STUDIES ON RETINAL ANTIGENS WITH SPECIAL REFERENCE TO AUTOIMMUNITY AND ANALYSIS BY MONOCLONAL ANTIBODY TECHNOLOGY

By

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TO MY FAMILY

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PROLOGUE

Whether or not a thing is measurable is not something to be decided a priori by thought alone but something to be decided only by experiment.

Feynam (1963)

SUMMARY

The ability of retina derived antigens to induce a strong immunological response resulting in immunopathological alterations in the retinae of experimentally immunised animals has been well established (reviewed by Faure, 1980). The photoreceptor specific soluble protein, known as S-antigen, has been implicated as one of the most uveitopathogenic components of the retina (Wacker et al., 1977). There is a growing number of reports suggesting immunological alterations in human retinal degeneration, such as retinitis pigmentosa (RP), and chorioretinal inflammatory diseases, either involving specific retinal autoimmunity or an imbalance in immunological control mechanisms (reviewed by Forrester et al., 1986). This thesis has sought to further characterise S-antigen, and to examine the specificity of autoimmunity in the RCS rat retinal dystrophic model, human uveitis, and RP.

Sensitive ELISA assays were developed in order to investigate humoral responses in RCS rats, and RP and uveitis patients to purified S-antigen, and rod outer segment membranes. In the rat study, it was possible to measure the antibody responses of individual animals at different times during the degenerative process, and the animals were tested at the ages of 3, 6 and 9 weeks, ie. early, middle and late stages. Four of the ten Campbell RCS rats exhibited positive titres to S-antigen, and weaker reponses to ROS in the middle to late stages of the degeneration. The pigmented Hunter RCS rats did not demonstrate responses significantly different from the controls. One Wistar albino control rat was also significantly positive to S-antigen at the age

of 6 weeks. It was interesting that the highest responses were obtained from the pink-eyed or albino animals, suggesting that light induced retinal changes could have a role in the initiation of autosensitisation. In immunoblots, two of the positive Campbell rat sera, and the one positive Wistar serum, identified S-antigen, plus a 40K antigen in the crude retinal extract. One Campbell rat also appeared to recognise a 35K antigen. Autoantibodies to opsin were not readily demonstrated in immunoblots, perhaps due to denaturation of the relevant epitopes during the procedure.

Five of the six uveitis patients' sera tested in the ELISA, demonstrated titres raised above those of the controls, either to S-antigen or to the ROS, but not to both antigens. The five RP patients tested, generated serum responses within the range of the ten normal sera. All of the positive sera, regardless of ELISA specificity, reacted with S-antigen and a 40K antigen in the crude retinal extract. Human antibodies to opsin were not detected in the immunoblots.

The potential humoral autoimmune responses to retinal antigens was further examined by the temporary immortalisation of the B lymphocytes of humans and rats. Circulating B lymphocytes from RP and uveitis patients were transformed by Epstein Barr virus (EBV), cultured and assayed for anti-retinal antibody secretion. Using this technology, several clones of transformed B cells producing antibody reactive against S-antigen and ROS could be detected in some RP and uveitis patients, but not in the healthy control subjects tested. The splenic lymphocytes of both strains of dystrophic RCS rats and pigmented PVG control rats were immortalised using the hybridoma technology of Köhler and Milstein

(1975), and similarly assayed. In the rat system, 14 out of 249 primary clones, derived from 5 fusions with RCS rat B lymphocytes, demonstrated reactivity in ELISA to S-antigen and/or ROS. Similar positive clones were not detected in the 3 control fusions.

Three Hunter RCS hybridoma lines secreting autoantibody to S-antigen and ROS were subcloned to attain stability and monoclonality, before further study. One of these clones, H9.1.C2, secreted antibody reactive with S-antigen. A second monoclonal autoantibody, H9.5.A4, was reactive against ROS in the ELISA but was unreactive in immunoblots, presumably due to the denaturation of the specific epitope upon binding to the nitrocellulose paper. The third antibody, H10.1.C2, crossreacted with S-antigen and opsin in ELISA in immunoblotting. The crossreaction may have resulted from a shared ammino acid sequence or a common carbohydrate group, or perhaps more likely, from dual specificity of the monoclonal antibody. All three monoclonal autoantibodies were of the IgM class and were of relatively low affinity, demonstrating low antibody titres.

In addition to the anti-retinal monoclonal autoantibodies, two monoclonal antibodies were produced from hyperimmunised DA rats. Monoclonal antibody, S2.4.C5 raised against S-antigen, indicated high specificity in immunoblots against crude retinal antigens and irrelevant antigens. This monoclonal antibody was shown to be of the IgG1 isotype, and demonstrated a relatively high titre and high affinity in the ELISA. This antibody was used in the immunocytochemical localisation of S-antigen at the ultrastructural level on human and pig photoreceptor ROS disc membranes visualised by gold probes. Labelling of the outer segments was more intense

compared with the inner segments, although the perinuclear region of the rods was fairly intensely stained. In addition the S-antigen was also localised on apparently newly phagocytosed ROS membranes within the retinal pigment epithelial (RPE) cells. IgM antibody, R1.2.D2, raised against ROS membranes, was reactive against an antigen with an apparent molecular weight of 39K in immunoblots. This antibody was also reactive at the electron microscopical level, specifically labelling the disc membranes of the rod cells in human and pig retinae. In contrast to the localisation of S-antigen, the 39K antigen appeared to be virtually absent from the inner segments.

In conclusion, certain patients with uveoretinal diseases might become sensitised to their retinal antigens, although it was not possible to deduce the immunopathogenicity of the response. This preliminary study using B lymphocyte transformations, in conjunction with ELISA assays, has indicated the potential of the technique to dissect the humoral immune response of patients without the complications of nonspecific antibody reactions present in low serum dilutions. Similar retinal autoimmune responses appear to develop in RCS rats. However, humoral autoimminity was only detected in a relatively small number of individuals, in particular the Campbell rats, suggesting that the phenomenon is not general and is unlikely to be involved in the initiation of the disease. S-antigen appears to be one of the dominant potential autoantigens in the retina, as indicated by the immunoblotting results. However, there seems to be at least one other important antigen, with an apparent molecular weight of 40K. This antigen may be the bearer of the epitope recognised by the IgM antibody, R1.2.D2.

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Abbreviations

The abbreviations used are those recommended by the Biochemical Journal in 'Instructions to Authors', 1981, with the following exceptions:

A	Aminopterin
A280	Absorbance at 280nm
A492	Absorbance at 492nm
Ab	Antibody
APC	Antigen presenting cell
BSA	Bovine serum albumin
CFA	Complete Freund's adjuvant
CIC	Circulating immune complexes
CsA	Cyclosporin A
EAU	Experimental autoimmune uveoretinitis
EBV	Epstein-Barr virus
ELISA	Enzyme linked immunosorbent assay
ERG	Electroretinogram
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Y-INF	Gamma interferon
HAT	Hypoxanthine aminopterin thymine
HT	Hypoxanthine thymine
Ig	Immunoglobulin
IgE,IgM,IgG	Immunoglobulin classes; E, M, G
IL-1,IL-2	Interleukin 1, 2
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
ONL	Outer nuclear layer (of retina)

PAS	Periodic acid Schiff's (reagent)
PEG	Polyethylene glycol
P/S	Penicillin/streptomycin
PVG	Piebald Virol Glaxo (strain of rat)
RCS	Royal College of Surgeons
RK	Rhodopsin kinase
ROS	Rod outer segment (of photo receptor rod cell)
RIS	Rod inner segment
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
RPMI	Rosewell Park Memorial Institute (medium)
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRBCs	Sheep red blood cells

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CHAPTER ONE

INTRODUCTION

Chapter 1

INTRODUCTION

1.1 LOCATION, STRUCTURE AND FUNCTION OF THE RETINA

1.1.1 The Anatomy of the Eye

The basic structure of the eye is similar in all classes of vertebrates (Figure 1.1). The outer coat of the eye is a tough, collagenous layer known as the sclera. The sclera is continuous with the transparent cornea. Adjacent to the sclera is a second layer of tissue, the uvea, which is pigmented and regionally differentiated to form the choroid, the ciliary body and the iris. A pigmented monolayer of cells, the retinal pigment epithelium (RPE) is intermediary between the vascular choroid and the neural retina. The neural retina forms a complex layer of cells, including the photoreceptors, which line the interior of the optic cup. The photoreceptor cells are closely associated with the RPE cells. The transparent vitreous body is an intraocular tissue with a gel-like structure, which occupies four-fifths of the volume of the globe. The transparent aqueous humour, located behind the cornea, is more fluid in form than the vitreous. The aqueous humour and vitreous assist in the maintenance of intraocular pressure. The corneal curvature and the lens are responsible for focussing transmitted light onto the retina.

1.1.2 The Neural Retina

The neural retina consists of a highly organised layer of six different kinds of neurons, including the photoreceptor rod and cone cells (Figure 1.2). The second order neurons, the horizontal and bipolar cells, synapse with the photoreceptors. The bipolar

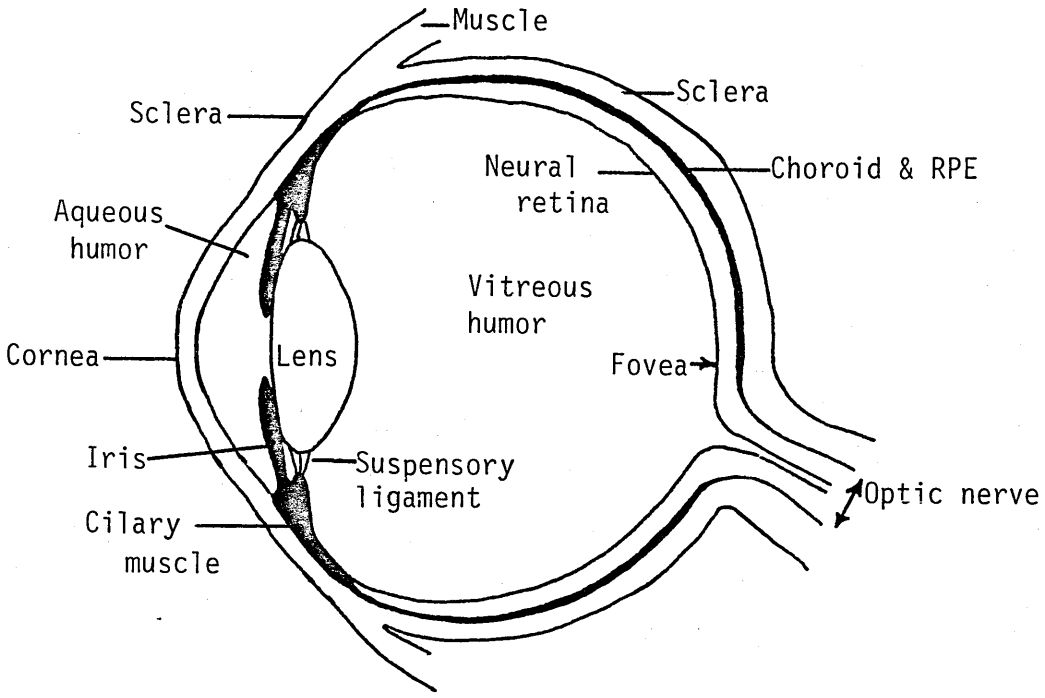


Figure 1.1 Diagram Showing the Gross Anatomy of the Mammalian Eye

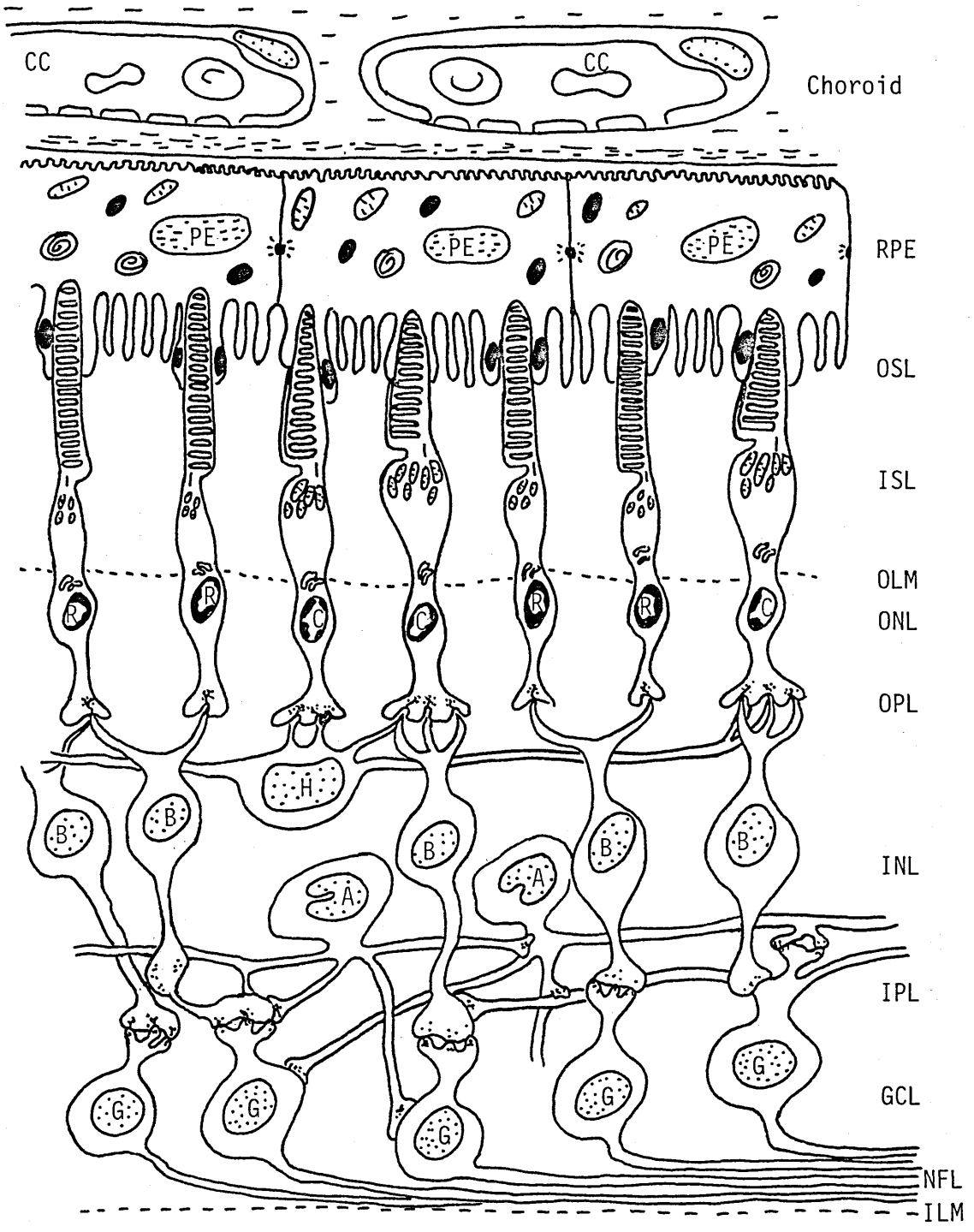


Figure 1.2 Diagramatic Representation of the Interrelationships of Cells in the Retina. Cell types: A=amicrine cell; B=bipolar cell; G=ganglion cell; H=horizontal cell; PE=pigment epithelial cell; CC=choriocapillaris; C=cone cell; R=rod cell. Layers of retina: RPE=retinal pigment epithelium; OSL=outer segment layer; ISL=inner segment layer; OLM=outer limiting membrane; ONL=outer nuclear layer; OPL=outer plexiform layer; INL=inner nuclear layer IPL=inner plexiform layer; GCL=ganglion cell layer; NFL=nerve fiber layer; ILM=inner limiting membrane. (Modified from Dowling and Boycott, 1966).

cells form synapses with the ganglion cells. The amacrine cells are accessory cells, and also synapse with the ganglion cells. The nuclei of the closely spaced photoreceptor cell bodies form the outer nuclear layer, and those of the second order neurons form the inner nuclear layer. The outer and inner plexiform layers consist of the axons and synapses between the cells of the neural retina (Figure 1.2). Incident light passes through these various layers before it reaches the light sensitive photoreceptor outer segments.

The photoreceptor cells are separated by Müller cell processes. The precise function of the Müller cells is unclear, although they may offer physical support to the retina. The tight junctions between the photoreceptor and the Müller cell membranes, form the external limiting membrane which is located below the photoreceptor nuclei (Figures 1.2 and 1.3). The photoreceptor cells and microvilli of the PRE do not completely fill the subretinal space, but are surrounded by the interphotoreceptor matrix (IPM). The IPM contains proteins which are distinct from those of the cells which it surrounds (Adler and Severin, 1981; Adler and Klucznik, 1982). The IPM proteins are synthesised and secreted by the RPE and, in particular, the retina (Adler and Severin, 1981) which is responsible for the production of interphotoreceptor retinol binding protein (Bridges et al., 1984).

1.1.3 The Photoreceptor Cells

Vertebrates may possess two basic photoreceptor cell types; the rods and the cones (Figure 1.3). These terms were originally coined by early nineteenth century biologists, to describe the morphological appearance of the distal tips of the cells at the level of the light microscope. However, the morphology of the two

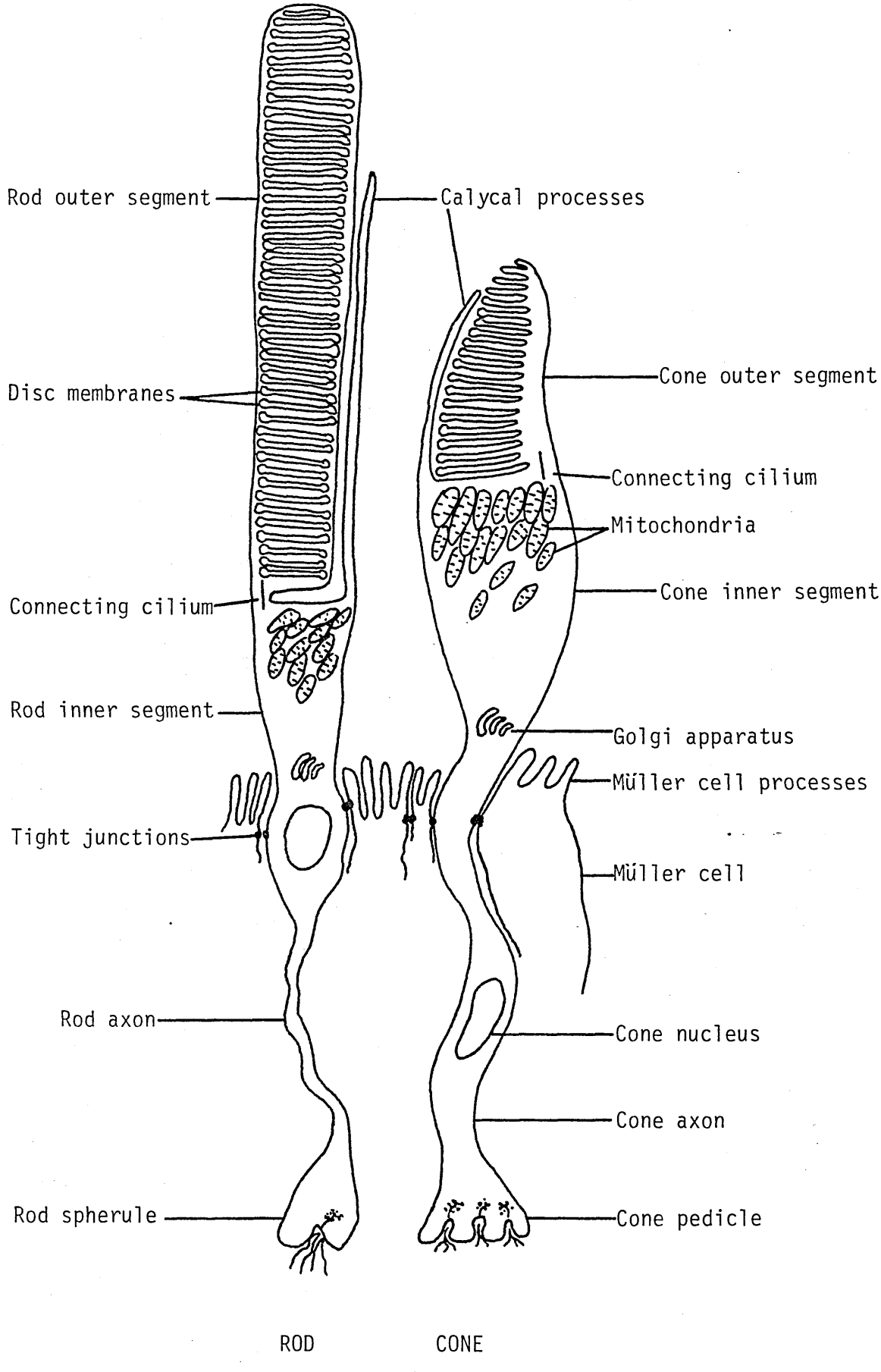


Figure 1.3 Diagram Showing the Main Features of Mammalian Photoreceptor Rod and Cone Cells (modified from Nilsson, 1964)

photoreceptor types is not always distinct, for example foveal cones are cylindrical rather than tapered, and are difficult to distinguish from rod cells. The rod cells are primarily responsible for vision in low light intensities, detecting different intensities of light, and are also involved in the perception of shape. The rods generally form the dominant cell type. A human retina has approximately 1.2×10^8 rods and 6×10^6 cones (Østerberg, 1935). The ratio of rods to cones is greater in nocturnal animals; for example in the rat the ratio is 4000:1 and in man it is 20:1 (La Vail, 1976c). The cone cells are the mediators of colour vision, and in mammals there appears to be distinct cone receptors which discriminate discrete bands of the spectrum, red, blue and green. The fovea, in man, is a region of the retina, 1.5mm in diameter, in the centre of the visual axis, which is composed virtually exclusively of cones (see Fein and Szuts, 1982). Towards the periphery of the visual field, the frequency of the cones decreases.

The photoreceptor cells are highly differentiated (Figure 1.3). The main regions of the photoreceptor cells are the outer segments, inner segments, nuclear regions, axons and synapses. The outer segments are modified non-motile cilia which are outgrowths of the inner segments. Finger-like structures, known as calycal processes extend from the inner segments and lie parallel and in close proximity to the outer segments, and may have a supportive role. The inner segments can be subdivided into specialised regions. The apical ellipsoid regions, of the inner segments contain large, densely packed mitochondria. Proximal to the ellipsoid region is the paraboloid region which contains vacuoles and presumptive glycogen granules. Below the mitochondrial area is the myoid

region, characterised by rough endoplasmic reticulum (RER), Golgi apparatus and free polyribosomes. This region is also rich in contractile proteins, actin and myosin, which are responsible for photomechanical movements in some species (see Fein and Szuts, 1982). The photoreceptor cell bodies house the nuclei, and from this region, slender axons of approximately 1μm in diameter, extend to the synaptic terminals. The rod synapse is known as the spherule, and the larger cone synapse is referred to as the pedicle. The dendrites of the horizontal and bipolar cells, insert into the single deep invagination of each spherule, and the numerous shallow invaginations of the pedicles.

The outer segments are packed with thousands of flattened disc membranes (Figure 1.3), containing a high concentration of the visual pigments and the other proteins associated with the biochemistry of vision (Sections 1.2.1). The discs are orientated perpendicular to the long axis of the outer segments. The disc membranes are formed by invagination in the plasma membrane at the base of the outer segment. In typical rod outer segments (ROS), the disc membranes are pinched off and separated from the plasma membrane, and only the lower 10 to 20 discs are continuous with the plasma membrane. In contrast, typical cone disc membranes remain continuous with the plasma membrane, and are filled with extracellular material. The rims of the discs are not free-floating, but are attached to the plasma membrane by proteins which have recently been partially characterised (Molday et al., 1986). Microtubules are also located within the outer segments.

1.1.4 Role of the Retinal Pigment Epithelium

The apical membrane of the retinal pigment epithelium (RPE) is

closely associated with the photoreceptor cell layer, and the two cell types are embryologically related. The RPE cells are vital to the normal functioning and health of the photoreceptor cells. The basal membrane surface of the RPE has numerous infoldings, and forms one boundary of Bruch's membrane. The membranes of the choriocapillaris of the choroid, form the other boundary to Bruch's membrane. The basal membrane of the RPE is specialised in the uptake of nutrients, oxygen, and other metabolites from the choriocapillaris, for the support of the photoreceptors as well as itself; both cell types are metabolically very active.

The apical surface of the RPE is specialised in the phagocytosis of shed rod outer segment (ROS) membranes and also in the exchange of material from the interphotoreceptor matrix (IPM), including nutrients and metabolites. Numerous microvillus processes, which are important in phagocytosis, extend from the apical surface of the RPE, for a distance equal to at least twice the height of the RPE cell. The thicker of these processes, which may contain melanin granules, encase the tips of the photoreceptor cell outer segments (Figure 1.2).

The RPE cell are involved in the metabolism of retinol, a vitamin A derivative. The RPE cells receive retinol from the circulation, which is transported tightly complexed with serum retinol binding protein (SRBP). The RPE monolayer forms a permeability barrier, similar to that found in the brain, which does not allow proteins and most low molecular weight substances, to cross to the interstitial space. Thus the apical surfaces of the RPE cells have receptors for SRBP (see Heller, 1976). The retinol is transported through the cytoplasm of the RPE cells associated with a cellular

retinol binding protein (Heller, 1976). In the photoreceptor cell, the vitamin A derivative, in the form of retinal, provides the chromophore group for rhodopsin, the visual pigment. When rodopsin absorbs a photon of light, the chromophore is released in the form of retinol (Dowling, 1960). The retinol is transported through the interphotoreceptor matrix, associated with interphotoreceptor retinol binding proteins (IRBP) (Adler and Martin, 1982). The retinol is transported to the RPE were it is esterified and stored (Berman et al., 1979). These transport processes are reversed in the dark. In light adapted retinae, the majority of retinol is bound to IRBP, in the IPM (Adler and Martin, 1982).

The outer segments of many vertebrates, including the rat, are continually renewed at their base and trimmed at their tips by the RPE (Young, 1967, 1971, 1974). The considerable proliferation of the rhodopsin containing membranes is one of the most characteristic features of the photoreceptor cells. New discs are formed at the base of the outer segments, thus displacing the existing membranes. The renewal process was determined by the use of tritiated amino acids, eg. methionine, leucine, histidine and phenalalanine, injected into live frogs, rats, mice and rhesus monkeys (Young, 1967; Young and Droz, 1968; Young and Bok, 1969; Bok and Hall, 1971). The animals were killed at different time intervals, their retinae fixed and sectioned for autoradiography. The results suggested that the precursors of the visual pigment, and other cellular proteins, were synthesised from the labelled amino acids, primarily in the myoid region of the photoreceptors. The labelled proteins appeared to migrate through the inner segment, many via the Golgi apparatus, through the connecting cilium and subsequently incorporated into the newly forming disc

membranes (Young and Droz, 1968). The labelled band of discs was displaced along the ROS as new, less intensely labelled discs were formed. The labelling of a band of discs more likely represented membrane proteins, such as rhodopsin, rather than the soluble proteins which would not remain confined to discrete regions. The older discs were discarded at the outer segment tips, and removed by phagocytosis. Young (1971) reported that in the rhesus monkey, groups of 8 to 30 discs were shed at a time. Electron microscopically, the edges of the discs appeared to curl upwards, displacing a small portion of cytoplasm into the space thus formed (Young, 1971). The plasma membrane of the outer segment indents, separating the shed package of discs and sealing the tip of the outer segment. Cytoplasmic extensions, originating from the RPE, surround the shed group of discs, and phagocytosis proceeds. Bok and Hall (1971) found that complete renewal of disc membranes was achieved after 8 days in rats, although the time varies with species.

Phagosomes containing ROS membrane material undergoing lysosomal degradation are found within the RPE cells (Young and Bok, 1969; Young, 1971). It has been suggested that the lysosomes of the RPE are specialised in their content of hydrolytic enzymes, for the degradation of ROS membranes (Zimmerman et al., 1983). At least some protein epitopes, eg. of S-antigen (Section 1.2), may be detected within the RPE phagosomes prior to complete digestion (Section 8.5).

In the developing retina, the RPE actively phagocytoses ROS membranes before the ROS have reached their maximum length (Herron et al., 1969; Bok and Hall, 1971; Tamai and Chader, 1979). The rate

of disc production must exceed the disc shedding until the maximum ROS length is reached, and a balance achieved. The adult pattern of shedding in the rat is reached at 3 to 4 weeks after birth. Under normal circumstances, there is a balance between synthesis and phagocytosis of disc membranes. The RCS rat model for inherited retinal degeneration, possesses an RPE which demonstrates a failure to phagocytose shed ROS membranes (Section 1.5.5). It is possible that some forms of human retinal degeneration have similar RPE abnormalities. Ripps et al. (1978) suggest that in some forms of human autosomal dominant retinitis pigmentosa (AD RP), there may be an imbalance in the renewal system such that the rate of disc shedding and phagocytosis is greater than their production, thus resulting in a progressive shortening of the ROS. Further investigations, however, are required to establish this.

Evidence suggests that the processes of photoreceptor disc shedding and phagocytosis in rats are light stimulated and follow a circadian rhythm, such that the packets of discs are shed at daybreak (La Vail, 1976b). However, these processes will continue in an animal which has already been exposed to the light cycle, if the light stimulus is absent. Maximal disc shedding in the rat, however, occurs 1 to 2 hours after the onset of light (La Vail, 1976a,b). Tamai and Chader (1979) present evidence that circadian rhythm of disc shedding and phagocytosis, is established at a very early age in the development of the rat retina, ie. when the eyes open at about 14 to 15 days. In addition, no difference was observed in the development of the ROS disc shedding and phagocytosis, in rats raised under dim or bright lighting conditions.

There is a growing number of reports supporting the view that there exists specific receptor-ligand interactions, which are involved in disc shedding and phagocytosis (see Colley and Hall, 1986). Colley and Hall (1986) produced evidence suggesting that the ligands on ROS surfaces do not vary in the relation to the shedding cycle, or that if cyclic alterations in components of the ROS surface do occur, they are not stable during the isolation procedure.

Finally, the RPE has a role in the absorption of 'stray' light particles which escape absorption by the photoreceptors. The is achieved by the numerous light absorbing pigment granules, containing melanin.

1.2 RETINAL S-ANTIGEN

1.2.1 Introduction

S-antigen is a soluble protein extractable from mammalian photoreceptor rod outer segments, with a molecular weight in the region of 50K. S-antigen is so called because of its high solubility. Since its discovery (Wacker, 1973), S-antigen has stimulated much interest with respect to its role in photoreceptor function and its ability to induce retinal autoimmunity (Sections 1.2.3 and 1.3.3). The protein was originally identified as a highly uveitopathogenic factor present within the photorecepter rod outer segments (Wacker, 1973) (Section 1.6.2).

Some groups have reported the absence of carbohydrate on S-antigen (Wacker et al., 1977; Beneski et al., 1984), however, the protein may have a small carbohydrate moiety, since it has been shown to stain positively with periodic acid-Schiff's (PAS) reagent in

SDS-polyacrylamide gels (Borthwick and Forrester, 1983). Wacker et al. (1977) suggested that there may be a small amount of lipid associated with the protein. There has been some discrepancy of the molecular weight of S-antigen (reviewed by Broekhuyse and Bessems, 1985) (Section 8.1). Recently, it has been suggested that S-antigen is the 48K protein, described by Kühn (1978), although the precise molecular weight is likely to vary between species.

There have been relatively few reports on the properties of human S-antigen, since the human tissue is difficult to obtain in quantity. Beneski et al. (1984), found that human S-antigen demonstrated similar properties to that of the bovine retina. The molecular weight was found to be 52K by SDS-PAGE and 48K by high pressure liquid chromatography (HPLC). The isoelectric point of the human protein was 5.7, which is similar to the value of 5.5 reported for bovine S-antigen (Borthwick and Forrester, 1983). The amino acid composition of bovine and human S-antigens were similar, except that there was almost twice the amount of glycine in the human protein (Beneski et al., 1984). In addition, the most common amino acids in the human S-antigen were aspartic acid and leucine, whereas in the bovine protein, aspartic acid and glutamine were the most abundant. The human S-antigen was demonstrated to have a similar capacity for the induction of experimental autoimmune uveitis (EAU) in guinea pigs (Beneski et al., 1984).

1.2.2 Cellular Localisation of S-Antigen and Species Specificty

S-antigen was first localised by Wacker et al., (1977) using antisera to purified S-antigen, throughout the photoreceptor rod cells in an immunofluorescence procedure. Using similar techniques, S-antigen has also been localised within the mammalian pineal gland

(Kalsow and Wacker, 1977; Korf et al., 1985), which is phylogenetically related to the retina (reviewed by Wiechmann, 1986). Furthermore, S-antigen has been identified within fixed sections of human photoreceptor derived retinoblastoma cells, using a monoclonal antibody in an immunoperoxidase technique (Donoso et al., 1985).

S-antigen appears to be well conserved; antibodies raised against the S-antigen from one species cross-reacts with the same protein of different species (Dorey and Faure, 1977; Wacker et al., 1977; Tuyen et al., 1982). Dorey and Faure (1977) obtained a reaction of identity in Ouchterloney immunodiffusion assays, with human, guinea pig, rabbit, bovine and possibly sheep, pig and horse S-antigens, using guinea pig antisera to homologous retina. Wacker et al. (1977) found that purified bovine S-antigen migrated as a doublet in SDS-PAGE, and that one of these bands gave a reaction of idenity with the guinea pig protein, and the other band gave a reaction of only partial identity with guinea pig S-antigen. De Kozak et al. (1981) found that S-antigen extracted from different species demonstrated similar capacities to induce EAU in rats. Tuyen et al. (1982) suggested that the conserved nature of S-antigen is not complete, since the sera of rats immunised with S-antigens extracted from different mammalian species, reacted most strongly with the immunising S-antigen in the enzyme linked immunosorbent assay (ELISA). Faure et al., (1984), Mirshahi et al., (1984) and Mirshahi et al., (1985) have studied the phylogenic distribution of distinct epitopes of S-antigen using mouse monoclonal antibodies. At least some epitopes seem to date back to the Cambrian period, being present in the ciliary type of photoreceptors (Mirshahi et al., 1984). These authors have detected epitopes of S-antigen in

the photoreceptors and pinealocytes of species from every class of vertebrates, and in the photoreceptors of certain invertebrates, using mouse monoclonal antibodies and rat polyclonal antisera to S-antigen, in immunofluorescence techniques. Epitopes of S-antigen could even be detected in annelids and molluscs, although not in the two species from the class of Arthropoda which were tested (Mirshahi et al., 1985). The photoreceptor outer segments of the retina and pineal organ were consistently stained, and the inner segments and synapses were usually also stained (Mirshahi et al., 1984). One of the monoclonal antibodies was specific for bovine S-antigen, which further suggested a diversion between the mammalian proteins (Faure et al., 1984).

At the level of the electron microscope, the protein has been localised primarily on the rod disc membranes (Broekhuyse et al., 1984a; McKechnie et al., 1986) using conventional polyclonal antisera with immunocytochemical techniques. The ultrastructural localisation of S-antigen is further discussed in the context of the findings of the present study (Section 8.5).

1.2.3 Physiological Role of S-antigen

The biological function of S-antigen remains obscure. Wacker et al. (1977) first suggested that it may be the 50K subunit of ROS cytosol retinol binding protein. Later, it was speculated that it might be the photoreceptor phosphodiesterase (Faure, 1980). Nussenblatt et al. (1981c) presented immunological evidence which suggested that S-antigen might be rhodopsin kinase (RK), a 67K protein (Kühn, 1978). The evidence was based on the crossreactivity of antiserum to S-antigen with RK, the presence of RK activity within preparations of S-antigen, inhibition of RK activity with

antiserum to S-antigen and the similar capacities of RK to induce EAU. The significant difference in the molecular weights of the two proteins was not discussed, and the experimental observations probably resulted from contaminating RK within the preparations of S-antigen, and vice versa.

At present, it is generally accepted that S-antigen is the soluble 48K ROS protein described by Kühn (1978). The 48K protein is characterised by its light-dependent binding to photoactivated, phosphorylated rhodopsin (the visual pigment) on disc membranes (Kühn, 1978; Kühn et al., 1984), and the inhibition of the light dependent activity of guanosine 3',5'-monophosphate (cyclic GMP)-phosphodiesterase. The 48K protein is purified from ROS using a procedure based on its ability to bind to illuminated disc membranes (Kühn, 1982). Evidence for the identity of S-antigen with the 48K protein, was based on their co-migration in SDS-PAGE with apparent molecular weights in the region of 50K, the ability for both proteins to bind to illuminated ROS disc membranes, the crossreactivity of monoclonal antibodies to S-antigen with the 48K protein, similar titration curves of specific antisera in radioimmunoassay (RIA) to both antigens, and the similarity in severity of ocular inflammation in EAU experiments (Pfister et al., 1985). The mechanism of interaction of the 48K protein (or S-antigen) suggested by Kühn et al., 1984) is described below.

