissociate cones from retinas is a second factor which may affect the integrity of unfixed cones. Three types of tissue grinders were evaluated, Thomas No. AA 85 (TA.85), Kontes No. 22 (K.22), and Kontes No. Duall 20 (Duall 20). As shown in Figure 6, all of these tissue grinders are composed of a test-tube shaped mortar and a pestle. The mortar of TA.85 is a round-

TABLE I

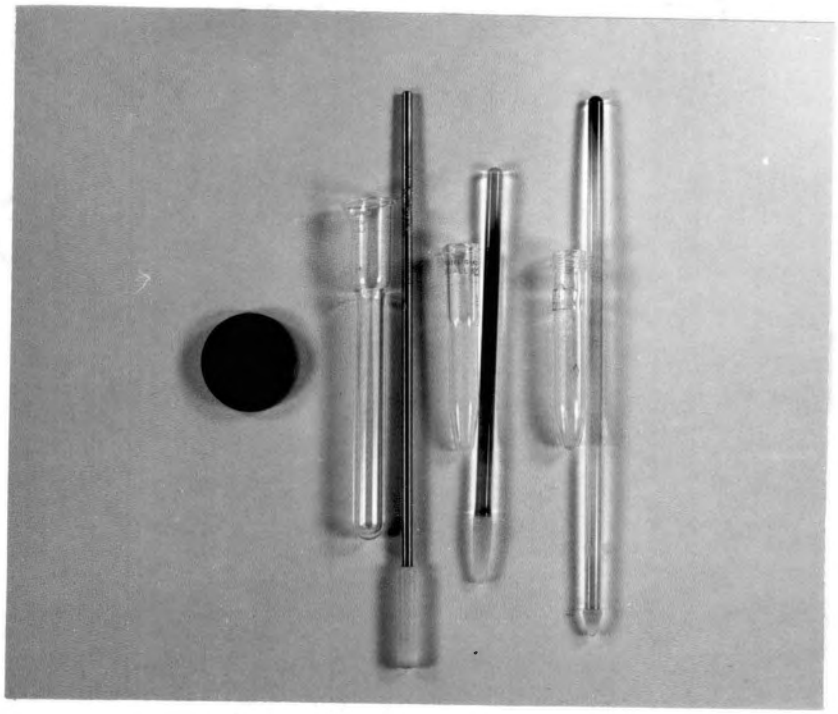
THE EFFECT OF THE OSMOLARITY OF HICKMAN'S TELEOST SALINE (HTS) ON THE INTEGRITY OF UNFIXED CONE CELLS

	mOsm	%Integrity of fixed cones*	%Integrity of unfixed cones
Exp 1	325	62.0 ± 13.8	33.2 ± 1.1
	410	69.8 ± 0.5	24.0 ± 0.8
Exp 2	325	N.D.**	33.3 ± 1.6
	280	N.D.	16.9 ± 2.5

* % Integrity was defined as the percentage of cones to which the outer segment remained attached.

** N.D.= Not done.

Figure 6. Tissue Grinders. (A) Plastic cap of 35 mm film; (B) TA.85; (C) Duall 20; (D) K22.


A
B
C
D

bottomed, smooth-walled tube of 95 mm in height and 12 mm in diameter. The pestle of TA.85 is a metal rod with a teflon tip of 37 mm length. The Duall 20 has a frosted mortar portion which is a conical-bottomed tube of 50 mm in height and 12 mm in diameter, and a pestle which is a glass rod with a frosted end of 25 mm length. The mortar of K.22 is 75 mm in height and 10 mm in diameter with a frosted conical bottom of 10 mm. The pestle of K.22 is a glass rod with a frosted pointed end of 10 mm length.

In the first experiment, one unfixed retina of each goldfish was disrupted with the TA.85 and another was done with the K.22. As shown in Table II, cone cells prepared by both tissue grinders contained about 30% intact cells. However, the cone yield from the TA.85 was twice that with the K.22. In experiment 2, using a Duall 20 tissue grinder, each eye of a goldfish was disrupted separatly. The percentage of intact cones was 34.8% from one retina and 31.7% from another. It was concluded that all three tissue grinders yielded preparations with comparable cone integrity levels (31-35%). Therefore, the cone integrity could not be improved by changing the type of tissue grinder used. However, the yields of total cones per retina did vary with the tissue grinder used. The K.22 gave the worst yield in cone number. The yields of the group using TA.85 and the group using Duall 20 were not comparable since those retina were obtained from two different fish. However, according to these observations it would appear that the Duall 20 gave

TABLE II

THE EFFECT OF TISSUE GRINDERS ON THE INTEGRITY OF UNFIXED CONE CELLS*

	Tissue grinder	% Integrity#	Yield+
EXP 1	TA.85	30.7	244×10^3
	K.22	31.9	113×10^3
EXP 2	Duall 20	34.8	310×10^3
	Duall 20	31.7	328×10^3

* This experiment was performed with HTS of 325 mosm.

As defined in Table I.

+ Total number of cones recovered per retina.

more complete disruption. In the rest of the experiments of this project the Duall 20 was used for antigen preparation.

Improvement of the Fusion Protocol

For a long time, low fusion rates were a major problem in our laboratory. To solve this problem several factors in the hybridoma protocols were carefully evaluated.

Polyethylene glycol concentration

Up to this point, all fusions performed in our laboratory used a fusogen containing 50% PEG-1000 and 7.5% DMSO. In considering the possible additive toxicity caused by the combination of DMSO and a high concentration of PEG, the composition of the fusogen was evaluated. In the first experiment (Table III) a single spleen cell and 653 cell mixture was divided into four fractions and each fused separately. The fusogen of the first three groups contains 7.5% DMSO, while the concentration of PEG was varied from 30-50 %. Spleen cells were obtained from a naive mouse and fused with 653 cells in a 4:1 ratio. Otherwise, the fusion procedure was the same as described in chapter II except for fraction 4 (line 4 of Table III). After fusion, cells were expanded to the density of $2x10^5$ splenocytes/ml. Peritoneal exudate cells were added as feeder cells to a final density of $1x10^4$ cell/ml and each fraction was plated into two 24-well tissue culture plates. During an incubation of 4 weeks, wells containing growing hybridomas were counted.