Rhodopsin, an integral membrane protein, has been extensively characterised (reviewed by Hargrave, 1982). The pigment is synthesised from a 39K glycoprotein, opsin, and the chromophore group 11-cis retinol, a vitamin A derivative, which dissociates from the protein upon the absorption of a photon, while rhodopsin

undergoes a series of stereoscopic and conformational transitions (see Kaupp and Koch, 1986). This results in a chain of amplified biochemical and electrophysiological events initiating vision (reviewed by Stryer et al., 1981 and Chabre, 1985). The α-subunit of transducin, the rod G protein, interacts with the photolysed rhodopsin intermediate, metarhodopsin II, which forms within a few milliseconds, to form a complex. Rhodopsin acts as a catalyst, causing the exchange of GDP bound to transducin, for GTP. A single molecule of rhodopsin, activated by one photon, has the capacity for 500 such conversions, this is the first stage in the amplification. The second step is not amplified. Each activated, GTP-transducin, then complexes with one phosphodiesterase (PDE), a peripheral membrane protein, which is then activated, probably by the release of an inhibitory factor, to rapidly hydrolyse cyclic GMP (cGMP). The hydrolysis of cGMP is highly amplified, since one activated PDE molecule is capable of hydrolysing 2000 molecules of cGMP per second. The absorption of a single photon can be amplified by the enzyme cascade, by a factor of 10^6.

The consequence of the decrease in the levels of cGMP is the closure of the ROS plasma membrane sodium channels, which results in the hyperpolarisation of the membrane. This is detected by the photoreceptor synapses, resulting in nerve signal firing and vision. The precise mechanisms of channel opening and closing are not yet clear. The light dependent closing of the Na^+ channels has also been found to be associated with a rise in the concentration of Ca^{2+} ions. Recent evidence suggests that the Ca^{2+} might have at least two effects on light-dependent conductance, either by directly regulating ion permeability or by regulation of cGMP concentration (see Kaupp and Koch, 1986).

The photochemical reactions are inhibited, or controlled, by hydrolysis of GTP bound to α-transducin, to GDP, as a result of a slow intrinsic GTPase activity (see Kaupp and Koch, 1986). At least two other proteins might be involved in the modulation of the light activation of PDE, rhodopsin kinase and the 48K protein. Rhodopsin kinase (RK) phoshorylates photobleached rhodopsin, transferring up to 9 phosphate groups onto the cytoplasmic surface of the protein (Wilden and Kühn, 1982). Wilden et al. (1986) studied the effect of ATP and the 48K protein, in the deactivation of the PDE system, using thoroughly washed disc membranes, whose rhodopsin had been previously phosphorylated and chromophore group regenerated. To this system, purified PDE and transducin were reassociated. It was found that such phosphorylated membranes demonstrated a significantly lower capacity compared to unphosphorylated control membranes. The addition of the 48K protein to the phosphorylated membranes further suppressed the activation of PDE. Depending on the concentration of the 48K protein and the intensity of the light flash, this suppression could be as high as 98%. The 48K protein was not able to influence the activation or deactivation of PDE activity on unphoshorlyated membranes (Wilden et al., 1986).

The binding of the 48K protein to the disc membranes was thought to be through phoshorylated rhodopsin, and appeared to be slow, and involved in inhibition of the interaction of transducin with rhodopsin, thus reducing the activation of PDE (Kühn et al., 1984; Wilden et al., 1986). Thus, if S-antigen is the 48K protein, it may have an important role in the regulation of phototransduction, in many species of vertebrates and invertebrates. Further work, however, is required in order to establish that S-antigen is, in

fact, the 48K protein, and to clarify the nature of the binding of the 48K protein to phosphorylated disc membranes.

1.3 EXPERIMENTALLY INDUCED RETINAL AUTOIMMUNITY

1.3.1 Introduction

A theory of ocular autoimmune reactions which might explain the observed histopathology was developed early this century in relation to human sympathetic uveitis (see Vannas et al., 1960). Since this time the theory has been extended to other human ocular conditions. Sympathetic uveitis is a condition which can develop in a healthy eye, a few weeks or months after injury to the other eye. The uninjured eye develops granulomatous uveitis, apparently in sympathy with the traumatised eye. The pathophysiology of this condition is unclear, although it is generally held that cell mediated immunity to uveal antigens is responsible (reveiwed by Wakefield et al., 1982). However, there is little evidence for autoimmunity to uveal rather than retinal antigens.

Hess and Römer (1906) first discovered the antigenicity of the ROS, and suggested that anti-ROS antibodies may be involved in human retinal disease. They further suggested that normally there exists a barrier at the level of the RPE or the choriocapillaris, which is now known as the blood-retinal barrier. S-antigen has been implicated as one of the potential autoantigens (Section 1.3.3) since, when injected into experimental animals it has the capacity to induce immunohistopathological changes similar to those seen in human sympathetic uveitis (Wacker et al., 1979).

1.3.2 Experimental Autoimmune Uveitis

Experimental allergic (or autoimmune) uveitis (or uveoretinitis)

(EAU) has a long history (reviewed by Faure, 1980). The term was introduced by Wacker and Lipton (1965), to describe the autoimmune experimental disease in guinea pigs induced by immunisation with homologous retina. EAU generally resulted in inflammatory cell infiltration of the vitreous, choroid and retina, with the eventual destruction of the photoreceptor cell layer. In 1968(a,b), Wacker and Lipton presented evidence for the superiority of the retina in inducing uveitis, compared with the uvea and other ocular antigens. The first evidence for the immunopathogenicity of the isolated ROS, in guinea pigs, was produced by Faure et al., in 1973.

It is important to identify and characterise the relevant ocular antigens in EAU, and to determine their involvement in human uveitis and retinitis pigmentosa. Proteins which have been partially characterised, eg. S-antigen and more recently, interphotoreceptor binding protein, have been found to be capable of inducing EAU (Sections 1.3.3 to 1.3.4) and provide models for human uveitis (Section 1.4.4). The similarities in the immunopathology and immune response of animals with EAU and human patients with certain diseases of the uvea and retina, imply that EAU may provide a useful model for research into the pathogenesis and therapy of such human conditions.

1.3.3 S-antigen induced EAU

S-antigen was first identified by Wacker in 1973, as the primary aetiological agent in EAU, being highly antigenic and capable of inducing the condition in microgram doses (Wacker, 1973; Wacker et al., 1977). There was a striking increase in immunopathogenicity with increasing purity of the S-antigen preparation, on a weight basis. An incidence of 100% EAU of mild to moderate severity was

obtained in guinea pigs immunised with as little as 1-5µg of the purified protein (Wacker et al., 1977). Several other laboratories have reported similar findings (Dorey and Faure, 1977; Faure et al., 1981; Forrester et al., 1985). This is in contrast to the much greater quantities (1 to 4mg) required to induce EAU with ROS, in guinea pigs (Faure et al., 1973; Meyers and Pettit, 1975; Meyers, 1976).

Forrester et al. (1985) describe the morphological changes at the ultrastructural level in the guinea pig retina induced by immunisation with purified bovine S-antigen. Single injections of S-antigen in adjuvant, 17 or 100µg of protein, resulted in focal chorioretinitis, characterised by the selective destruction of the outer retinal layers. The inflammation observed in the choroid and retina consisted almost entirely of monocytes. Although each focal lesion rapidly progressed to the end stage after 2 to 3 weeks, new lesions continued to appear and the inflammatory activity in the vitreous became chronic. Forrester et al. (1985) supported the view that the ROS are the immune target tissue in EAU, which appeared swollen with the loss of discrete discs. Large macrophages were identified within the subretinal space and appeared to phagocytose the ROS debris. EAU provides a useful model for certain human ocular conditions, for example uveitis (Brinkman et al., 1980a; Newsome and Nussenblatt, 1984; Chant et al., 1985) (Section 1.4.4). The pathology of S-antigen induced EAU is focal in rats and guinea pigs (Broekhuyse et al., 1986; Forrester et al., 1985), as are many posterior human uveitis conditions, and human RP may also be focal in its early stages.

1.3.4 Other Uveitopathogenic Antigens

1.3.4.1 Rhodopsin

Early reports on the uveitopathogenic activity of rhodopsin (or opsin) was based on experiments with isolated ROS (Wong et al., 1975; Faure et al., 1973). However, although these antigen preparations may represent over 80% opsin, recent evidence suggests that S-antigen, a highly uveitopathogenic agent (Section 1.3.2), is not readily removed form the ROS (Broekhuyse et al., 1986). More recently, the ability of purified rhodopsin or opsin, to induce EAU has been investigated. Marak et al. (1980), reported chorioretinitis and inflammation in guinea pigs immunised with 1mg of rhodopsin in adjuvant. The inflammation extended into the outer retina only in the most severe cases, sparing the inner retina. The abilities of rhodopsin and opsin, to induce EAU, were compared and no significant difference was found (Marak et al., 1980). Meyers-Eliott et al. (1983), reported similar findings in guinea pigs injected with smaller doses of purified rhodopsin (250 or 500µg, in adjuvant). The outer retinal layer was destroyed, without substantial inflammation of the retina.

Broekhuyse et al. (1984), investigated the rat as a model for rhodopsin induced EAU, since this animal, like man, has a vascular retina. In Lewis rats, high doses, 250µg of rhodopsin, were found to induce moderate to severe disease, with retinitis predominating after 2 weeks. Within a few days, the photoreceptor cell layer was destroyed. Lymphocytes obtained from these animals, generated high responses in the lymphocyte transformation assay in the presence of rhodopsin. Lower doses of rhodopsin seldom induced severe retinitis (Broekhuyse et al., 1984). The authors also pointed out that the method of solubilisation of the rhodopsin could be important in uveitopathogenesis, since they found lithium dodecyl sulphate

solubilised rhodopsin to be less antigenic and immunopathogenic compared with Triton X-100 solubilised material.

The morphology of S-antigen and rhodopsin induced EAU, described by different groups varies in some respects, perhaps due to differences in the animal species or strain, anatomy of the retina, preparations of S-antigen or the immunisation schedules (see Broekhuyse et al., 1984; Forrester et al., 1985; Broekhuyse et al., 1986).

1.3.4.2 Rhodopsin kinase

There is evidence that rhodopsin kinase (RK) may have a similar uveitopathogenic capacity to that of S-antigen (Nussenblatt et al., 1981c). The similarities between the histopathological pictures of EAU induced by RK and S-antigen led the authors to suggest that they are one and the same protein (see also Section 1.2.3). Both antigens induced a focal degeneration of the photoreceptor cell layer when 30µg of protein, in adjuvant, was injected into Lewis rats. However, since no evidence for the purity of these preparations was presented, and given that S-antigen has been found to be capable of inducing EAU with as little 1 to 5µg (Wacker et al., 1977), further work is required to establish the uveitopathogenicity of RK.

Broekhuyse et al. (1986) found Lewis rats to be more susceptible to the interphotoreceptor retinal binding protein (IRBP) induced disease, compared with Wistar, PVG, and BN rats. Thus, it is possible that different strains of rat may be more or less sensitive to EAU induction by RK and S-antigen, thus differentiating between the two antigens.

1.3.4.3 Interphotoreceptor retinol binding protein

Interphotoreceptor retinol binding protein (IRBP) (see Section 1.1.4), derived from the matrix surrounding the photoreceptor cells, has recently been found to have a capacity to induce EAU in Lewis rats, in microgram quantities, similar to S-antigen (Broekhuyse et al., 1986). Doses of 15 to 80µg of protein in complete Fruend's adjuvant, induced moderate to severe inflammation in almost all rats after 9 days. The disease induced by IRBP demonstrated an earlier onset, progressed more rapidly, and appeared slighly more severe than S-antigen induced EAU. Like S-antigen, IRBP induced EAU affected the anterior segment of the eye initially, followed by severe inflammation of the posterior segment, 1 to 2 days later. In opsin induced disease, where very much larger doses are required (Section 1.3.4.1), milder reactions occurred in the anterior segment, and retinitis was the predominant feature (Broekhuyse et al., 1986). The common feature in S-antigen, rhodopsin and IRBP induced EAU was the total destruction of the photoreceptor cell layer.

1.3.5 Evidence for T Cell involvement in EAU

There is strong evidence that EAU, whether induced by S-antigen or other retinal antigens, is dependent on T cell mediated mechanisms (Nussenblatt et al., 1980c; Nussenblatt et al., 1983b; Nussenblatt and Chan, 1985). In the presence of retinal antigens, macrophage migration inhibition and positive lymphocyte stimulation indices have been found in animals with EAU (de Kozak et al., 1974; Nussenblatt et al., 1980c), in addition to serum antibody. Moreover, EAU can be adoptively transferred to naive guinea pigs, by the intraperitoneal injection of peripheral blood lymphocytes or

spleen cells from donors immunised with S-antigen, providing that these in vivo sensitised cells were stimulated in vitro with S-antigen or concanavalin A (Mochizuki et al., 1985). Passive transfer of ROS induced EAU, has been successfully obtained in guinea pigs injected with freshly harvested lymphoid cells (Aronson et al., 1971; Meyers, 1976). Adoptive transfer of EAU, with hyperimmune serum has been largely unsuccessful (Quinby and Wacker, 1967; Aronson et al., 1971).

Cyclosporin A, an immunosuppressive drug derived from a fungal product, has been found to be effective in the prevention of EAU in rats, further supporting the importance of T cell mediated mechanisms (Nussenblatt et al., 1981b; Nussenblatt et al., 1982; Nussenblatt et al., 1983b). A large amount of information, sometimes conflicting, has accumulated over the last decade, on the mechanisms of action of CsA (reviewed by Shevach, 1985). The most dramatic effect ot the drug is the prevention of T cell proliferation in the presence of mitogens and alloantigens. The drug probably acts on T-helper cells by preventing the induction of cell-surface receptors for IL2, or by the inhibition of its secretion (see Shevach, 1985).

Further evidence that the induction of EAU requires T cell mediated mechanisms comes from studies with the athymic nude rat (Salinas-Carmona et al., 1982). These animals were incapable of producing humoral antibody or cell-mediated responses to S-antigen after immunisation, and were refractory to the induction of EAU. However, the athymic rats developed EAU when injected with sensitised, in vitro stimulated lymphocytes. The disease could not be transferred by serum alone (Salinas-Carmona et al., 1982).

1.4 OCULAR AUTOIMMUNITY IN HUMAN EYE DISEASE

There is evidence that the immune system may be involved in human retinal inflammtory and degenerative diseases (Sections 1.4.3.3 to 1.4.4). Little is known of the precise effector mechanisms involved in retinal degeneration, such as RP, and inflammatory eye diseases, and autoimmune mechanisms have been implicated, particularly in uveitis.

1.4.1 Aetiology of Autoimmunity

Autoimmune disease is thought to arise from defective mechanisms of tolerance to self-antigens. Since the immune system has access to the majority of self proteins, and other potential autoantigens, there must exist a mechanism for the prevention of autosensitisation. Burnett's clonal deletion theory has, for several years, been central to discussion of autoimmune disease. During development after birth, a state of tolerance or unresponsiveness to self antigens is achieved, in particular to the major common antigens. This theory holds that certain tissue specific antigens, such as thyroglobulin, and lens, retina and brain proteins, are sequestered early during development and escape tolerisation. These antigens, if exposed to the immune system in the adult, are not recognised as self, but are treated as foreign. The destruction of certain cells, such as the photoreceptors, and the release of their antigens, may stimulate the immune system. For example, Gregerson *et al*. (1982) detected a raised serum antibody response to bovine S-antigen in some diabetic retinopathy patients after argon laser photocoagulation treatment. The proliferation of the *Toxoplasma gondii* protozoons within retinal cells, and their subsequent rupture releasing the organisms, has also been found to

raise serum responses to S-antigen, the P-antigen, and the P59 antigen in the ELISA (Abrahams and Gregerson, 1982). These findings support the view that nonspecific retinal damage can initiate humoral immune resonses. The immunopathological significance of such autoantibodies is not known.

The concept of clonal deletion has been found to be too simple, since there is evidence that self reactive T and B lymphocytes normally exist within the adult. This is shown by the induction of experimental autoimmune disease in animal injected with autoantigens, for example thyroglobulin or retinal antigens in adjuvant. Furthermore, many autoantigens are 'seen' by circulating immunocompetent cells. For instance, thyroglobulin circulates in concentrations of 10 to 100µg per ml (Roitt, 1984).

Smith and Steinberg (1983) have emphasised the importance of a number of key observations in relation to autoimmune disease, namely, that self reactivity is normal, and that both normal immune responsiveness and normal immune regulation are based on self recognition. The general acceptance of the network theory of immune regulation (Jerne, 1974) has lead to the concept of homeostatic mechanisms which prevent the triggering of the normally present autoreactive T and B cells. The key to this system is the helper T cell (Th cell), which is activated by specific antigen presented, in the context of self histocompatibility Class II antigens, on the surface of antigen presenting cells (APC) (Unanne, 1984). APCs make up a group of cell types, and are primarily located in the skin epidermis, lymph nodes, spleen and thymus, where they are known as Langerhan's cells (skin), macrophages (lymph nodes and other tissues) and interdigitating cells and follicular dendritic cells

(lymphoid tissues) (see Roitt et al., 1985). The Th cells are stimulated by the APCs to help or induce T-effector or cytotoxic cells and B cells to proliferate. This requires the interaction of two leukocyte growth factors, macrophage derived interleukin I which induces Th cell proliferation, and interleukin II, secreted by Th cells, which stimulates the T-effector and B cells to divide. These mechanisms result in antibody production and/or autoaggressive cytolytic reactions, if the original antigen was an autoantigen.

The anti-self lymphocytes, which are normally present, are quiescent due to a lack of recognition of the autoantigens by specific helper T cells, ie. the helper cells are functionally absent, either due to clonal deletion or to activation of suppressor T cells (Ts cells). The Ts cells are activated simultaneously by the APCs, and probably prevent autoimmune disease by active suppression of potentially autoreactive T and B cells, and are either specific for antigen or idiotype, or they may also be nonspecific. Lack of T suppression leads to severe autoimmune disease, and defects in stem cell T precursors specific for Ts cells have been reported in certain animal models. Autoimmunity arises when this complex regulatory mechanism is circumvented, either as a defect in one or more Ts cell types, eg. antigen or idiotypic specific (Delves and Roitt, 1984), by direct activation of T-effector or Th cells by autoantigen, or by activation of T contrasuppressor cell (Tcs cell), which render the autoreactive Th cells resistant to suppression or by acting on the Ts cells (Roitt et al., 1985). However, the existence of Tcs cells is still equivocal (Roitt et al., 1985). Several mechanisms have been suggested in which the tolerised Th cells could be bypassed (see

Roitt et al., 1985). A foreign antigen or organism might bear an epitope which crossreacts with host antigen. Under these circumstances, Th cells recognising the foreign epitopes could supply help to the potentially autoreactive T and B lymphocytes, which recognise the crossreactive determinants. In another situation, drugs or other haptens can bind to autoantigens, forming a foreign determinant recognisable by Th cells, which may then provide help, inappropriately to lymphocytes reactive with the self antigen. Similarly, an alteration in the ammino acid sequence or structure of a self protein might initiate autoimmunity. Polyclonal activators, such as lipopolysaccharide (LPS) or the Epstein Barr virus (EBV) are able to stimulate lymphocytes directly and nonspecifically, bypassing T cell help. Although the latter phenomena are utilised experimently in vitro, there is little evidence for the importance of such mechanisms in vivo.

Finally, inappropriate expression of MHC Class II antigens (HLA-DR in humans or Ia in certain rodents) on a cell carrying an autoantigen could convert that cell into an antigen presenting cell, and enable it to stimulate autoreactive T-effector and Th cells. Recently, such a mechanism has been implicated in S-antigen induced EAU (Chan et al., 1986) where an inappropriate expression of Ia antigens was induced on the surface of the RPE cells.

Autoimmunity can result from quantitative imbalance in these homeostatic regulatory mechanisms, for example the amount of antibody produced or the degree of proliferation of an autoantigen. An autoimmune disease, however, is unlikely to result from a single 'factor', eg. clinically unaffected relatives of systemic lupus erythromatosis (SLE) patients, share with the patients a defect in

the generation of nonspecific Ts cells (see Roitt et al., 1985). Some of these factors may be nonimmunolgical, eg. genetic, environmental and even hormonal. This clearly has particular relevance in RP.

1.4.2 Tests of Immune Function

A wide variety of tests are available for the clinical assessment of immune function in disease states. These are classified as assays of humoral (B cell) or cellular (T cells and macrophages) immunity. However, it is clear that since immune responses involve the interactions of several cell types, making this classification too simple. Most tests of humoral immunity involve the assay of serum for autoreactive antibody, either in tissue sections (immunocytochemistry) or against purified antigens in complement fixation or solid phase immunoassays, eg. the enzyme linked immunosorbent assay (ELISA). In immunohistochemical assays, ideally the sera should be tested against the patient's own tissue, otherwise there may be nonspecific reactions due to differences in histocompatibility of the non-self antigens (Leopold, 1973). Testing for anti-retinal antibody, for example, is hindered because of the low titres of the patients tested, and the need for a highly sensitive assay (Rahi, 1973). The ELISA offers exquisite sensitivity. However, low affinity autoantibodies may be discouraged from binding due to the washing steps necessary in this assay.

The most frequently used assays for cell mediated immunity test T-effector cell functions, eg. the secretion of lymphokines by effector T cells (macrophage migration inhibition factor, chemotactic factor, procoagulant activity, etc.); the ability of

lymphocytes to proliferate in the presence of autoantigen or nonspecific mitogens, and the activity of cytotoxic T (Tc) cells. The detection of proliferation of lymphocytes to antigens does not give precise information about the type of cell responding, ie. T or B cells. Assays for Th or Ts cell activities have not been widely applied to clinical studies although indirect information regarding specific T cell functions has been extrapolated from data on T cell subset counts in peripheral blood.

Although the cell mediated immunity assays might be useful in detecting cellular hypersensitivity in ocular diseases, their diagnostic and prognostic value have yet to be proved, and the relationship between the in vitro lymphocyte reactions and immunity in vivo is yet to be established (Meyers, 1975). The relevance of finding sensitised cells in vitro can not be accurately interpreted until the nature of the cell relative to the disease under study and in which in vitro assay best reflects the function of the cell is known (Meyers, 1975).

1.4.3 Autoimmunity in Retinitis Pigmentosa

1.4.3.1 Retinitis Pigmentosa

Retinitis pigmentosa (RP) is a term which encompasses a group of hereditary progressive degenerations of the retina, characterised by night blindness, early loss of visual field eventually including the central vision, and typical pigmentary (bone-spicule) fundus changes with late attenuation of retinal arterioles and optic atrophy. Marmor et al. (1983) have defined RP as a set of progressive hereditary disorders that diffusely and primarily effect photoreceptor and pigment epithelial function. The electroretinogram is abnormal from an early stage and is eventually

lost. Expression of RP has been found to be extremely diverse in both clinical course and mode of inheritance. RP is the most frequently encountered inherited eye disease, affecting approximately 1 to 2 persons in every 5 thousand of the general population (Boughman et al., 1980).

RP is classified by generic type, topography and retinal involvement and severity of disease. The majority of cases are sporadic (simplex), without known affected relatives. However, almost 50% of cases show a marked familial tendency, although only a few cases are clearly dominant, recessive or X-linked recessive (Fishman, 1978; Berson et al., 1980; Jay, 1982; Hu, 1982). When a profound, progressive hearing loss is associated with RP, the condition is known as Usher's syndrome, and is usually a recessive condition. As more clinical and biochemical studies on RP are made, further subsets of the disease emerge and the classification becomes more complex (Fishman et al., 1985). The considerable heterogeneity of RP renders comparisons of laboratory studies difficult, and results often appear to conflict, for example in investigations of the immune mechanisms (Newsome and Fishman, 1986).

Although the modes of inheritance and clinical features of the disease are quite well known, the pathophysiology of the disease remains obscure. Pathological studies of human RP have been relatively infrequent, and for obvious reasons, the cases studied have usually demonstrated advanced disease (Mizumo and Nishida, 1967; Kolb and Gouras, 1974; Szamier et al., 1979; Goebel et al., 1985). These studies have shown extensive loss of the photoreceptor rod and cone cells, with changes in the RPE. The inner nuclear, and

outer plexiform layers also show disorganisation, and large pigment-laden macrophages are present in the areas corresponding to the bone-spicule pigmentation seen ophthalmoscopically. In these areas, the pigment epithelial cells appear devoid of melanin granules. Epiretinal membrane formation is also a constant feature of RP pathology (Szamier, 1981), and appears to develop at an early stage. Such membranes have a high content of laminin (Newsome and Hewitt, 1985) possibly laid down by migrating retinal glial cells.

1.4.3.2 Physiological alterations in RP

It is possible that the retinal degeneration is a result of a primary metabolic defect or physiological disorder to which the retina is particularly sensitive. Useful information has been gained by studies on more accessible tissues such as blood. For example, some groups of RP patients have been found to have subnormal concentrations of plasma taurine (Uma et al., 1983), a sulphonated amino acid, which exists in the retina in high concentrations, and has been found to be essential for its viability (Voaden, 1982). Furthermore, several forms of RP are associated with known metabolic diseases such as abetalipoproteinemia, Rafsum's disease (disorder of phytanic acid metabolism). In addition, it has been reported that the majority of RP men over the age of 35 years and also X-linked RP males, are hyperlipidaemic, compared to the unaffected spouses and siblings (Converse et al., 1983; Converse et al., 1985). In addition, serum docosahexaenoic acid (C22:6), thought to be specifically required by the photoreceptors, has been reported to be reduced in two X-linked and one autosomal dominant RP patient from three families (Converse et al., 1983). In view of these findings, it is interesting that photoreceptor degeneration in the RCS rat can be

significantly retarded by specific control of the diet, feeding with a mixture containing corn starch and 4% corn oil rather than with commercial lab chow (Pautler and Ennis, 1984).

Hussain and Voaden (1985) have reported that there may be an increase in erythrocyte osmotic fragility in the recessive forms of RP, and also certain forms of autosomal dominant RP. This may be a reflection of a defect in lipid metabolism as suggested by Converse et al. (1985), or an abnormality in a cytoskeletal protein such as spectrin. Both these factors are relevant to the visual cells, which have a specific membrane lipid composition, and also possess a spectrin-like cytoskeletal protein, thought to be involved in the maintenance of the normal photoreceptor outer segment structure (Wong and Molday, 1986). Thus, defects in either lipid metabolism or the synthesis of specific retinal proteins could produce defective visual cell function.

Recent studies on isolated Bruch's membrane have indicated another possible dimension to the RP problem (Hewitt and Newsome, 1985). Hewitt and Newsome (1985) found that the proteoglycans extracted from autosomal dominant RP Bruch's membrane were larger in size, than from aged matched controls. In addition, there was a dramatic increase in the proportion of heparan sulphate proteoglycan in the RP Bruch's membrane. Considering the structural and filtration properties of the proteoglycans, alterations such as these could affect the transport of nutrients and metabolites from the choriocapillaris to the RPE, and thus affect the whole retina (Hewitt and Newsome, 1985). Although the alterations in the Bruch's membrane may be secondary, such changes could exacerbate the retinal degeneration.

Much of the research into RP involves the *in vitro* culture of normal and dystrophic human or animal RPE cells, in order to investigate their behaviour. In view of the difficulties involved in differentiating between primary and secondary defects, and the infrequent availability of retina undergoing the early stages of degeneration, *in vitro* tissue culture systems, such as that described by Boulton *et al*. (1983), provide a valuable model system for both RP and normal RPE cells, and those derived from retinal dystrophic animal models. However, even in these systems data must be carefully interpreted since the culture environment may alter the cell to cell interactions. In addition, important factors and nutrients present *in vivo*, may be absent.

It has been suggested that the retinal damage might have an autoimmune component, although this is more likely to be secondary to biochemical or physiological cause. At present, the evidence for retinal autoimmunity in RP is somewhat tenuous (Newsome and Fishman, 1986), however there is evidence that abnormal immune regulation may play a role in certain forms of RP (Sections 1.4.3.4 to 1.4.3.6).

1.4.3.3 Evidence for Retinal Autoimmunity in RP

The hypothesis that there may be some immunological and/or inflammatory basis to RP is not new. Indeed, RP was originally thought to be post-inflammatory (hence its name) since it showed strong similarity to other post inflammatory or traumatic retinal pigmentary disturbances (otherwise known as pseudoretinitis pigmentosa). Vitreous cells are a constant feature of RP (Marmor *et al*., 1983), and there is frequent evidence of peripheral vascular

sheathing, suggestive of retinal vasculitis. Recent studies have shown that there is breakdown of the blood retinal barrier in RP, with inward transport of fluorescein (Mallick et al., 1984). Retinal vascular leakage in RP and other retinal/choroidal diseases, which could result form low grade inflammatory changes, will permit communication between retinal tissue and circulating immunocompetent cells. Thus, the chances of stimulating the normally present autoreactive cells to previously isolated antigens, will be increased. ROS antigens could break a possible state of immune tolerance, although the resulting immune response may not necessarily have a pathogenic role.

Yamamoto and Yamori, (1966), were among the first to discuss the possibility that autoimmune phenomena may play a role in the pathogenesis of RP. They suggest that opsin might enter the bloodstream of the patients and act as an autoantigen stimulating the production of anti-opsin antibody. The antibodies could then bind with opsin of the ROS and the resulting immune complexes could initiate inflammation.

1.4.3.4 Evidence for altered humoral immunity in RP

Total serum IgM and IgG in RP patients has been estimated in several studies. Raised serum IgM in some RP patients has been reported (Fessel, 1962; Rahi, 1973; and Spalton et al., 1978a,b). More recently, Garcia-Calderon et al. (1984), reported raised levels of IgM in only 2 out of 46 RP patients, and rheumatoid factor in 3 patients. In addition, Galbraith and Fundenberg (1984) reported a high frequency of raised serum antibody to human IgG (rheumatoid factor), which seemed confined to a subgroup of patients and was unrelated to mode of inheritance of the RP or to

severity of the disease. A rise in the level of serum IgM is indicative of chronic infection (Ganfors, 1979), and may suggest that the aetiology of RP may be associated with infectious/chronic inflammation. However, larger studies have not confirmed these findings (Newsome, 1985; and Spiro et al., 1984). The range of values for serum immunoglobulin concentrations, among the normal population, is probably too wide to identify subtle changes.

The presence of circulating immune complexes (CIC) in RP patients was investigated in a single study reported by Heredia et al. (1984) and Garcia-Calderon (1984). CIC were found in approximately 20 of 46 RP patients, but not in 100 control subjects. Furthermore, it was reported that there was a fall in the levels of circulating complement components C3 and C4. However, Spalton et al. (1978a,b) in earlier work, did not detect the breakdown products of complement in the serum of RP patients. It was suggested that the complexes could lodge in the ocular tissues and activate the complement system, thus resulting in cell lysis and also an increase in vascular permeability, which may serve to exacerbate the disease (Heredia et al., 1984). It is possible that in some cases, small amounts of immune complexes are formed and deposited locally and would only be detected in tissue studies (Spalton et al., 1978a,b). However, CIC could have a protective role in the sequestering of potentially lytic antibodies. The CIC identified by Heredia et al. (1984) were measured by polyethylene glycol (PEG), a method which has many inherent flaws. In addition, CIC can be demonstrated in the normal population, therefore strict attention must therefore be paid to the adequacy of controls in such studies.

Autoantibodies to retinal antigens in the sera of RP patients has

been sought by several investigators. Spalton et al. (1978a) observed that the sera of RP patients can react, at a 1 in 5 dilution, with rat photoreceptors, in an indirect fluorescent antibody assay on frozen, fixed rat photoreceptors. Chant et al. (1985) report that the undiluted sera of 43 out of 116 (37%) RP patients demonstrated reaction in the immunofluorescence assay, with the photoreceptor cells, or more frequently the whole retina. This was compared with 21 positive fluorescence reactions out of 64 (33%) non-RP ocular patients and 1 out of 40 controls (2%). There were, however, several experimental variables which the authors did not investigate, in particular serum dilution. The interpretation of these results is difficult since sera from some normal, healthy adults contains low titres of anti-smooth muscle antibody, and also fluoresces with rat photoreceptors due to crossreactions with the actin and myosin within the myoid region of the photoreceptors. Thus, it is difficult to distinguish autoantibody to retinal antigens from non-tissue specific antibody.

The complement fixation assay has also been used to investigate the humoral immune responses of RP patients to the soluble and insoluble fractions of human and bovine retinae, but no significant difference was found in comparison to controls (Brinkman et al., 1980). More precise information may be derived from antibody assays, using purified retinal antigens. Antibodies to retinal S-antigen (Section 1.2) have been found by some authors (reviewed by Newsome and Fishman, 1986). However, significant numbers of normal people have also been found to secrete antibody which reacts (or crossreacts) with S-antigen (Forrester, personal communication, 1986).

1.4.3.5 Evidence for altered cell mediated immunity in RP

There have been several studies of the cellular immune response in RP. Char et al. (1974), found anti-retinal cell mediated immunity in 17 of 20 patients with pigmentary retinal degenerations, including RP patients, using a ^{125}I-iododeoxyuridine (^{125}I-UDR) cytotoxicity assay against a long term tissue culture line derived from human retinoblastoma. However, no further work on this system has since been reported.

Lymphocyte proliferative responses of some RP patients to crude retinal extracts (Brinkman et al., 1980), and to S-antigen, particularly in Usher's patients (Newsome and Nussenblatt, 1984) have been reported. The lymphocytes of some RP patients were significantly stimulated when incubated in the presence of human soluble antigens and bovine ROS, but not to human insoluble antigens and bovine uveal antigens (Brinkman et al., 1980). In macrophage migration inhibition assays, the migration of leukocytes from patients with non-recordable ERGs were significantly more inhibited with purified rhodopsin than those from patients with subnormal ERGs and the controls (Brinkman et al., 1980). It is interesting that no significant difference between the patients and controls was obtained for either assay when bovine ROS, which contains mainly opsin, and human crude retinal insoluble antigens. Newsome and Nussenblatt (1984) reported that the lymphocytes from only 1 of 15 RP and 1 of 6 Usher's patient responded to crude human retinal antigen, and that a second Usher's patient responded to crude human choroid antigen. The choroid, however, contains many antigens with which any type of serum might react, for example histocompatibility antigens. When the responses to purified S-antigen were tested, 5 RP and 2 Usher's patients appeared

positive. However, others have been unable to confirm these results using purified bovine S-antigen (Hendricks and Fishman, 1985) and bovine ROS or human crude retinal extract (Chant et al., 1985).

There are two reports suggesting that the lymphocyte responsiveness to nonspecific mitotgens, such as phytohaemaglutinin, may be diminished in RP (Heredia et al., 1981; Galbraith and Fundenberg, 1984). These results have not been confirmed in a study involving 12 RP patients (see Forrester et al., 1986). Significant discrepancies were observed between tests performed on samples from the same individuals, taken on different days (Hendricks and Fishman, 1985), which rather casts doubt on the findings of previous studies. These authors do not support the hypothesis that retinal autoimmunity is associated with RP. Very similar observations were made by BenEzra et al. (1984), who performed lymphocyte stimulation assays using bovine retinal S-100 antigen in addition to S-antigen.

1.4.3.6 Lymphocyte subsets and lymphokines production in RP

There are recent reports relating an imbalance in the helper/suppressor T cell subsets, and also abnormal lymphokine production and Class II antigen expression in RP. These reports, however, have been contradictory. It has been suggested that there might be a subset of RP patients with a subnormal level of circulating T lymphocytes, and a reduction in T cell function (Galbraith and Fundenberg, 1984). In addition, Galbraith et al. 1984 reported a decrease in the ability of helper T cells, derived from RP patients, to form rosettes with transformed human B cells.

Hooks et al. (1983) reported normal levels of T and B lymphocytes

in RP and Usher's syndrome patients, but subnormal levels of γ-INF were detected in 11 out of 12 RP patients, although normal values were obtained for the Usher's patients. It was suggested that there may be a reduced number of γ-INF producing cells in RP or that there was a defect in its production. Alternatively there may be a fault in the population of macrophages, which are involved in the production of γ-INF by T cells. However, others have been unable to repeat these results, using a similar biological assay (BenEzra et al., 1984) or a sensitive RIA method for measuring γ-INF (Hendricks and Fishman, 1986). In addition, the production of IL-1 and IL-2, by RP patients' lymphocytes, was not obviously different from that of the lymphocytes from the controls (BenEzra et al., 1984). Furthermore, there was a marked varibility in the production of the lymphokines between the different individuals tested, and also within individuals, evident upon re-testing (BenEzra et al., 1984; Hendricks and Fishman, 1986). BenEzra et al. (1984) made recommendations for a large double masked longitudinal study of lymphokine production in patients and controls. However, even in such a study the results may still be equivocal, since these tests have such a large inherent variability, and are of limited value in studies involving heterogeneous populations.

The T lymphocyte subsets and γ-INF production were investigated by Hendricks and Fishman (1985; 1986). In their initial study they report a significant decrease in the number of helper T cells, using the Leu series of mouse monoclonal antibodies with specificities for human lymphocyte cell types, and the fluorescence activated cell sorter (FACS). However, the data suggested that although the mean of the RP group was below that of the controls, the majority of the points appeared within the normal range.

However, later Hendricks and Fishman (1986) reported the absolute number of the helper T cells per ml of blood in RP patients was normal in 33 RP and 16 Usher's patients. In addition, the natural killer (NK) cell activity in the patients was evaluated and found not to be significantly different from the controls (Hendricks and Fishman, 1986). It was concluded that there were no significant immunological abnormalities in RP and Usher's patients.

Detrick et al. (1985) have reported for the first time, the presence of Class II antigens (HLA-DR) on the surfaces of cultured normal human RPE cells (derived from 4 individuals). This expression appeared diminished on the surfaces of RPE cells derived from 4 RP post mortem eyes. Furthermore, the expression of HLA-DR antigens on the surfaces of circulating monocytes was diminished in all 19 RP patients tested, compared to 22 control subjects. This was correlated to a subnormal production of γ-INF, a potent regulator of HLA-DR antigen expression, since the expression of HLA-DR on the monocytes and RPE cells of RP patients could be raised to normal levels by incubation in the presence of exogenous γ-INF. Detrick et al. (1985) suggested that the genetic disorder of RP may be linked with a defect in γ-INF production. Further studies, however, have failed to verify these results (Detrick et al., 1986).

Thus, it is thus clear that the data from immunological studies in RP are conflicting, and the relevance of positive responses to the aetiology of RP is unclear. The evidence in support of retinal autoimmunity in RP is somewhat tenuous at present, not least due to the paucity of subjects and controls involved in the studies. Many of the contradictions in the published work arise form the

heterogeneity among the patient group, and inconsistency in the techniques between laboratories (Newsome and Fishman, 1986).

1.4.4 Autoimmunity in Uveitis

1.4.4.1 Uveitis

The term uveitis refers to the inflammation of the uveal tract. Uveitis is a heterogeneous group of diseases classified according to the major site of involvement. Anterior uveitis is frequently unilateral (affecting only one eye) and involves the iris and/or ciliary body. Anterior uveitis is associated with pain, blurred vision and other symptoms. The disease is usually relatively short lived, 2 to 4 weeks, with a tendency to recur. Posterior uveitis is usually bilateral, involves an inflammation of the choroid, retina and vitreous, and tends to form a chronic disease. The annual incidence of uveitis is 15 new cases per 100,000 of the general population. Thus, uveitis is a relatively common condition (see review by Wakefield *et al.*, 1982).

Uveitis may be associated with infectious agents such as viruses, bacteria, fungi, *Toxoplasma gondii* (protozoon) and *Toxocara canis* (dog round worm larvae), or with systemic diseases, for example sarcoidosis. However, 50% of cases are of unknown aetiology (Wakefield *et al.*, 1982). The inflammatory infiltrate into the uveal tissues and vitreous humour, consists of macrophages and lymphocytes, perhaps predominantly T lymphocytes (Kaplan *et al.*, 1982; De Abreu *et al.*, 1984). The means by which the inflammatory responses localise in the eye are not fully understood, and the role of autoimmune mechanisms are often investigated. Healing of the inflammatory lesions can result in scarring and a decrease in

visual acuity, if the central macular/foveal region is involved.

Associations' with the major histocompatability antigens, the human leucocyte antigens (HLA) may determine susceptibility to disease. There have been several reports of an association between the HLA-B27 antigen and susceptibility to different forms of the disease, including anterior uveitis (Andrews et al., 1979; Saari et al., 1981; Wakefield et al., 1982; Kahn et al., 1983; and McCoy et al., 1984). Wakefield et al. (1982), report that 37% of patients with uveitis were HLA-B27 positive, in an Australian population. Crews et al. (1979) reported an association between Bechet's disease, a form of uveitis, and HLA-B5.

Over the last 20 years, there have been several reports which have examined the potential autoimmune mechanisms involved in uveitis. Clearly the immune system is involved, although the specificity of the responses are yet to be established. The possible immune mechanisms include (1) exposure to an antigen, exogenous or endogenous, that causes proliferation of autoimmune effector cells, (2) an altered suppressor cell modulation, eg. a defect in suppressor cell activity, or a non-reponsiveness of the effector cells to their signals or, (3) the production of inducer cells that are not modulated (Nussenblatt et al., 1985). Research, either involving the experimental disease in animals, or the immune responsiveness of patients' lymphoid cells or serum antibody, has sought to dissect the nature of the immune mechanisms of uveitis. The striking success of immunosuppressive therapy with cyclosporin A (CsA) in preventing the development of EAU (Section 1.3.5), prompted preliminary use of the drug in human ocular inflammatory disease, and CsA has been used to treat uveitis patients with

varying success (Nussenblatt et al., 1983a; Karjalainen, 1984). The responses however, are likely to be complex, and perhaps variable due to the diverse nature of the disease (Nussenblatt et al., 1985). The present evidence suggests that the immune mechanisms involved in anterior and posterior uveitis may be different (reviewed by Nussenblatt et al., 1985). In general, however, evidence for altered immune mechanisms, and/or specific ocular autoimmunity, is somewhat contradictory, probably due to the great diversity of uveitis syndromes and also differences in the immunological methodology between laboratories.

1.4.4.2 Alterations in circulating immunoglobulins and autoantibody

Uveitis patients may demonstrate raised serum IgM, but the significance of this remains unknown, and may reflect a substantial systemic immune response (Kahn et al., 1983). Kahn et al. (1983), found that 62% of 975 uveitis patients demonstrate significant raised immunoglobulin levels, of either IgM (43.8% of cases), IgA (34%) or IgG (11.9%). Literature concerning the existence of raised Ig levels is contradictory, and the studies limited (see Kahn et al., 1983).

In seeking ocular autoimmunity, early investigators concentrated primarily on crude uveal antigen, which included choroid, RPE cells and retina. Later, after the establishment of the superior antigenicity of the retina, and in particular certain individual antigens (Sections 1.3.2-4), the potential humoral, and cell mediated immune responses of the patients could be dissected in greater detail. Using crude uveal antigen and a simple, but relatively insensitive, Ouchterlony immunodiffusion technique, Aronson et al. (1964) reported that 52% of patients possessed

anti-uveal serum antibody. This seemed to be related to the severity and duration of the disease. However, 22% of patients with ocular diseases not involving the retina (controls) also had such antibodies (Aronson et al., 1964; Aronson et al., 1966). Thus, the antibodies could be relatively nonspecific, and could be recognising non-tissue specific antigens. Using immunofluorescence, Audain et al. (1977) found that 4 out of 23 patients had anti-uveal antibody activity, and one had anti-nuclear activity, and a second anti-smooth muscle activity, and three anti-mitochondrial activity. Thus, the apparent anti-uveal antibody response could have been nonspecific. It was not clear if control sera were tested, or the serum dilution, if any, employed.

Recently a useful study has been made on the reactivity of 893 uveitits patients' sera to non-ocular antigens (Murray, 1986). Out of the whole group, over 40% had detectable levels of serum autoantibody. Antibodies to smooth muscle antigens were found in 23.1% of patients, 12.7% had autoantibody to nuclear material, 3% demonstrated antibody to gastric parietal cells, 1.8% had antibody to reticulin and 0.2% had serum reactivity to mitochondria. When the patients were categorised into 9 groups, only one clinical entity, patients with uveitis associated with juvenile chronic arthritis, showed reactions significantly different from the controls. Anti-nuclear antibody was detected in 77% of these patients. These findings have relevance to studies involving the testing of patients' sera on sections of retina in immunohistochemical assays since the photoreceptor cells contain nuclear and mitochondrial antigens, and also elements similar to those found in smooth muscle.

Better defined antigens have also been studied, using more sensitive techniques (Gregerson et al., 1981). Circulating antibody, reactive against ROS antigen, the particulate (P), both of which contain rhodopsin, the previously unreported P59 antigen, and S-antigen were detected in the sera of certain uveitis patients using the ELISA assay. However, although 91 patients were involved in the study, there were only 13 controls which probably was an insufficient number to cover the wide variability in the normal population. In addition, the patients' responses were highly variable, and the data rather open to interpretation. The majority of the patients' responses appear to lie well within the normal range.

The specificity of serum antibody from uveitis patients with reactivity to retinal antigens has also been studied by means of immune precipitation techniques (Gregerson and Abrahams, 1983). The authors found that the sera were capable of recognising at least 2 other antigens, in addition to S-antigen. However, only 6 patients and 3 controls were studied, rendering the data rather unconvincing. In addition, there were a few problems with the methodology employed, especially the use of crude human choroid and retina, which were heavily cross-contaminated containing large amounts of human immunoglobulin, which could also react with the rabbit anti-human IgG antibody employed to detect the patients antibody, after the addition of the labelled protein A.

1.4.4.3 Immune complexes

Immune complex mediated ocular tissue injury has been reported in rabbits with EAU (Howes and McKay, 1975). Char et al. (1979), have demonstrated a significant increase in immune complexes (of the IgM

type) in 10 of 12 patients with diffuse uveitis, most of whom did not have detectable autoantibody. Andrews et al. (1979) reported the presence of circulating immune complexes in 49% of all acute anterior uveitis patients. Immune complexes have been found in the sera and aqueous humour in a number of uveitis conditions (Dernouchamps et al., 1977). Depositions of Ig and C3, as well as Ig containing inflammatory cells, have been identified in iris biopsies (Audain et al., 1977). Although iris biopsies can be taken, and also samples of vitreous and aqueous humour removed, choroidal biopsies are more hazardous, and the situation in the choroid and retina may not reflect that in the vitreous. Further work, however, is required before the significance of immune complexes in the human eye can be determined.

1.4.4.4 Cell mediated immune mechanisms

In posterior uveitis, it is possible that mast cell factors induce an increase in vascular permeability of the choroidal blood vessels and choriocapillaris, thus facilitating the entry of immunocompetent cells. Under these circumstances the ROS antigens could break a possible state of immunological tolerance. It is perhaps significant that in rats with S-antigen induced uveitis, mast cells accumulate in the choroid, and degranulate, resulting in the influx of lymphocytes (de Kozak et al., 1981). Certain uveitis patients have been found to possess cell mediated immunity to purified bovine and human S-antigen and crude human retinal antigens, but not human choroid (Nussenblatt et al., 1980b). Out of 31 posterior uveitis patients, only 7 (22%) responded to S-antigen. This group had either active or inactive lesions, also suggesting that they might become sensitised to retinal antigens. However, only 9 control subjects were included (patients with anterior

uveitis) which renders the data relatively invalid. A larger control group is necessary before the significance of these results can be evaluated.

It has been suggested that there may be an altered suppressor cell function in uveitis patients, including an increase in the suppressor cell population (Nussenblatt et al., 1980a; Nussenblatt et al., 1983a; Nussenblatt et al., 1985) or a decrease in the helper T cells (de Abreu et al., 1984). However, Ts cells can be antigen specific (see Roitt et al., 1985) and these assays only involved the estimation of the quantity of Ts cells and T cell ratios. In addition, the population of circulating lymphocytes can vary on different days and also according to the physiological state of the subject. Several samples should be taken from each of a large number of well classified cases of uveitis, and age matched healthy controls, in order to establish these observations.

Clearly, further well controlled studies are required to answer many of the questions with respect to autoimmunity in uveitis.

1.5 RCS RAT MODEL FOR RETINITIS PIGMENTOSA

1.5.1 Value of Natural Animal Models for Retinitis Pigmentosa

Several potential animal models for human retinal degeneration have been discovered during the last 25 years (reviewed by Matuk, 1984). The RCS rat and the various mouse models for human retinal degeneration, have been extensively studied, offering the advantage of easy animal husbandry and short periods between generations. Two other, less well studied, forms of retinal degeneration in rats have been described (Lai et al., 1979; Lai et al., 1980). The

degeneration in the RCS rat, like RP, extends from the anterior retina. The peripheral retinal degeneration in aging Fisher 344 rats might also provide a suitable model for human RP (Lai et al., 1979). Progressive retinal atrophy (PRA) in the dog (Setter) was first described in 1911 (Magnusson, 1911). Since this time, PRA has been described in many breeds of dog throughout the world. Recently, progressive retinal degeneration, or atrophy, has been described in the Abyssinian cat (Narfstrom, 1983; Barnett and Curtis, 1985), a breed of Friesian cows (Bradley et al., 1982) and in the Rhode Island chicken (Ulshafer and Allen, 1985).

It is difficult to determine how relevant the RCS rat model, or any other animal retinal degenerations, is for human RP, since only very rarely are the early stages of the human disease available for histopathological investigation, and most studies have been made on the late stages. RP appears to present a variable clinical course, although this could be related to other genetic or environmental factors, whereas retinal dystrophic rats and mice are inbred, and therefore genetically fairly homogeneous. The similarities between human RP and animal progressive retinal degeneration, or dysplasia, include their genetic transmission, bilateral expression, retinal blood vessel attenuation, depigmentation of the RPE, similar end stage histologic picture and progressive loss of ERG and vision. However, at the electron microscopical level, the various retinal dystrophies may differ (Matuk, 1984), and could develop from completely different pathophysiological courses. It is possible that in RP the fault lies with the RPE, or photoreceptors or both. The photoreceptor cell is morphologically, physiologically and biochemically unique and complex, and a large number of genes are expected to maintain such a cell. There are likely to be several

avenues along which retinal instability and degeneration could proceed. There is a limited number of reactions which a given tissue can undergo in response to harmful genotypic changes. Thus, a common phenotype could develop from different mutations or environmental factors. As an example, severe vitamin A deficiency looks like the end stages of inherited retinal dystrophy of man, rats, mice and dogs (Dowling and Sidman, 1962).

During the last decade, with the aid of technical advances in molecular biology and physiology, much has been learned about the underlying mechanisms of photoreceptor degeneration. The use of animal models has also enabled a better understanding of the normal physiological processess of healthy retinal tissue. In addition, it is possible that research involving animal models, which may not be identical to the human condition, may reveal methods of controlling secondary pathological changes, possibly including retinal autoimmunity, which might be common to several forms of retinal degeneration, and thus slow the disease process.

The RCS rat model was utilised in the present study, in order to investigate further the possiblity of associated retinal autoimmunity (Sections 1.5.6 and 4.1).

1.5.2 The RCS Retinal Dystrophic Rat

Hereditary degeneration of the retina in the Royal College of Surgeons (RCS) rat was first described by Bourne et al. (1938a), although the strain was initially developed as a model for cataract. The degeneration is a recessive condition resulting in the progressive destruction of the photoreceptor cell layer (Section 1.5.3). The histopathology that develops in the RCS rat

is similar to that which develops in human retinitis pigmentosa (Bourne et al., 1938b). The original RCS rat is known as the Campbell strain and is a tan-hooded animal with pink eyes and a non-pigmented RPE. Since 1938, pigmented strains of the RCS rat have been developed (Herron et al., 1974; Yates et al., 1974; La Vail et al., 1975). The pigmented rat is perhaps a more useful model for human RP, since humans normally have a pigmented RPE. Several studies have been conducted in the search for the site of action of the recessive mutant gene causing retinal dystrophy in the RCS rat.

1.5.3 Retinal Degeneration in the RCS Rat

Rats, like other rodents, are born blind and vision develops when they are 9 to 19 days old (Dowling and Sidman, 1962). At 9 days, the nuclear layers of the retina are formed, and small inner segments project beyond the limiting membrane. The connecting cilia extend in the direction of the RPE, but there is little or no outer segment material until day 10. The outer segments rapidly develop, reaching the adult length of 23.5μm by day 39 (Dowling and Sidman, 1962). The normal relationship between the photoreceptors and the RPE cells is described in Section 1.1.4. The importance of the RPE cells in the health of the visual cells has been further exemplified by the investigations of the RCS rat RPE. The RCS rat demonstrates an almost absolute inability to phagocytose shed ROS tips (Dowling and Sidman, 1962; Herron, et al., 1969; Bok and Hall, 1971; Edwards and Szamier, 1977). The site of action for the defective gene causing retinal dystrophy in the RCS rat is now known to be expressed within the RPE (Mullen and La Vail, 1976; Edwards and Szamier, 1977).

Visual cell development in the RCS rat is normal until day 12, when the shed ROS membrane material begins to accumulate between the outer segments and the RPE. The accumulated material causes the retina to thicken. At day 22, when some inner segments and visual cell nuclei degenerate, and the ERG decreases in sensitivity (Dowling and Sidman, 1962). By day 32, the inner segments have almost entirely disappeared and thus the ability to synthesise ROS proteins is lost, and the rhodopsin content markedly decreases. The RPE cells have been reported to break away from the Bruch's membrane by day 40, and migrate through the ROS debris (Dowling and Sidman, 1962). However, others suggest that these cells are more likely to be macrophages (Essner and Gorrin, 1979). Cytoplasmic processes, emanating from the wandering RPE-like cells, extend through the debris. However, at no time has phagocytosis of the shed ROS tips been seen, either by the RPE cells in situ, or wandering RPE-cells seen in late stages of the degeneration (Dowling and Sidman, 1962; Herron, et al., 1969; Bok and Hall, 1971; Essner and Gorrin, 1979).

Through the second month of life, degeneration of nuclei and synapses occurs with complete loss of ERG, no normal photoreceptors can be seen, and the rat becomes completely blind (Dowling and Sidman, 1962; Herron et al., 1969). The eventual cause of photoreceptor cell death might, at least in part, result from a poor nutritional supply, since the presence of the photoreceptor material blocks the flow of nutrients from the choriocapillaris (Dowling and Sidman, 1962). After one year, the ROS debris zone almost disappears and there is the complete absence of a visual cell layer, although the RPE and layers of nerve cells showed no significant change (Dowling and Sidman, 1962; Eisenfeld, et al.,

1984).

1.5.4 Defective RPE Cells in the RCS Rat

The elegant work of Mullen and La Vail (1976), using experimental chimeras of albino RCS and pigmented normal rats, clearly suggests that the mutant gene is expressed in the RPE cells, and not in the visual cells. The difference in pigmentation allowed the identification of the two genotypes of the RPE cells. The retinae of the chimeras were a mosaic of mutant and genetically normal cells, and degenerating photoreceptors only occurred opposite the non-pigmented RPE. Patches of non-pigmented RPE greater than two cells, always had degenerating photoreceptors opposite them, although there was less disorganisation and death of photoreceptors opposite areas of only one mutant RPE cell.

Additional evidence that the mutant RCS gene is expressed in the RPE was obtained from *in vitro* phagocytic studies (Edwards and Szamier, 1977). Normal RPE began phagocytosing ROS one hour after incubation, irrespective of their origin. In contrast, most of the RCS RPE cells did not phagocytose the ROS fragments, even after 18 hours incubation.

1.5.5 Speculation on the Expression of the Dystrophic Gene

The mode of expression of the mutant gene, within the RCS RPE, has not yet been determined. Although the RCS RPE rarely phagocytoses ROS material, the cells appear to recognise it by extending microvillus processes in vivo (Dowling and Sidman, 1962; Herron *et al.*, 1969; and Bok and Hall, 1971) and *in vitro* (Edwards and Szamier, 1977). There are probably different genes for recognition, attachment and ingestion, the RCS rat lacking the latter gene (see

Colley and Hall, 1986). Edwards and Szamier, (1977) have shown that the mutant RCS RPE cells do not have a general defect in phagocytosis· since they are capable of phagocytosing polystyrene spheres when cultured in vitro, at a rate similar to that of normal RPE cells.

Early workers suggested that the inability to phagocytose may be a result from the lack of a diffusible substance, or an enzyme deficiency (Herron et al., 1969; Young, 1971; Mullen and La Vail, 1976). The presence of acid phosphatase has been demonstrated within the interphotoreceptor matrix (IPM), the activity being greatest near the RPE surface, suggesting that some predigestion of the ROS membranes, exposing new determinants, may be required for phagocytosis (Alder and Martin, 1983). There is evidence that the acid phosphatase positive lysosomes of RCS rat RPE, are fewer in number compared to normal, and that this enzyme may be inappropriately shunted to either the RPE microvilli or melanosomes, instead of the phagosomes (Seyfried-Williams and McLauchlin, 1984).

It has also been suggested that the rat retinal dystrophy might be initiated by the presence of lytic enzymes originating from the RPE (Burden et al., 1971). An increase in the activity of the albino RCS rat RPE lysosomal enzymes was detected, beginning after 4 weeks and progressing with degeneration. In rats as young as one week old, the lysosomes of the RCS RPE were less stable than those of control animals, and this instability appeared to increase with disease progression.

Recent evidence suggests that there may be an alteration in a

ligand-receptor mechanism involving mannose, contributing to the RCS rat RPE phagocytic defect, since cultured RCS RPE cells phagocytose fewer mannose coated latex beads than the controls (Seyfried-Williams and McLaughlin, 1983). In addition, the RCS RPE cells phagocytosed greater numbers of latex beads coated in lectins specific for mannose, suggesting that more mannosyl residues are accessible on the RCS RPE microvilli than normal RPE microvilli (McLauchlin et al., 1984).

1.5.6 Retinal Autoimmunity in the RCS Rat

The antigenicity of the retinal ROS has been well established (Section 1.3). In view of the accumulation of the shed photoreceptor tips in the RCS rat, it seems possible that the antigens released could stimulate the immune system in these animals. In addition, there may be a breakdown in the tight junctions between the RCS RPE cells (Caldwell and McLaughlin, 1984; Caldwell et al., 1984)), which may predispose the rats to a breaching of the blood-retinal barrier. There has been conflicting evidence, however, for the sensitisation of these rats to retinal proteins, and there is little evidence for an inflammatory reaction in the RCS rat retina.

IgM autoantibodies in RCS rat sera were detected using immunofluorescence techniques, from day 8 and throughout the degenerative process, but not in animals older than 7 to 12 months where degeneration is complete (Chant and Meyers-Elliott, 1982). The autoantibodies appeared to stain mainly the photoreceptor cell bodies, and not the ROS, perhaps recognising precursors of the ROS structures or certain cytoplasmic proteins. Furthermore, positive stimulation indices to bovine ROS and rhodopsin, were obtained with

RCS rat lymphocytes, in comparison with congenic controls (Chant and Meyers-Elliott, 1982). However, there are several problems in the interpretation of these results, for example the use of undiluted sera, which maximises the opportunity for nonspecific antibody interactions in immunocytochemistry. In addition, studies involving lymphocyte assay have been found to have a wide inherent variability, and in a study involving splenic lymphocytes, large numbers of animals would have to be employed, even in an inbred strain.

In contrast to these findings, Brinkman and Broekhuyse (1981), were unable to detect autoantibody to retinal antigens in neat sera from pink eyed RCS rats, using similar techniques. In addition, no difference was found between the dystrophic and control rats, when cell mediated immunity to rhodopsin, uveal pigment granules and soluble retinal proteins, was measured by a radiometric ear assay. The RCS rats were shown to be capable of responding to bovine rhodopsin when immunised (Brinkman and Broekhuyse, 1981). However, the radiometric ear assay was not compared with the more commonly used lymphocyte stimulation assay, and only 2 animals were tested from each selected age grouping (15, 40, 65, and 120 days). Similarly, no evidence has been found for a humoral autoimmune response to the RPE, in RCS rats (Reich-D'Almeido and Rahi, 1974). However, this study was very limited, since only animals of 3 weeks and 1.5 to 2 months old were investigated, and it was not clear how many rats in each group were studied, nor the dilution, if any, of the RCS rat sera used.

The concept of retinal autoimmunity in the RCS is further discussed in Sections 4.9 and 8.4, in the context of the findings of the present study.

1.6 EXPERIMENTAL OBJECTIVES

This study was undertaken in order to further investigate the association of retinal autoimmunity with ocular disease. S-antigen is one of the most potent mediators of EAU, and has been implicated in human ocular disease (Sections 1.3 and 1.4). In addition, there is evidence that the RCS retinal dystrophic rat might present anti-retinal immune reactivity (Section 1.5.6). However, the data concerning specific retinal autoimmune reactions in ocular patients, and RCS rats, is generally unconvincing and often contradictory.

In order to shed more light on the problem of humoral autoimmunity in the RCS rat, a detailed serum study was made, involving primarily a sensitive ELISA assay and immunoblotting, to test for antibodies to S-antigen and crude retinal antigens. The pink-eyed and pigmented strains of RCS rat involved in the investigation were inbred and therefore genetically fairly homogeneous. Thus, in the rat study it was more feasible to make comparisons between individuals, than in a heterogeneous human population. The RCS rat study also sought to isolate and partially characterise monoclonal autoantibodies with specificity for retinal antigens, from the potentially autoimmune animals.

This study also sought to investigate the value of techniques for the immortalisation of circulating B lymphocytes derived from uveitis and RP patients, in the analysis of their antibody specificity. The number of patients involved in this preliminary study was small, and thus the additional analysis of their ELSIA titres to retinal antigens was much more limited in comparison to

the rat study.

A further aspect of this work involved the development and characterisation of a specific rat monoclonal to S-antigen, for use in the further analysis of this protein, including its ultrastructural localisation. The effect of the purified monoclonal antibody on the progress of S-antigen induced EAU has also been studied. A second antibody, to a 39K retinal antigen was also produced, and used in electron immunocytochemical localisation studies.

CHAPTER TWO

MATERIALS AND METHODS

Chapter 2

MATERIALS AND METHODS

The materials and methods employed during the course of this study are described below.

2.1 MATERIALS

2.1.1 Major Suppliers

B.D.H. Chemicals Ltd., Poole, Dorset, England

Becton Dickinson Co. Ltd., Dublin.

Boehringer-Mannhein, Sussex, England

Costar Inc., Northumbria Biologicals Ltd., Northumbria, England

DIFCO Laboratories, Surrey, England

Dynatech Laboratories Ltd., Sussex, England

Fisons Scientific Apparatus, Loughborough, England

Flow Laboratories Ltd., Irvine, Scotland

Gibco Europe Ltd., Paisley, Scotland.

Koch-Light Laboratories Ltd., Haverhill, Suffolk, England

May and Baker Ltd., Dagenham, England

Miles Scientific Ltd., Slough, England

Nunc, Trident House, Paisley, Scotland

Pharmacia Fine Chemicals, Hounslow, Middlesex, England

Polaron Equipment Ltd., Watford, Hertfordshire, England

Sigma Chemical Company Ltd., Dorset, England

Sterilin Ltd., Teddington, Middlesex, England

2.1.2 Bovine Eyes

Cattle eyes were supplied by Sandyford Abattoir Accessories, Paisley, Scotland.

2.1.3 Protein Purification Materials

DEAE-Sephacel and also Chromatofocusing materials, in the form of a kit containing Polybuffer Exchanger 94 and Polybuffer 74, were obtained from Pharmacia Fine Chemicals. PD10 Sephadex, G25, prepacked columns, 9ml bed volume, were also supplied by Pharmacia. QAE-Sephadex was supplied by Sigma Chemical Co.

Dialysis tubing was obtained from Visking Tubing, Scientific Instruments Ltd., London.

2.1.4 Electrophoretic Materials

Acrylamide, N,N'-methylene bis acrylamide, N,N,N'N'-Tetramethylene diamine (TEMED) and ammonium persulphate were obtained from B.D.H. Chemicals. Koch-Light Laboratories supplied the 2-mercaptoethanol, and the low molecular weight protein markers were supplied by Pharmacia Fine Chemicals. Schleicher and Schuell, Surrey, England, supplied the 0.45μm nitrocellulose membrane filter paper.

2.1.5 Stains and Staining Reagents

Coomassie Blue (G and R forms) and Naphthol Blue black were obtained from Sigma Chemical Co. Schiff's reagent was also supplied by the same company. Johnson Matthey Chemicals, U.K. supplied the silver nitrate. Polaron Equipment Ltd. supplied the uranyl acetate, and the lead citrate was supplied by TAAB Laboratories, Reading, England.

2.1.6 Enzymes

Electrophoretically pure bovine trypsin and chymotrypsin were obtained from Worthington Biochemical Corporation, Freehold, New Jersey, USA. *Staphylococcus aureus* V8 protease was supplied by Sigma Chemical Co.

2.1.7 Radiochemicals

The Western Infirmary, Glasgow, Scotland, supplied the ^{125}I-NaI, with a specific activity of 7.5PBq/μmole. The iodogen was supplied by Pierce-Warriner, Chester, England.

2.1.8 Tissue fixing, Embedding and Mounting Materials

2.1.8.1 Fixatives

Paraformaldehyde, Sychemica grade, was obtained from Hopkin and Williams, Chadwell Heath, Essex, England. Gluteraldehyde, EM grade, was supplied by Sigma. Johnson Matthey Chemicals Ltd., UK, supplied the osmium tetroxide.

2.1.8.2 Materials for Light Microscopy

Xylene and D.P.X. mounting medium were supplied by BDH Chemicals Ltd.

2.1.8.3 Materials for Electron Microscopy (EM)

Lowicryl K4M, Dimethylformamide (D.M.F), Araldite CY212, dodecenyl succinic anhydride (D.D.S.A.), Dilutyl phthalate, benzyldimethylamine (B.D.M.A.), and nickel grids, G300 mesh size, were obtained from Polaron Equipment Ltd. LR White was supplied by Agar Aids, Hampshire, England. Propylene oxide (1,2-Epoxypropane) was obtained from Koch-Light Ltd.

2.1.9 Acids

Citric acid was obtained from BDH Chemicals Ltd. Glacial acetic acid and hydrochloric acid were supplied by May and Baker Ltd. Periodic acid was obtained from Sigma Chemical Co. Ltd. Phosphoric acid was supplied by BDH Chemicals Ltd. Sulphuric acid was obtained from Riedel-De Haen AG, Seelze-Hannover.

2.1.10 Detergents

Tween 20 (polyoxyethelene sorbiton monolaurate 20) was obtained from Sigma Chemical Co. Triton-X 100, cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) were supplied by BDH Chemicals Ltd.

2.1.11 Enzyme Substrates

Ortho-phenylenediamine (OPD), diaminobenzidine (DAB), and ortho-dianisidine were supplied by Sigma Chemical Co. Hydrogen peroxide was supplied by Fisons.

2.1.12 General Chemicals

Agarose (gel electrophoresis grade) was supplied by Bethesda Research Laboratories Ltd. 2-amminoethylisothiouronium bromide (AET), bovine serum albumin (BSA), histidine and poly-L-lysine (molecular weight 70K to 150K) were obtained from Sigma Chemical Co. Butan-2-ol was supplied by BDH chemicals Ltd. Complete Freund's adjuvant (H37 Ra) was supplied by DIFCO. EDTA (ethylenediaminetetra-acetic acid) was supplied by BDH Chemicals Ltd. Ammonia, ammonium sulphate (low in heavy metals), ammonium chloride and EDTA were obtained from BDH Chemicals Ltd. Formaldehyde, acetone and glycerol were obtained from May and Baker Ltd. Glycine was obtained from BDH Chemicals Ltd. Gold colloid, 10nm particle size, was obtained from Janssen Pharmaceutica Ltd., Beerse, Belgium. Hydrogen peroxide was obtained from Fisons. Ovalbumin was obtained from Pharmacia Fine Chemicals Ltd. Polyethylene glycol (PEG) 6000 was obtained from Serva Laboratories Ltd. Protein A (Cowan strain) was supplied by Sigma Chemical Co., and Miles Scientific Ltd. Formachem, Strathaven, Scotland, supplied the sucrose. Tris (hydroxymethyl) aminomethane (trizma base) was

obtained from Boehringer-Mannhein.

2.1.13 Preservatives

Benzamidine hydrochloride hydrate was supplied by Aldrich Chemical Company Ltd. PMSF (phenylmethylsulphonyl fluoride) was supplied by Boehringer-Mannhein. Sodium azide was obtained from Sigma Chemical Co.

2.1.14 Sodium Salts

Sodium chloride was obtained from Research International Ltd., Strathaven, Scotland. Fisons Scientific Apparatus supplied the sodium carbonate and sodium bicarbonate. Sodium di-hydrogen phosphate and di-sodium hydrogen phosphate were supplied by Riedel-De Haen AG, Seelze-Hannover. Sodium hydroxide was obtained from Koch-Light Ltd. May and Baker Ltd. supplied the sodium metabisulphite. Sodium periodate was obtained from BDH chemicals Ltd.

2.1.15 Materials for Immunological Assays

The microtitration ELISA plates were supplied by Dynatech Laboratories Ltd. The BSA blocking agent was obtained from Sigma Chemicals Ltd. Automatic micro-pipettes and multichannel micro-pipettes were obtained from Gilson Anachem Ltd., Luton, England, and from Flow Laboratories Ltd., respectively.

2.1.16 Immunological Reagents

Goat anti-human IgG(H+L) horseradish peroxidase conjugate, rabbit anti-human IgG(H+L), rabbit anti-human IgG+IgM+IgA, rabbit anti-rat IgG(H+L), rabbit anti-rat IgG(H+L) horseradish peroxidase and fluorescein conjugates, rabbit anti-rat IgM (μ chain specific), rabbit anti-rat IgG (γ chain specific) sheep anti-rat IgG1, goat

anti-rat IgG2a, rabbit anti-rat IgG2b, goat anti-rat IgG2c, were supplied by Miles Scientific Ltd.

Gold adsorbed protein A and goat anti-rat IgG(H+L), 5 and 10nm gold particle sizes, were obtained from Janssen Pharmaceutica Ltd., Beerse, Belgium.

Normal goat and normal rabbit sera were obtained from the Scottish Antibody Production Unit, Wishaw, Scotland

2.1.17 Animals

Sibling pairs of DA rats were obtained from OLAC Ltd., Blackthorn, Bicester, U.K., in 1982, and propagated by sibling mating. Piebald Virol Glaxo (PVG) rats, and the pink-eyed Campbell and pigmented Hunter strains of RCS rat, were supplied by Mrs Jean Hunter of the Pharmacology Department, Edinburgh University, Scotland. Wistar rats and Balb C mice were obtained from colonies propagated in the University of Glasgow. Experimental work generally required animals no younger than 3 weeks of age.

2.1.18 Cell Lines

The rat myeloma cell line, Y3-Ag.1.2.3 (Galfre et al., 1979), was obtained from Professor C. Milstein, MRC, Cambridge, England The KR4 human plasmacytoma cell line (Kozbor et al., 1982), and the EBV secreting marmosette cell line, B95-8 (Miller and Lipman, 1973), were obtained from Dr Dorothy Crawford, Hammersmith Hospital, London.

2.1.19 Disposable Cell Culture Plasticware

Cell culture plates of both 24 and 96-wells, and sterile petri dishes were supplied by Costar Inc., Northumbria Biologicals.

Screwcap plastic ampoules (capacity 1.8ml) were also obtained from Costar Inc. Tissue culture flasks of 50, 250 and 500ml were obtained from Nunc. Sterile universals and disposable pipettes of 1 and 5ml in capacity were obtained from Sterilin Ltd. Sterile 10 ml pipettes were obtained from Costar Inc. Sterile V-bottom tubes, 50ml capacity, were supplied by Becton Dickinson. The sterile o.2µm sterile filters were supplied by Flow. Northumbria Biologicals Ltd. Sterile plastic syringes of 1, 2, 5, 10 and 20ml in volume, and sterile needles gauge size 21, were obtained from Becton Dickinson Co. The gauge 25 needles were supplied by Gillette Surgical, Middlesex, England. A microaliquoter and fitting 5ml sterile syringes were obtained from SMI Scientific Manufacturing Industries Inc. Lithium heparin tubes were supplied by Sterilin Ltd.

2.1.20 Cell Culture Media and other Materials

Flow Laboratories, supplied the RPMI-1640 medium (constituents shown in Table 2.1). Gentamycin was obtained from Sigma Chemical Co. Foetal calf serum (virus and mycoplasma negative), the sheep red blood cells, penicillin and streptomycin, glutamine were supplied by Gibco Ltd. Ficoll-Paque and Percoll was supplied by Pharmacia Fine Chemicals.

2.1.21 Cell Culture Chemicals

Aminopterin was obtained from Flow laboratories Ltd. Dimethylsulphoxide (DMSO) was supplied by Koch-Light Laboratories. Hypoxanthine, thymidine and ouabain-octahydrate were obtained from Sigma Chemical Co. Cyclosporin A was obtained from Sandoz International, Switzerland. Polyethylene glycol (PEG) 1500 and 4000 were obtained from Serva, Heidelberg, Germany.

2.1.22 Photographic Materials

Table 2.1 Composition of RPMI 1640 medium

Amino acids	mg/litre
L-Arginine	200.00
L-Aspargine H_2O	56.82
L-Aspartic acid	20.00
L-Cystine disodium salt	59.15
L-Glutamic acid	20.00
L-Glutamine	300.00
Glycine	10.00
L-Histidine	15.00
L-Hydroxyproline	20.00
L-Isoleucine	50.00
L-Leucine	50.00
L-Lycine HCl	40.00
L-Methionine	15.00
L-Phenylalanine	15.00
L-Proline	20.00
L-Serine	30.00
L-Threonine	20.00
L-Tryptophan	5.00
L-Tyrosine	20.00
L-Valine	20.00
Vitamins	
Biotin	0.20
D-Calcium pantothenate	0.25
Choline Chloride	3.00
Folic acid	1.00
i-Inositol	35.00
Nicotinamide	1.00
p-Aminobenzoic acid	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20
Thiamin HCl	1.00
Vitamin B 12	0.005
Inorganic salts	
$Ca(NO_3)_2$	69.49
KCl	400.00
$MgSO_4.7H_2O$	100.00
NaCl	6000.00
$NaHCO_3$	2000.00
Na_2HPO_4	800.70
Other compounds	
Glucose	2000.00
Glutathione	1.00
Sodium phenol red	5.00

X-ray film (Kodak X-omats), SX-80 developer, FX-40 X-ray liquid fixer, and Kodak Ektachrome (ASA 160 and 400, for tungsten light) were supplied by Kodak Ltd., Hemel Hempstead, England.

2.1.23 Miscellaneous

Precision optic quartz cells were obtained from Thermal Syndicate Ltd., England. Plastic disposable cuvettes were supplied by Sastedt, Germany. Eppendorf tubes (capacity 1.5ml) were supplied by Geratebau, Netheler and Hinz, U.K. Scalpel blades were supplied by Swann Morton, Sheffield, England. The haemocytometer was obtained from Weber Scientific International Ltd., Sussex, England.

All other reagents were of analytical grade or of the highest available purity.

2.2 STANDARD BUFFERS AND SOLUTIONS

The solutions and buffers required for antigen isolation, protein purification, electrophoretic techniques immunological procedures and cell culture are specified below.