The result show that the group fused with 30% PEG and 7.5% DMSO had the best fusion frequency, with no growth appearing in the group fused with 50% PEG and 7.5% DMSO, the condition previously used. An intermediate value of 2.1% was obtained with 40% PEG plus 7.5% DMSO. Therefore, reducing the PEG from 50% to 30% caused a 4-fold increace in the yield of positive growth wells. With another fraction fromthe same pool of splenocytes and 653 cells, a different fusion protocol as described by Gefler et al.(1977) was tested and results of this protocol appear in line 4 of Table III. This fusion was performed by slowly adding 1 ml of fusogen containing 35% PEG in RPMI-1640 and centrifuging at 550 xg for a total of 8 min exposure time. The fusogen was then diluted with Enr-RPMI/10% FCS. The other factors of this fusion were the same as those of the other three groups. This fusion gave a hybridoma frequency of 2.1% which is the same as that obtained with 40% PEG and 7.5% DMSO. Although lowering the concentration of PEG did improve our yield of fusion from 2.1 to 8.3%, it was obvious that the concentration of PEG was not the only problem since the fusion frequency was still very low.

Feeder cell density

In a second experiment (Table IV), the contribution of feeder cells was evaluated by varying their density. Spleen cells were obtained from a mouse immunized with the cone preparation. Spleen cells ($1x10^8$)were fused with 653 cells

TABLE III

FUSION I : EFFECT OF PEG CONCENTRATION

Composition of fusogen		Yield of positive growth wells@
PEG-1000	DMSO	
* 30%	7.5%	8.3%
* 40%	"	2.1%
* 50%	"	0.0%
# 35%	0%	2.1%

@ Yield is defined as:
$\frac{\text{No. of wells containing hybridomas}}{\text{total well No. of each group}} \times 100\ \%$

* Fusion protocol is the same as described in materials and methods.

This fusion was performed by slowly adding 1 ml of fusogen containing 35% PEG in RPMI-1640 and centrifuging at 550 xg for a total of 8 min exposure time. The fusogen was then diluted with Enr-RPMI/10% FCS. The other factors of this fusion were the same as those of the other three groups.

in a 5:1 ratio using fusogen containing 35% PEG and 7.5% DMSO. After fusion, the cell suspension was diluted to a density of $5x10^5$ splenocytes/ml and divided into four groups. Murine peritoneal exudate cells were added to each fraction at different densities ranging from $2x10^3$ to $2.5x10^5$ cell/ml and the cells dispensed into 24-welled culture plates. The number of wells containing growing hybridomas were counted after 3 weeks of incubation. Under the conditions of this fusion, feeder cell densities ranging from $2x10^3$-$5x10^4$ cell/ml provided optimal conditions for growth of hybridomas. Introduction of a larger number ($2.5x10^5$ cell/ml) of feeder cells did reduce the growth of hybridomas by 40%. Interestingly all these conditions resulted in significantly higher hybridoma outgrowth rates than the previous experiments suggesting that some factor other than feeder cell density was contributing to the high hybridoma yield. It was noted that another difference between the experiments in Table III and IV was the immune state of the splenocyte donating mouse. In fusion I (Table III) splenocytes had come from a naive mouse while in fusion II (Table IV) the spleen cells came from a hyperimmunized mouse. Therefore two additional fusions were set up using spleen cells from a naive mouse and a hyperimmunized mouse respectively compare the fusion rates.

TABLE IV

FUSION II : EFFECT OF FEEDER CELL DENSITY

Feeder cell density (cell/ml)	Yield of positive growth wells* (%)
2×10^3	77.1 ± 2.1
1×10^4	81.3 ± 6.3
5×10^4	75.0 ± 4.2
2.5×10^5	47.9.± 2.1

* As defined in Table III.

Immune state of splenocyte donor

Spleen cells of fusion III were obtained from a naive mouse and fused with 653 cells in 5:1 ratio. The composition of the fusogen and final spleen cell density were the same as those of the fusion II (Table III). Peritoneal exudate cells of $1x10^4$ cell/ml were added into culture. As shown in Table V, the yield from this fusion was only 7.6% of positive growth wells.

Fusion IV followed all the conditions of fusion III except that spleen cells were obtained from a mouse immunized with cone preparation. This time a positive growth rate of 52% was obtained. The data from both fusion III and IV are shown in Table V. They show that a seven-fold increase in hybrid outgrowths occured when an immunized mouse was used. Therefore we may tentatively conclude that a major cause of failure to get high yields of hybridoma outgrowth in fusion I and III was the failure to use properly immunized mice as spleen cell donors.

Monoclonal Antibodies Against the Retinal Preparation

Using the conditions finally determined and described in the materials and methods section, two fusions were performed for the purpose of generating mAbs against the cone preparations. After culture of two to three weeks, growth of hybridomas appeared in 48 out of 184 wells (26.1%)

TABLE V

EFFECT OF IMMUNE STATUS OF SPLEEN CELL DONOR ON FUSION EFFICIENCY

Fusion	Spleen donor	PEG (%)	DMSO (%)	Feeder cell (cell/ml)	Yield of positive growth wells (%)@
III	Naive	35	7.5	1×10^4	7.6 ± 0.0
IV	Immunized	35	7.5	1×10^4	52.2 ± 16.3

* In each experiment, two separate fusion procedures were performed. In each fusion 5×10^7 spleen cells were fused with 1×10^7 653 cells and expanded to four 24-welled tissue culture plates.

@ As described in Table III.

TABLE VI

SCREENING AND CLONING OF SPECIFIC MONOCLONAL ANTIBODIES

Original wells (+)* in screening	Name of the 1st clones	Screening of the 1st clones	Name of the 2nd clones	Screening of the 2nd clones
B1A3**				
B1A4	B1A4C	(-)	N.D.@	N.D.
B2A5				
B4C6				
B5B1				
B5B3				
B5D5	B5D5C	(+)	B5D5D4d	(+)
B6A5				
B6B4	B6B4C	(+)	B6B4D2a	(+)
B6C2				
B7A4	B7A4C	(+)	B7A4D1e	(+)
B7D4				
B8C6				
B8D2				
C1C1				
C2A6				
C2C5	C2C5C	(+)	C2C5D1b	(+)
C3A5	C3A5C	(+)	C3A5D1a	(+)
C4A5	C4A5C	(+)	C4A5D1a	(+)
C4B4	C4B4C	(+)	C4B4D1a	(+)
C5A2				
C5D5				
C6C1	C6C1C	(+)	C6C1D1a	(+)
C6C6	C6C6C	(+)	C6C6D1a	(+)
C7C2				
C8B3				
C8C4				

* (+) = positive; (-) = negative.

** Wells from the fusion B were prefixed by "B" and those from the fusion C were prefixed by "C".

@ N.D.= Not done.

of the first fusion, called fusion B. The supernatants of the 48 wells containing hybridomas were screened by FLICA on immobilized cone preparation. As shown in the Table VI, fourteen of those forty-eight wells (29%) possessed antibodies reactive with the antigen. Cells of these wells were cloned using the soft agar cloning method. After incubation for 10 to 14 days, clones of hybridoma appeared in four of the fourteen soft agar plates. From each of these four plates, one to ten clones, called the first clones and named in Table VI, were picked up and expanded in a 24-welled tissue culture cluster. The supernatant of the first clones were collected and screened by FLICA on immobilized cone preparation. In this assay, positive responses showed in the first clones coming from the original wells B5D5, B6B4, and B7A4 with no signifigant differences in the staining pattern between the clones and the original wells. However, all of the first clones from the original well B1A4 failed to produce specific antibodies. Cells of clones B5D5C, B6B4C, and B7A4C were recloned and 1-4 clones of each soft agar plate were picked and expanded after 10-14 days incubation. The supernatant of these clones, called the second clones and named in Table VI, were tested with FLICA as before. For each original well, one to two positive second clones were expanded in Enr-RPMI/10% FCS. The supernatants of these monoclonal hybridoma cultures containing 77-182x10^4 cell/ml were collected and analysed as described later.

The second fusion, called fusion C, was performed using the same protocol as fusion B. Growing hybridomas appeared in 59 out of 184 wells (32.1%) after incubation. By FLICA on immobilized cone preparation, thirteen wells (22%) were identified to be positive for secreting antibodies against our antigen. These wells are shown in Table VI. After cloning, the first clones appeared in plates from six original wells, namely C2C5, C3A5, C4A5, C4B4, C6C1, and C6C6. From each plate, four clones were picked and expanded. The supernatants of these first clones were screened and positive clones were recloned. Two clones from each soft agar plate of the second cloning were picked up and expanded. After screening, one to two clones from each original well were expanded in tissue culture flaskes and supernatants from them were collected as with fusion B.

Staining pattern of cone preparation

Table VII shows the FLICA profile of mAbs on the immobilized cone preparation. For each mAb, three to seven cells of each type were randomly chosen in white light and then observed in UV light. The staining degree of each cell was assigned with an arbitrary scale (i.e., -, +, ++, +++). According to this analysis the following descriptions of the individual mAbs were drawn.

B5D5D4d. This mAb showed slight to moderate fluorescein binding to OS of rods, blue cones, red cones and

TABLE VII

THE FLICA ANALYSIS OF MONOCLONAL ANTIBODIES TO THE CONE PREPARATION#

mAb	FL *	*** %	Major components of the cone preparation ##								
			Rod		Blue		Red		Green		SSB
			IS	OS	IS	OS	IS	OS	IS	OS	**
B5D5	-	100								20	
D4d	+			50				40		40	25
	++			50	100	100	100	60	100	40	50
	+++										25
B6B4	-	100				75		40		60	
D2a	+			20		25		20			
	++			80	100		100	40	100	40	
	+++										100
B7A4	-	75				50		50		50	
D1e	+	25				50	25	25	25	25	
	++			100	100		75	25	75	25	50
	+++										50
C2C5	-				25						
D1b	+	100	100		75	100	50	100	50	100	50
	++						50		50		50
	+++										
C3A5	-	100					17		20	20	60
D1a	+				100	100	83	100	60	80	40
	++								20		
	+++			100							

Three to seven cells of the components were randomly chosen in white light and observed in UV. A staining degree was assigned to each cell with an arbitrary scale.

The red blood cell is also a major component in our cone preparation, while none of these mAb stained it.

* FL, fluorescein ; -, negative; +, slightly stained; ++, moderately stained; +++, strongly stained.

** SSB, small spherical bodies; LSB, large spherical bodies; ND, not done.

*** Percentage of cells stained by antibodies.

TABLE VII (CONTINUED)

mAb	FL / %	Major components of the cone preparation								
		Rod		Blue		Red		Green		SSB
		IS	OS	IS	OS	IS	OS	IS	OS	
C4A5 D1a	-	40			100	40	80	20	80	20
	+	60	40			20	20	40	20	
	++		40	100		20		40		60
	+++		20			20				20
C4B4 D1A	-	75	100		100	50	100	50	100	
	+	25				25		25		25
	++			100		25		25		75
	+++									
C6C1 D1a	-	100	100	100	100	100	100	100	100	100
	+									
	++									
	+++									
C6C6 D1a	-	67	14						33	
	+		57	40	40	17	83	17	50	
	++	33	29	60	60	83	17	83	17	67
	+++									33

Three to seven cells of the components were randomly chosen in white light and observed in UV. A staining degree was assigned to each cell with an arbitrary scale.

The red blood cell is also a major component in our cone preparation, while none of these mAb stained it.

* FL, fluorescein ; -, negative; +, slightly stained; ++, moderately stained; +++, strongly stained.

** SSB, small spherical bodies; LSB, large spherical bodies; ND, not done.

*** Percentage of cells stained by antibodies.

most of the green cones. It reacted moderately with IS of all cones but did not bind rod IS. It caused moderate fluorescein staining of small spherical bodies (SSB), which were suspected to be neurons.

B6B4D2a. This mAb bound to OS of all the rods but only 25-60 % of cones. This mAb appeared to react with IS of all cones but of no rods. It caused strong fluorescein staining of all SSB (see Fig.7-A).

B7A4D1e. This mAb stained OS of all rods moderately but only 50% of cones. It bound to IS of all cones and IS of 25% rods. It gave strong fluorescence in SSB.

C2C5D1b. The OS of both rods and cones were slightly stained when the cone preparation was incubated with this mAb. The IS of all rods, red cones, and green cones were slightly to moderately stained, while about 25% of blue cone IS were not stained. All SSB were slightly to moderately stained.