2.2.1 Retinal S-Antigen Purification Buffers

2.2.1.1 Hypotonic extraction buffer

2.5mM Tris

0.01% (w/v) Sodium azide

2mM Benzamidine

0.5mM PMSF

HCl to pH 7.9

2.2.1.2 Column starter buffer

20mM Tris

0.01% (w/v) Sodium azide

HCl to pH 7.5

2.2.1.3 Histidine/HCl

25mM Histidine

HCl to pH 6.2

2.2.2 Column Buffer for IgG Purification

1M Tris

HCl to pH 6.4

2.2.3 Solution for isolation of Rod Outer Segments

2.2.3.1 42% Sucrose solution

42% (w/v) Sucrose in distilled H O

2.2.3.2 Tris acetate buffer

1.0M Tris

Acetic acid to pH 7.4

2.2.3.3 Sucrose homogenising solution

1.14M sucrose

5mM Tris acetate buffer, pH 7.4

0.2mM MgCl

65mM NaCl

2.2.4 ELISA Buffers

2.2.4.1 PBS

145mM NaCl

9.0mM Na_2HPO_4

1.3mM NaH_2PO_4

Distilled H_2O to 1l

pH 7.6 for ELISA

pH 7.2 to 7.4 for immunocytochemistry

2.2.4.2 PBS-Tween wash buffer

PBS pH 7.6, with 0.05% (v/v) Tween 20

2.2.4.3 McIlvaines buffer, pH 6.0

Stock 0.1M Citric acid 17.9ml

Stock 0.2M Na_2HPO_4 32.1ml

Distilled H_2O to 100.0ml

2.2.4.4 ELISA peroxidase substrate

McIlvaines buffer; 10ml/ELISA plate

Orthophenyldiamine 4.0mg/10ml

Stock 30% Hydrogen peroxide 3.2µl/10ml

(Make up immediately prior to use)

2.2.5 Electrophoresis Buffers and solutions

2.2.5.1 30% Acrylamide

30% (w/v) Acrylamide

0.8% (w/v) Bisacrylamide

Distilled H_2O to 100ml

2.2.5.2 Resolving gel buffer

Stock solution

3.0M Tris

HCl to pH 8.8

2.2.5.3 Stacking gel buffer

0.5M Tris

HCl to pH 6.8

2.2.5.4 Reservoir buffer

0.25M Tris

1.92M Glycine

1% (w/v) SDS

pH 8

2.2.5.5 Sample buffer

0.0625M Tris

2% (w/v) SDS

10% (v/v) glycerol

5% (v/v) 2-mercaptoethanol

0.001% (w/v) Bromophenol blue

HCl to pH 6.8

2.2.5.6 Coomassie Blue stain and destain

Stain

0.1% (w/v) Coomassie Blue G250

25% (v/v) Methanol

10% (v/v) Glacial acetic acid

Destain

5% (v/v) Methanol, 7% (v/v) acetic acid in distilled H_2O

2.2.6 Immunoblotting Buffers

2.2.6.1 Electro-transfer buffer

25mM Tris

0.19M Glycine

0.03% (w/v) SDS

20% (v/v) Methanol

HCl to pH 8.6

Distilled H_2O to 3 litres

2.2.6.2 Wash buffer

20mM Tris

0.015M NaCl

0.05 or 0.5% (v/v) Tween 20

HCl to pH 7.2

2.2.7 Cell Culture Media

2.2.7.1 Complete medium

Foetal calf serum (FCS) (Section 2.1.20) was heat inactivated at 56°C for 30 minutes, and stored froozen at -20°C. Frequent freezing and thawing was avoided. FCS 2, 5, 10 or 20% (v/v) was added to RPMI (Section 2.1.20). The medium was usually supplemented with glutamine (Section 2.1.20), to give a final concentration of 50µg/ml of medium. Penicillin and streptomycin were also usually added to routine culture medium, to give a final concentration of 10^2I.U./ml and 100µg/ml, respectively.

2.2.7.2 HAT medium

The concentrations of hypoxanthine (H), thymine (T) and aminopterin (A) (Section 2.1.21) in the complete medium (Section 2.2.7.1) were as described by Littlefield (1964). 100 fold concentrated stocks of HT were made by dissolving 135.1mg of H, and 38.7mg of T in 100ml distilled H_2O. H was dissolved by stirring the mixture at 50°C for 60 minutes. The solution was filter sterilised through 0.2µ sterile filters (Section 2.1.19), and stored at -20°C in the dark. 1ml of the HT stock was added to 100ml of complete medium to give a final

concentration of 1 x 10^{-3}M of H and 1.6 x 10^{-4}M of T. HAT medium was made by adding 0.4ml of A stock to give a final concentration 4 x 10^{-7}M.

2.2.7.3 Freezing medium

20% (v/v) FCS, 15% (v/v) DMSO in RPMI

2.2.7.4 PEG medium

Polyethylene glycol (PEG) (Section 2.1.21) was sterilised by autoclaving at 15lb/sq.in. for 15 minutes. While it was still liquid, the PEG was mixed with warm RPMI to give a final concentration of 45 to 50%. Aliquots of 2ml were stored at -20°C.

2.2.8 Immunocytochemistry Buffers

2.2.8.1 Tris/HCl buffers

Wash buffer: 20mM Tris/HCl, pH 7.4

Incubation buffers: 20mm Tris/HCl 0.1% BSA, pH7.4

20mM Tris/HCl 1.0% BSA, pH8.4

2.2.8.2 Cacodylate buffer

0.2M Sodium cacodylate	33.0ml
2.0M Sucrose	12.5ml
HCl to pH 7.2	
Distilled H_2O to	100.0ml

2.2.8.3 Reynolds lead citrate

1.76g of sodium citrate, and 1.33g of lead nitrate were each dissolved separately in 15ml of distilled water and then combined. A white precipitate was formed. The mixture was agitated for 20 to 30 minutes, before the addition of 8ml of 1N NaOH. The volume was

then made up to 50ml with distilled H_2O. To minimise the formation of carbonates, the solution was passed through a millipore filter (Section 2.1.19).

2.2.8.4 DAB peroxidase substrate

3,3'-Diaminobenzidine tetra hydrochloride (Section 2.1.11) was dissolved in 20mM Tris/HCl, pH 7.6 to give a concentration of 0.75mg/ml. H_2O was added give a concenration of 0.01%.

2.3 GENERAL METHODS

2.3.1 Measurement of Total Protein by Reading Absorbances at 280nm

To give an approximate estimation of the total protein concentration, the samples were placed in quartz cells (Section 2.1.23) and their absorbances read at 280nm in a spectrophotometer. A reading of 1 absorbance unit at 280nm (A280) is approximately equivalent to a protein concentration of 1mg/ml. When the protein concentration of column fractions was measured, the column buffer served as a blank for the readings. Several substances are known to interfere with the absorbance at 280nm, for example detergents such as Triton X-100 (2.1.14) and chemicals like Benzamidine (Section 2.1.13).

2.3.2 Bradford's Protein Assay

The Bradford microprotein assay (Bradford, 1976) was employed to assess total protein during the purification of S-antigen and of monoclonal antibodies.

2.3.2.1 Solutions

1. Coomassie Blue in alcohol

Coomassie Brilliant Blue (G250), 0.1g in 50ml 95% (v/v) Ethanol

2. Phosphoric acid 85% (w/v), in distilled water

3. Protein reagent

The two solutions were mixed in a ratio of one part of solution 1 to two parts solution 2, and filtered. The volume was made up with distilled water to give the proportions of 5% solution 1 and 10% solution 2.

4. BSA protein standards

1mg/ml BSA in the appropriate buffer

2.3.2.2 Procedure

The protein standards were prepared in test tubes, in the same buffer as that of the sample, for either the mg or µg protein assay. For the mg assay, the dilutions were made from 1mg/ml to 0.1mg/ml, in volumes of 100µl. 5ml of the protein reagent was added to each tube and mixed by inversion or vortexing. 100µl of the protein samples were added to 5ml volumes of the protein reagent. The absorbances at 595nm were read in a spectrophotometer, and a standard curve was plotted in order to determine the protein concentration of the samples.

The procedure for the µg protein assay was similar; the standards were made up to give 0.1 to 0.01 mg/ml, and 2ml of protein reagent were added to each tube.

2.3.3 The Lowry Assay

The Lowry protein assay (Lowry *et al.*, 1951) was utilised to

estimate the total protein content of purified retinal ROS (Section 3.6).

2.3.3.1 Solutions

1. 2% (w/v) $NaCO_3$ in 0.1M NaOH

2. 0.5% (w/v) $CuSO_4.5H_2O$ in 1% potassium tartarate

3. Reagent A: 1ml of solution 2 mixed with 50ml of solution 1. This reagent was made up fresh before use.

4. Folin's reagent

5. BSA standard solution (1mg/ml)

2.3.3.2 Procedure

Tests tubes were set up in duplicate, containing 100µl volumes containing 10 to 100µg of BSA, in the appropriate buffer. 1ml of reagent A (Section 2.3.3.1) was added to each tube, followed by a 10 minute incubation. Then 0.1ml of Folin's reagent was added, the tubes mixed well, and incubated for a further 30 minutes. 100µl of buffer containing no protein, similarly treated served as a reagent blank. The optical densities were read at 750nm, and a standard curve plotted. The test samples were treated in the same manner and their protein concentrations determined from the standard curve.

2.3.4 SDS-Polyacrylamide Gel Electrophoresis

Protein samples were resolved according to their molecular weights, under the denaturing conditions of SDS-PAGE, with a discontinuous gel buffer system (Table 2.2), as described by Laemmli (1970). The

Table 2.2 Recipe for gel preparation

Stock Solutions	Stacking gel	Final acrylamide concentration in resolving gel*			
	3.3%	15%	12.5%	10%	8.5%
Acrylamide-bisacrylamide+ (30:0.8) ml	2.5	15.0	12.5	10.0	8.5
Stacking gel buffer stock+ ml	5.0	-	-	-	-
Resolving gel buffer stock+ ml	-	3.75	3.75	3.75	3.75
10% SDS ml	200	300	300	300	300
15% ammonium persulphate μl	100	150	150	150	150
Distilled water ml	12.3	10.45	12.95	15.45	16.95
TEMED μl	15	15	15	15	15
Total Volume ml	20	30	30	30	30

* Final concentration of buffers: Stacking gel; 0.125M Tris-HCl, pH 6.8
Resolving gel; 0.375M Tris-HCl, pH 8.8

+ Section 2.2.5

chemicals and reagents required for electrophoresis are listed in Sections 2.1.4 and 2.1.12. This system of protein detection provided a criterion for purity in the purification of S-antigen (Section 3.4.2) and one of the monoclonal antibodies (Section 6.5). The procedure is a prerequisite to the immunoblotting of antigens described in Section 2.3.9. Preparative gels were also employed for the excision of specific bands for Cleveland gel experiments (Section 2.3.8). Variations in the technique described below involve only the change in porosity of the gel matrix or in the size of the gel slab.

2.3.4.1 Gel and sample preparation and electrophoresis

The stock solutions for the preparation of SDS polyacrylamide gels are described in Section 2.2.5. The recipes for the various gel porosities employed for the experiments described in this work are described in Table 2.2, and are classified according to their percentages of acrylamide composing the gel. The gels were cast between glass gel plates at room temperature, or 40°C. Samples to be electrophoresed were mixed with sample buffer (Section 2.2.5.5) in a ratio of 4:1 and boiled for 2 minutes to solubilise with SDS.

The gels were assembled onto the electrophoretic apparatus and the reservoir buffer (Section 2.2.5.4) was added. Finally, the samples were applied to the wells of the upper stacking gel. Electrophoresis was achieved at 40 mamps per gel, at a constant voltage of 200, at room temperature.

2.3.4.2 Drying of gels

After electrophoresis as described above, and staining (see below), selected gels were photographed and dried between filter paper and

cling film under a vacuum at 80°C. To avoid gel cracking the gels were pre-soaked in the destain to which glycerol (Section 2.1.12) had been added to give a final concentration of 5% (v/v). this increased the drying time from 1 hour to 3 hours. The silver stained gels (Section 2.3.6) were soaked in a more aqueous solution before drying, eg. 10% methanol.

2.3.5 Coomassie Blue Staining of SDS-PAGE Gels

Routine protein staining was achieved by staining with Coomassie Blue G250 (Section 2.2.5.6), followed by destaining (Section 2.2.5.6). When results were required quickly (eg. for obtaining gel chips for Cleveland experiments; Section 2.3.8), the gels were stained for 30 to 60 minutes at 40°C, followed by destaining with gentle continuous agitation. Generally, however, the gels were stained for 4 hours or left to stain overnight, before destaining.

2.3.6 Silver Staining for Protein

The silver staining method described below was employed when a more sensitive detection procedure was required. The method is essentially the same as that described by Wray et al. (1981). This staining technique was used to assess the purity of fractions obtained during protein purification procedures, and also to detect low concentrations of protein in Cleveland gels (Section 2.3.8).

2.3.6.1 Solutions and reagents

The solutions and reagents required for the silver staining were made up in deionised water which had been filter sterilised.

Solution A

Silver nitrate, 0.8g in 4ml deionised H_2O

Solution B

0.36% (w/v) NaOH 21ml

14.8M Ammonia 1.4ml

Solution C

Solution A was added dropwise to solution B, while stirring vigorously. The volume was made up to 100ml with deionised water.

Developer

1% (w/v) Citric acid 2.5ml

38% Formaldehyde 250µl

Deionised H_2O to 500ml

2.3.6.2 Staining procedure

The gels were placed in solution C and stained for 15 minutes with constant gentle agitation. High percentage gels, eg. 15%, required a few minutes longer in the stain. The gels were rinsed in deionised water for approximately 5 minutes, with 3 changes. The bands were visualised with the developer, again with constant, gentle agitation for up to 15 to 20 minutes. The reaction was terminated after placing the gel into 50% methanol, although this did not stop the reaction immediately. The destain used for Coomassie Blue stained gels was used to lighten overstained gels. Stain C was collected in a bottle and the silver was precipitated as AgCl by the addition of NaCl. Once the precipitate had settled, the supernatant was poured off to avoid the formation of explosive compounds.

2.3.7 PAS Staining for Carbohydrate Groups

The periodic acid Schiff's (PAS) stain (Section 2.1.5) is used to identify glycoproteins, glycolipids, polysaccharides and other materials which are high in carbohydrate.

2.3.7.1 Solutions and reagents

1. 7.5% (v/v) Acetic acid
2. 10% (v/v) Acetic acid
3. 0.2% (w/v) Periodic acid
4. Schiff's reagent
5. Destain: Na metabisulphite 5.0g
   1M HCl 50.0ml
   Distilled H_2O to 1 liter

2.3.7.2 Procedure

All incubations were at room temperature unless otherwise stated. The samples were electrophoresed in the normal fashion (Section 2.3.4.1), and the gel slab placed in 7.5% acetic acid (Sections 2.1.9 and 2.3.7.1) for 1 hour. The gels were then placed into 0.2% periodic acid (Sections 2.1.9 and 2.3.7.1) and incubated at 4°C for 45 minutes. The gels were removed and placed immediately, without rinsing, into the Schiff's reagent and refrigerated for a further 45 minutes. The gels were destained in two or three changes of 10% acetic acid, or for more rapid destaining, the destain recipe described above was used, and care taken not to bleach the bands.

The gels were stored at 4°C for a longer preservation of bands.

2.3.8 Cleveland Gels

Peptide maps of S-antigen were produced by enzymatic proteolysis and separation by SDS-PAGE employing the method of Cleveland et al., (1977). Three proteolytic enzymes were utilised; trypsin, chymotripsin and Staphalococcus aureus V8 protease (Section 2.1.6).

2.3.8.1 Preparation of samples

The S-antigen sample to be subjected to peptide mapping was prepared for SDS-PAGE as usual (Section 2.3.4.1). The samples were resolved in 8% SDS-polyacrylamide gels without the use of a comb in the stacking gel. After electrophoresis, the gels were briefly stained and destained (Section 2.3.5). The stained band of S-antigen was excised using scalpel blades and cut into pieces of dimensions 2 x 3mm. These gel chips were rinsed in 0.125M Tris/HCl buffer, pH 6.8, with 0.1% SDS; 4 changes in 30 minutes, with occasional swirling. The chips could be stored frozen at this stage.

2.3.8.2 Method of digestion

Peptide mapping requires gels of small porosity in order to visualise the small fragments of protein. 15% SDS-polyacrylamide slab gels were cast (Table 2.2), with a 5cm 3% stacking gel using the buffers described in Section 2.2.5.

The stock solutions of trypsin, chymotrypsin and V8 protease were at a concentration of 1mg/ml. These were diluted to 0.25 to 2.5µg/ml in the chip wash buffer (Section 2.3.8.1), to which glycerol had been added to 10%, and bromophenol blue to 0.01%.

The S-antigen gel chips were loaded to the slots in the stacker of the 15% gels with the aid of a small spatula. The chips were overlaid with chip wash buffer containing 20% glycerol. Finally 20µl, containing 5 to 50ng of the enzyme samples were applied above the glycerol layer. The gels were electrophoresed at 10 to 45 mamps just until the band of bromophenol blue had almost reached the base of the stacking gel. At this time the current was switched off for 30 to 60 minutes, before running again to approximately 1cm from

the bottom of the main gel. Electrophoresis and digestion were carried out at room temperature.

The gels were either silver stained for total protein (Section 2.3.6), since this method is more sensitive compared with Coomassie Blue, or elecrophoretically transferred to nitrocellulose paper for use in immunoblotting experiments (Section 2.3.9.9).

2.3.9 Immunoblotting

During the course of this work, both enzyme and the ^{125}I protein A immunoblotting procedures were employed. The technique followed was essentially that of Batteiger et al. (1982).

2.3.9.1 Electrotransfer of proteins

The protein samples were electrophoresed in the normal manner (Section 2.3.4.1). The gels were handled with gloves and placed carefully on filter paper, avoiding air bubbles, soaked in the transfer buffer (Section 2.2.6.1), which was in turn lying upon a gauze square soaked in the same buffer. The gel was wetted with transfer buffer and over laid with a piece of nitrocellulose paper, soaked in the buffer, avoiding air bubbles. The nitrocellulose paper was finally covered over with a second piece of filter paper and then the second gauze square, both soaked in the transfer buffer. The described assembly was then encased within the plastic cassette and quickly fitted into the transfer tank containing 3 litres of the transfer buffer, in such a way that the nitrocellulose paper was nearest to the positive electrode. Electro-transfer was achieved at room temperature after 4 hours at 350 mamps, or overnight at 35 mamps, followed by 1 hour at 350

mamps.

After electro-transfer and dissassembly of the apparatus, the nitrocellulose strips were trimmed to the size of the original gel and the orientation was marked by the cutting of one corner. The strips were then either stained with Amido black (Section 2.3.9.2) for total protein or immunoblotted as described below (Section 2.3.9.6).

2.3.9.2 Staining for total protein

The Amido black strips were stained for total protein with 0.1% Naphthol Blue-black (Amido black) (Section 2.1.5) in 40% methanol, 10% acetic acid in distilled water. Staining was achieved after a 2 minute incubation in the stain, and the strips were destained in 40% methanol, 10% acetic acid in water. The nitrocellulose strips were dried flat between filter paper.

2.3.9.3 Primary antibodies

The dilutions of the primary antibodies used was dependent upon their titres in ELISA. Generally the rat antisera to bovine S-antigen and ROS demonstrated a titre of several thousand to their appropriate antigens in ELISA (Section 3.8.5-6), and were used at dilutions of 1:200 to 1:1000 in immunoblotting experiments.

When autoantibodies were sought using the immunoblotting technique, higher concentrations were required (eg. 1:80) due to the low serum titres in ELISA. Sera from RCS rats (Section 4.1.8), and uveitis and RP patients (Section 4.2.7), were tested by this method, for autoantibodies. Controls included normal human and normal rat sera, in addition to the omission of primary and/or secondary antibodies.

2.3.9.4 Secondary antibodies

Primary rat or human antibody was detected by means of rabbit anti-rat IgG(H+L) and rabbit anti-human IgG(H+L) respectively (Section 2.1.16) diluted 1:200. The ^{125}I protein A (Section 2.1.12) detection system allowed the omission of the second antibody in the case of the rat IgG monoclonal antibody S2.4.C5 (Chapter 6). Almost all the nonspecific reactions observed were attributed to the second antibody (Section 6.3.3). The second antibody was retained, however, for protein A detection in Cleveland blots (Section 2.3.9.9) since it may increase the sensitivity, and no nonspecific reaction was observed betweeen the S-antigen and the second antibody (Section 3.8.1, Figure 3.9).

2.3.9.5 Iodination of protein A

1mg of iodogen (Section 2.1.7) was dissolved in 0.5g of chloroform in a vial. The vial was coated with the iodogen by slowly blowing off the chloroform. 1mg of the protein A was dissolved in 0.5ml of the blot wash buffer (Section 2.2.6.2), and transferred to the iodogen coated vial. 27MBq of Na ^{125}I in 100µl, was added to the protein A and allowed to stand for 15 minutes at room temperature. The solution was then transferred to a separate clean vial. The iodinated protein A was passed through a 10ml G50 column (Section 2.1.3), previously equilibrated with the blot wash buffer and 1ml fractions were collected. 10µl samples of each fraction were counted in an LKB 1275 Minigamma counter, and the fractions containing the first peak were pooled, aliquoted to give 10 counts per aliquot, and stored at -20°C under lead.

2.3.9.6 Immunoblotting methodology

The methodologies for the enzyme and ^{125}I immunoblotting were almost identical. The Tris/HCl, pH7.2, blocking and wash buffer contained 0.5% Tween 20 (Section 2.1.10). Blocking was achieved ater a 1 hour incubation at 40°C or overnight at 4°C. Further incubations were for 60 to 90 minutes at room temperature. To conserve reagents, the incubations were made in sealed plastic bags in volumes of 2.5 to 10ml, depending on the size of the nitrocellulose strips. Washing was carried out with 5 x 200ml of buffer (Section 2.2.6.2); each wash was incubated on a shaker for 5 to 10 minutes. Monoclonal antibodies were applied as cell-free hybridoma culture supernatant. After further washing, the nitrocellulose strips were incubated with the second antibody, which was conjugated to horseradish peroxidase in the case of the enzyme method. In the protein A system, the labelled protein A was able to bind to the IgG1 rat monoclonal antibody S2.4.C5, allowing the omission of the second antibody (Section 2.3.9.4). Monoclonal antibodies of the IgM class and all sera involved the incubation with a second antibody, before washing and the final incubation with the protein A. After the final incubation with either the labelled protein A, or the peroxidase conjugate, the nitrocellulose strips were finally washed in the washing buffer.

2.3.9.7 Development of the reaction

Blots treated according to the ^{125}I labelled protein A methodology were developed on X-Omat film (Kodak) (Section 2.1.22). The length of time necessary to develop the bands varied with the stock of the labelled protein A. For this reason, all blots which were to be compared, were treated with the same preparation. At the end of the reaction detection the nitrocellulose strips could be stained with Amido black (Section 2.3.9.2), allowing the overlay of the

developed film on stained strips and thus the identification of the antigenic bands was facilitated.

Blots treated according to the peroxidase enzyme system were incubated with freshly made peroxidase substrate, described below. The reaction was almost instantaneous when specific antibodies had been used in the first antibody incubation. The reaction was terminated by washing in running deionised water. Washing was continued for several minutes before drying between filter paper. The bands tended to fade with time, thus important blots were photographed promptly.

Stock Solutions

1. 1% (w/v) O-dianisidine (Section 2.1.10) in methanol
2. 10mM Tris/HCl, pH7.4
3. H_2O , o.2% (v/v)

Peroxidase Enzyme Substrate, 20ml

O-dianisidine stock	0.5ml
H_2O stock	1.0ml
Tris/HCl	18.5ml

2.3.9.8 Blotting with Cleveland gels

Only the protein A technique was used for the Cleveland blotting experiments, since varying the exposure to the film facilitated the identification of epitope bearing bands. Shorter exposure times were required to visualise the higher molecular weight bands than the smaller peptides. The methodology for the electro-transfer and blotting of Cleveland gels was almost identical to that of conventional gels. Similar transfer times were used since, although

the gels were of a higher percentage of acrylamide (15% instead of 8 or 10%), the molecular weights involved were all below 50K (not including the protein standard markers). After transfer, the nitrocellulose papers were blocked with 0.5% Tween 20 (Section 2.2.6.2) as usual. However, the concentration of Tween was reduced to 0.05% for all subsequent washing since this was found to reduce the loss of the lower molecular weight peptides during the immunoblotting procedure. When antiserum was used in place of a monoclonal antibody, the reduced Tween concentration sometimes resulted in an increase in background reaction, probably due to the immunoglobulins nonspecifically interacting with the nitrocellolose paper. The second antibody step (Section 2.3.9.4) was preferred for the Cleveland gels since it could amplify the reaction.

2.4 IMMUNOCYTOCHEMISTRY AT THE LIGHT MICROSCOPE LEVEL

2.4.1 Tissue Fixation

Rat eyes were dissected and the anterior segments removed together with the vitreous. Some eyes were fixed overnight in pre-cooled (4°C) 70% alcohol and embedded in paraffin wax for light microscopy either according to conventional procedures or to the method of Sainte-Marie (1962). Other eyes were fixed in 4% gluteraldehyde (Section 2.1.8.1) in PBS (Section 2.2.4.1) and processed for light microscopy according to conventional methods.

2.4.2 Conventional Paraffin Embedding

Fixed tissue was dehydrated through a graded series of alcohols in distilled water to xylene and finally paraffin wax, in a Histokinette automatic tissue processor, as described below.

1. 8% phenol in 50% (v/v) alcohol 2 hours
2. 8% phenol in 70% (v/v) alcohol 2 hours

3.	8% phenol in 90% (v/v) alcohol	2 hours
4.	Methanol I	1 hour
5.	Methanol II	1 hour
6.	Absolute alcohol I	1 hour
7.	Absolute alcohol II	1 hour
8.	Absolute alcohol: chloroform (1:1)	1 hour
9.	Chloroform I	1 hour
10.	Chloroform II	2 hours
11.	Wax I (Paraplast)	2 hours
12.	Wax II (Paraplast)	4 hours

2.4.3 Cold Embedding Method

The alcohol fixed tissue (Section 2.4.1) was dehydrated in 4 changes of pre-cooled absolute alcohol, 1 hour each incubation. The tissue was cleared by passing through consecutive baths of pre-cooled xylene for 1-2 hours each, at 4°C. The tissue, in xylene, was allowed to come up to room temperature and taken through consecutive baths of filtered paraffin at 56°C for 1-2 hours each. The blocks were stored at 4°C, and sectioned at room temperature.

2.4.4 Immunoperoxidase Staining Procedure

Wax sections were cut on a rotary microtome at 5µm, collected on polylysine (Section 2.1.12) (10µg/ml) coated (for histochemistry only) slides. The sections were dewaxed in xylene and hydrated through a graded series of ethanol dilutions, to water.

The endogenous peroxidase was exhausted by incubation with 0.03% hydrogen peroxide in methanol for 30 minutes. After blocking with 10% normal rabbit serum in PBS, the sections were incubated for 60

minutes with antiserum (diluted 1:200 in the blocking buffer) or monoclonal antibodies to retinal antigens, in the form of cell free culture supernatants. Negative controls consisted of pre-immune serum (diluted 1:200) and irrelevant rat monoclonal antibodies. The sections were washed in PBS before a 30 minute incubation with fresh rabbit anti-rat peroxidase conjugate (Section 2.1.16), diluted 1:100 in the blocking buffer. After washing, the sections were incubated with the diaminobenzidine substrate (Sections 2.1.11 and 2.2.8.4) for 5 minutes.

The sections were washed and counterstained with haematoxylin to visualise nuclei. The sections were then dehydrated through a graded series of alcohols (ethanol) to xylene. The sections were mounted with DPX (Section 2.1.8.2) and coverslips. The results were recorded by photography at the light microscopical level using Kodak Ektachrome colour film for tungsten light, ASA 160 or 400 (section 2.1.22).

2.4.5 Immunofluorescence Staining Procedure

Immunofluorescence was performed on frozen sections of rat eye and whole of mounts fresh rat retina. Whole albino rat eyes were enucleated and snap frozen in liquid nitrogen, and embedded in Tissue Tek O.C.T. (Raymond Lamb, London). Sections were cut at 8µm in a cryostat, and stored at -20°C before use.

The staining procedure was the same for the whole mounts and the frozen sections. All steps were carried out at room temperature. The sections were briefly fixed in acetone or 95% alcohol for 2 minutes and air dried before blocking for 10 minutes in 20% normal rabbit serum in PBS (Section 2.2.4.1). The sections were drained

and the primary antibody samples applied and incubated in a humid chamber for 1 to 3 hours. The sections were then gently washed 4 times with PBS, before incubation with the FITC conjugated second antibody (Section 2.1.16), diluted 1:20 in PBS, for 1 hour in the humid chamber. The sections were finally gently rinsed 4 times in PBS and once in water, and mounted in the fluid specified below. The sections were visualised in a Leitz Orthoplan fluorescence microscope equiped with a Leitz MPV compact photometer.

Mounting Fluid, pH 8-9

$NaHCO_3$	71.5mg
Na_2CO_3	16.0mg
Distilled H_2O	10.0ml
Glycerol	90.0ml

2.5 IMMUNOCYTOCHEMISTRY AT THE ELECTRON MICROSCOPE LEVEL

2.5.1 Araldite Embedding

2.5.1.1 Tissue fixation

Rat eyes were dissected as described in Section 2.4.1 and fixed for 1 hour in 0.75% gluteraldehyde and 3% formaldehyde, freshly prepared from paraformaldehyde (Section 2.1.8.1) in PBS (Section 2.2.4.1). After fixation the tissue was washed in 3 changes of PBS and 3 changes of cacodylate buffer (Section 2.2.8.2) over the period of an hour. The fixed tissue was cut into pieces of 1mm by 2mm using a razor blade.

2.5.1.2 Araldite embedding mixture

i) Araldite CY212	10.0ml
ii) D.D.S.A.	10.0ml

iii) Dilutyl phthalate 1.0ml

iv) B.D.M.A. 0.5ml (accurate)

Substances i), ii) and iii) (Section 2.1.8.3) were mixed thoroughly, yet gently to avoid bubbles, before iv) was added.

2.5.1.3 Embedding procedure

The pieces of fixed retina were placed in 25% ethanol (Burrough's absolute alcohol) for 10 minutes. This was exchanged for 50% ethanol for a further 10 minutes, and followed by third 10 minute incubation with 75% ethanol. Then the tissue samples were incubated with absolute alcohol for 20 to 30 minutes. The absolute alcohol was changed 3 more times before 2 incubations with propylene oxide for 10 minutes each. The samples were then incubated overnight in an Araldite propylene oxide 50:50 mixture. Then the pieces of tissue were incubated with a mixture of Araldite and propylene oxide in a ratio of 75:25 for 2 to 6 hours on a spinning device. Finally the pieces of retina were placed in fresh Araldite in rubber moulds and cured at 60°C for 24 to 48 hours.

2.5.2 Gold Conjugation of Antibodies and Protein A

Rabbit anti-rat IgG(H+L) (Section 2.1.16) and protein A (Miles, Section 2.1.12) were conjugated to 10nm colloidal gold particles (Section 2.1.12), according to the method of Roth (1982).

2.5.2.1 Preparation of the reagents

The rabbit anti-rat sera was diluted 1:5 in 20mM Tris HCl, pH 8.4; the optimal pH for the stabilisation of immunogold (De Mey, 1983). A stock solution of 2mg/ml protein A was made in distilled

water. An 800µl volume of distilled water was added to 20µl of the stock solution to obtain a concentration of 50µg/ml.

2.5.2.2 Titration to determine concentrations of reagents

The gold colloid was the colour of red wine. Upon destabilisation in the presence of salt, the gold particles precipitate, assuming a mauve to blue colour. Protein has the ability to stabilise the colloidal gold particles in the presence of sodium chloride. The optimal concentrations of the antibodies and protein A required to stabilise the gold was determined as follows:

For each reagent, protein A or rabbit anti-rat IgG(H+L), to be conjugated to gold, 10 wells of a 96 well tissue culture plate (Section 2.1.19) were filled with 200µl of gold colloid. From the first to the tenth well, 5 to 90µl of the 50µg/ml protein A solution or 5 to 90µl of 1/100 dilution of antisera, was added. In the case of the 1:5 diluted rabbit anti-rat IgG(H+L), 5 to 90µl samples were applied, one to each of the 10 wells of gold. For the purified monoclonal antibody, 5 to 90µl were added to the 10 wells. Finally, 50µl of 10% NaCl was added to every well containing gold and the differing amounts of stabilising proteins.

The blue precipitate indicated destabilisation, and the mauve colour indicated partial destabilisation, while the wine colour signified stabilisation. The quantity of the antibody or protein A taken to be optimal in stabilisation, was that amount, 2 wells away from the mauve coloured well.

Generally, 20µl of protein A (50µg/ml) stabilised 200ul of

colloidal gold. Thus 50µg (30µl of 2mg/ml stock) of protein A was used to stabilise 12ml gold colloid. In the case of antisera, 20µl (1/100 dilution) stabilised 200µl of colloidal gold, or 60µl of 1/5 dilution, stabilised 12ml of gold.

2.5.2.3 Gold conjuation procedure

25µl of protein A (2mg/ml), or 60ul of rabbit anti-rat(H+L) (1/5 dilution), was added in drops around the edge of a tube for a Ti60 rotor, containing 12ml of colloidal gold. The tube was then spin mixed and left for 2-3 minutes. Then 1ml of 1% PEG (molecular weight 20,000) in distilled water, was added. This mixture was then centrifuged at 48,000g for 60 minutes, at 4°C. The supernatant was discarded and the dark red pellet of protein A-gold or immunogold complexes, resuspended in 1% PEG-H_2O, and centrifuged as before. The pellet was resuspended in 1.5ml of 1% PEG in PBS and stored at 4°C, for up to 1 year. Before use, the protein A gold suspension was diluted to give an absorbance reading of 0.2 at 520nm, and passed through a 0.2µm membrane filter (Section 2.1.19).

2.5.3 Immunostaining Procedures

Sections were cut at 800A and mounted on 300 mesh nickel grids (Section 2.1.8.3). The grids were floated on droplets of the reagents, on sheets of dental wax or Nescofilm and incubated in a moist chamber. All procedures were carried out at room temperature. All grids were pre-incubated on PBS for 10 minutes and then blocked with 5% normal goat serum (immunogold method), or 1% ovalbumin (protein A technique), in PBS for 30 minutes. The grids were drained and incubated for one hour on the rat monoclonal antibodies (hybridoma supernatants) or rat primary

antisera, diluted 1:200 in PBS with 5% normal goat serum. The protein A staining method yielded best results when a second antibody step was included, this involved the incubation of the grids with rabbit anti-rat IgG(H+L) (Section 2.1.16) diluted 1:100, for 1 hour. The grids were then jet washed in PBS (Section 2.2.4.1) followed by incubations through droplets of Tris/HCl buffer pH 7.2 (Section 2.2.8.1) 5 changes, 4 minutes each. This was followed by two, 5 minute incubations in the Tris buffer with 0.1% BSA. Then the grids were transferred to Tris buffer, pH 8.4, with 1% BSA, for 5 minutes. The grids were then transferred to droplets of gold adsorbed goat anti-rat immunoglobulin or gold labelled protein A (Section 2.1.16) diluted to give an optical density reading of 0.2 at 520nm, in Tris/HCl with 1% BSA, pH 8.4, and incubated for one hour. The grids were then transferred to Tris buffer with 0.1% BSA, two changes, 4 minutes each. This was followed by washing through droplets of Tris buffer, eight changes, 3 to 4 minutes each. Finally, the grids were rinsed in distilled water, three changes, 3 to 4 minutes each and counterstained with filter sterilised solutions of uranyl acetate (saturated in 50% ethanol) (Section 2.1.5) and lead citrate (Section 2.2.8.3).

The following control experiments were performed:

1. Omission of the primary antibody, to control for non-specific binding of secondary antibody or gold conjugate to the sections.
2. Incubation with preimmune rat serum, diluted 1:200 or 1:100, instead of the primary antibody.
3. Incubation of grids with an irrelevant rat IgG monoclonal antibody, either as hybridoma supernatant or as a 5 to 10 fold concentrate.

4. Incubation of grids with antisera or monoclonal antibody which had been pre-incubated with saturating amounts of purified bovine S-antigen.

5. Antiserum from a rat immunised with bovine opsin (purified by SDS polyacrylamide gel electrophoresis) diluted 1:200 was included as a disc staining control since opsin is known to be located on the disc membranes.

The grids were viewed in a transmission electron microscope (Phillips) and a photographic record was made of the results.

2.6 PRODUCTION OF RAT MONOCLONAL ANTIBODIES

2.6.1 Immunisation of Experimental Animals

The DA strain of rat (Section 2.1.17) was chosen for the production of rat monoclonal antibodies. Although the fusion partner for the rat B lymphocytes, was originally derived from a Lou rat (Galfre et al., 1979), the DA strain has been found to be suitable, and these rats produce a good immune response upon immunisation. Rats were immunised with bovine retinal S-antigen (Section 3.2-3) or photoreceptor rod outer segment (ROS) membranes (Section 3.6). Generally 10 to 50µg of protein (according to the Bradford's assay, Section 2.3.2) in a volume of 0.5ml, was homogenised with an equal volume of complete Freunds adjuvant (CFA) (Section 2.1.12) immediately prior to immunisation. The animals were anaesthetised with ether and injected subcutaneously in the neck and one or both hip regions. Usually the animals received two or three such immunisations in CFA, with a minimum period of one week between injections. It is generally considered desirable to administer an intraperitoneal or intravenous (tail vein) boost of the antigen in

buffer or saline, 4 days prior to the day of fusion (see Campbell, 1984). The protein content of these boosts was approximately 1 to 10µg in volumes of 0.1 to 0.4ml.