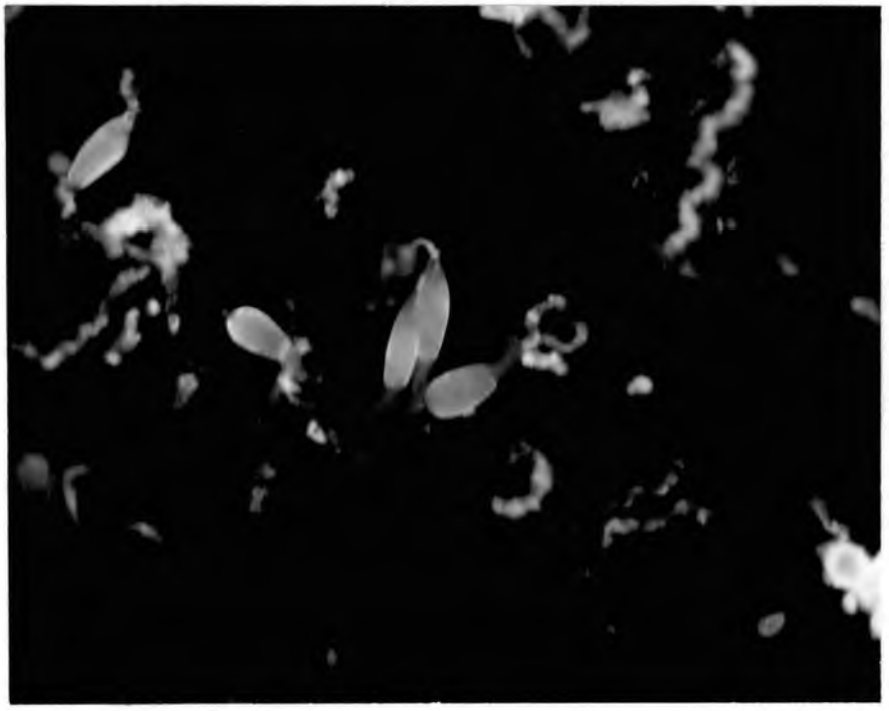
C3A5D1a. The OS of all rods were strongly stained, while only slight fluorescence appeared in the cone OS. No IS of rods were stained, while slight fluorescence showed in those of most cones. Forty percent of SSB were slightly stained.

C4A5D1a. This mAb slightly stained about one half of the rod IS and significantly stained all rod OS. It stained

Figure 7. The FLICA Pattern of mAbs on Cone Preparation, 1. A, B6B4D2a; B, C4A5D1a.

A

B



most of the cone IS, expecially that of blue cones, and OS of a small fraction of red and green cones. Most of the SSB were moderately to strongly stained (see Fig.7-B).

C4B4D1a. No OS of either rods or cones was stained. The IS of 25% of rods , 50% of red and green cones were stained slightly to moderately, while the IS of all blue cones were moderately stained. The myoids of some cones were specifically stained. This mAb stained all the SSB slightly to moderately (see Fig. 8-A).

C6C1D1a. This mAb only bound to the outer layer of some unidentified large spherical bodies (LSB)(see Fig. 8-B) in the cone preparation. It stained no other retinal components.

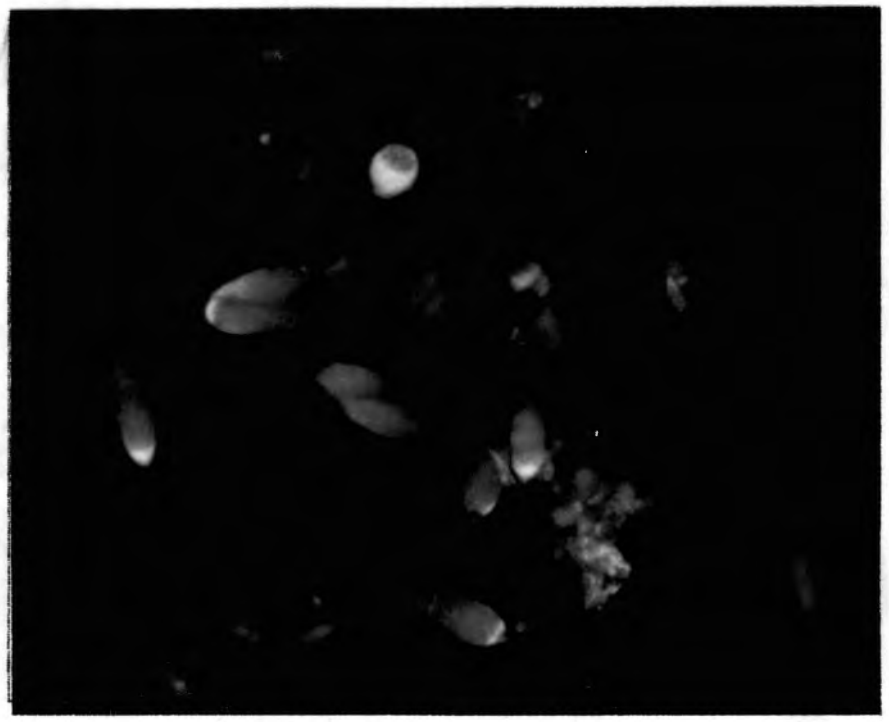
C6C6D1a. This mAb stained OS of all blue cones, red cones and those of most green cones as well as rods. The IS of all cones but only 30% of rods were stained. The SSB were significantly stained.

Staining pattern of retina section

In order to obtain more information about the specificity of these nine mAbs for components of the retina, a FLICA screening was performed on cryostat sections (8 μ) of goldfish retina. In this assay, the fluorescein densities on six layers of retina sections were determined using an arbitrary staining scale (from - to +++). The

Figure 8. The FLICA Pattern of mAbs on Cone Preparation, 2. A, C4B4D1a; B, C6C1D1a.

A



B

staining pattern of eight mAbs to six retina layer, namely the outer segment (OS), the inner segment (IS), the outer nuclear layer (ON), the inner nuclear layer (IN), the inner plexiform layer (IPL), and the combination of ganglion layer and inner limiting membrane (G&IL) are shown in Figure 9. Monoclonal antibody C6C1D1a, which is not shown in this figure, stained nothing in this assay. From this figure, four different staining categories, named A, B, C, and D, can be established according to the distribution and density of fluorescein.

Pattern A. Monoclonal antibies B5D5D4d, C2C5D1b, and C3A5D1a are included. The mAbs of this category slightly stained some OS and IPL, while strongly staining the ON and IN layers. In this category, the G&IL was slightly to moderately stained (Fig. 9 and 10-A).

Pattern B. Monoclonal antibodies B6B4D2a, and C6C6D1a are included. The mAbs of this category moderately stained OS and G&IL and strongly stained ON and IN (Fig. 9 and 10-B).

Pattern C. Monoclonal antibodies C4A5D1a and C4B4D1a are included. The mAbs of this category strongly stained the IN layer but exibited much less staining of the ON and other layers. In FLICA of C4B4D1a IN was the only layer with significant fluorescence (Fig. 9 and 10-C).

Pattern D. Only mAb B7A4D1e is included. This mAb

Figure 9. The Fluorescein Distribution on Goldfish Retina Sections Stained by Eight different mAbs. The fluorescene density was desighed with an arbitrary scale (-, +, ++, +++). (A), pattern A; the monoclonal antibodies of this category slightly stained some OS and IPL, while strongly staining the ON and IN layers. (B), pattern B; the monoclonal antibodies of this category moderately stained OS and G&ILM and strongly stained ON and IN. (C), pattern C; the monoclonal antibodies of this category strongly stained the IN but exibited much less staining of the ON and other layers. (D), pattern D; the monoclonal antibodies of this category stained OS, IN, and G&ILM, while the IS and ON were moderately stained. G&ILM, ganglion layer and inner limiting membrane; IN, Inner nuclear layer; IPL, inner plexiform layer; IS, inner segment layer of photoreceptor; ON, outernuclear layer; OS, outer segment layer of photoreceptor.

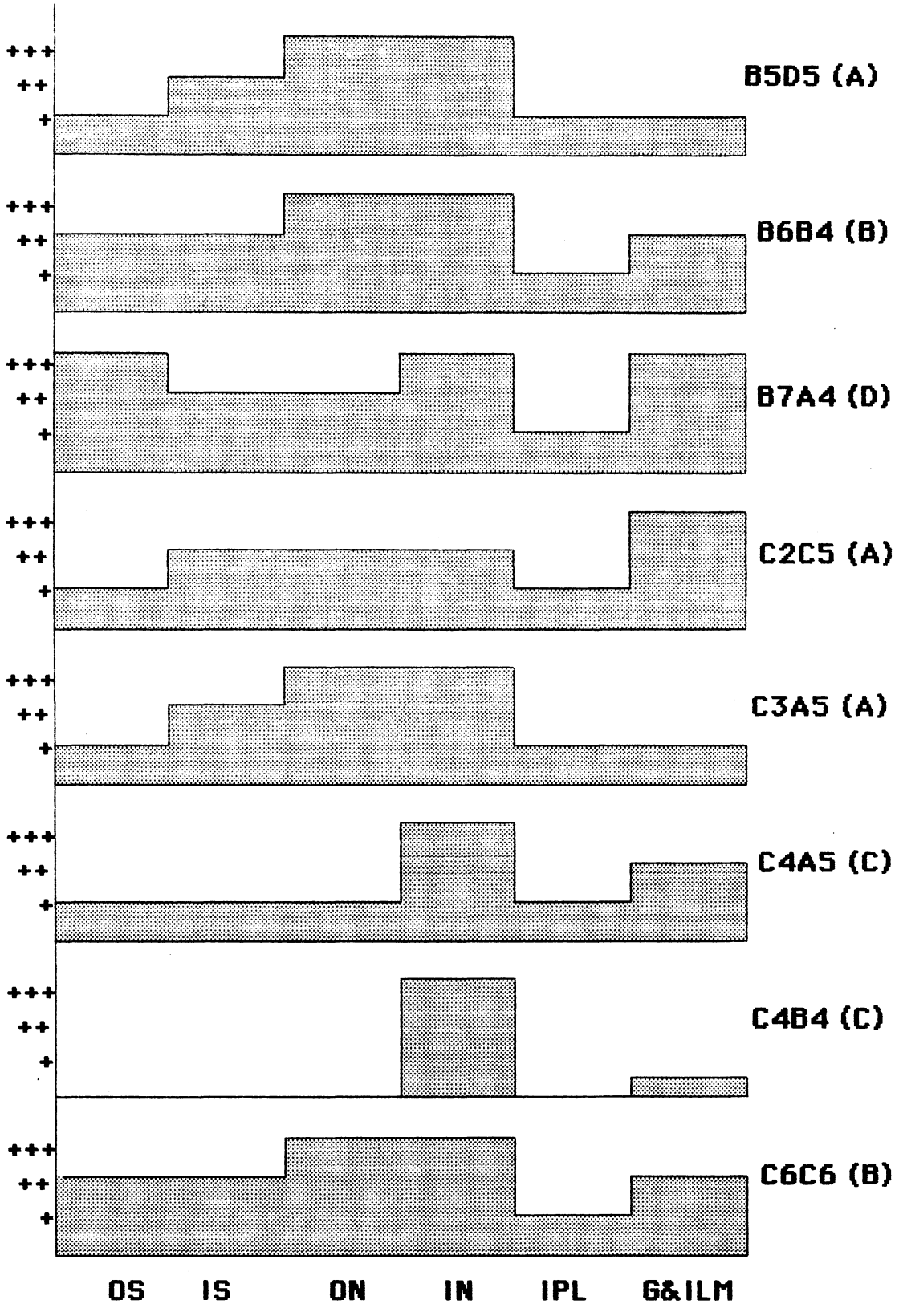
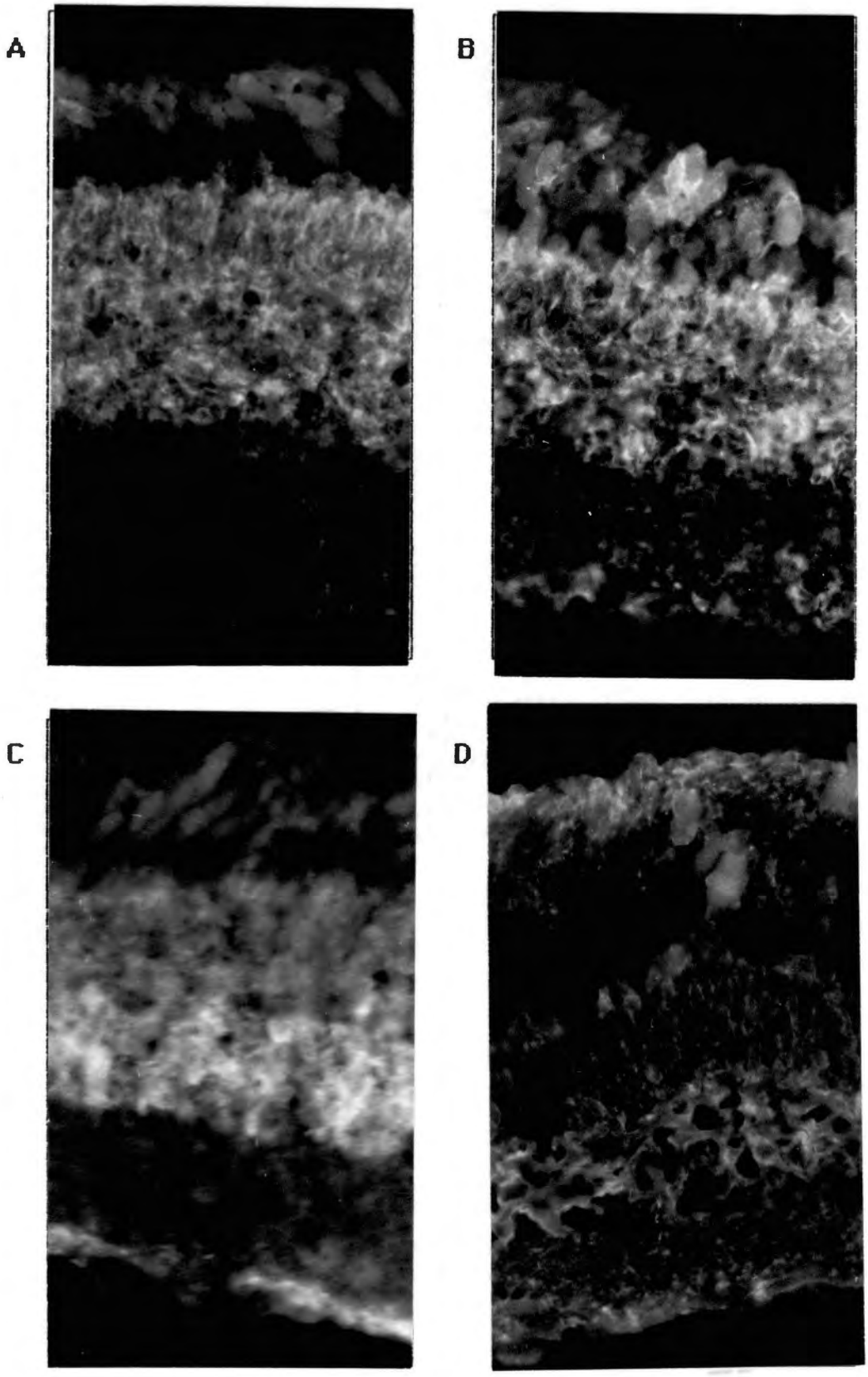