2.6.2 Assay of Sera and Hybridoma Supernatants

2.6.2.1 Bleeding and assessment of serum titre

The animals were anaesthetised with ether prior to the tail bleed. Using a fresh scalpel blade for every second animal, the tails were cut at minimum distance from the tail tip. A few drops of blood were collected in Eppendorf tubes (Section 2.1.23). The blood was allowed to clot at room temperature for an hour, and then kept at 4°C overnight. The serum was removed after centrifugation and stored at -20°C.

The sera were diluted 1:100 in PBS Tween 20 buffer (Section 2.2.4.2) with 0.05% (w/v) BSA (Section 2.1.12), for titre determination by ELISA. The ELISA plates (Section 2.1.15) were coated with 1µg/ml of S-antigen and blocked with 50mg/ml BSA. Doubling dilutions of the sera were made across or along the plates, beginning with a dilution of 1:100. The plates were incubated for 30 minutes at room temperature before completion of the ELISA protocol, as described in Section 3.8.3.

Animals demonstrating a serum titre in ELISA of greater than 1:2000 were considered to be suitable for fusion purposes.

2.6.2.2 Assay of fusion hybridoma supernatants

The ELISA system was selected for the screening of fusion supernatants since it is rapid, and convenient to use. It also tends to select for relatively high affinity antibodies since it

involves washing steps.

Samples of hybridoma supernatant were removed by means of sterile tips and a micropipette, set at a volume of 100µl, and applied directly to coated and blocked ELISA plates. The plates were incubated for 2 hours at room temperature or kept at 4°C overnight. The results were developed in the usual manner (Section 3.8.3).

2.6.3 Rat Parent Cell Line and its Care

The rat myeloma cell line Y3-Ag.1.2.3 (Y3) (Section 2.1.18) (Figure 2.1) was employed as the fusion partner for the rat splenic lymphocytes (Galfre et al., 1979). The rat hybridoma system was selected because of the greater stability of both uncloned and cloned hybrids, in terms of continual antibody secretion, compared with the mouse system. The fusion methodology and treatment of the cells is almost identical to that of the mouse system. The Y3 cells were maintained on 10% FCS in RPMI (Table 2.1) (Section 2.2.7.1), and were usually grown in a spinner flask for three or four days prior to fusion. Care was taken not to allow the Y3 cells to overgrow or become too crowded in the few days before fusion, and the cells were taken to a low cell density about 24 hours prior to the time of the fusion.

2.6.4 Dissection of Spleen and Removal of Blood

The rats were killed by over-anaesthetising in ether. The abdomens were soaked in iso-propanol before incision. A transverse lower abdominal cutaneous incision was made exposing the anterior abdominal muscles, which were cut with the parietal peritoneum. The intestines were lifted aside and any fat cut away to expose the dorsal aorta. Blood was withdrawn from the aorta by means of a

gauge 21 (G21) needle and a 5ml syringe (Section 2.1.19). Volumes obtained in this manner ranged from 1 to 5ml. The serum was isolated and stored as described in Section 2.6.2.1.

The spleens were dissected out, leaving minimal fatty tissue adhering to the organ, and placed immediately into a sterile plastic universal container (Section 2.1.19).

2.6.5 Method of Cell Counting

An estimate of cell density was obtained by counting the cells on grids within the haemocytometer chamber. Cells growing in flasks usually required diluting 1:10 or 1:20 before they could be counted. A 50µl sample of the diluted spleen cells was added to a 300ul volume of 0.9% (w/v) ammonium chloride (Section 2.1.12) in distilled water, and incubated at room temperature for 5 minutes. This procedure lysed the red blood cells present in the rodent spleen cell suspension, thus facilitating counting of the lymphocytes. The value of the mean of two counts, taken from different regions of the grid, was multiplied by 10 to obtain the number of cells per ml within the sample. This number was multiplied by the dilution factor in order to obtain the density of cells within the original cell suspension.

2.6.6 Fusion Protocol

The fusion protocol employed was essentially that of Köhler and Milstein (1975).

2.6.6.1 Preparation of the spleen cells

The spleen was rinsed in approximately 5ml of RPMI in a small petri dish and transferred to a fresh petri dish (Section 2.1.19), to

which a further 5ml of RPMI had been added. The spleen tissue was teased to form as fine a suspension as possible using sterile G21 needles (Section 2.1.19). The petri dish was tilted and the spleen cell suspension drawn into a 5ml syringe. The syringe contents were then passed into a fresh petri dish. The spleen cell suspension was again taken up into the syringe and re-expelled. This syringe passage was repeated at least four times. Then the spleen cell suspension was passed through fine gauge 25 (G25) needles, at least three times, and finally expelled into a sterile universal container. The universal was topped up with RPMI and centrifuged for 5 minutes at approximately 500g. The supernatant was discarded and the pellet resuspended in RPMI and centrifuged as before. The cell pellet was resuspended in 10ml RPMI and the cells counted as described in Section 2.6.5. It was shown that approximately 1×10^8 cells can be obtained by this method.

2.6.6.2 Preparation of the Y3 cells

Universals of Y3 myeloma cells were centrifuged for 5 minutes at 500g, and the cell pellets resuspended in RPMI. The cells were re-centrifuged and resuspended in 10ml of RPMI for counting (Section 2.6.5). Approximately half the number of Y3 cells to splenic lymphocytes was required, ie. usually 0.5×10^8 cells.

2.6.6.3 Cell fusion

Suspensions of Y3 and spleen cells were mixed, ensuring that a small sample of each was retained to provide spleen and Y3 cell controls. The Y3 cell control wells were included, to ensure that these cells were killed in the HAT medium (Section 2.2.7.2). Spleen cell control wells were included since the spleen cells may secrete antibody for several days, and this could result in the detection

of false positives, unless the hybridoma medium was changed at least twice before assay. The cell mixture was pelleted in the usual manner, and the supernatant discarded.

Over a period of 30 seconds, 2ml of warm (37°C) 45-50% PEG (molecular weight 1500 or 4000) in RPMI (Section 2.2.7.4), was added dropwise to the cell pellet, with constant agitating of the tube to disperse the cells in the PEG medium. The mechanism of cell fusion is complex and, as yet, not fully understood. The PEG is hydrophilic in nature and is thought to occupy the 'physical free water' space, leading to cell agglutination. The cells were gently resuspended for 30 seconds, and allowed to rest for another 30 seconds. Then 5ml of RPMI was added over a period of 90 seconds, while the tube was agitated to mix the media. A further 5ml of RPMI was then added over 2 seconds, while the tube was rotated. Finally, the universal was allowed to stand for 2 to 3 minutes before centrifugation in the usual manner.

The supernatant was tipped and the pellet gently resuspended in a few ml of HAT medium (Section 2.2.7.2), and transferred to a 100ml volume of the same medium. The cell suspension was seeded at approximately 1ml per well, into four 24-well Costar plates (Section 2.1.19), apart from those reserved for the spleen and Y3 cell controls. The fusion plates were then placed in a 37°C incubator with 5% CO_2. The plates were briefly inspected after approximately 24 hours to ensure that no contamination had occurred.

The HAT medium allowed the growth of the hybridoma cells, while the Y3 cells did not survive. The drug aminopterin, inhibits the enzyme

dihydrofolate reductase, which is essential to the main pathways of purine and pyrimidine de novo biosynthesis. However, salvage pathways exist, where exogenous nucleotides are used; the purine pathways involve exogenous hypoxanthine with the enzyme, hypoxanthine phosphoribosyl transferase (HPRT). The pyrimidine pathway utilises exogenous thymidine and thymidine kinase (TK). The Y3 cells cannot survive in HAT medium unless they have acquired the HPRT gene by fusion with the antibody secreting spleen cells.

2.6.7 Care of Fusions and Observations of Wells

2.6.7.1 Feeding

The fusion plates were fed after four to seven days with HAT medium, 0.5ml per well. The fusion plates were fed a second time after approximately 10 days post fusion. At the second feed, 0.5ml of medium was removed to be replaced with 0.5ml of fresh medium. By this time the Y3 cells had died, and aminopterin was no longer required in the medium. The HT was retained at this stage, however, since residual aminopterin will remain with the emerging hybridomas. Subsequent feeding depended on the emergence of clones.

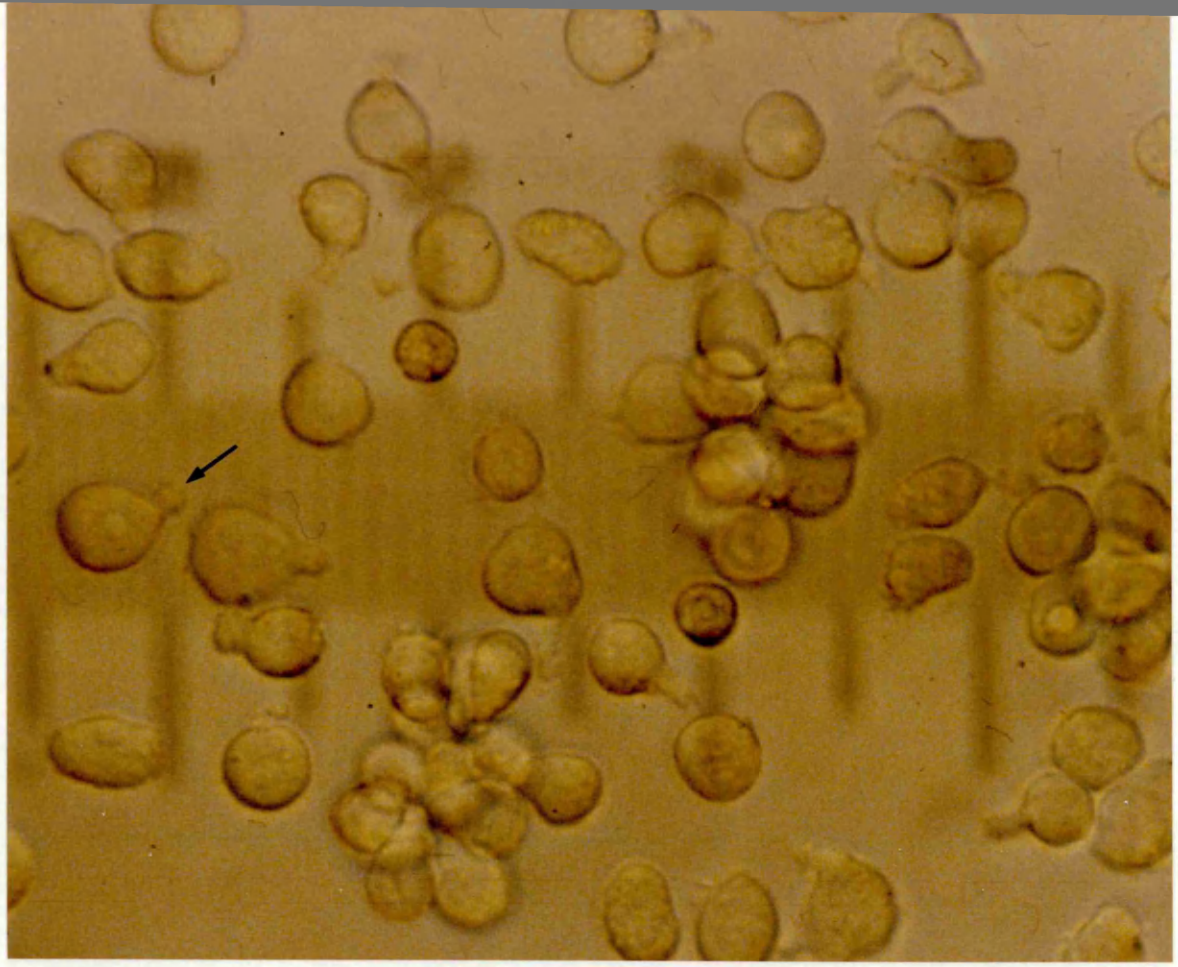
2.6.7.2 Characteristics of a fusion well

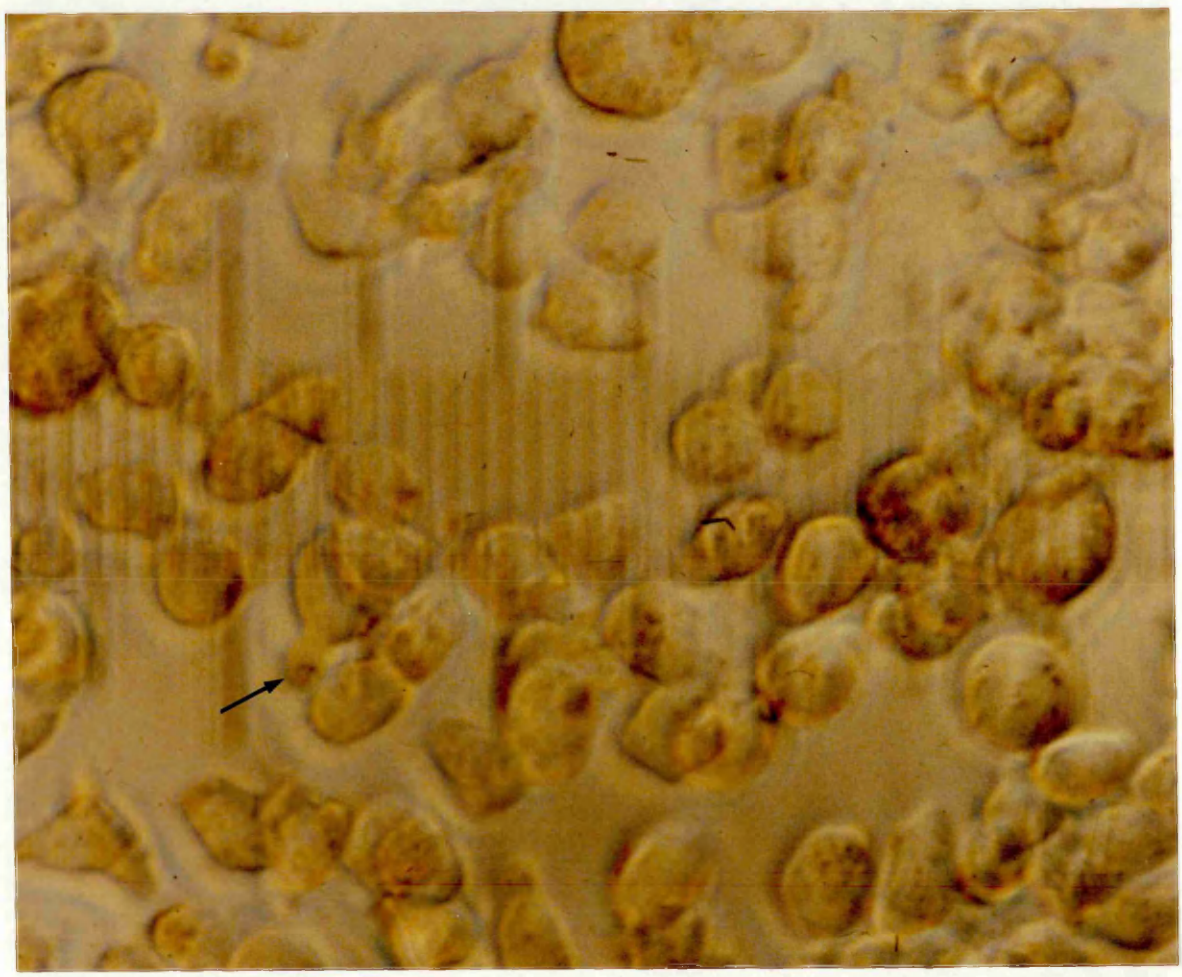
After three or four days the Y3 cells appeared dead, having become granular and tending to clump together. Several types of spleen cell remained alive for some time, in particular the fibroblasts which would occasionally take over the well and cause the medium to turn acidic (indicated by the yellow colouration). Over a few days post fusion, the background population of cells, dying Y3s, red blood cells, and other spleen cells began to clear away and tended to form a large mass of cell debris at the well centres. Such a clearing process facilitated detection of the emerging

Figure 2.1 High Magnification of the Rat Myeloma Cells

Figure 2.2 High Magnification of Rat Hybridoma Cells

Arrows indicate cell blebs





hybridomas.

2.6.7.3 The emergence of the hybridomas

The rat hybridomas appeared virtually indistinguishable from their Y3 parent cells; they were relatively large cells with variable and irregular shapes and were non-gregarious in behaviour (Figure 2.2).

The hybrids generally emerged by 14 days post fusion. In 'good' fusions, the hybrids were detected as early as 6 days after fusion. Such early emergence of clones was generally indicative of a high fusion frequency, and conversely when clones were slow to appear, there tended to be fewer wells with growth. If no hybrids appeared by the third or fourth week post fusion, the plates were discarded.

2.6.7.4 Assay and expansion of clones

Once a well became approximately 70-80% confluent with the hybridomas, it was assayed by the asceptic removal of 100µl of supernatant, and directly applied to ELISA plates, which had previously been coated with antigen. The ELISA assay procedure is described in Section 3.8.3. Positive clones were expanded into 25ml flasks and fed with approximately 3ml of complete medium. The flasks were generally left upright overnight to allow the cells to further expand before feeding with 3 to 5ml of medium and then being placed horizontal. The cells were fed every 24 to 48 hours until enough cells were obtained to make a frozen stock (Section 2.6.8).

2.6.8 Cell Freezing

Aliquots of positive, but as yet uncloned cells, were frozen and stored in liquid nitrogen as insurance against loss of cultures.

Suspensions of the cells to be frozen in liquid nitrogen were counted (Section 2.6.5) and centrifuged at 500g for 5 minutes and the cell pellets resuspended in a volume of freezing medium (section 2.2.7.3) to give 5-10 x 10^6 cells per ml. 1 to 1.5ml of the cell suspensions were placed into screw cap plastic Costar ampoules of 1.8ml capacity (Section 2.1.19). The ampoules containing the cells were frozen for 24 to 48 hours at -70°C, before transfer to liquid or gas phase nitrogen storage facilities.

2.6.9 Subcloning to Establish Monoclonality

Monoclonality is achieved by successive subcloning at 0.5 or 0.25 cells per well, in the presence of spleen feeder cells. Monoclonality may be assumed after the third subcloning at such cell densities.

2.6.9.1 Procedure

Suspensions of spleen cells were obtained from an unimmunised rat or Balb C mouse (Section 2.1.17) as described in the fusion protocol in Section 2.6.6.1. These cells act as feeders for cells cloned at very low densities, or for cells exhibiting poor growth. These spleen cells probably affect the hybrids by providing growth factors and physical support.

The feeders were resuspended in complete medium (Section 2.2.7.1) and seeded at 1 x 10^5 cells/well in 100µl volumes, into the wells of two Costar 96-well plates (Section 2.1.19), per cell line to be subcloned. The hybrid cells to be cloned were washed and counted (Section 2.6.5), and diluted to give 1 x 10^5 cells per ml. Consecutive 1:10 dilutions of the cells were made through universals containing 5ml volumes of RPMI to achieve a

concentration of 1 x 10^2 cells per ml. This cell suspension was diluted 1:10 in complete medium to obtain 10 cells per ml. This was then diluted 1:2 in complete medium to give 5 cells per ml, and diluted 1:2 again, to give 2.5 cells per ml. Using a microaliquoter or a one ml pipette (Section 2.1.19), 0.1ml of the 10 cells per ml suspension was applied to the wells of one 96-plate, in which feeders had been laid down. To the second 96-well plate, the 5 cells per ml suspension was added to half of the plate and the 2.5 cells per ml to the other half. Thus, there was one plate with 1 cell per well, and another plate with 0.5 and 0.25 cells per well.

The plates were briefly inspected approximately 24 hours after subcloning, to ensure no contamination had occurred. The plates were incubated for 10 to 14 days at 37°C to allow the clones to develop. The wells were considered ready for assay once the cells covered half or more of the well floor. Generally, a plate seeded at 1 cell per well was not assayed if there were enough wells with growth in the plate seeded at the lower cell densities. The wells were assayed in the same manner as the fusion wells, described in Section 2.6.7.4.

2.6.10 Bulk Culture of Monoclonal Hybridomas

Once monoclonality had been achieved through subcloning procedures, unlimited quantities of antibody could be generated for further analysis and experimentation. For the purposes of this study, up to 1 litre volumes were generated as required.

The hybrids were usually expanded to the normal volume of one or two 250ml flasks, prior to transfer into a 1 litre, siliconised, sterile glass spinner culture flask. The flasks were incubated at

37°C over a magnetic stirring device either in a 37°C water bath or in a 37°C hot room. The hybrids usually grew exponentially and required sub-culturing on a daily basis.

2.6.11 Determination of Monoclonal Antibody Isotype

The immunoglobulin classes and subclasses of monoclonal antibodies were determined by means of Ouchterlony immunodiffusion assays in agarose (Section 2.1.12), against antisera with specificty for rat immunoglobulin isotypes.

2.6.11.1 Solutions

1. 1% agarose (w/v) in PBS (Section 2.2.4.1)
   with 0.02% sodium azide
2. 0.9% (w/v) NaCl, 0.02% sodium azide
   in distillied H_2O
3. 0.025% (w/v) Coomassie Blue R (Section 2.1.5)
   in destain solution
4. Destain

Acetic acid	25ml
Methanol	25ml
H_2O	350ml

2.6.11.2 Preparation of Ouchterlony slides

The agarose was dissolved in PBS by boiling, and while hot 3ml applied to clean glass microscope slides using a pre-heated 5ml glass pipette. When cool, 1mm diameter wells were made using a template, consisting of a central and 6 surrounding wells. The prepared slides were stored in a moist chamber at 4°C, if not immediately used.

2.6.11.3 Procedure

The rat monoclonal antibodies were assayed with rabbit anti-rat IgM, and IgG, sheep anti-rat IgG1, goat anti-rat IgG2a, rabbit anti-rat IgG2b and goat anti-rat IgG2c (Section 2.1.16). Serial dilutions (1:1 to 1:32) of the hybridoma supernatant, in volumes of 5µl, were applied to the wells surrounding the central well, to which 5µl of 1:10 dilution of the anti-rat Ig class specific sera was added. In addition, the undiluted hybridoma supernatant was applied to the centre well of separate slides, and assayed with serial dilutions (1:10 to 1:320) of the different antisera to the rat immunoglobulin isotypes, in the outside wells. The slides were then incubated in a moist chamber, for 18 to 24 hours at 4°C. The immunoprecipitation reaction were visible between the centre well and the outer wells containing the higher concentrations of antibody.

2.6.11.4 Staining of the immunoprecipitates

The Ouchterlony slides were washed in at least 3 changes of 100-200ml saline solution (Section 2.6.11.1), at 4°C, to remove excess proteins. The slides were then dried between filter paper, under a weight for 24 to 48 hours. The precipitates were stained with Coomassie blue for up to 10 minutes, and destained as necessary (Section 2.6.11.1). The slides were air dried and stored.

2.7 THE HUMAN TRANSFORMATION AND HYBRIDOMA SYSTEM

2.7.1 Production of the Transforming Virus

The Epstein-Barr virus (EBV) utilised for the transformation of human peripheral blood lymphocytes was obtained from the marmoset cell line, B95-8 (Miller and Lipman, 1973) (Section 2.1.18). The virus has been classified as a category 3 pathogen, by the British Medical Research Council, thus routine microbiological practice was taken during handling.

The B95-8 cells were grown in 5% FCS in RPMI (Section 2.2.7.1) at a density of $1x10^6$ cells/ml. For virus secretion, the cells were diluted to a density of 0.2×10^6 cells/ml in RPMI with the FCS reduced to 2%. In addition, the temperature at which the cells were incubated was dropped to 33°C. The cells were cultured under these conditions for 2 weeks before the virus particles were harvested. The B95-8 cells were allowed to settle at 4°C and then the supernatant containing the virus was collected after centrifugation at 200g. The supernatant was filter sterilised and aliquoted at volumes of 1ml in 1.8ml capacity plastic screw cap freezing ampoules (Section 2.1.19) and stored in liquid nitrogen until required.

2.7.2 Preparation of Peripheral Blood Lymphocytes

Heparinised (Section 2.1.19) human blood samples, of 10 to 40ml, were diluted 1:1 with warm (37°C) 2% FCS in RPMI, and gently layered onto equal volumes of Ficoll-Paque (Section 2.1.20) in 50ml Falcon tubes (Section 21.1.19). The tubes were centrifuged at 500g for 15 minutes. The lymphocyte layer was removed at the interface, and the pelleted red cells were discarded. The lymphocytes were

washed twice, in universals, in the 2% FCS in RPMI. The cells were counted as described in Section 2.6.5, and recentrifuged and resuspended in RPMI to give a cell density of 10^7 cells/ml.

2.7.3 Separation of the B Lymphocytes

2.7.3.1 AET-treatment of sheep red blood cells

Human T lymphocytes are able to bind to sheep red blood cells (SRBCs), and this capability can be exploited for the removal of these cells from the B cells. The SRBCs (Section 2.1.20) were washed three times with RPMI. 1ml of the red cell pellet was removed and placed into 4ml of a filter sterilised solution of 2-aminoethylisothiouronium (AET) (Section 2.1.12); 102mg in 10ml water, adjusted to exactly pH 9.0, with sodium hydroxide solution. This pre-treatment was used in order to improve the reproducibility of the technique (Kaplan and Clark, 1974). The cells were incubated at 37°C for 20 minutes with occasional mixing, then washed 5 times with RPMI (Section 2.1.20) and made up to a concentration of 10%, i.e. to 1ml of packed cell volume 9ml of RPMI was added.

2.7.3.2 Preparation of Percoll

The human peripheral B lymphocytes were separated from the rosetted T cells by means of floatation on a Percoll (Section 2.1.20) cushion (Section 2.7.3.3). The Percoll was prepared as follows: 10ml of filter sterilised PBS was added to 90ml of sterile Percoll; 6.3ml of the Percoll-PBS was mixed with 3.7ml of RPMI prior to use, to attain a density of 1.08 for the B cell separation.

2.7.3.3 Procedure for the isolation of B lymphocytes

The PBLs, prepared as described above, were diluted to obtain 10^7 cells per ml. An equal volume of 2 % AET-SRBCs was added to the

cell suspension. Then a volume of FCS, equal to that of the original volume of the cell suspension was added. This combination was lightly centrifuged at 200g for 10 minutes. The tube was placed on ice for 1 to 2 hours, without the removal of the supernatant. After this time, the tube was inverted or rocked gently and a sample removed for examination by haemocytometer to check for the presence of the T cell rosettes.

The sample was then gently layered over Percoll and centrifuged at 500g for 10 minutes. The B lymphocytes were collected from the interface and washed twice in RPMI-2% FCS.

2.7.4 Transformation of Human Peripheral Blood B Lymphocytes

Procedure

The pelleted B lymphocytes, separated from the rosetted T cells (Section 2.7.3.3), were resuspended in 1ml of virus stock supernatant (per 20ml blood sample). The cells were incubated with the virus, at 37°C for 1 hour. Finally, the cells were washed once and resuspended in complete medium with 20% FCS (approximately 12ml per 20ml blood sample). The cells were seeded into the inside wells of one 96 well plate (Section 2.1.19) per 20ml blood sample, at approximately 200µl/well. The peripheral wells were filled with 200µl each of RPMI; this was to protect the inside wells containing the cells, from evaporation.

2.7.5 The Cyclosporin A Method

Cyclosporin A (CsA) is a drug which is thought to specifically inhibit the activity of T lymphocytes (reviewed by Shevach, 1985). The drug inhibits the potential activity of T cells against in vitro transformed B lymphocytes (Bird et al., 1981). In this study

the rosetting and CsA methods of removing the T cell influence were compared in some samples.

The procedure for the CsA technique was essentially the same, with the omission of the rosetting steps to remove the T cells, and the inclusion of CsA at 1 to 2µg/ml in the complete medium in which the cell were finally resuspended and seeded into 96-well plates. The CsA method, reduced the time of the transformation procedure by at least 4 hours.

2.7.6 Care and Appearance of Emerging Transformed Cells

The transformed cells were first fed after 7 days, by the removal of approximately 50µl of the original medium, and replacing with fresh complete medium (Section 2.2.7.1). The CsA treated transformants were treated in the same manner. The cells were fed weekly for up to 3 or 4 weeks, by which time the cells were well established within the wells and ready for assay (Section 2.7.7).

The transformed cells appeared much larger than the original B lymphocytes. The cells tended to be asymmetrical; rounded at one end, with cytoplasmic protrusions at the other (Figure 2.3). The cells tended to be gregarious in behaviour, forming discrete clumps ranging from two cells to collections up to 1mm in diameter. These clumps were not clones since the cells could be separated by gentle shaking; within an hour the clumps would reform.

2.7.7 Assay and Expansion of Positives

The transformations were usually ready for assay when they were approximately three weeks old. The wells were assayed in a similar manner to the rat hybridomas (Section 2.6.4.7), however, generally

only 70µl of supernatant was removed for assay due to the small amount available, and the need to assay against both S-antigen and ROS. The medium was replaced by feeding, after removal for assay. Positive clones were expanded into 24-well plates (Section 2.1.19), reassayed and expanded into 50ml flasks (Section 2.1.19), if they remained positive. The cells could be further expanded into 250ml flasks. Fusions were performed with transformed cells grown in either 50 or 250ml flasks (Section 2.7.8).

2.7.8 Human Fusion Protocol

2.7.8.1 Preparation of parent cells

The patients' EBV transformed cells which remained positive, albeit weakly, after expansion in 50 or 250ml flasks, were harvested and washed 3 times in serum-free RPMI. The cells were resuspened in 10ml of RPMI and counted as described in Section 2.6.5. The fusion partner employed was the KR-4 human lymphoblastoma cell line (Kozbor et al., 1982) (Section 2.1.18). These cells were also harvested, washed and counted. Equal numbers, eg. 1×10^7, of each cell type was required.

2.7.8.2 Fusion procedure

The transformed cells and the KR-4 cells, prepared as above were pooled and centrifuged together in a sterile universal container, at 500g for 5 minutes. The supernatant was discarded and 0.5ml of warm (37°C) 50% PEG in RPMI was added dropwise over 1 minute, while gently agitating the tube. The cells were gently mixed with the PEG for 90 seconds, and 5ml of warm RPMI was added over a period of 6 to 10 minutes. The first 1ml of RPMI was believed to be the most critical and was added over 3 minutes. The universal container was then capped and incubated at 37°C for 20 to 60 minutes, during

which time more fusions could be made.

The cells were then centrifuged and washed twice in RPMI, before finally being resuspended in HAT medium (Section 2.2.7.2) to which oubain (Section 2.1.21) has been added to give a final concentration of $1x10^{-5}$M. This medium was also supplemented with spleen feeder cells from a young Balb C mouse (Section 2.1.17) prepared as described in Sections 2.6.6.1 and 2.6.9.1. The cells were seeded into one 96 well plate per fusion, at 100µl/well. Approximately 10 wells were reserved for the controls, these consisted of 5 wells of the unfused transformed cells and 5 wells of the unfused KR-4 cells. Generally, after 8 to 10 days, the parent cells were dead. The fusion plates were maintained at 37°C.

2.7.8.3 Care of human fusions and appearance of hybrids

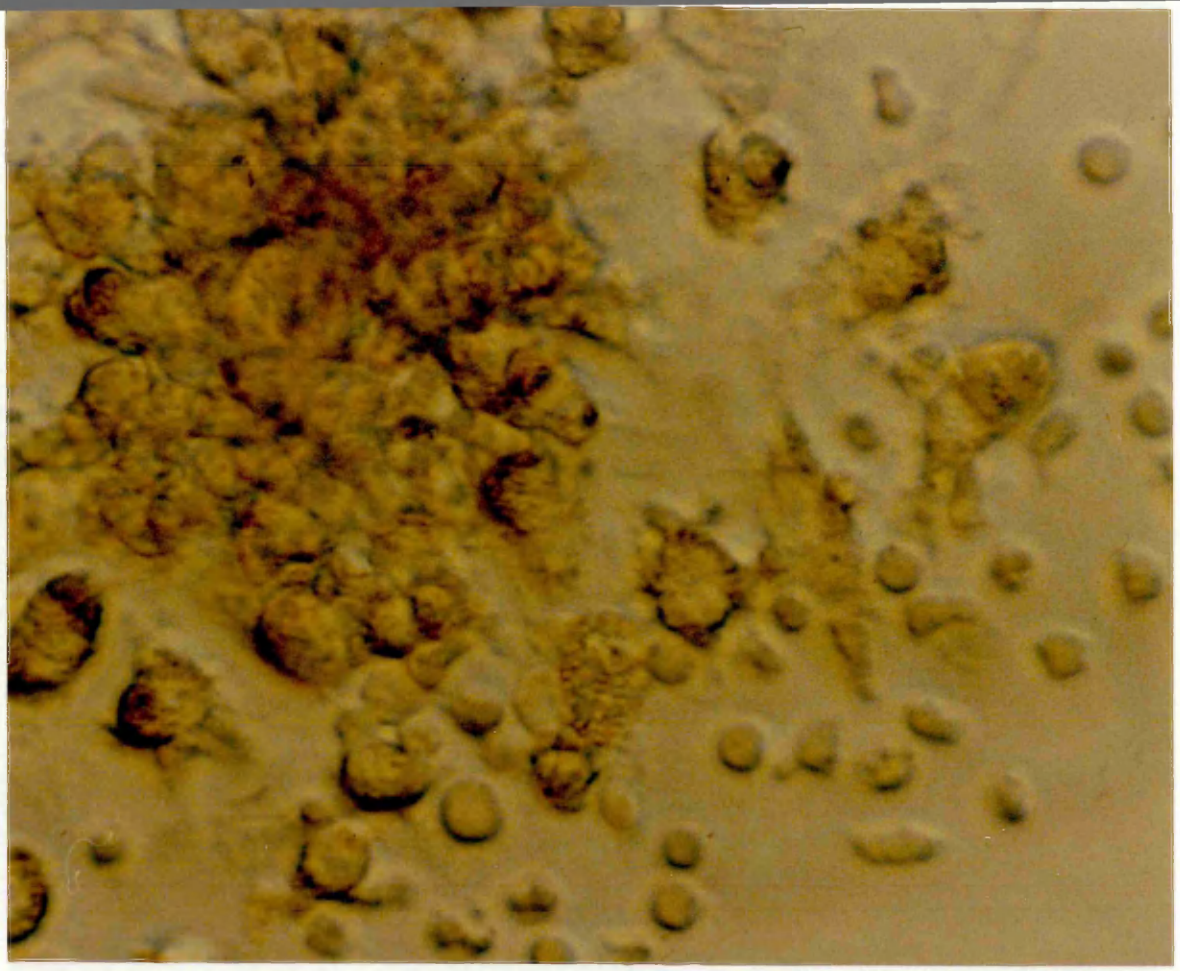
The wells of the fusion plates were fed after 24 hours with 100µl of complete medium containing twice the usual concentration of HAT (Section 2.2.7.2), although the concentration of oubain remained the same. Three days after fusion, approximately 150µl of the medium was aspirated and replaced with 100µl of HAT-oubain medium, at the original concentrations. The cells were fed every 4 to 6 days depending on the growth rate of the cells. The oubain was omitted from the medium after about 2 weeks and the HAT was omitted after 3 weeks.

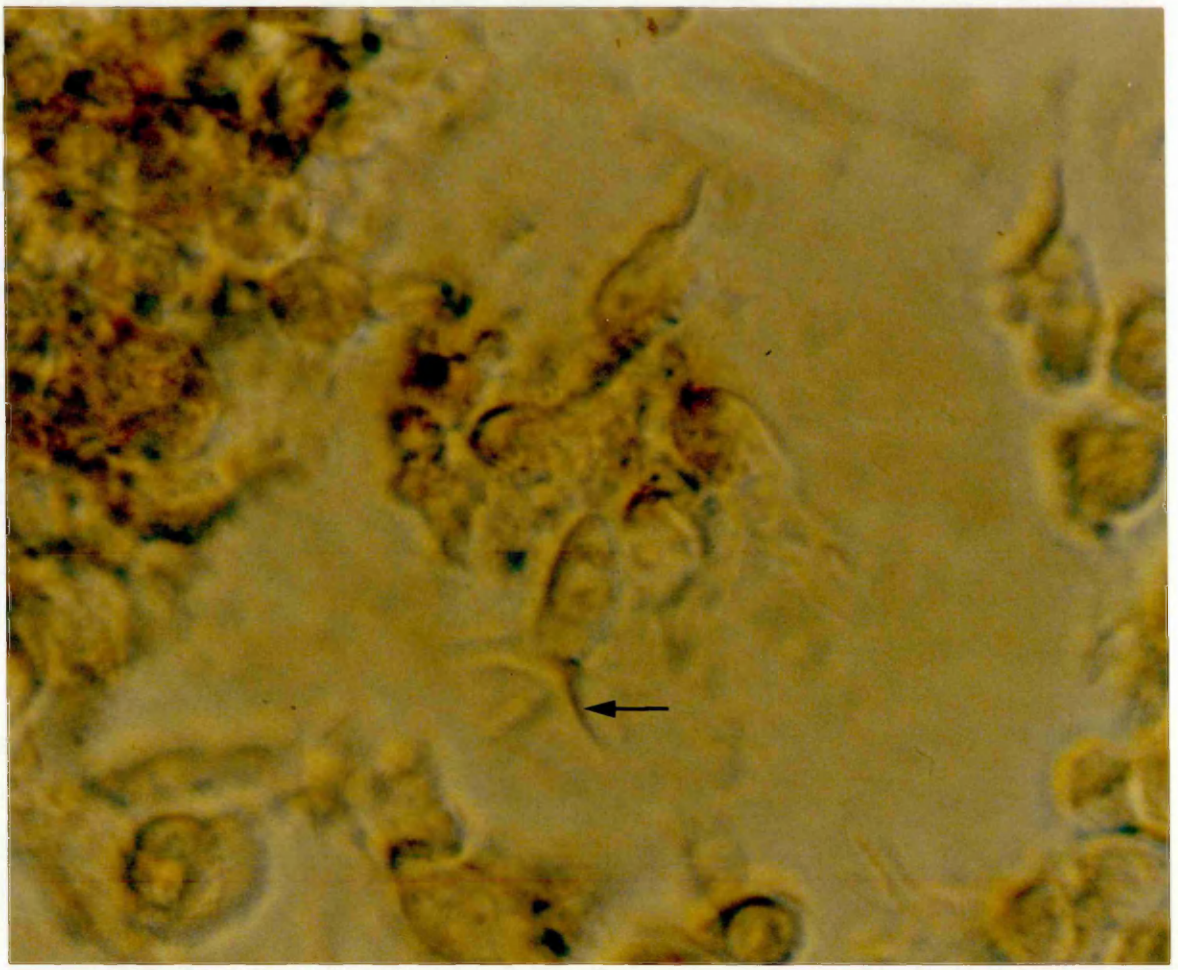
The hybrids could first be detected after as early as 4 days in one fusion, although 6 to 12 days was more usual. The hybrids appeared about 1.5 times larger than the parent cells, and were also gregarious. These cells tended to be rounded at one end, tapering at the other and often fish like in appearance (Figure 2.4).

Figure 2.3 High Magnification of Transformed Human B Lymphocytes

Figure 2.4 High Magnification of Human Hybrid Cells

Arrows indicate cell protrusions





2.7.8.4 Assay of human hybrids

The human fusion plates were ready for assay by ELISA, after 3 to 4 weeks when the hybrids has grown to a number that could be expanded into the wells of a 24 well plate. Positive human hybrids can be expanded and subcloned to achieve monoclonality, as in the rat system (Section 2.6.9). However, in this study no suitable positives were detected after fusion.

CHAPTER THREE

PREPARATION OF ANTIGENS AND DEVELOPMENT OF IMMUNOASSAY SYSTEMS

Chapter 3

PREPARATION OF ANTIGENS AND DEVELOPMENT OF IMMUNOASSAY SYSTEMS

The methods for the preparation of S-antigen and ROS are described below. The development of the ELISA assay systems involving these antigens, is also described.

3.1 PREPARATION OF RETINAL ANTIGENS

The principal retinal antigens investigated in this study were the rod photoreceptor outer segment (ROS) membrane proteins and the soluble protein, S-antigen, extractable from the ROS. Bovine retinae were utilised since the eyes were readily obtained in large quantities, and common inter-species determinants have been found for retina specific proteins (Brinkman et al., 1979; Brinkman et al., 1980), including S-antigen (Dorey and Faure, 1977).

It is known that ROS membranes consist of 80 to 90% opsin; other membrane or membrane associated proteins account for the remainder (Heitzmann, 1972; Papermaster and Dreyer, 1974) (Figure 3.8). Although a mixture of potential antigens, the ROS antigens will be referred to as the ROS antigen.

The ROS were prepared according to the method of Papermaster and Dreyer (1974) with minor alterations, and the S-antigen was purified by a modification of the method of Borthwick and Forrester (1983). The procedures involved in the preparation and purification of the retinal antigens are described below.

3.1.1 Eye Dissection

The sclera was incised with a scalpel blade and the anterior

portion of the eye (containing cornea, lens, and iris) together with the vitreous was removed. The posterior eye cup was everted and the retina gently detached from the RPE using a pasteur pipette. The retina was suspended from its attachment at the optic nerve head and rinsed with the hypotonic buffer, into a beaker containing the appropriate buffer (Section 2.2.1.1 and 3.6.1). The retina was then removed with scissors and dropped into the beaker. All procedures were carried out on ice.

3.2 PURIFICATION OF RETINAL S-ANTIGEN BY ION EXCHANGE CHROMATOGRAPHY

3.2.1 Preparation of Crude Retinal Extract

It has been shown that hypotonic buffer aids the extraction of soluble proteins from the ROS (Godchaux and Zimmerman, 1979). The retinae were dissected into the 2.5mM Tris/HCl hypotonic buffer, pH 7.5 (Section 2.2.1.1), on ice. Approximately 5 to 10ml of buffer were used for each retina. The suspension was homogenised by hand with a glass homogeniser, with about 5 or 6 passes, followed by ultracentrifugation at 80,000g for 60 minutes, at 4 °C. The supernatant containing the soluble material was collected and the pellet discarded.

An equal volume of cold saturated ammonium sulphate solution, adjusted to approximately pH 7.5 with ammonia, was slowly added to the supernatant, with constant stirring. The precipitate was allowed to settle overnight. The suspension was centrifuged at 12,000g for 30 minutes and the antigenically rich pellet was dissolved in water. The sample was then dialysed against 0.15M saline to remove the ammonium sulphate, followed by dialysis against the 20mM Tris/HCl column starter buffer, pH 7.5 (Section

2.2.1.2). All procedures were performed at 4°C.

Prior to ion exchange chromatography, the crude soluble extract was centrifuged at 80,000g for 60 minutes to remove aggregated protein.

3.2.2 Preparation of Column

DEAE-Sephacel (Section 2.1.3) was selected since it offers volumetric stability over the ionic concentrations employed, and also allows relatively high flow rates to be used. The column was assembled in an upright position at 4°C. A slurry of the Sephacel gel in starter buffer, was poured into the column and allowed to settle under gravity. The column was then equilibrated with at least 5 column volumes of the starter buffer (Section 2.2.1.2) at a flow rate of 50ml per hour.

3.2.3 Sample Application and Elution

Crude retinal extract, prepared as described above, was applied to the column. The column capacity was 160mg/ml of gel, and generally 100mg of crude protein was applied to a 10ml column. The column was then washed through with at least 5 column volumes of starter buffer to remove unbound material. A gradient consisting of 250ml of starter buffer and 250ml of 1.5M NaCl in starter buffer was pumped through the column at the same rate as before. Fractions of 3 to 5ml volumes were collected.

Total protein concentration in the fractions was estimated by reading the absorbance at 280nm and the Bradford's protein assay (Section 2.3.2). S-antigen was detected in the column fractions by use of an ELISA system developed for S-antigen (Figure 3.1). SDS-PAGE was used to test the purity of the fractions collected.

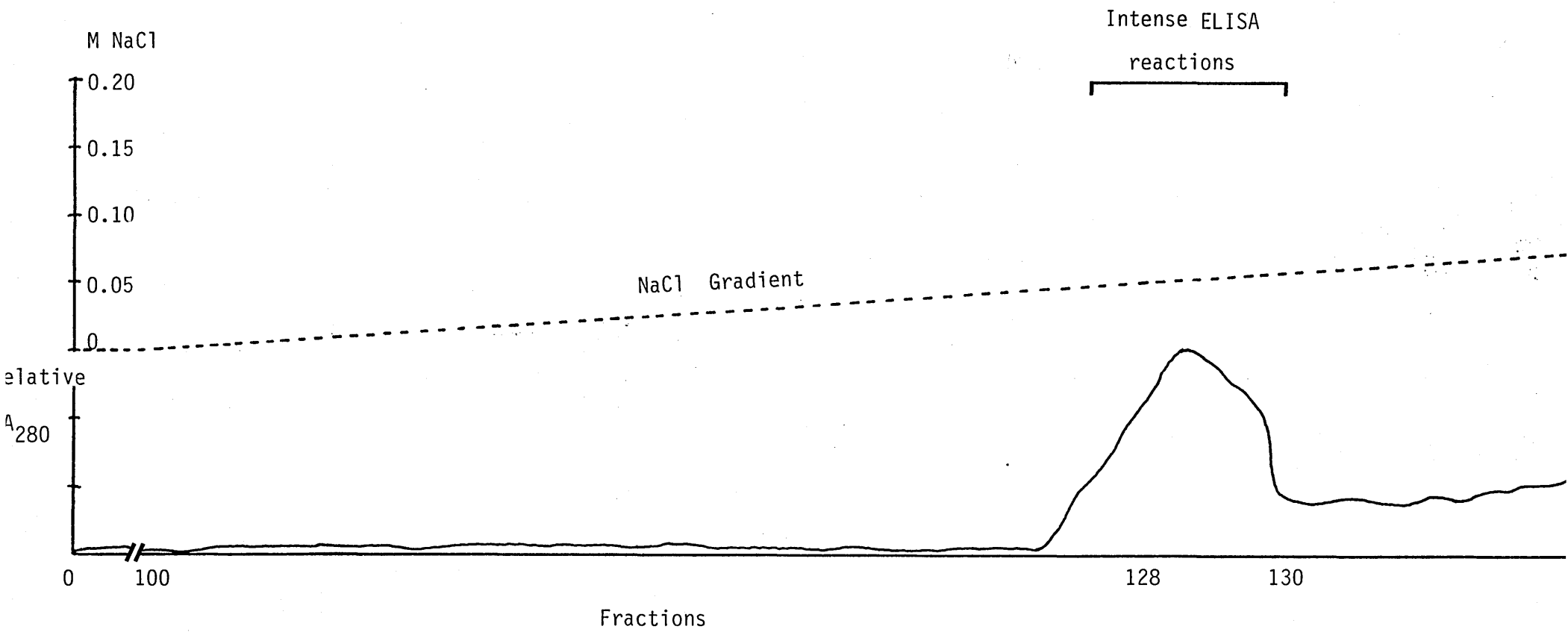
Figure 3.1 Detection of S-Antigen in Fractions of DEAE-Sephacel Column

The absorbance of the eluent of the DEAE-Sephacel ion exchange column, was measured at 280nm (A280), during the purification of S-antigen. The antigen was detected in most column fractions, using the ELISA assay, with the rat monoclonal antibody S2.4.C5, specific for S-antigen. The majority of the S-antigen, however, was obtained in the protein peak (A280) in the region of 0.05M NaCl as demonstrated by ELISA, and also in SDS-PAGE (Figure 3.3).

KEY

—— Protein concentration (A280)

— — NaCl gradient


Intense ELISA
reactions
M NaCl
0.20
0.15
0.10
0.05
0
NaCl Gradient
elative
A280
0
100
128
130
Fractions

3.3 CHROMATOFOCUSING

When highly purified S-antigen was required for immunisation of DA rats for monoclonal antibody production (Section 2.6.1), further purification was achieved by a means of a second, chromatofocusing column (Borthwick and Forrester, 1983). Chromatofocusing is a chromatography technique which separates proteins according to their isoelectric points (pI). The technique was first described by Sluyterman and Wijdenes (1977), who demonstrated that a pH gradient could be produced on an ion exchanger by taking advantage of the buffering capacity of the exchanger's charged group. A pH gradient is formed within a column when a buffer, adjusted to a specific pH, is applied to an ion exchanger, equilibrated to a different pH. This pH gradient can be used to elute proteins in order of their isoelectric points.

In the present study, Polybuffer Exchanger 94 (PBE 94) and Polybuffer 74 (Pharmacia, Section 2.1.3) were utilised. The pH range in this system is 7 to 4, which was suitable for the purification of S-antigen with its pI of 5.5 (Borthwick and Forrester, 1983). The PBE 94 was equilibrated to pH 6.2 and the S-antigen sample to be further purified was dialysed against the column buffer at the same pH. The sample containing S-antigen was loaded onto the column, and at the starting pH of 6.2 the S-antigen was negatively charged and bound to the exchanger. During the application of the eluent (PB 74) pH 4, a pH gradient was created. As the pH of the buffer surrounding the S-antigen decreased to a value below the pI of the protein, S-antigen became positively charged and was repelled from the exchanger and migrated rapidly through the column until it reached a position where the pH of the

buffer was above its pI and the protein became bound once more. This process occurred for all proteins present in the sample. The attraction and repulsion processes were repeated until the proteins were eluted from the column in order of their isoelectric points.

3.3.1 Preparation of Column

The gel was dispersed in the 25mM histidine/HCl, pH 6.2 starter buffer (Section 2.2.1.3) to make a slurry. The slurry was degassed prior to packing. A column of dimensions 1 x 13cm was used. The slurry was applied dropwise to keep the column full to the brim with buffer, while the gel was packing. Packing was continued at a linear flow rate of 100ml/hour until the gel bed had completely settled, with a final bed volume of 10ml. It was convenient to equilibrate overnight with a reservoir of excess buffer, over night at 4°C. The column was equilibrated when the pH of the eluent matched that of the starter buffer.

3.3.2 Preparation of Sample

A sample of S-antigen purified by ion exchange chromatography, was dialysed against the histidine/HCl, pH 6.2 buffer (Section 2.2.1.3) at 4°C. The dialysis also removed the NaCl, present in the S-antigen eluted from DEAE-Sephacel columns (Section 3.2), which would interfere with the chromatofocusing.

3.3.3 Sample Application and Elution

A 5ml volume of PB 74 (Section 2.1.3) eluent, pH 4.0, diluted 1:8 was applied to the equilibrated column, prior to the application of the sample in order to avoid exposure to extremes of pH. Approximately 10mg of S-antigen was applied in a volume of 10ml. The sample was eluted with 12.5 column volumes of the diluted PB 74

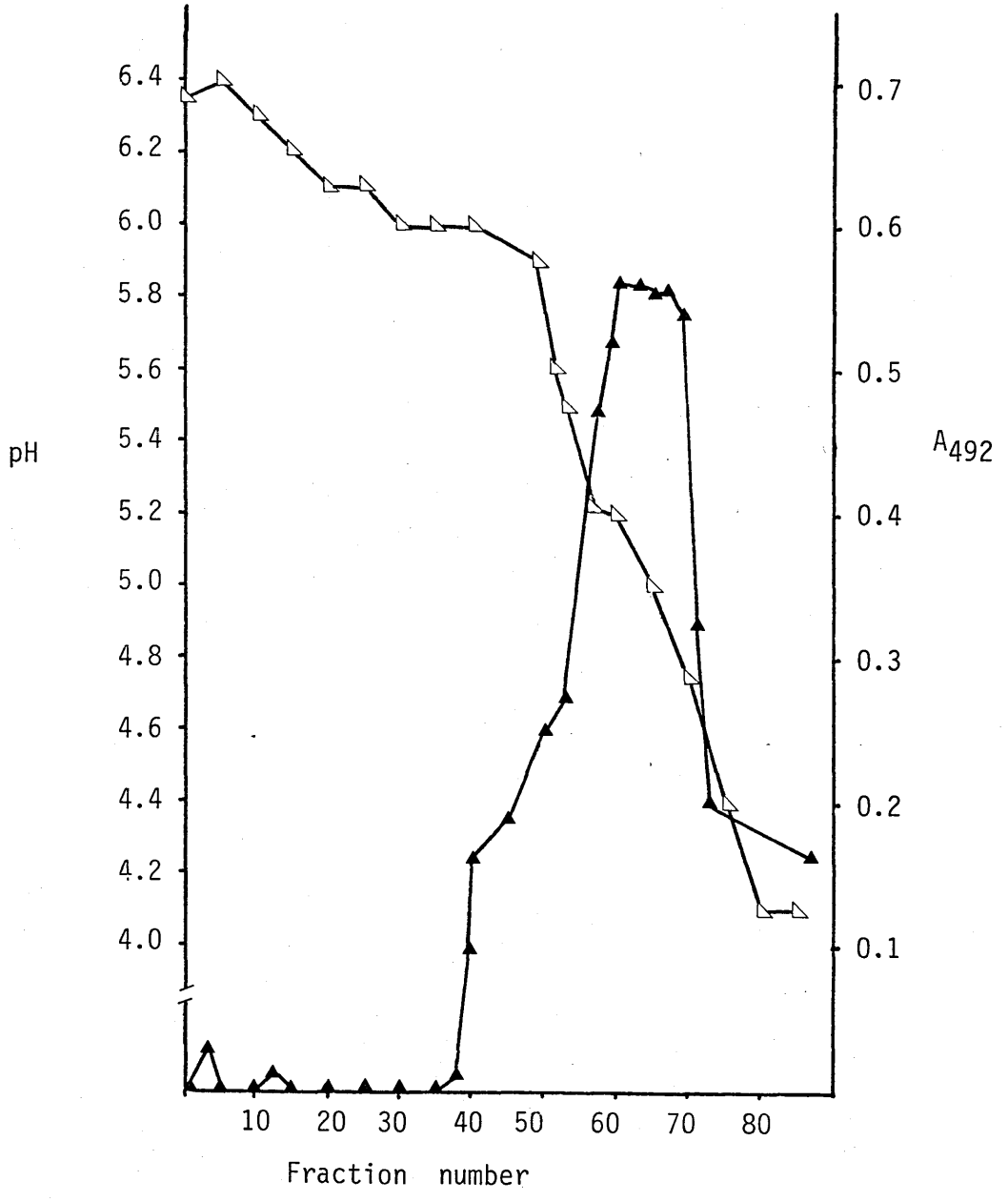
Figure 3.2 Detection of Chromatofocusing Purified S-Antigen

S-antigen, purified by Chromatofocusing, was detected in the ELISA assay using a 1:1000 dilution of rat antisera, with specificity for S-antigen. The majority of S-antigen was detected in the pH range of 4.8 to 5.6.

KEY

◺ pH gradient

◣ ELISA reaction


pH
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A492
10
20
30
40
50
60
70
80
Fraction number

at a flow rate of 30 to 40ml per hour and 1ml fractions were collected. During the elution procedure the pH of the column fractions were measured (Figure 3.2). All procedures were carried out at 4°C.

The chromatofocusing PBE 94 was regenerated by the passage of two to three bed volumes of the starter buffer through the column, and was stored in 20% ethanol at 4°C, when not in use.

3.4 DETECTION OF S-ANTIGEN, YIELD AND PURITY

3.4.1 Detection of S-Antigen

S-antigen, purified by ion-exchange chromatography and chromatofocusing, was detected immunologically in ELISA (Sections 3.8.2 and 3.8.3), by applying directly to the plate, 50µl of each of the column fractions to be tested, or a 10 fold dilution of the fractions. Initially, S-antigen was detected using the rabbit antisera of Borthwick and Forrester (1983), raised against bovine S-antigen. In subsequent preparations, the protein was detected by means of antibody raised in rats against S-antigen, either polyclonal antiserum (Section 3.8.1) or monoclonal antibody S2.4.C5 (Chapter 6), when it became available. The monoclonal antibody was preferable since it has been shown to be specific to S-antigen in immunoblotting experiments, against crude retinal proteins and irrelevant proteins (Figures 6.7 and 6.11).

Immunologically S-antigen could be detected in most column fractions after ion exchange chromatography, although it tended to remain bound to the column during the wash prior to the application of the gradient, providing that the column had not been overloaded with the crude material. The most intense reactions in ELISA were

obtained for the fractions around the peak absorbance at 280nm, in the region 0.05M NaCl (Figure 3.1), and showing S-antigen to form the major band after SDS-PAGE (Figures 3.3 to 3.5). The detection of S-antigen in the ELISA over such a wide range of fractions was a reflection of the sensitivity of the assay system, which is capable of the detection of antigen at nanogram quantities. Minute quantities of S-antigen dissociating from the column before the major peak, were thus easily detected.

The majority of the S-antigen, purified by chromatofocusing, was detected by ELISA in the column fractions within the pH range of 4.8 to 5.6 (Figure 3.2). The isoelectric point of S-antigen is known to be 5.5 (Borthwick and Forrester, 1983), thus, this is the pH region in which it is likely to be eluted.

3.4.2 Electrophoretic Identification of S-Antigen

Column fractions were subjected to SDS-PAGE (Section 2.3.4) as a criterion for purity. Both Coomassie Blue (Section 2.3.5) and silver staining (Section 2.3.6) methods were utilised. S-antigen purified by ion exchange chromatography (Section 3.2), was identified as the major band with an apparent molecular weight in the region of 50K, after SDS-PAGE (Figures 3.3 to 3.5). Figure 3.3 shows a Coomassie Blue stained gel of column fractions obtained from the protein peak in the region of 0.05M NaCl, which gave intense readings in the ELISA assay (Figure 3.1). Due to the high protein concentration in this region, the contaminating proteins are more easily seen. Some of these contaminants might represent breakdown products of S-antigen, and micro-aggregates of these. This was shown by the reaction of several minor bands in immunoblots (Section 2.9) of the S-antigen containing column

Figure 3.3 SDS-PAGE of S-Antigen Purified on a DEAE-Sephacel Column

Column fractions, of volume 5ml, containing S-antigen purified by ion-exchange chromatography on a DEAE-Sephacel column, were subjected to SDS-PAGE as criterion of purity. The purity of 15μl samples of fractions, obtained from the S-antigen containing peak (Figure 3.1), was shown in a 12.5% gel stained with Coomassie Blue.

LANES

1 to 4, 7 to 9. Consecutive column fractions

5. Pharmacia low molecular weight standard proteins

6. S-antigen from a previous preparation

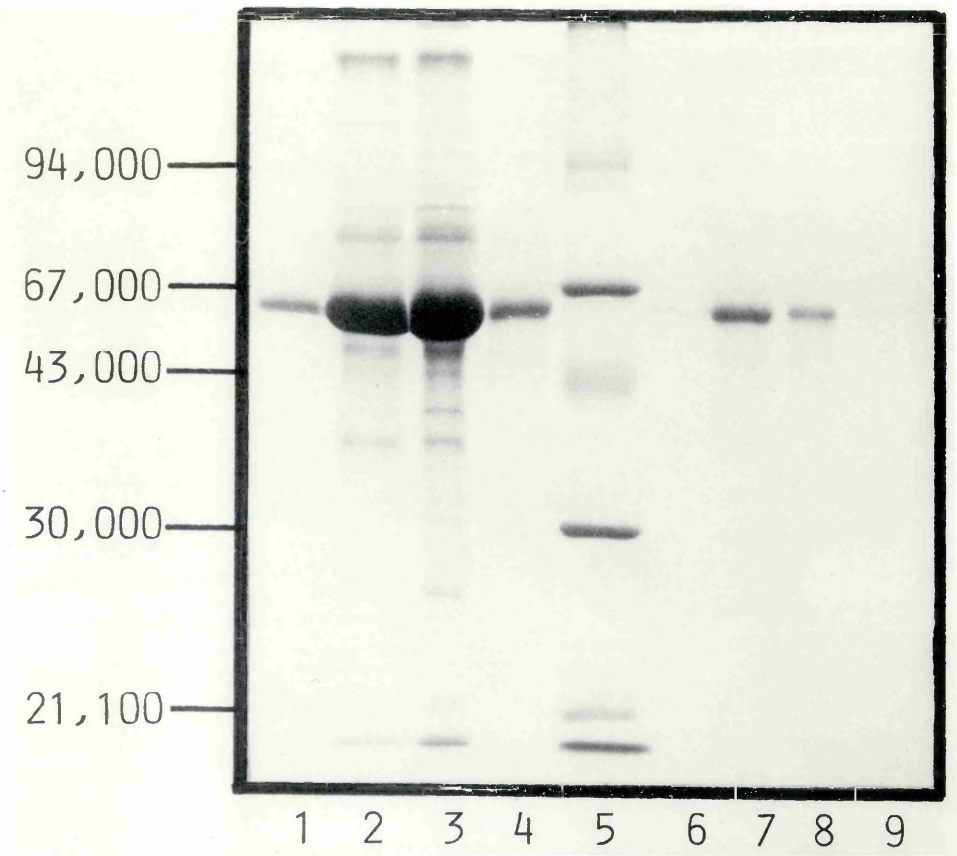

94,000
67,000
43,000
30,000
21,100
1 2 3 4 5 6 7 8 9

Figure 3.4 SDS-PAGE of S-Antigen Purified on a DEAE-Sephacel Column

Column fractions, of volume 5ml, containing S-antigen purified by ion-exchange chromatography on a DEAE-Sephacel column, were subjected to SDS-PAGE as criterion of purity. The purity of 5µl samples of fractions, obtained from the S-antigen peak, was shown in a 12.5% gel stained with Coomassie Blue.

The S-antigen was the only band detected in the Coomassie Blue stain, however, silver staining of similar fractions on the same gel, revealed a few contaminating bands (Figure 3.5).

LANES

1. Pharmacia low molecular weight standard proteins
2 to 5. Consecutive column fractions
6. Crude retinal extract

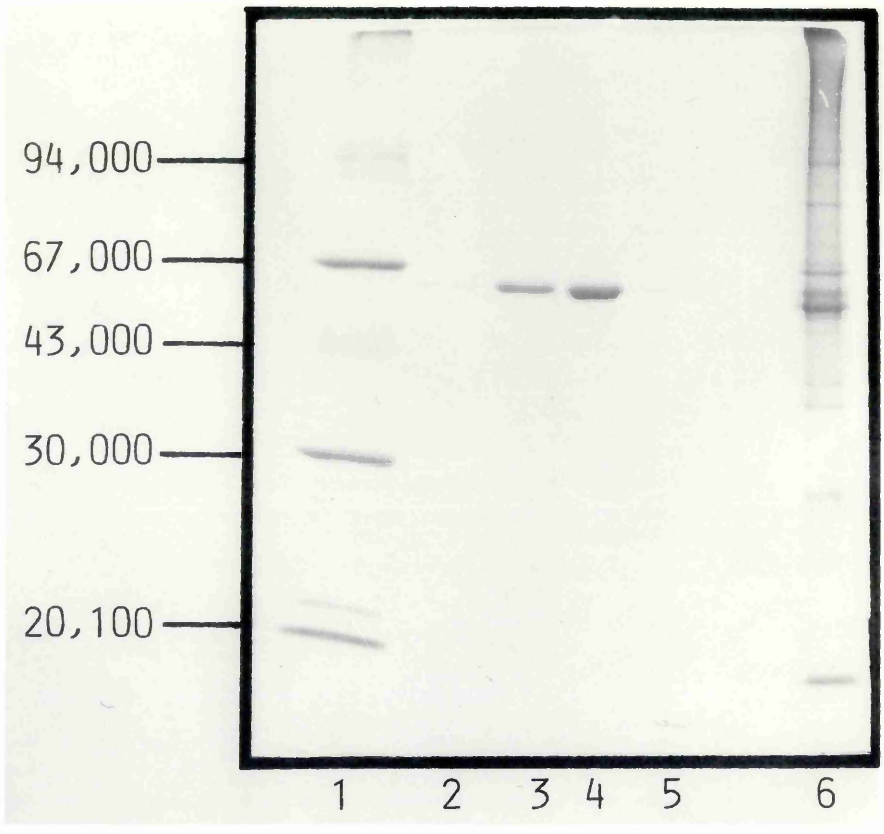

94,000
67,000
43,000
30,000
20,100
1
2
3
4
5
6

Figure 3.5 Purity of S-Antigen in a Silver Stained Gel

Samples of 5µl were obtained from fractions containing S-antigen purified on a DEAE-Sephacel column, and electrophoresed in a 12.5% gel, followed by sensitive silver staining (Wray et al., 1981).

The S-antigen was the major band detected after silver staining, however, a few contaminating bands were evident, compared with the less sensitive Coomassie Blue stain (Figure 3.4).

LANES

1. Crude retinal extract
2. Pharmacia low molecular weight standard proteins
3, 4 and 5. Consecutive column fractions

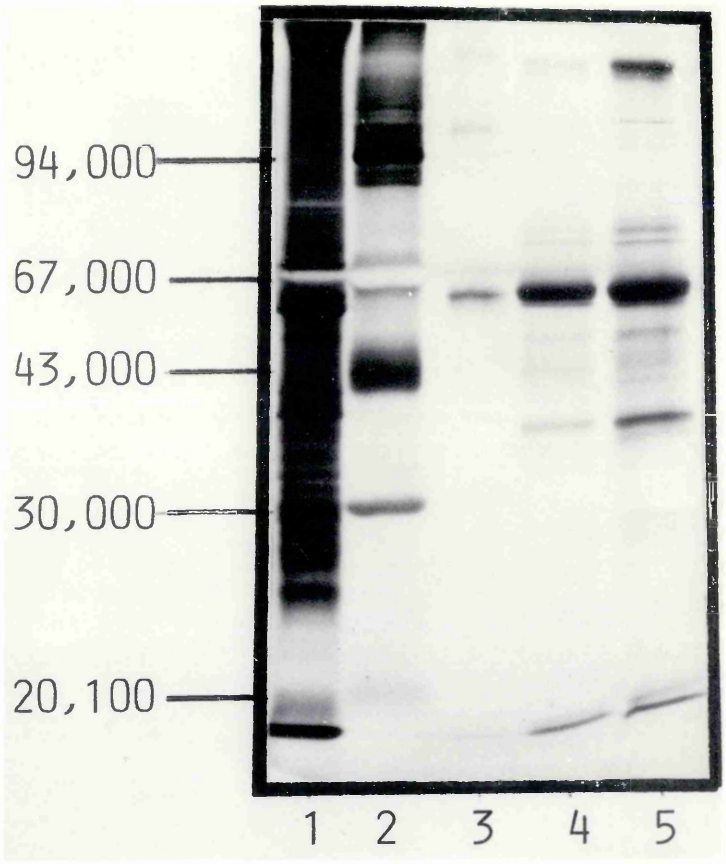

94,000
67,000
43,000
30,000
20,100
1
2
3
4
5

Figure 3.6 SDS-PAGE of S-Antigen Purified by Chromatofocusing

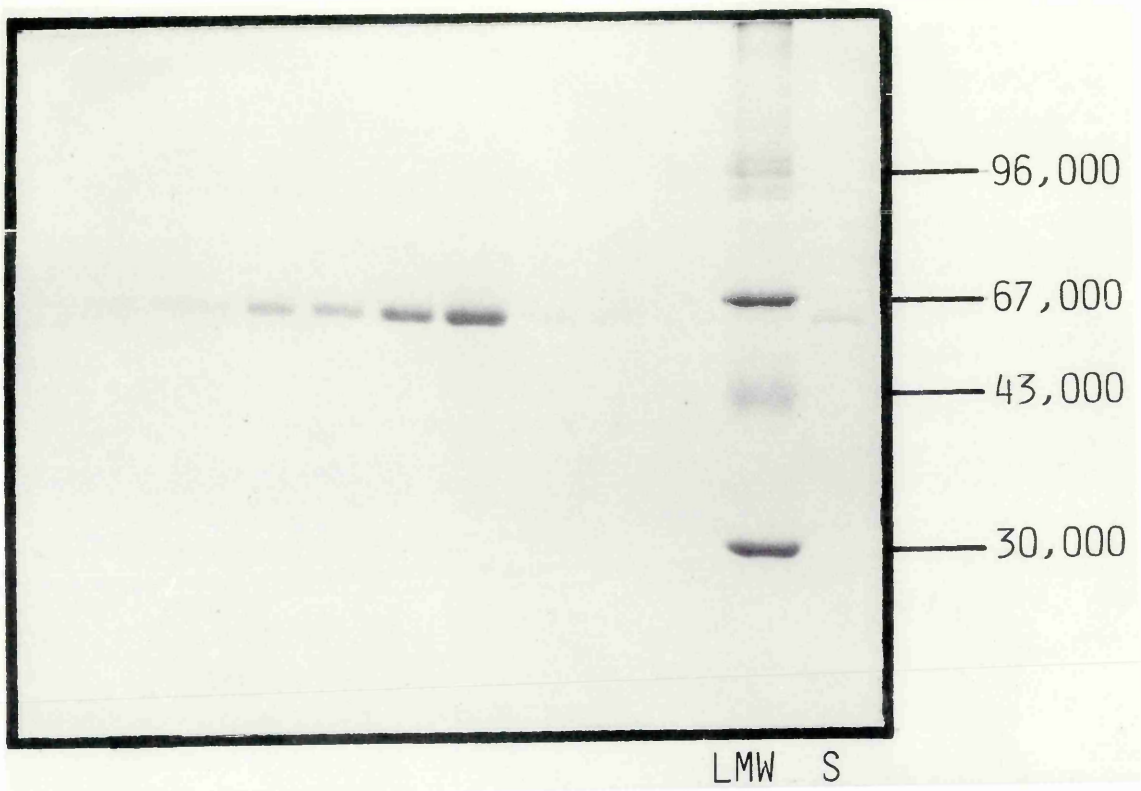
Samples of 10μl were obtained from chromatofocusing fractions which generated the most intense reactions for S-antigen in the ELISA, and electrophoresed in a 12.5% gel, and stained with a sensitive silver stain.

LANES

1 to 8. Consecutive chromatofocusing fractions

LMW: Pharmacia low molecular weight standard proteins

S: S-antigen, used as a marker


96,000
67,000
43,000
30,000
LMW
S

fractions with the IgG monoclonal antibody, S2.4.C5 (Chapter 6). Figures 3.4 and 3.5, compare similar gels of S-antigen, purified by ion-exchange chromatography, stained with Coomassie Blue and by a silver staining technique. The silver stain of the purified S-antigen showed that it was about 90% pure.

The majority of the S-antigen, purified by chromatofocusing, was recovered in the column fractions within the pH range of 4.8 to 5.6 (Section 3.4.1 and Figure 3.2). Figure 3.6 shows a Coomassie Blue stained gel of S-antigen obtained from this region. The presence of the elution Polybuffer, which contains a mixture of salts, caused diffusion of the samples during the initial stages of SDS-PAGE. The resolution was apparently unaffected by the initial diffusion (Figure 3.6). The major protein detected after SDS-PAGE had a molecular weight in the region of 50K (Figure 3.6).

The column fractions were pooled according to their purity and protein content evident in SDS-PAGE.

3.4.3 Protein Estimation

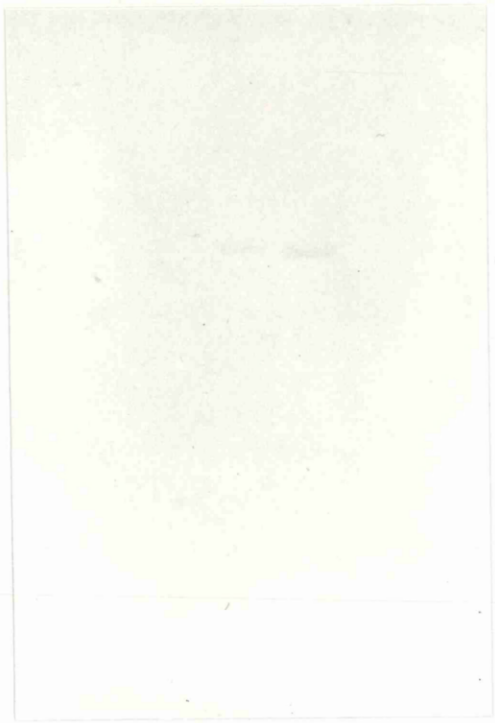
During ion-exchange chromatography, an estimate of protein content was given by measuring the absorbances of the column fractions at 280nm (Figure 3.1). Unbound material was eluted during the wash of the column prior to the application of the gradient. As the salt gradient progressed, S-antigen was eluted in a peak in the region of 0.05M NaCl (Figure 3.1).

Pooled column fractions of both ion exchange and chromatofocusing columns were subjected to the Bradford's protein assay (Section 2.3.2) for a more accurate value of protein concentration. When

Figure 3.7a SDS-PAGE of Purified S-Antigen stained with PAS Reagent

The PAS reaction was determined for approximately 20µg S-antigen, in an 8.5% SDS gel (Section 2.3.7).

The reaction was very weak, and sharply contrasted to that obtained for opsin (Figure 3.7b).