+++
++
+
B5D5 (A)
B6B4 (B)
B7A4 (D)
C2C5 (A)
C3A5 (A)
C4A5 (C)
C4B4 (C)
C6C6 (B)
OS
IS
ON
IN
IPL
G&ILM

Figure 10. The FLICA Patterns Produced by mAbs on Retina Sections. A, Pattern A (B5D5D4d); B, Pattern B (B6B4D2a); C, Pattern C (C4B4D1a); D, Pattern D (B7A4D1e).


A
B
C
D

strongly stained OS, IN, and G&IL, while the IS and ON were moderately stained. The IPL was only slightly stained by this mAb (Fig. 9 and 10-D).

Western blot assay

In order to reveal more information about the molecules these mAbs were reacting with, two western blot assay were performed as described in Chapter II. In the first experiment, individual mAb was applied to a nitrocellulose strip containing retina protein blotted in advance. A conjugate solution containing 1:2000 peroxidase-conjugated sheep anti-mouse IgG antiserum were used as secondary antibodies to indicate the specific binding between retina proteins and specific mAbs. As shown in Table VIII, no bands appeared in any of the strips except the one incubated with 1:50 diluted mouse anti-retina polyclonal antibodies which was used as the positive control. In the second experiment, nine mAbs was applied to strips as in experiment I. However in this case, a 1:1000 diluted peroxidase-conjugated goat anti-mouse IgG, IgM, and IgA antiserum was used as the conjugate. As shown in Table VIII, none of these mAbs gave positive response.

TABLE VIII

THE WESTERN BLOT ASSAY OF NINE MONOCLONAL ANTIBODIES

Primary Antibodies	Conjugate	Results
B5D5D4d	*I	(-)
	II	(-)
B6B4D2a	I	(-)
	II	(-)
B7A4D1e	I	(-)
	II	(-)
C2C5D1b	I	(-)
	II	(-)
C3A5D1a	I	(-)
	II	(-)
C4A5D1a	I	(-)
	II	(-)
C4B4D1a	I	(-)
	II	(-)
C6C1D1a	I	(-)
	II	(-)
C6C6D1a	I	(-)
	II	(-)
1:50 Mouse anti-retina antibodies	I	(+)
	II	(+)
Condition medium	I	(-)
	II	(-)

* I, peroxidase-conjugated sheep anti-mouse IgG antibodies. II, peroxidase-conjugated goat anti-mouse IgG, IgM, and IgA antibodies.

CHAPTER IV

DISCUSSION

Cone Preparation

In this project, hybridoma technology was applied to faciliate the study of color-specific photoreceptors (cones) and their respective photopigments. This technique is especially applicable because of it can be applied to impure antigens.

Cones and their outer segments contain the antigens of major interest to the long-range objectives of our laboratory. Therefore, a cone-enriched preparation will be helpful in restricting the number and categories of unwanted antigens in order to increase the probability of obtaining cone-specific hybridoma clones. However, since cones are fragile, especially at the site between the inner and outer segments, our laboratory had found it is necessary to fix them with an aldehyde fixative to retain their integrity. Unfortunately, the reaction with aldehyde fixatives, which causes covalent crosslinkage between proteins, also renders fixed cones unsuitable as a source for opsin purification. Further, fixation may alter antigen determinants, such that the mAb produced against fixed tissue may not bind to native

tissue. These problems caused us to seek a method to prepare cones from unfixed tissue. Two alterations of the procedure we tried were changing the osmolarity of the HTS and using different tissue grinder types.
Unfortunately, neither of these alterations improved the integrity of unfixed cones. However, this does not lessen the need for eliminating the fixation step from our antigen preparation. Future efforts may have to look for ways to avoid mechanical grinding of retinas altogether such as scraping the photoreceptor off the retina with a glass slide. This would require using much larger fish than currently used.

Improvement of Fusion Efficiency

Since cell fusions are random events, the probability of obtaining the desired mAbs depends upon the ability to generate large numbers of hybridoma clones. In order to resolve the problem of low fusion efficiency in our laboratory, two important factors (i.e., the PEG concentration and feeder cell density) in our fusion protocol were evaluated.

The fusion of antibody-secreting lymphocytes with myeloma cells is a critical step in hybridoma technique. High frequencies of PEG-mediated hybridization depend upon the optimal balance between toxicity and enhancement of fusion (Gefter et al., 1977). Factors affecting this balance include the concentration and exposure time to PEG.

Davidson and Gerald (1976) have tested the effect of PEG concentration on hybridization by fusing 3T3 and RG6 cell lines with various concentration of PEG-6000 for 1 min and obtained the optimal yield of hybridomas with 50% PEG. Gefter et al. (1977), fusing MPC-11 and MOPC 315 cell lines with PEG-1000 for 8-9 min, obtained the highest outgrowth of hybridomas using 35% PEG. The differences in their results could be a reflection of the cell types and molecular weight of PEG they used. Whatever the reason, their results would suggest that the optimal PEG concentration should be determined for each new system.