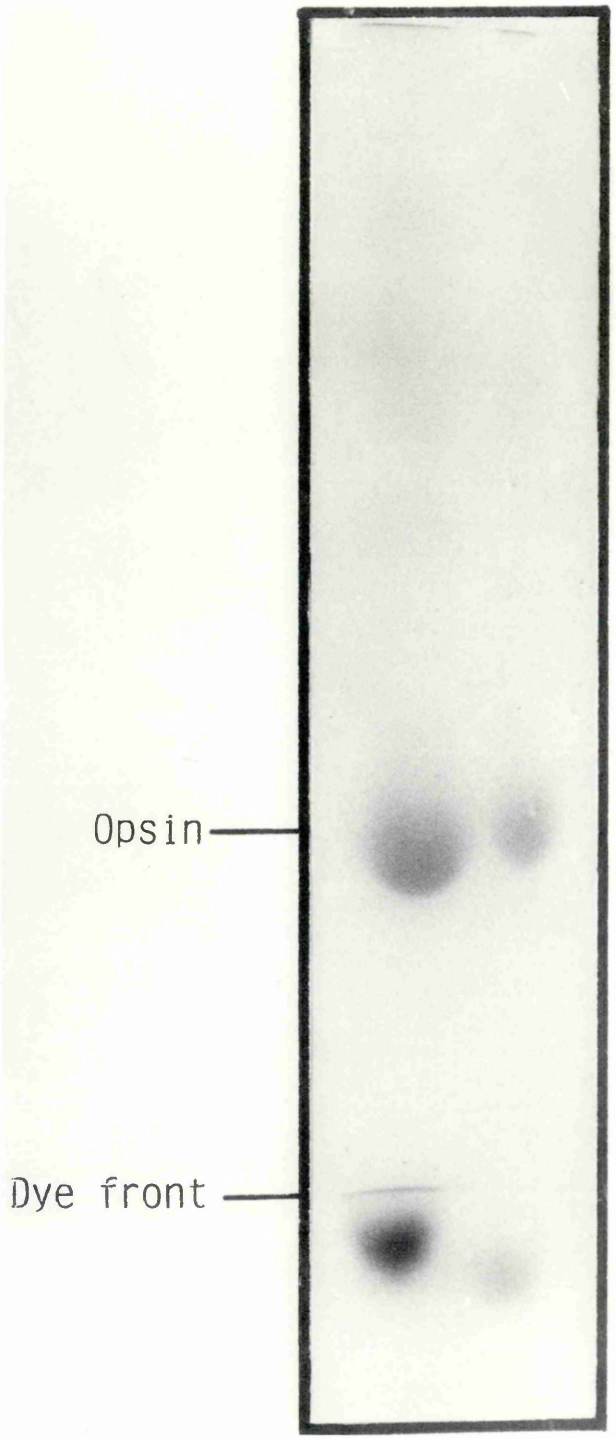


— **S**-antigen

Figure 3.7b SDS-PAGE of Bovine ROS Membranes stained with PAS Reagent

The PAS reaction of bovine ROS (Section 3.6.1), electrophoresed in an 8.5% gel and stained as described in Section 2.3.7.

An intense reaction was obtained for opsin, which provided a positive staining control for testing of S-antigen (Figure 3.7a). The reaction observed below the dye front, may represent glycolipid material which was not visible in the Coomassie Blue stain for protein (Figure 3.8).


Opsin
Dye front

100mg of crude protein was applied to a 10ml column of DEAE-Sephacel, approximately 3.4mg of S-antigen could be obtained, giving a yield of 3.4%. The preparation was reasonably pure, forming the only one major band in SDS-PAGE, even when the highly sensitive silver stain (Wray et al., 1981) (Section 2.3.6) was employed (Figure 3.5).

When 10mg of S-antigen was applied to the chromatofocusing column, slightly less than 10mg were recovered in the pooled fractions. This was presumably due to the loss of protein during the procedure, in addition to the removal of contaminating protein.

3.5 CHARACTERISTICS OF PURIFIED S-ANTIGEN

3.5.1 Periodic Acid Schiff's Staining of S-Antigen

Purified S-antigen was tested for carbohydrate content by the periodic acid Schiff's (PAS) reaction (Section 2.3.7), after SDS-PAGE. A very faint staining was obtained for S-antigen, even when 20µg of the protein was applied to the gel (Figure 3.7a), which may indicate the presence of a small carbohydrate moiety. The PAS reaction obtained for S-antigen contrasted with the intense reaction observed for opsin (Figure 3.7b). The ROS provided a positive staining control since the N-terminal of opsin is known to be rich in carbohydrate residues (Heller, 1968).

3.5.2 Uveitopathogenicity of S-Antigen

The uveitopathogenic activity of bovine S-antigen purified according to the procedures described above was verified by the induction of EAU in Duncan-Hartley guinea pigs, following the injection of S-antigen (Section 6.6).

3.6 ISOLATION OF PHOTORECEPTOR ROD OUTER SEGMENTS

The retinal photoreceptor rod outer segments (ROS), of cattle and rats, were prepared on a discontinuous sucrose gradient, essentially by the method of Papermaster and Dreyer (1974), with minor modifications.

3.6.1 Procedure

The retina were dissected out (Section 3.1.1) into the 34% sucrose solution (Section 2.2.3.3), in order to float the ROS (eg. from 50 bovine eyes). The retinae were gently stirred and swirled. The suspension was centrifuged at 1,600g for 5 minutes in a Sorvall SS34 rotor and the supernatant containing the ROS was retained. The pellets were resuspended together in about 25ml of the 34% sucrose solution. Smaller volumes were required for preparing rat eyes. The suspension was centrifuged for approximately 8 minutes at 1,600g and the supernatant was collected, and added to the first supernatant. Twice the volume of 10mM Tris acetate (pH 7.4) (Section 2.2.3.2) was then added to the pooled supernatants. The mixture was then centrifuged in a Beckman Ti60 rotor at 21,000g for approximately 30 minutes. The supernatant was collected and frozen at -70°C for subsequent use in the preparation of S-antigen (Section 3.2). The pellets containing the ROS membranes were resuspended in 18ml of density 1.10 sucrose solution (Table 3.1). Aggregates of the crude ROS material were dispersed by passage through fine gauge G21 needles (Section 2.1.19).

Sucrose solutions for density gradients are displayed in Table 3.1. The sucrose gradients were made in six tubes according to the capacity of the SW40 rotor. The layers of sucrose solutions were applied using a pasteur pipette, in volumes of 3ml. The ROS samples

Table 3.1 Solutions required for the preparation of sucrose gradients*

Stock solutions	Sucrose solutions for density gradients: Density 1.15	Density 1.13	Density 1.11	Density 1.10
42% sucrose solution	93.00g	81.40g	68.40g	62.40g
1.0M Tris acetate, pH7.4	100µl	100µl	100µl	100µl
0.1M MgCl	100µl	100µl	100µl	100µl
To final weight with distilled H_2O	115.00g	113.00g	111.00g	110.00g

*Papermaster and Dreyer (1974)

were applied to the top of the gradients in volumes of 3ml. The tubes were topped up and balanced using either additional crude ROS in density 1.10 sucrose, or the density 1.10 sucrose solution alone. The tubes were ultracentrifuged at 95,000g for 2 hours. The purified ROS forming bands between density 1.11 and density 1.13 sucrose layers, were collected. The samples were diluted with 10mM Tris acetate buffer at twice the sample volume. The suspensions were centrifuged in a Beckman centrifuge, for 30 minutes at 21,000g. The pellets were resuspended in the Tris buffer, and centrifuged as before. This washing procedure was repeated several times when required. The pelleted ROS were stored in Eppendorf tubes (Section 2.1.23) at -20°C or -70°C.

3.6.2 Reduction of S-Antigen Content

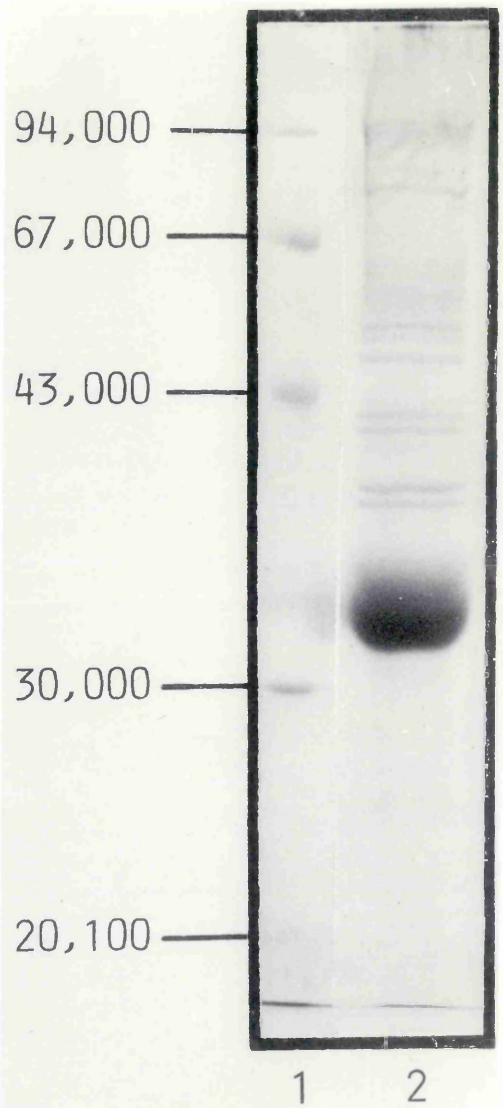
Some experiments performed during the course of this study required a reduction in the S-antigen content of the ROS so that antibody responses to rhodopsin and other insoluble proteins could be more readily induced or measured. The final step in the method of Papermaster and Dreyer (1974) involved two washes of the ROS. The soluble proteins of the ROS may be reduced by repeated washing of the purified membranes (Section 3.6.1), especially after lysis by freezing and thawing. Membrane associated proteins, such as S-antigen, may be more resistant to removal by washing, especially under illuminated working conditions. The supernatants from the repeated washing of the ROS were therefore tested for S-antigen in ELISA using initially antisera and subsequently monoclonal antibody S2.4.C5 (Chapter 6), raised against bovine S-antigen, to determine the degree of contamination of the ROS antigen with soluble proteins.

Figure 3.8 SDS-PAGE of isolated ROS Membranes

Bovine ROS were prepared according to a method based on that of Papermaster and Dreyer (1974) (Secton 3.6.1). After washing the ROS membranes 5 times, in 10mM Tris acetate buffer, pH7.4, very little S-antigen could be detected in the Coomassie Blue stained 8.5% gels after SDS-PAGE. Opsin appeared to represent over 80% of the protein (Lane 2).

LANES

1. Pharmacia low molecular weight standard proteins
2. Bovine solubilised ROS membranes


94,000
67,000
43,000
30,000
20,100
1
2

S-antigen was found to persist at low levels in the supernatants after as many as 25 washes in 15ml volumes of the ROS (1ml pellet), by the application of supernatant to ELISA plates and use of specific antisera for the detection. Figure 3.8 shows an SDS polyacrylamide gel of ROS which were washed 5 times. In Coomassie Blue stained gels, no S-antigen could be detected in ROS which had been washed 10 times. Generally, 5 washes of the ROS pellet were found to remove most of the S-antigen, although it could still be detected in immunoblotting procedures using the monoclonal antibody S2.4.C5 (Chapter 6).

3.7 STABILITY OF STORED RETINAL ANTIGENS

Puified S-antigen frozen at -70°C remained stable for long periods. Stocks in routine use, stored at -20°C, were found to degrade upon re-freezing and thawing; degradation products were observed upon SDS-PAGE and the major band decreased in molecular weight. In addition, the protein concentration decreased, especially when the original amount of protein was low, presumably due to the protein forming insoluble precipitates, or adhering to the walls of the vessels. Consequently, repeated freeze-thawing was avoided.

The ROS membranes stored at -70°C were similarly stable. The ROS, when subjected to repeated freeze-thawing, was apparently more stable than the S-antigen, probably due to the higher protein content of the preparation of ROS. The ROS in sample buffer (Section 2.2.5.5), however, tended to aggregate during storage at 4 °C.

3.8 DEVELOPMENT OF THE ELISA ASSAY SYSTEMS

The ELISA assay, first described by Engvall and Perlman (1971), was

employed throughout the course of this study for the screening of column fractions after the purification of S-antigen (Section 3.4.1) and of the monoclonal antibody, S2.4.C5 (Section 6.5.2) and also for the screening of human and rat sera and cell culture supernatants against S-antigen and ROS (Chapters 4, 5 and 6). The ELISA assay was selected because of its high sensitivity and reproducibility. It also provided a rapid and convenient assay, enabling large numbers of samples to be screened simultaneously. The methodology was similar to that of Tuyen et al. (1982), with modifications.

3.8.1 Specificity of Antisera

Rat polyclonal antisera, raised against bovine S-antigen and ROS, were obtained upon the sacrifice of immunised animals (Section 2.6.1) for the purposes of fusions (Section 2.6.4). The antisera, with specificity to S-antigen, was used in the screening of column fractions, in the ELISA assay (Sections 3.8.3), during the purification of the protein. Rat antisera to S-antigen and ROS insoluble proteins, were also used as positive ELISA reaction controls, for the screening of rat and human sera, and cell culture supernatants (Chapters 4, 5 and 6).

All antisera obtained demonstrated a serum titre in ELISA of at least 2000 (eg. Figure 3.10). The specificities of antisera to S-antigen and ROS were confirmed in immunoblotting experiments (Section 2.3.7). Figure 3.9 shows the specificity of one rat polyclonal antiserum to S-antigen, when immunoblotted against crude retinal proteins and purified S-antigen using the ^{125}I protein A (Sections 2.1.7,12) detection system (Sections 2.3.7.6,7). In this experiment it was shown that the second antibody employed, rabbit

Figure 3.9 Specificity of a Rat Antiserum to S-Antigen in Protein A Immunoblots

The specificity of the one antiserum used in the ELISA assays, was tested by immunoblotting. In the experiment shown in this Figure, the ^{125}I protein A detection system was utilised (Section 2.3.9.6,7). This involved the use of a second antibody; rabbit anti-rat IgG(H+L).

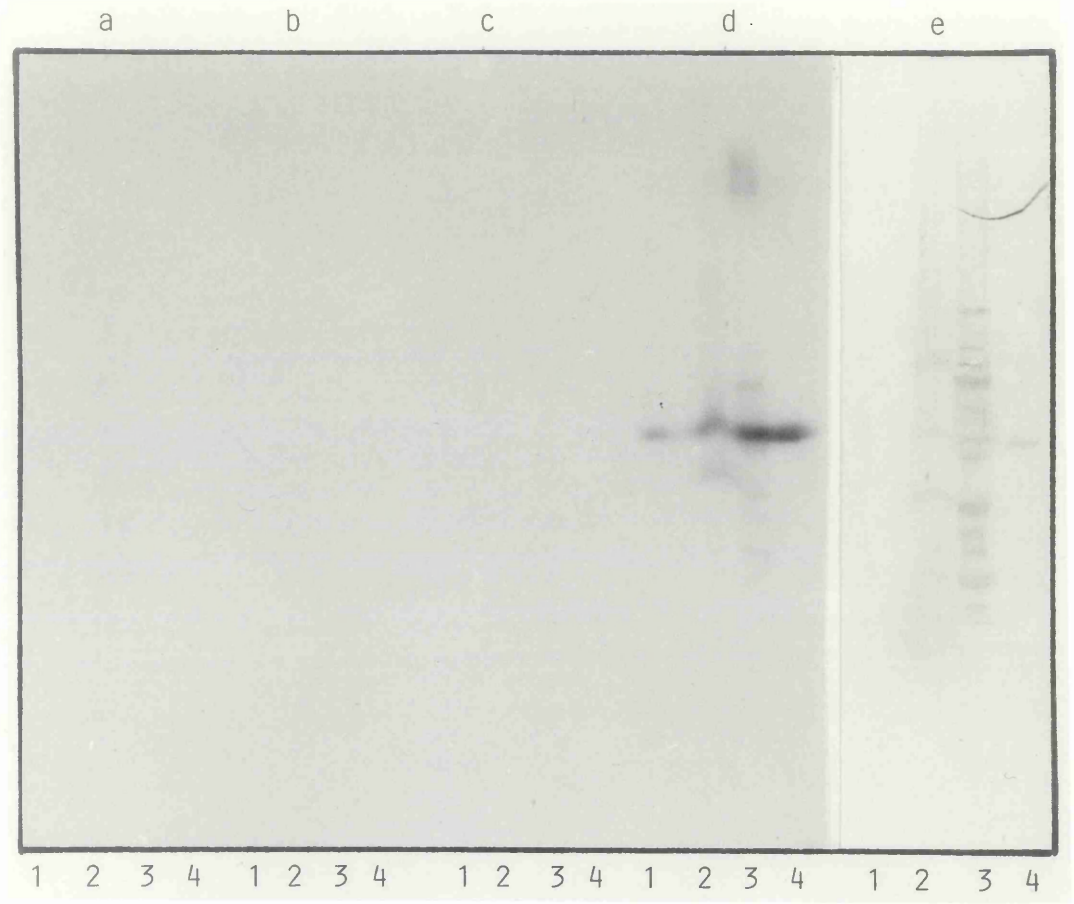
Neither the protein A (a) or the second antibody, rabbit anti-rat IgG(H+L) (b), nonspecifically interacted with the retinal proteins, when the primary antibody was omitted. No reaction could be detected with the rat antiserum, raised against S-antigen, when the second antibody was omitted (c). The use of the rabbit second antibody enabled the reaction of the rat antiserum to be detected (d).

The rat antiserum was fairly specific to S-antigen. The contaminating bands might indicate the presence of antibodies to proteins which contaminated the immunising antigen, or they may represent breakdown products of S-antigen and micro-aggregates of these.

An Amido black stained blot is shown at (e).

LANES

1. Pharmacia low molecular weight standard proteins
2. Solubilised ROS membranes
3. Crude retinal extract
4. S-antigen


a
b
c
d
e
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4

anti-rat IgG(H+L) (Section 2.3.7.4) and the protein A, did not react with the retinal antigens or the standard marker proteins.

The antisera employed in the experiment, shown in Figure 3.9, reacted intensely with the purified S-antigen and the S-antigen within the crude retinal extract. S-antigen was also detected within the resolved ROS proteins, which had been washed 5 times in order to remove most of the soluble antigens (Section 3.6.2). A few bands, in addition to the S-antigen band, were apparent in the autoradiograph. This may have indicated the presence of antibodies raised against minor contaminants present within the immunising antigen, or the bands could be breakdown products of S-antigen, and microaggregates of these.

3.8.2 Antigen Coating

Dynatech microtitration plates (Section 2.1.19) were pre-rinsed in distilled water and dried before use. The plates were then coated with S-antigen diluted in the column start buffer (20mM Tris/HCl, pH 7.5, Section 2.2.1.2) since this was the buffer of the stock protein. Where bovine ROS were used as the antigen, the plates were pre-treated with poly-L-lysine (Section 2.1.12), 10µg/ml in PBS (Section 2.2.4.1), 100µl per well, for one hour. The plates were then rinsed in PBS and dried before coating with ROS solubilised as described in Section 3.8.6. Generally the plates were coated with 50 to 100µl per well of a solution containing 1µg/ml of S-antigen or 5µg/ml of ROS protein. Antigen coating was achieved after an incubation of one hour at room temperature, or 18 hours at 4°C.

A high pH of coating buffer was not used to increase antigen binding (Engvall and Perlman, 1971) since the neutral pH of the

rest of the ELISA procedure may facilitate leaching of antigen.

3.8.3 The ELISA Procedure

The antigen coated ELISA plates were rinsed three times in the wash buffer, and dried by vigorous application of the plates onto towelling. The plates were then blocked with 100µl per well of the BSA blocking buffer with either normal rabbit serum (for ELISA assays with rat serum samples) or normal goat serum (for assay of human serum). The plates were washed and dried as before. The antibodies to be tested were diluted in PBS with 0.05% Tween 20 and 0.5% BSA (Section 2.2.4.2) and applied to the plates at 100µl per well. The plates were then incubated at room temperature for 30 to 60 minutes, or overnight at 4°C. The plates were washed thoroughly in the PBS-Tween buffer and dried in the usual manner. A 1:1000 dilution of the rabbit anti-species (rat or human) IgG(H+L) peroxidase conjugate (Section 2.1.16), in the diluting buffer, was applied at 100µl per well, and incubated at room temperature for 30 minutes. They were washed thoroughly, and dried. The plates were then incubated in the dark with 100µl per well the substrate, for approximately 10 minutes, with occasional but brief inspection. The reaction was terminated by the addition of 50µl per well of 4N H_2SO_4 (Section 2.1.9), including the first column of the plate which served as a blank on which to read the absorbances at 492nm, in a Titertrek Multiskan spectrophotometer. The results were obtained in the form of a numerical printout of the absorbances.

3.8.4 The ELISA Chequerboard

Suitable antigen concentrations for ELISA plate coating were determined using a chequerboard system. Doubling dilutions of the antigen concerned were made either across or down the plate, and

incubated as described in Section 3.8.3. After washing and blocking the plates (Section 3.8.2), the plates were treated with doubling dilutions of the appropriate antibody made in the opposite direction to that of the antigen. The ELISA protocol was continued, as described in Section 3.8.3, and the results examined in order to select a concentraton of antigen suitable for detecting antibody at a chosen dilution. Figure 3.10 shows the results obtained for a chequerboard experiment with S-antigen and a rat antiserum raised against this protein (see below).

3.8.5 The S-Antigen ELISA

In this system, a useful concentration of S-antigen for the coating of ELISA plates, was determined using chequerboard systems (Figure 3.10) (Section 3.8.4). Figure 3.10 shows the effect of decreasing antigen concentration on the ELISA reaction at constant antibody concentration, and also the effect of increasing antibody dilution while the concentration of antigen remained constant. Generally microtitre plates are coated with a solution of 1 to 10µg/ml of soluble protein. A useful concentration of S-antigen was found to be in the region of 1µg/ml, when the plates were coated with 50 to 100µl volumes of antigen. At this concentration, rat antiserum titres of several thousand could be detected, above the same dilutions of pre-immune serum (Figure 3.10). In Figure 3.10 the ELISA reaction is shown to decline fairly rapidly, as the concentration of the coating antigen is reduced, while the serum dilution remains constant at 1:200. this suggests that antigen concentrations below 1µg/ml, might be less suitable for the detection of low antibody concentrations.

Figure 3.10 also demonstrates the sensitivity of the ELISA

Figure 3.10 Chequerboard ELISA Experiment with S-Antigen and Rat Antiserum

The determination of a suitable concentration of S-antigen, and dilution of rat polyclonal antiserum, to use in the ELISA assays, involved a chequerboard experiment (Section 3.8.4,5).

The intensity of the ELISA reaction (A494 value) decreased when the concentration of S-antigen was reduced, and the dilution of the rat polyclonal antiserum remained constant, at 1:200.

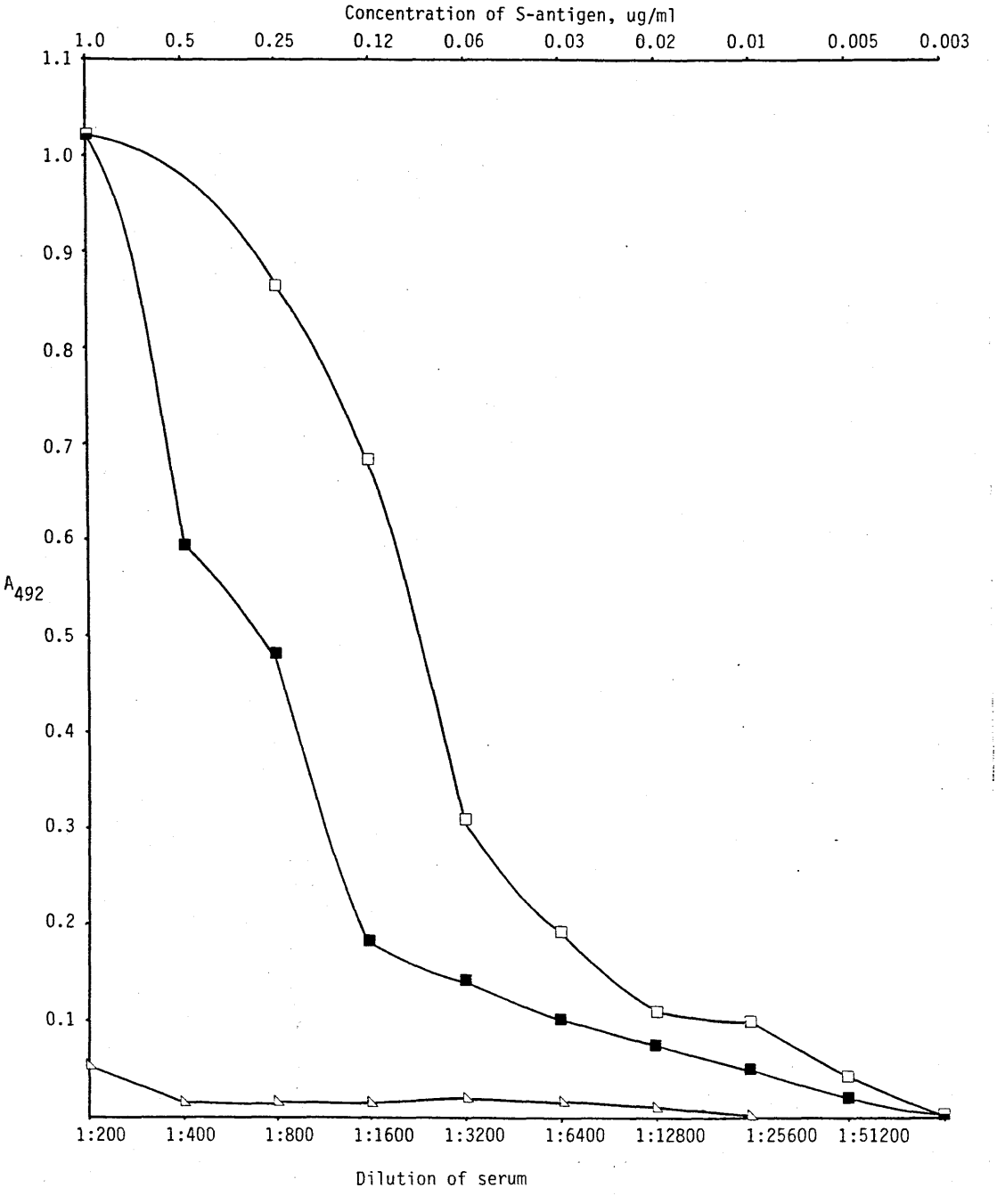
The ELISA reaction also decreased when the dilution of the antiserum was increased, while the antigen concentration remained constant, at 1µg/ml. A similar experiment was performed with pre-immune rat serum. The high titre of the antiserum is evident, in comparison with the reaction obtained with the pre-immune serum. The titre of this antiserum appeared to be greater than 50,000, ie. generating an ELISA reading above that of the pre-immune serum, at a dilution of 1:51,200.

KEY

□ Dilution of rat polyclonal antiserum

■ Concenration of S-antigen

◺ Dilution of pre-immune rat serum


Concentration of S-antigen, ug/ml
1.0
0.5
0.25
0.12
0.06
0.03
0.02
0.01
0.005
0.003
1.1
1.0
0.9
0.8
0.7
0.6
A492
0.5
0.4
0.3
0.2
0.1
1:200
1:400
1:800
1:1600
1:3200
1:6400
1:12800
1:25600
1:51200
Dilution of serum

technique, since the 1:200 dilution of antisera was able to detect S-antigen in nanogram quantities. The ELISA assay with S-antigen was found to give reproducible results.

3.8.6 The ROS ELISA

The ROS employed in the ELISA assay were stored frozen, were lysed upon thawing, and were solubilised by means of mild detergents. Thus whole ROS were not applied to the plates. During the development of an ELISA suitable for the ROS antigens, three different detergents were investigated, for the solubilisation of the ROS. In addition, chequerboards, with and without the pre-coating of the ELISA plates with 10µg/ml polylysine, (Section 2.1.12) were made.

Volumes of 100µl, of pelleted ROS membranes were solubilised in either 7% Tween 20, Triton X-100 or CTAB (Section 2.1.10) in a total volume of 3ml of PBS (Section 2.2.4.1). Even after vigorous stirring, complete solubilisation of the ROS membranes was not achieved. The membranes apparently became more fully solubilised upon dilution, to give approximately 5µg/ml of protein, for the ELISA, in PBS containing 0.05% the appropriate detergent. The Lowry assay (Lowry et al., 1951) (Section 2.3.3) was employed to give an estimate of the total protein concentration of the solubilsed ROS, since the detergents interfered with the Bradford protein assay (Section 2.3.2).

Table 3.2 shows the effects, on the ELISA reactions, of different detergents used for solubilisation of the ROS, and the effect of pre-treating the plates with polylysine. Tween 20 (Section 2.1.10) was found to be unsuitable for their solubilisation, giving rise to variable readings while the concentrations of polylysine, antigen

TABLE 3.2 The efficiency of different detergents and pre-coating of the ELISA plates in assays involving ROS* as antigen

		ELISA reaction recorded as A492			
Anti-ROS Serum**		No Detergent	Tween 20	Triton X	CTAB
+polylysine	X	1.563	1.568	1.836	1.776
	SD	0.434	0.331	0.013	0.138
-polylysine	X	0.797	0.325	0.717	1.624
	SD	0.516	0.174	0.101	0.278
Pre-immune Serum**					
+polylysine	X	0.057	0.073	0.069	0.100
	SD	0.140	0.051	0.009	0.318
-polylysine	X	0.055	0.028	0.024	0.030
	SD	0.018	0.006	0.005	0.011

* 5µg/ml

** Dilution 1:500

X Mean (8 assays)

SD Standard Deviation (n-1)

\+ Polylysine 10µg/ml

\- No polylysine

and antibody remained constant. The pre-coating of the ELISA plates with polylysine, 10μg/ml in PBS, was found to improve the binding of the Tween 20 solubilised ROS. This was indicated by the increase in the ELISA reading from 0.3 to 1.5 absorbance units at 492nm (A492). When no detergent was utilised to solubilise the ROS, similar results were obtained (Table 3.2).

ELISA plates, pre-treated with polylysine and coated with the ROS, solubilised in CTAB and Triton X-100 (Section 2.1.10), gave rise to stronger reactions, with smaller standard deviations compared with ROS solubilised in Tween 20. Triton X solubilised ROS, gave lower readings compared to the CTAB solubilised material, when polylysine was not used.

Pre-treatment of the plates with polylysine increased the signal to noise ratio, ie. the reactions were increased to a greater extent with the antiserum, than with the pre-immune serum (Table 3.2). Polylysine was also found to improve reproducibilty, as shown by the smaller standard deviations (Table 3.2), perhaps by facilitating a more even coating of the ELISA plates with ROS. Thus polylysine was utilsed in all subsequent ELISA assays involving ROS.

Triton X-100 was selected for the solubilisation of the ROS membranes, since it gave rise to more reproducible reactions compared to Tween 20 and CTAB. A useful concentration of ROS protein for the coating of the ELISA plates was found to be in the region of 5μg/ml, as indicated by chequerboard experiments (Section 3.8.4).

The preferred wash buffer contained 0.05% Tween 20 since the ROS tended to be more poorly solublised in this buffer compared with the same concentration of Triton X-100. Thus, the amount of antigen leaching from the ELISA plates, might be reduced when Tween 20 is used, rather than Triton X-100.

In comparison with the S-antigen ELISA, the assay with the ROS antigen showed similar sensitivity and reproducibility.

3.7 SUMMARY

Bovine retinal antigens were utilised in this study since, they were readily available in large quantities, and similarities of certain retinal antigens between different species have been found, including opsin (O'Tousa *et al.*, 1985) and S-antigen (Section 1.2.2). Rod outer segment (ROS) membranes were prepared by a similar procedure to that of Papermaster and Dreyer (1974) (Section 3.6). S-antigen was purified to give the major band upon silver staining (Figure 3.5), by a method modified from that of Borthwick and Forrester (1983) (Sections 3.2 to 3.3). The apparent molecular weight of S-antigen was calculated to be 48K from the Pharmacia low molecular weight standard protein markers (Figure 6.14), however, some variation was observed in the molecular weights calculated from gels with different percentages of acrylamide.

A faint reaction was obtained for S-antigen after the PAS reaction for carbohydrates indicating the presence of a small carbohydrate moiety (Figure 3.7a). The S-antigen and ROS antigens were found to be highly antigenic, inducing high serum titres in DA rats subcutaneously immunised with approximately 50μg of protein in CFA (Section 2.6.1). The DA rats, however, remained refractory to the

induction of EAU under these conditions. The uveitopathogenicity of the S-antigen prepared for this study was confirmed upon injection of 50 to 100µg of the protein in CFA into the hind foot pads of Duncan-Hartley guinea pigs and Lewis rats (Section 6.6).

The optimal concentrations of antigen and antibody for use in the ELISA were determined by means of the chequerboard system (Section 3.8.4). The ELISA assay systems for both antigens were found to be reproducible, generating very similar readings for the same antibodies, both within one ELISA plate and between plates.

CHAPTER FOUR

STUDIES ON THE HUMORAL RETINAL AUTOIMMUNITY IN RATS AND HUMANS

Chapter 4

STUDIES ON THE HUMORAL RETINAL AUTOIMMUNITY IN RATS AND HUMANS

In this chapter the extent of the humoral autoimmune response to retinal antigens is examined in rats and humans. Section 4.1 presents a detailed serum analysis of two inbred strains of the RCS rat model for human retinal degeneration, and three control strains of rat. In this study it was possible to compare genetically similar individuals, raised under identical conditions. Section 4.2 describes the results obtained from a much more limited study of the serum antibody responses in human RP and uveitis patients, and healthy controls.

4.1 STUDIES ON RETINAL AUTOIMMUNITY IN RATS

The potential immunopathogenicity of the rod outer segment components has been well established (Section 1.3) (reviewed by Faure, 1980). In view of the accumulation of the highly antigenic ROS debris in the subretinal space of rats with inherited retinal dystrophy, it has been suggested that these rats might become auto-sensitised to their retinal components (Chant and Meyers-Elliott, 1982). Conflicting evidence has, however, been reported with respect to the contribution of an autoimmune component to the degenerative process of the RCS rat retina (Section 1.5.6).

In an attempt to examine this problem further, experiments were conducted in order to investigate the extent of autosensitisation to the retina. The study was confined to the humoral immune response of the dystrophic and control rats.

4.1.1 Confirmation of Ocular Histopathology and presence of S-Antigen

Retinal dystrophy of both strains of RCS rat employed in this study, was confirmed by a histological investigation of paraffin embedded sections of alcohol and gluteraldehyde fixed eyes (Section 2.4.1). Figures 4.1a,b show the histopathology at an advanced stage of the degenerative process, for a 9 week old, pigmented Hunter RCS rat. The section shown in Figure 4.1a was stained with monoclonal antibody S2.4.C5 to S-antigen (Chapter 6), in an indirect immunoperoxidase procedure (Section 2.4.4) demonstrating the presence of the protein within the RCS rat photoreceptor ROS. S-antigen was apparently absent from the ROS membrane debris lying between the RPE cell layer and the ROS. Figure 4.1b shows a section of RCS rat retina treated with an irrelevant monoclonal antibody as a negative control. The nuclei were counterstained with haematoxylin.

At the limit of the light microscope, the histopathology resembled the earliest stages of experimental autoimmune uveitis (EAU). The most obvious feature was the advanced dissolution of the outer nuclear layer (ONL), which had been reduced to a double layer of nuclei from the normal twelve (Figure 4.1b). The photoreceptor layer had become homogeneous and eosinophilic, in sections stained with eosin. Changes in the retinal pigment epithelium (RPE) included cytoplasmic swelling and an increase in the nuclear content. The RPE cells also had attenuated processes containing melanosomes (Figure 4.1b). The RPE monolayer remained intact suggesting that the blood retinal barrier was still in operation, and that there had been no major exposure of the antigenic ROS debris to the immune system.

Figure 4.1 Sections of a 9 Week old Hunter RCS Rat Eye showing characteristic Retinal Histopathology and the presence of S-Antigen within Rod Photoreceptors

a) Localisation of S-antigen in RCS retina with MAb S2.4.C5 Photographed at Magnification X 100

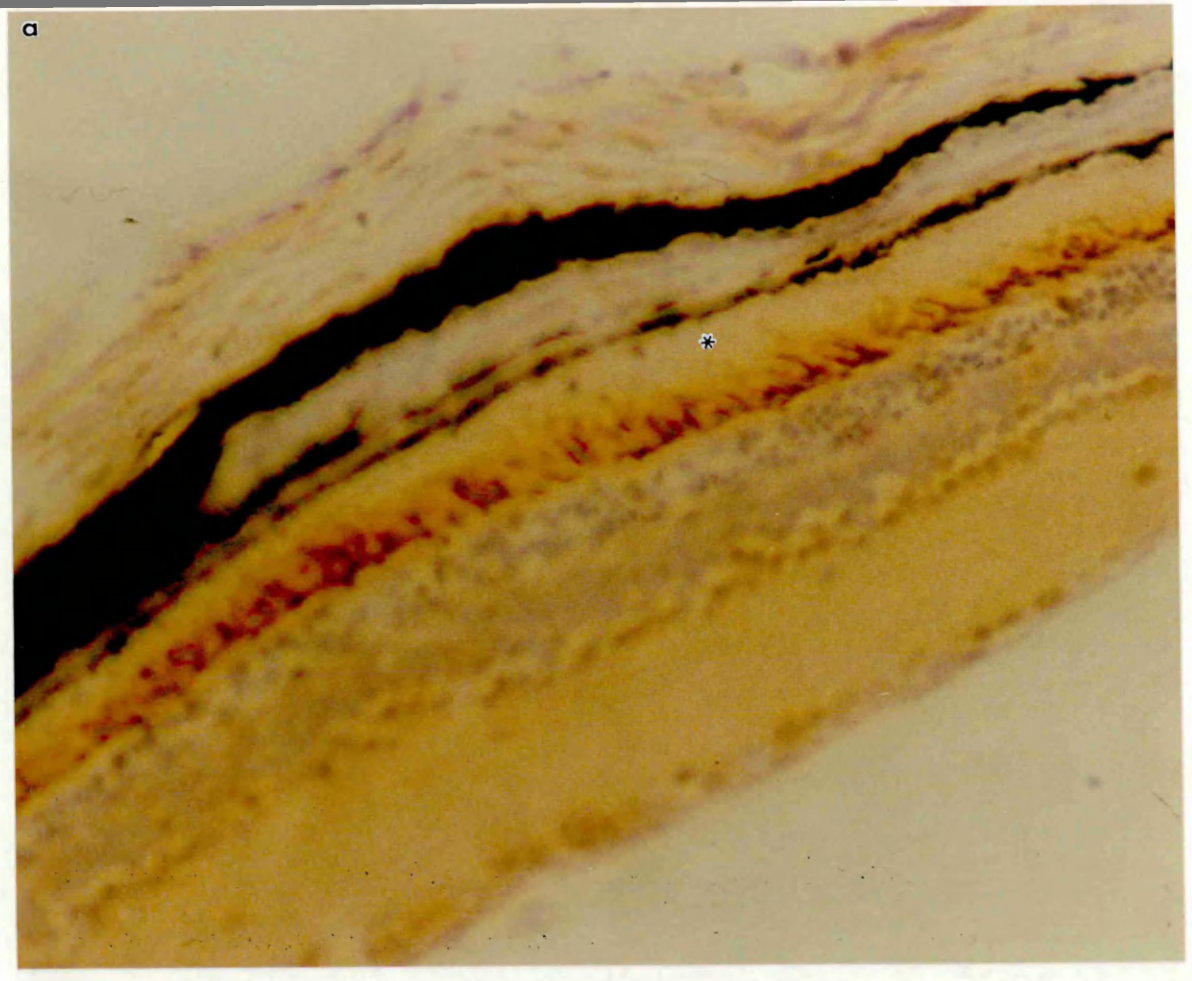
b) Immunocytochemistry control with an irrelevant rat MAb Photographed at Magnification X 400

S-antigen was localised in dewaxed sections of gluteraldehyde (4%) fixed Hunter RCS rat eye, using an indirect immunoperoxidase technique. The reaction was mainly confined to the ROS. The band of ROS membrane debris appeared unstained.