Dimethyl sulphoxide (DMSO) is a well-known cryoprotective and radioprotective agent having some toxic effects on cells. Norwood et al. (1976) have suggested that the addition of 15% (v/v) DMSO to 42% (w/v) PEG-6000 results in better fusions. DeSt. Groth and Scheidegger (1980) introduced 5% DMSO into the fusogen they used based on the observation that addition of DMSO was beneficial when the PEG solution or diluting medium were added too quickly. Klebe and Mancuso (1982) found that a fusogen containing 25% PEG-600 and 19.5% DMSO produced the optimal fusion yield. Our results demonstrate that with the addition of 7.5% DMSO to the fusogen, the lower concentration (30-35%) of PEG is optimal for fusion.

In early culture of hybridomas, the introduction of feeder cells may increase the yield of positive growth since lymphoid cells often grow poorly or die when grown at low

density (Goding, 1983). Several types of cells have been used as feeder cells including splenocytes, thymocytes, and peritoneal exudate cells (Galfre and Milstein, 1981; Lernhardt et al., 1978; DeSt. Groth and Scheidegger, 1980). Peritoneal exudate cells, mainly half lymphocytes and half macrophages (Goding, 1983) were used as feeder cells in this project since macrophages have been reported to enhance hybridoma yield (DeSt. Groth and Scheidegger, 1980). The results of this project show that using peritoneal exudate cells a feeder cell density ranging from $2x10^3$ to $5x10^4$ cell/ml was suitable in a fusion culture containing $5x10^5$ spleen cells and $1x10^5$ myeloma cells per mililiter. A higher density of feeder cells was inhibitory to hybridoma outgrowth. The reason for this is unclear but may be the result of nutrient depletion or the production of toxic products.

A comparison of the results of the experiments to test the effect of PEG concentration with that to test feeder cell density shows that under similar conditions of PEG concentration and feeder cell density (line 1 of Table III and line 2 of Table IV), two very different efficiencies were observed (i.e., 8.3% and 81.3% respectively). This caused us to look for another possible difference between these experiments. The spleen cell donor for fusion I was a naive animal while that for fusion II had been hyperimmunized with cone preparation. Based on this observation we hypothesized that this could have contributed

to the difference in efficiency between the experiments. This hypothesis is supported by additional fusions performed in this study. Table V shows that for the third fusion using an immunologically naive spleen cells donor the hybridoma efficiency was 7.6% while for the fourth fusion using an immunized mouse as spleen cell donor the hybridoma efficiency was 52.2%, almost 7 fold greater. This hypothesis is further supported by the study of Andersson and Melcher (1978). In their experiment, lymphocytes were sorted into two fractions by size (i.e., fraction of small resting cells and fraction of large activated cells) and fused with myelomas separately. The results showed that only large (i.e., activated) lymphocytes offered good outgrowth of hybridomas after fusion.

FLICA Analysis of mAbs

The Enzyme-linked immunosorbent assay (ELISA) is commonly used for screening mAbs. However, this procedure requires at least a small amount of purified antigen. Since purified antigen is unavailble in our project, we chose to use a fluorescein-labelled immunocytochemistry assay (FLICA) as the screening process instead of ELISA. A widely used procedure in other areas, ELICA has rarely been used as a primary screening process for mAb production because it is technically much more demanding. The suitability of FLICA in our research was based on the fact that the cells of interest can be definitively identified on morphological

grounds. We took advantage of the fact that the red, green, and blue photoreceptors can be identified on the basis of morphology in certain fish species including the common goldfish. Perhaps the most significant observation of this study is that FLICA proved to be a very valuable and easy procedure for use as a primary screening procedure in mAb production where the antigen in question can be morphologically localized. It proved to be no more technically demanding than ELISA and yielded much more information. We can easily screen 20 to 30 positive growth wells per day. Besides being easy to perform, a single screen identified not only the presense of antibodies but the cell components it reacted with (i.e., rods, cones, neurons, red blood cells, etc.) as well.

Analysis of Nine Monoclonal Antibodies Reactive with Goldfish Retina

The mAb produced by the nine clones identified in Table VI were carefully examined by FLICA procedures on both immobilized photoreceptor preparation and cryostat sections of intact retina. The results are shown in Table VII and Figure 9. Further analysis of these data is shown in Table IX and Figure 11. In Table IX, the nine mAbs were sorted into several groups according to their staining patterns on OS or IS of photoreceptors. Seven parameters were used: rod outer segment (ROS), blue cone OS (BOS), red cone OS (RdOS), green cone OS (GOS), blue cone IS (BIS), red cone IS (RdIS),

TABLE IX

ANALYSIS OF THE STAINING PATTERN OF NINE MONOCLONAL ANTIBODIES TO OS AND IS OF PHOTORECEPTORS

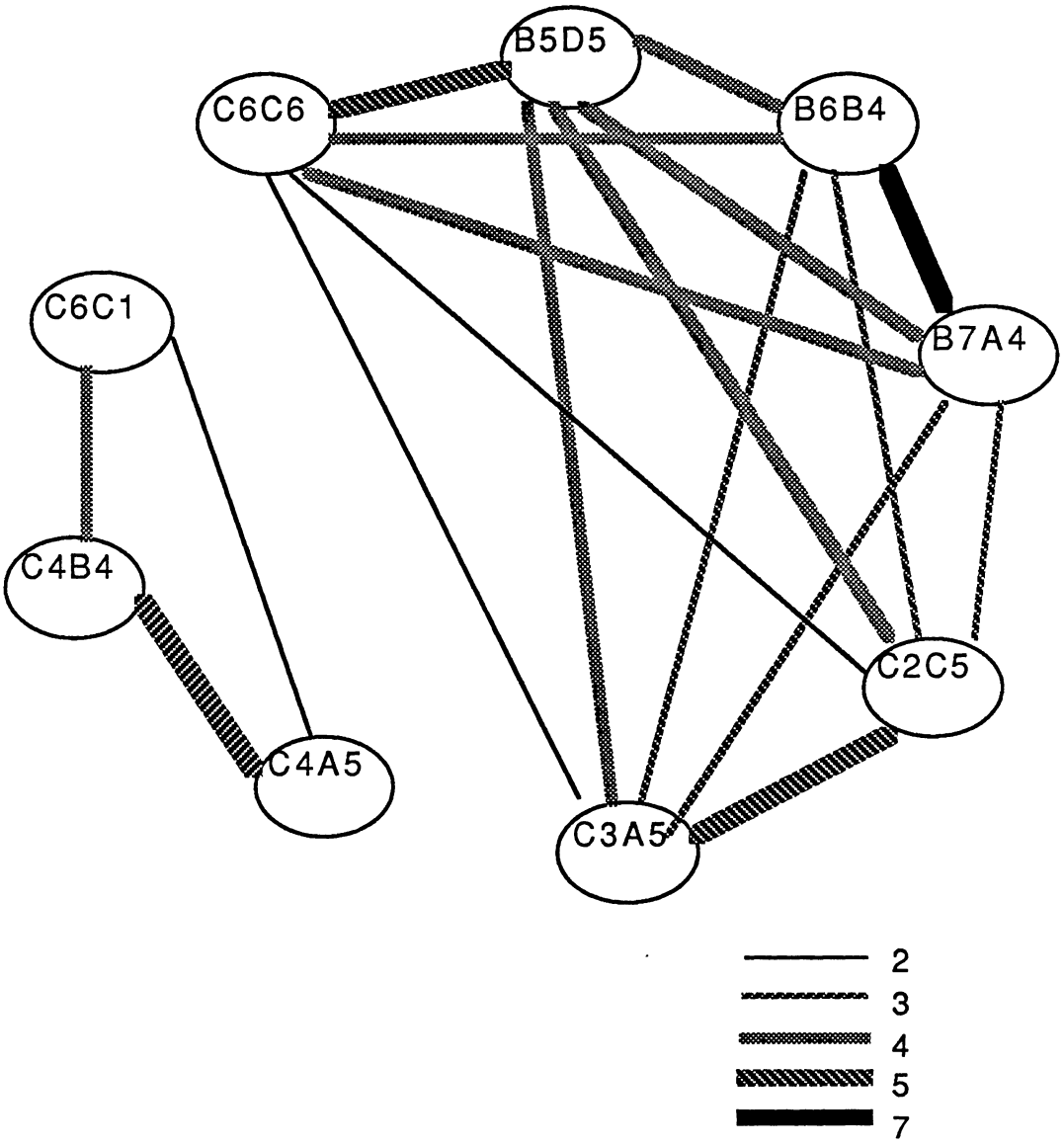
ST@ / mAb	ROS	BOS	RdOS	GOS	BIS	RdIS	GIS
Group* 1	B5D5 B6B4 B7A4 C3A5 C6C6	B5D5 C6C6	B7A4 B6B4 C4A5	B5D5 C2C5 C3A5	B5D5 B6B4 B7A4 C2C5 C3A5	B5D5 B6B4 B7A4 C2C5 C3A5 C6C6	B5D5 B6B4 B7A4 C2C5 C6C6
Group 2	C2C5	B6B4 B7A4	C2C5 C3A5	B6B4 B7A4 C6C6	C4A5 C4B4 C6C6	C4A5 C4B4	C3A5 C4A5 C4B4
Group 3	C4B4 C6C1	C2C5 C3A5	B5D5 C6C6	C4A5 C4B4 C6C1	C6C1	C6C1	C6C1
Group 4		C4A5 C4B4 C6C1	C4B4 C6C1				

@ ST, structure

* Groups are set arbitrarily.

and green cone IS (GIS). Staining of other components such as neurons was not included in this analysis. The grouping was constructed independently for each of the antigen regions using subjective evaluation of the intensity of staining produced by each of the nine mAbs. After the grouping displayed in Table IX was generated, an analysis was performed to determine how many times the same two mAbs were found to segregate into the same group and a line of identity was drawn between them (Fig. 11). The more often two mAb were found in the same group the more lines of identity were drawn between them. As an example mAbs C6C6 and B7A4 were found in the same group four times, group 1 under ROS, group 1 under RdIS, group 1 under GIS, and group 2 under GOS. The full result of this analysis is shown in Figure 11. The nine mAbs were separated into two distinct groups, while subgroups may exist in both. From this figure, a relatively high pattern of identity was found between mAbs B6B4 and B7A4. A slightly lesser index of identity was found between mAbs C6C6 and B5D5; C3A5 and C2C5; as well as between C4B4 and C4A5. A less close relationship was shown between B5D5 and B6B4, B7A4, C2C5, and C3A5; as well as C6C1 and C4B4. It is interesting that the results of this grouping are matched fairly well by obtained with FLICA on retina section as shown in Figure 9. We may hypothesize that the more similar the staining pattern is between two mAbs, the more likely that they are reacting with similar or identical antigenic determinants.

Figure 11. Relationship among 9 mAbs in Terms of Their Specificity to OS and IS of Photoreceptors. The code of lines represents the frequency that two mAbs have been sorted into the same group. There is no line shown between mAbs which have been sorted into the same group only once.


B5D5
C6C6
B6B4
C6C1
B7A4
C4B4
C2C5
C4A5
C3A5
2
3
4
5
7

For example, the antigenic determinants recognized by B6B4 and B7A4 may either be identical or located on the same protein molecule. However, differences in staining pattern between two mAbs could also be due to differences in antibody titer or affinity.

It was hoped that the Western blot experiments would shed some light on the possible similarity or even identity of antigens with which these mAbs reacted. Two critical pieces of information that can be determined from Western blot analysis are: 1) the number of protein subunits with which a specific mAb reacts and 2) the possibility that two or more mAbs may react with the same protein(s). Unfortunately, our limited attempts at Western blot analysis produced negative results. None of our nine mAbs was found to react with homogenates of whole retina separated by SDS-PAGE and blotted onto nitrocellulose paper. Positive reactions were observed between a mouse polyclonal anti-goldfish photoreceptor serum, demonstrating that the basic assay was functional. These negative results for the Western blot assay may be caused by any of a variety of reasons: first, there may have been no effective antigenic site existing in the nitrocellulose paper for mAbs to bind to. Loss of effective antigenic sites is a common result in SDS-PAGE procedures because of their protein denaturing properties, thus destroying all secondary, tertiary and quarternary epitopes. Our initial thought that it is unlikely that all nine mAbs would be reactive with such

epitopes may not be well founded. The observed similarities in staining pattern suggest fewer than nine epitopes may be involved. Denaturation of maybe as few as three to four epitopes on target proteins could have produced our negative results. A second possiblility for the negative result may be too low a concentration of mAbs in the culture supernatant. This hypothesis, testable by concentrating the antibodies, is not very likely because of the significant staining potential of these mAbs as shown by FLICA. A third possibility is that none of these mAbs belong to either the IgM, IgG, or IgA isotypes. This hypothesis can also be easily tested. However, it is most unlikely since IgM and IgG are the most common isotypes among mAbs (Anderson and Melchur, 1978; DeSt. Growth and Scheidegger, 1980).

Finally, the most significant value of this series of mAbs may be to help in the preparation of a more homogeneous cone antigen preparation thereby improving the likelihood of finding cone-specific clones. According to the staining pattern shown in Figure 9, IN and ON layer cells contain many of the potent antigenic determinants of the retina. The use of monoclonal antibody C4B4 which is specific to IN, in affinity procedures should be useful in removing these unwanted antigens from the current cone preparation.

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VITA 2

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