The essential histopathological features included: a layer of ROS debris; reduction in the number of photoreceptor nuclei; change in nuclear and melanin content of the RPE.

KEY

1. Sclera
2. Choroid (pigmented and vascular)
3. Retinal pigment epithelium
4. Photoreceptor outer segment layer (with ROS debris *)
5. Photoreceptor inner segment layer
6. Photoreceptor nuclei (outer nuclear layer)
7. Outer plexiform layer (synapses)
8. Nuclei of second order neurons (inner nuclear layer)
9. Inner plexiform layer (synapses)
10. Ganglion cell layer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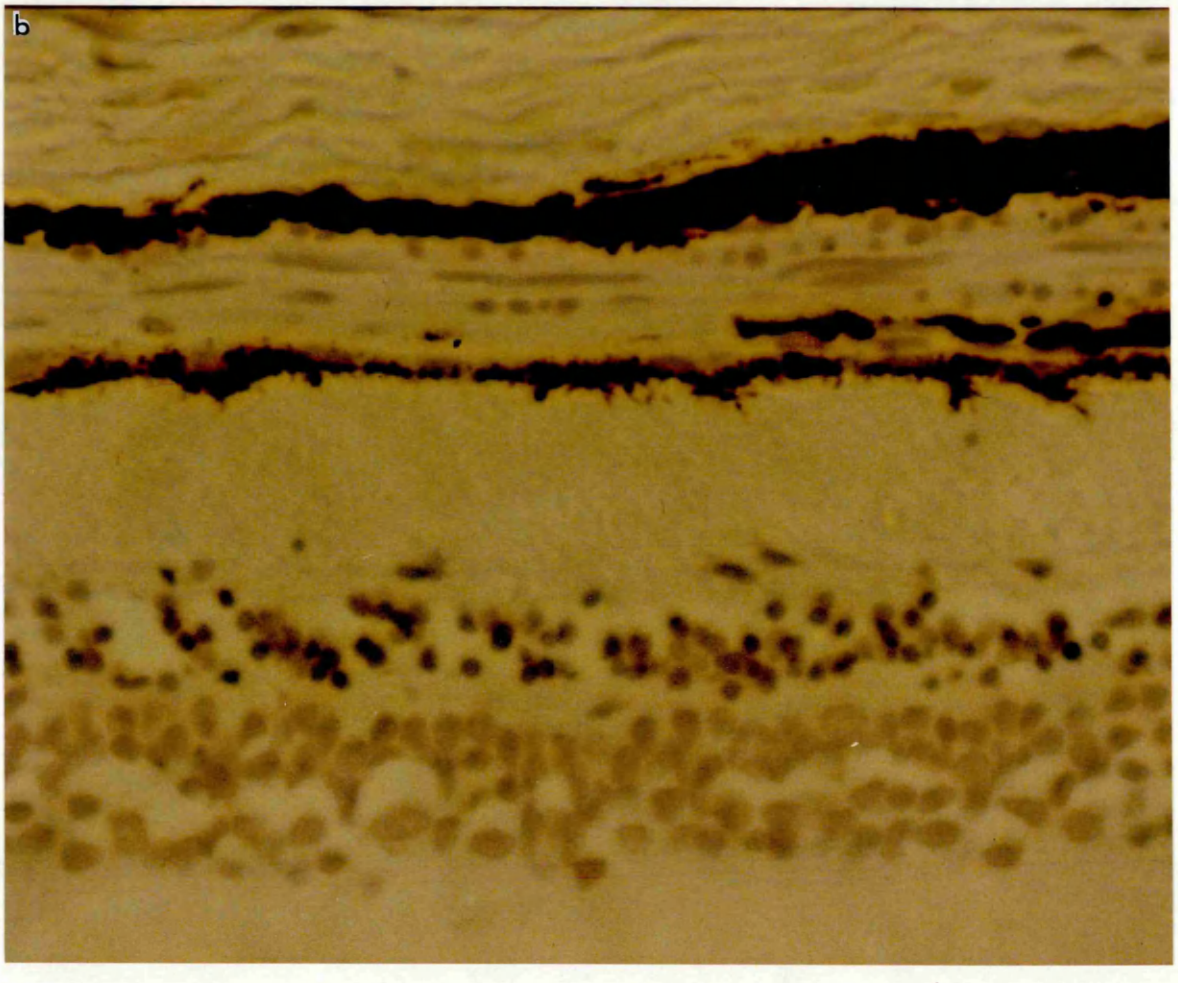
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The RCS rat was originally employed as a model for cataract (Bourne et al., 1938). Cataracts were observed in only two animals of the approximately 50 RCS rats involved in this study. These animals were several months old, and most of the other animals were sacrificed before the cataracts could develop. It has been suggested that the cataracts which occur in the RCS rats may be initiated by the toxic lipid peroxidation products, released by the degenerating photoreceptor cells, which diffuse through the vitreous to the lens (Zigler and Hess, 1985).

4.1.2 Experimental Approach

4.1.2.1 Animals

Populations of 10 pigmented Hunter and 10 pink eyed Campbell RCS rats were used for the study (Section 2.1.17). Control rats included 5 pigmented DA and 5 albino Wistar, as well as 10 rats of the PVG strain (Section 2.1.17) from which the Hunter strain of RCS rat derives its pigmentation (Yates et al., 1974). Pigmented rats were included in the study since the presence of melanin within the pigment epithelium is thought to slow the degenerative process (Yates et al., 1974; La Vail and Battelle, 1975). Such a slowing of the process might also be reflected in any concomitant immune response. The small amount of pigment in the RPE cells of the Campbell rats has been found to have an insignficant effect in delaying the disease (La Vail and Battelle, 1975). The eyes of the Campbell rats were grossly albino and indistinguishable from albino eyes. Wistar rats were included as controls since albino animals are prone to a light induced retinal degeneration (Noell et al., 1966; Noell, 1979). Pigmented DA rats were also included as an additional normal control.

Each rat was identified by a system of ear marking so that the reaction profiles could be monitored at the individual level. The animals were bled, from the tail artery, at weekly intervals from the age of 3 weeks, the time of weaning, to 10 weeks, by which time the RCS rat photoreceptors have lost their outer segments (Dowling and Sidman, 1962); the sera were stored at -70°C before use. The data presented shows readings for the early (3 week), middle (6 week) and late (9 week) stages of the retinal degenerative process.

4.1.2.2 Choice of assays

Three similar types of assay were employed for the serum study; the ELISA, immunocytochemistry and immunoblotting. The assays were similar in that they all involved antigens in the solid phase, which were first incubated with the serum, followed by an incubation with a rabbit anti-rat IgG(H+L) antibody, peroxidase conjugated in the case of the ELISA and immunoperoxidase techniques. The reactions were detected after incubation with the enzyme substrate, or with ^{125}I protein A, in the case of the immunoblots. The IgG(H+L) second antibody detects all rat isotypes including IgM, due to the light chain epitopes. These techniques revealed different information about the antibodies in question, although they varied in their sensitivity.

The ELISA assay (Section 3.8) was selected because of its high sensitivity and reproducibility. This assay was also sparing on antigen and allowed the simultaneous screening of large numbers of samples over a wide range of dilutions, in duplicate. However, the assay does not give biochemical or cytological identification of the specific antigenic proteins.

The ELISA plates were coated with approximately 5µg/ml of solubilised, washed ROS membranes (Section 3.8.6) or 1µg/ml of purified bovine S-antigen (Section 3.8.5). The optimal concentrations were determined by the chequerboard system with the appropriate antiserum and preimmune serum (Section 3.8.4). The plates were blocked with 50mg/ml of BSA in order to avoid possible nonspecific reaction at the lower serum dilutions. All serum samples were assayed against the same preparations of S-antigen and were subject to identical experimental conditions.

All titrations were made in duplicate to ensure reproduciblity. Variability between plates for the S-antigen ELISA was assessed by using monoclonal antibody S2.4.C5, with specificity to S-antigen (Chapter 6), and preimmune serum obtained from a DA rat, as positive and negative controls respectively. These control antibodies were titrated on each plate. Variability between the ELISA plates coated with ROS was assessed by the titration of rat anti-ROS serum and preimmune DA serum (positive and negative controls respectively). The readings obtained for the omission of the primary antibody, gave an indication of background. If the background reaction was over 0.08 absorbance units, at 492nm (A492), then the value was subtracted from all other A492 values before the calculations of means and standard deviations were made (Appendix, Tables 4.1 to 4.30). Generally the background reaction was in the region of 0.05 A492, and is shown as a dotted line in Figures 4.2 to 4.3.

Immunocytochemical assays were also performed (Sections 2.4 and 4.1.7). These techniques allow the localisation of autoantigens

within sections of tissue. They also provide a range of potential antigens with which serum antibody might react. However, these techniques are much less sensitive than the ELISA, requiring lower serum dilutions, and generally require antibody of high avidity (strength of binding) and high specificity (Polak and Van Noorden, 1984).

Serum samples which demonstrated a positive response in the ELISA assay to either S-antigen or ROS (Section 4.1.5) were tested for their reactivity in sections of albino rat retina in an indirect immunoperoxidase procedure (Section 2.4.4), employing diaminobenzidine as the peroxidase substrate (Section 2.2.8.4). Albino rat eyes were utilised since the peroxidase reaction product may nonspecifically adhere to the melanin granules present within the retinal pigment epithelium and choroid. The eyes were fixed in 70% alcohol and processed according to the method of Sainte-Marie (1962) (Section 2.4.3), which is thought to allow a greater preservation of native antigens than conventional fixation and embedding techniques.

Indirect immunofluorescence (Section 2.4.5) was also employed to allow a comparison with the studies made in other laboratories. In these experiments frozen sections of an albino rat eye were utilised (Section 2.4.5).

The protein A immunoblotting (Section 2.3.9) technique was utilised in order to assess the specificities of the positive serum responses detected in the ELISA study (Sections 4.1.4-5). This technique enables the molecular weights of the resolved antigens to be calculated. The immunoblotting procedure has the disadvantage in

that the antigens are denatured. However, this can facilitate the identification of relevant epitopes which are not exposed in the ELISA or immunocytochemical techniques.

The sera were diluted 1:40 and incubated with the resolved antigens on the nitrocellulose strips, which had previously been blocked with 0.5% Tween 20 (Section 2.2.6.2). The amount of Tween in the wash buffer was reduced to 0.05%, which was thought to reduce the leaching of the lower affinity autoantibodies. The protein A detection system was employed, with the intermediate antibody, rabbit anti-rat IgG(H+L) (Sections 2.3.9.4,6). Controls consisted of age matched normal rats, the DA and PVG strains. Age matched RCS and Wistar rats which gave no indication of a response in the ELISA, were also tested by immunoblotting. The omission of a primary antibody provided a control to assess any nonspecific interaction of the intermediate rabbit antibody with the resolved retinal antigens, or the Pharmacia low molecular weight standard protein markers (Figure 3.9).

4.1.3 Method of ELISA Data Presentation

The responses of sera from populations of RCS and control rats at, 3, 6 and 9 weeks of age, to bovine solublised rod outer segment membranes (Section 3.8.6) and purified S-antigen (Section 3.8.5) in ELISA assays are recorded in Tables 4.1 to 4.30 (Appendix). The results for the individual animals are recorded as the mean of two assays with standard deviations (S.D.). In addition, the mean and S.D. for each strain are recorded for each time interval. The mean and S.D. for Campbell strain of RCS rat were calculated with and without the apparently positive sera (Tables 4.1 to 4.6, Appendix), since these animals were found to have a significant affect on the

mean for the whole strain. The means which do not include the positive Campbell rats are used only in Figures 4.3a,b,c where the mean including all of the Campbells is also shown. Figures 4.3a to 4.3c, presents the ELISA titres of the Campbell rat sera, which generated reactions to S-antigen and/or ROS above those of the apparently unresponsive littermates. The responses for the rats are shown at 3, 6 and 9 weeks to both antigens, to allow comparisons to be made. The results are described Section 4.1.5.

In Figures 4.2a to 4.2e, the mean responses of RCS and control rat sera to S-antigen and ROS at 3, 6 and 9 weeks are graphically portrayed. Figure 4.2 enables the comparison of the mean responses of the different strains of RCS and control rats to the different antigens at the different ages (Sections 4.1.4 to 4.1.6).

Figures 4.4 and 4.5 compare the ELISA responses to both antigens of all the strains of rat, at serum dilutions of 1:20 and 1:80, respectively (Sections 4.1.4 to 4.1.6). The points represent the mean of two assays for each individual rat. The mean for each strain, at each particular dilution, is indicated by a bar.

At the 1:20 dilution, the A492 readings are more scattered (Figure 4.4), as well as being higher, than those at the 1:80 dilution (Figure 4.5). The range of scatter of the points at the 1:20 dilutions, was wider for S-antigen than ROS (Figure 4.4). At the 1:20 dilution, the potentially positive sera were most obvious. The 1:80 dilution was considered to be important for comparison with the 1:20 dilution, since responses which were no longer significantly raised at 1:80, may have generated nonspecific reactions at the lower dilutions.

4.1.4 Analysis of the ELISA Results for the Control Rats

The DA and PVG rats showed no significant change in mean titre to either antigen at the different age intervals tested (Figures 4.2c,d). These rats gave generally lower readings to both antigens at lower serum dilutions, to both antigens, in comparison with the RCS and Wistar rats (Figures 4.2a,b,e). There was no obvious change in the mean titres of the Wistar rats to either antigen, although the mean to S-antigen at 6 weeks was slightly raised at the 1:20 serum dilution, due to a higher serum response of two rats (W4 and W2) (Figures 4.2e and 4.4b; Table 4.26, Appendix). At the age of 9 weeks, Wistar rat W4 also gave a reaction to S-antigen above the level of the littermates, at a serum dilution of 1:20 ie. over 0.5 A492, (Table 4.27, Appendix). Although at a dilution of 1:80, the sera of these Wistar rats gave readings above those of the littermates, the reactions were not striking. Immunoblotting experiments with the W4 sera gave more information on the specificity of the response (Section 4.1.8; Figure 4.8).

4.1.5 Analysis of the Responses of the Campbell RCS Rats

The mean reactions for the Campbell RCS rats to both antigens, at low dilutions, at all ages tested (Figure 4.2a), were above those of the pigmented sighted control rats (Figures 4.2c,d). The mean responses to ROS at 3 and 9 weeks were comparable to those of the Wistars (Figure 4.2e). The readings were highest to both antigens at 6 weeks (Figure 4.2a).

Four Campbell rats were identified, which gave higher ELISA readings to retinal antigens, than the controls and apparently unresponsive littermates, at 6 and/or 9 weeks (Tables 4.2, 4.3, and

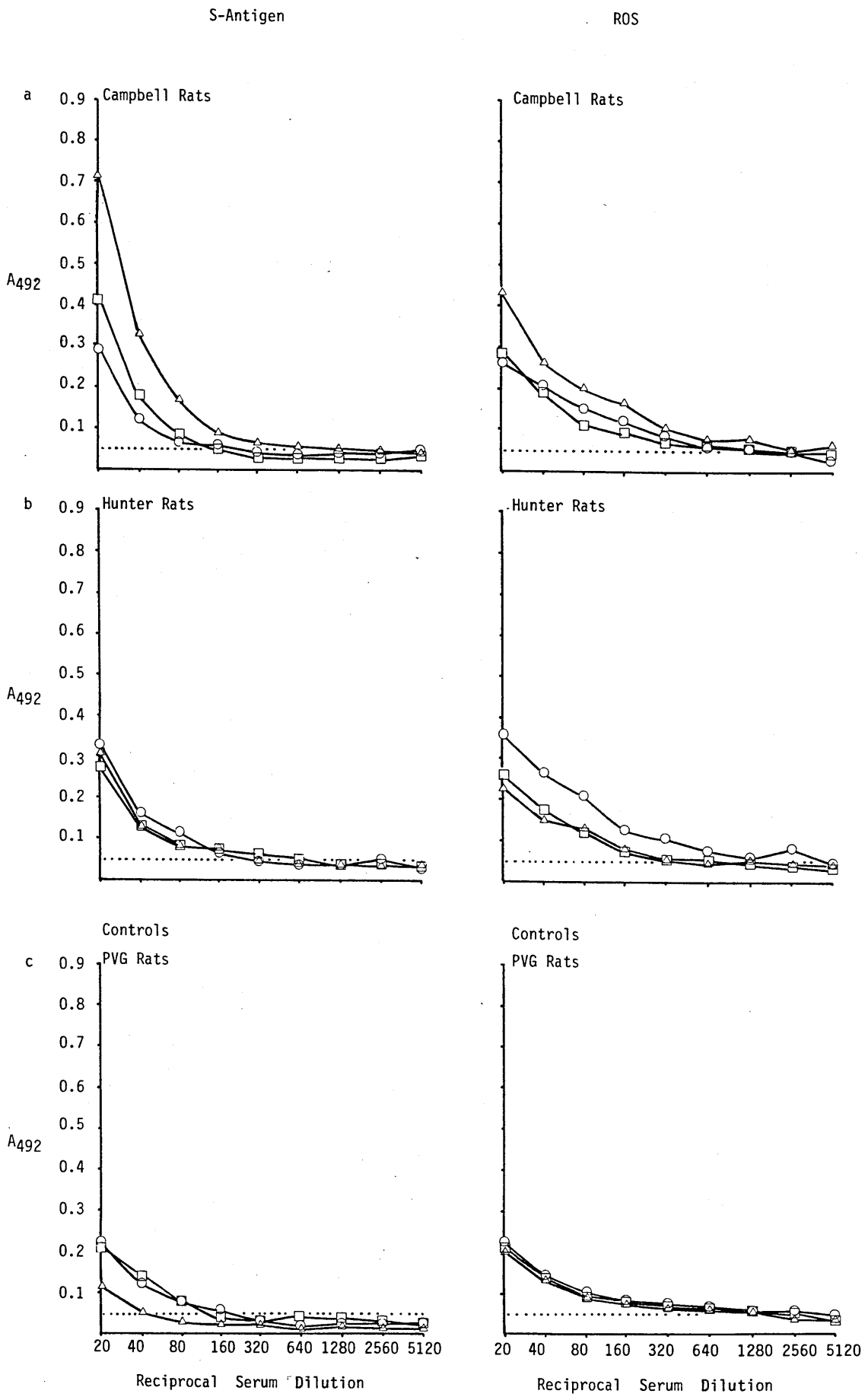
Figure 4.2 Mean ELISA Responses of Sera from 3, 6 and 9 Week old RCS and Control Rats to S-Antigen and ROS

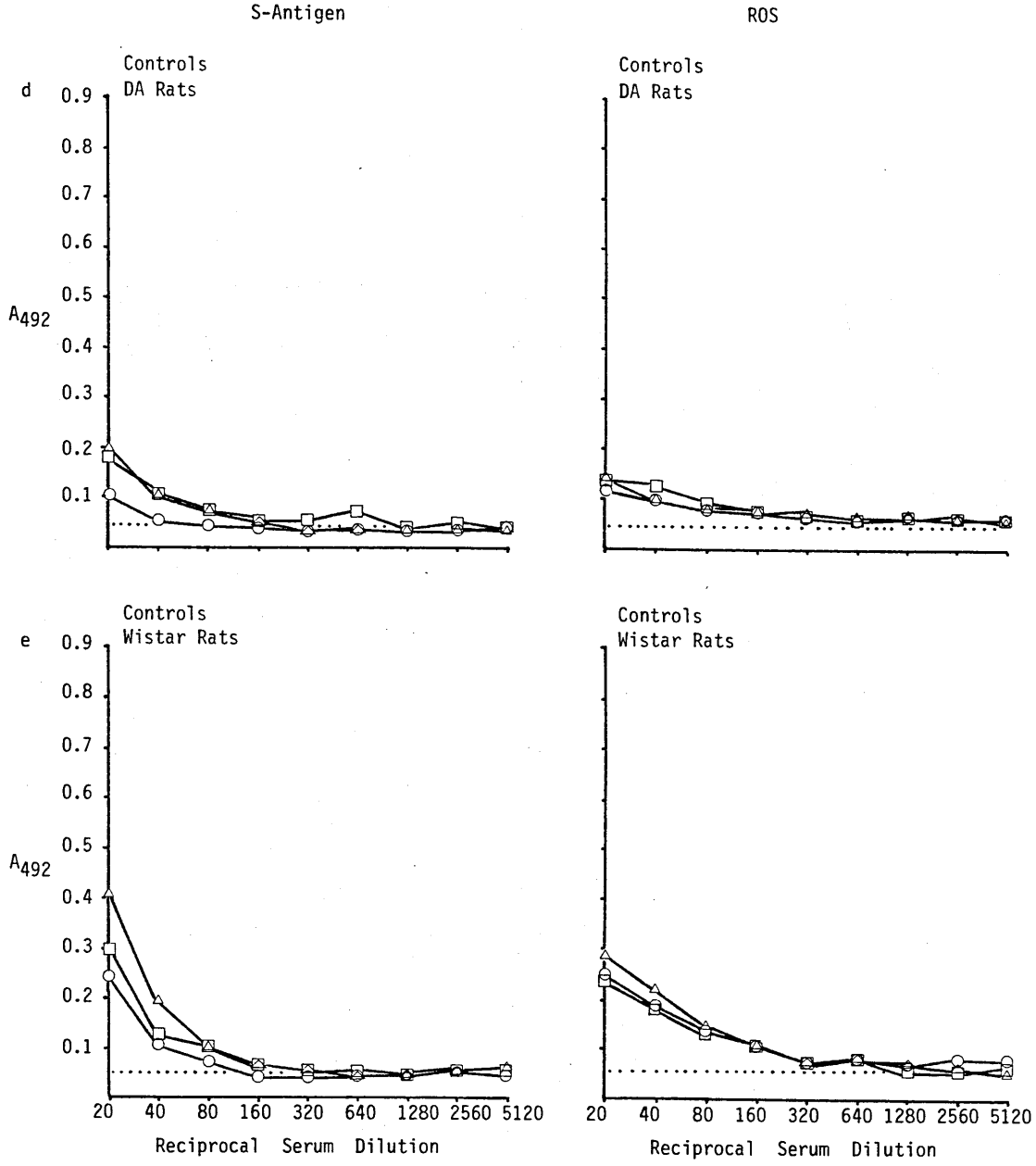
Sera obtained from 10 Campbell and 10 Hunter RCS rats, and 10 PVG, 5 DA and 5 Wistar control rats rats, at the ages of 3, 6 and 9 weeks, were tested in the ELISA against 1µg/ml purified S-antigen and 5µg/ml ROS. The mean reponses obtained for each strain were calculated from the means of two readings for the individual animals. The means and standard deviations of the individual animals, and for each strain as a whole, at the ages of 3, 6 and 9 weeks, are also recorded in Tables 4.1 to 4.30, in the Appendix.
The results are described in Sections 4.1.4 to 4.1.6.

a) Mean Responses of Campbell RCS rats to S-antigen and ROS
b) Mean Responses of Hunter RCS rats to S-antigen and ROS
c) Mean Responses of PVG control rats to S-antigen and ROS
d) Mean Responses of DA control rats to S-antigen and ROS
e) Mean Responses of Wistar control rats to S-antigen and ROS

KEY

○ Responses of 3 week old rats
△ Responses of 6 week old rats
□ Responses of 9 week old rats
...Omission of primary antibody


S-Antigen
ROS
a
b
c
Campbell Rats
Hunter Rats
Controls
PVG Rats
A_{492}
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
20 40 80 160 320 640 1280 2560 5120
Reciprocal Serum Dilution


S-Antigen
ROS
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Controls
DA Rats
Controls
Wistar Rats
A_{492}
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
20 40 80 160 320 640 1280 2560 5120
Reciprocal Serum Dilution

Figure 4.3 Serum Data from Individual Campbell Rats, giving Elevated ELISA Responses to Retinal Antigens

Sera obtained from four Campbell RCS rats (C1, C4, C5 and C8) generated ELISA responses to S-antigen, significantly above those of the apparently unresponsive littermates. The responses of these positive sera are compared with the mean results for the 10 Campbell rats, and also the mean of the six unresponsive littermates (see Key below).

At 3 weeks, only rat C1, indicated a response to S-antigen, at a serum dilution of 1:20 only, above the mean for the Campbells (a). The responses of rats C1, C4 and C8 to ROS at the age of 3 weeks, were not significantly raised compared with the mean for the Campbell rats at this age (a). Campbell rats C1, C4 and C8 appeared to have high responses to S-antigen at the age of 6 weeks (b). These individuals also demonstrated responses to ROS above those of the littermates, albeit lower than the S-antigen response (b). At 9 weeks, only rat C8, demonstrated a response to S-antigen, significantly above the level of the unresponsive littermates (c). However, at this time a forth rat, C5, indicated a raised response to S-antigen (c). At 9 weeks, no rats generated a response to ROS significantly above the mean for 10 the Campbell rats (c).

The results are described in Section 4.1.5.

The standard deviations and means of the individual animals, and for each strain as a whole, at the ages of 3, 6 and 9 weeks, are also recorded in Tables 4.1 to 4.30, in the Appendix.

a) Responses of 3 week old Campbell rats

b) Responses of 6 week old Campbell rats

c) Responses of 9 week old Campbell rats

KEY

■ Responses of Campbell rat C1 to S-antigen and ROS

▲ Responses of Campbell rat C4 to S-antigen and ROS

△ Responses of Campbell rat C5 to S-antigen and ROS

◣ Responses of Campbell rat C8 to S-antigen and ROS

--- Mean responses of all 10 Campbell rats to S-antigen and ROS at (a) 3(b) 6 and (c) 9 weeks

-·-· Mean responses of 6 unresponsive Campbell rats to S-antigen and ROS at (a) 3 (b) 6 and (c) 9 weeks

···· Omission of primary antibody

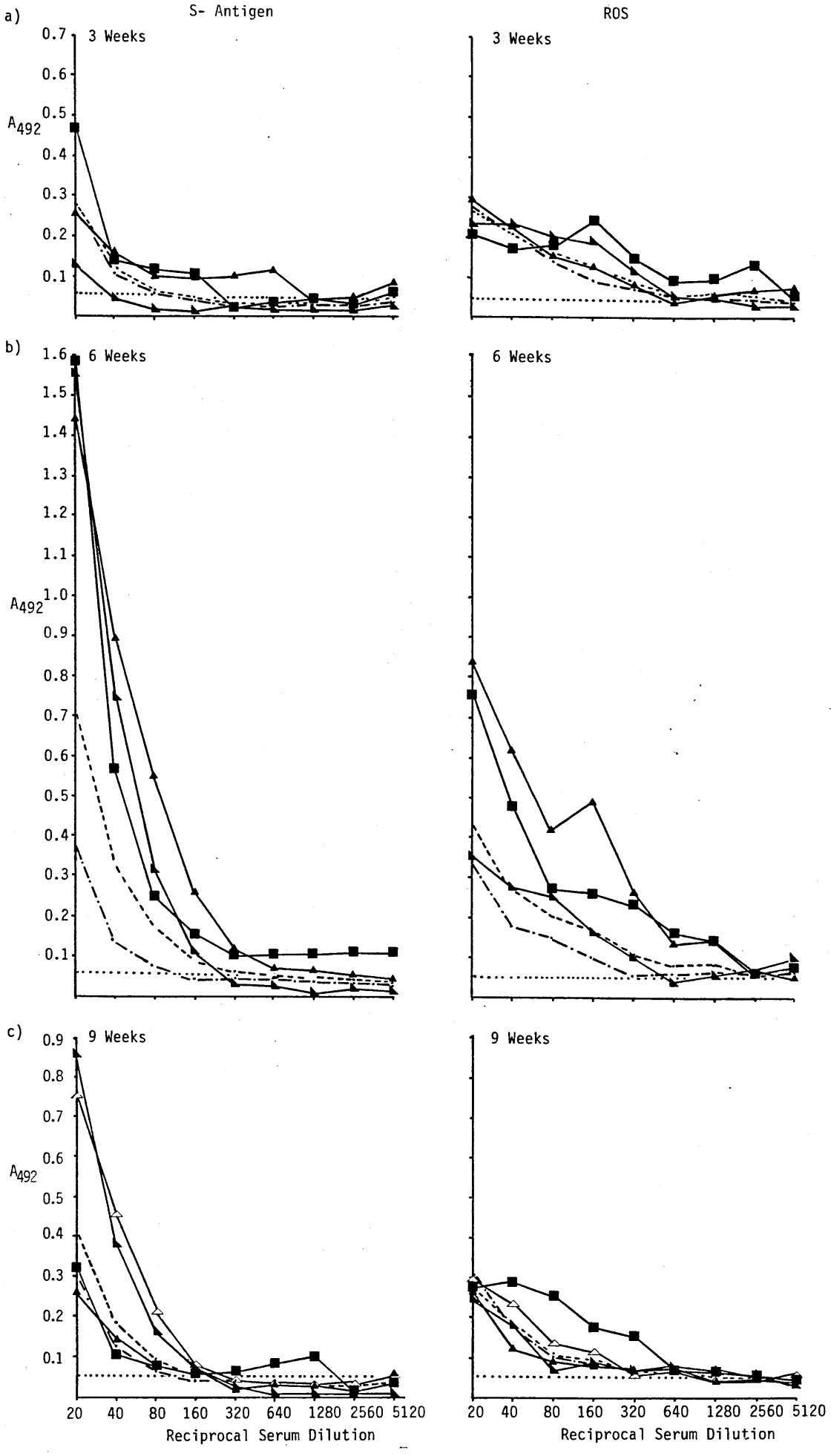

a)
S- Antigen
ROS
3 Weeks
3 Weeks
A_{492}
0.7
0.6
0.5
0.4
0.3
0.2
0.1
b)
6 Weeks
6 Weeks
A_{492}
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
c)
9 Weeks
9 Weeks
A_{492}
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
20 40 80 160 320 640 1280 2560 5120
Reciprocal Serum Dilution
20 40 80 160 320 640 1280 2560 5120
Reciprocal Serum Dilution

Figure 4.4 ELISA Readings of RCS and Control Rat Sera diluted 1:20 at 3, 6 and 9 Weeks in Age to S-Antigen and ROS

a) Responses of 1:20 dilution of RCS and control rat sera to S-antigen and ROS, at the age of 3 weeks

b) Responses of 1:20 dilution of RCS and control rat sera to S-antigen and ROS, at the age of 6 weeks

c) Responses of 1:20 dilution of RCS and control rat sera to S-antigen and ROS, at the age of 9 weeks

ELISA A492 readings to S-antigen and ROS are shown for the 1:20 dilution of the sera obtained from 10 Campbell and 10 Hunter RCS rats, and 10 PVG, 5 DA and 5 Wistar control rats rats at the ages of (a) 3 (b) 6 and (c) 9 weeks.

The points represent the mean of two assays at the 1:20 dilution (Appendix, Tables 4.1 to 4.30). The bars indicate the means obtained for each strain at (a) 3 (b) 6 and (c) 9 weeks.

The results are discussed in Sections 4.1.4 to 4.1.6, in the text.

KEY

C: Campbell RCS rats

H: Hunter RCS rats

PVG: Control rats

DA: Control rats

W: Wistar control rats

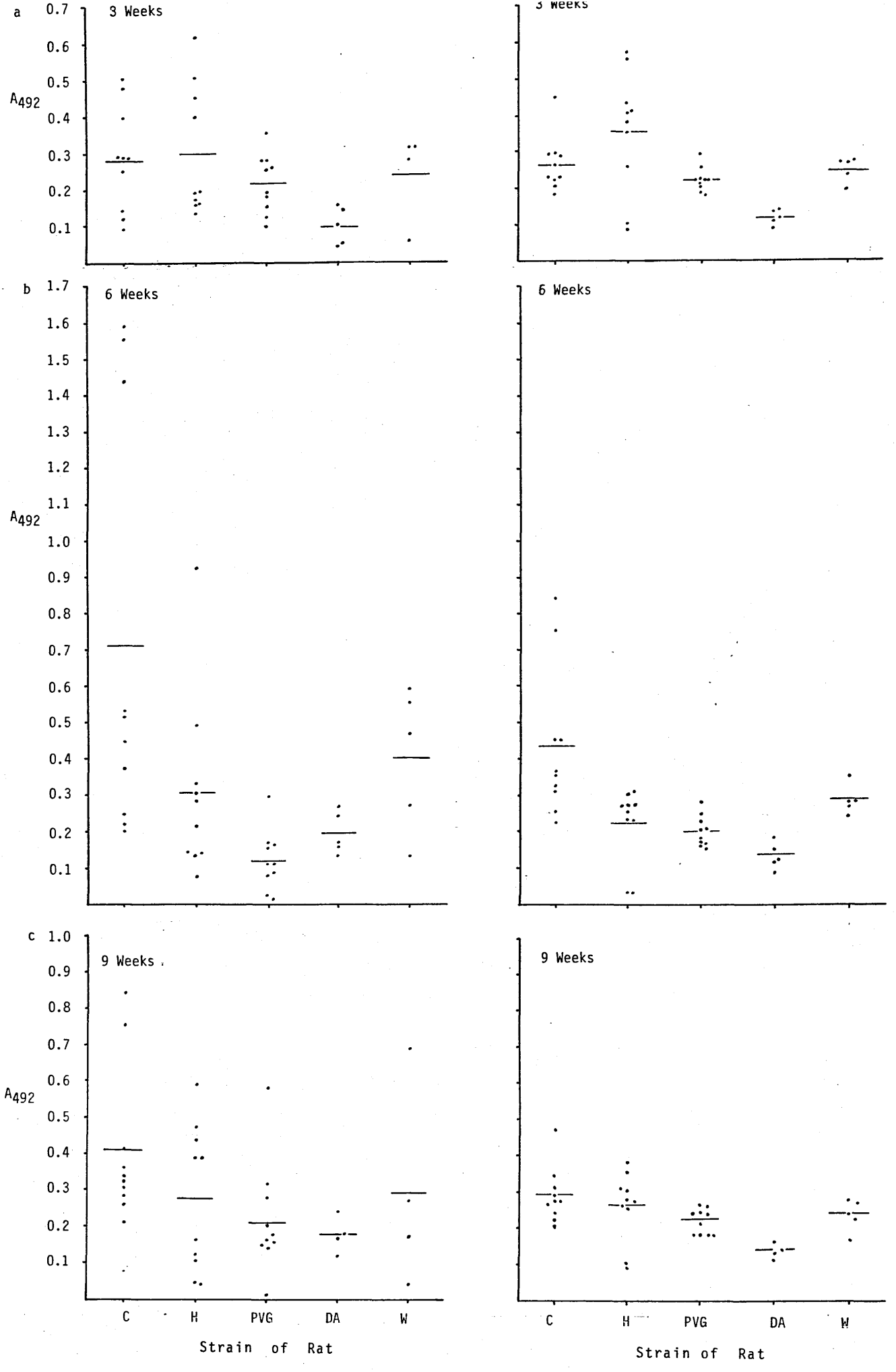

a
3 Weeks
A_{492}
0.7
0.6
0.5
0.4
0.3
0.2
0.1
b
6 Weeks
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
c
9 Weeks
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
C
H
PVG
DA
W
Strain of Rat

Figure 4.5 ELISA Readings of RCS and Control Rat Sera diluted 1:80 at 3, 6 and 9 Weeks in Age to S-Antigen and ROS

a) Responses of 1:80 dilution of RCS and control rat sera to S-antigen and ROS, at the age of 3 weeks

b) Responses of 1:80 dilution of RCS and control rat sera to S-antigen and ROS, at the age of 6 weeks

c) Responses of 1:80 dilution of RCS and control rat sera to S-antigen and ROS, at the age of 9 weeks

ELISA A492 readings to S-antigen and ROS are shown for the 1:80 dilution of the sera obtained from 10 Campbell and 10 Hunter RCS rats, and 10 PVG, 5 DA and 5 Wistar control rats rats at the ages of (a) 3 (b) 6 and (c) 9 weeks.

The points represent the mean of two assays at the 1:80 dilution (Appendix, Tables 4.1 to 4.30). The bars indicate the means obtained for each strain at (a) 3 (b) 6 and (c) 9 weeks.

The results are discussed in Sections 4.1.4 to 4.1.6, in the text.

KEY

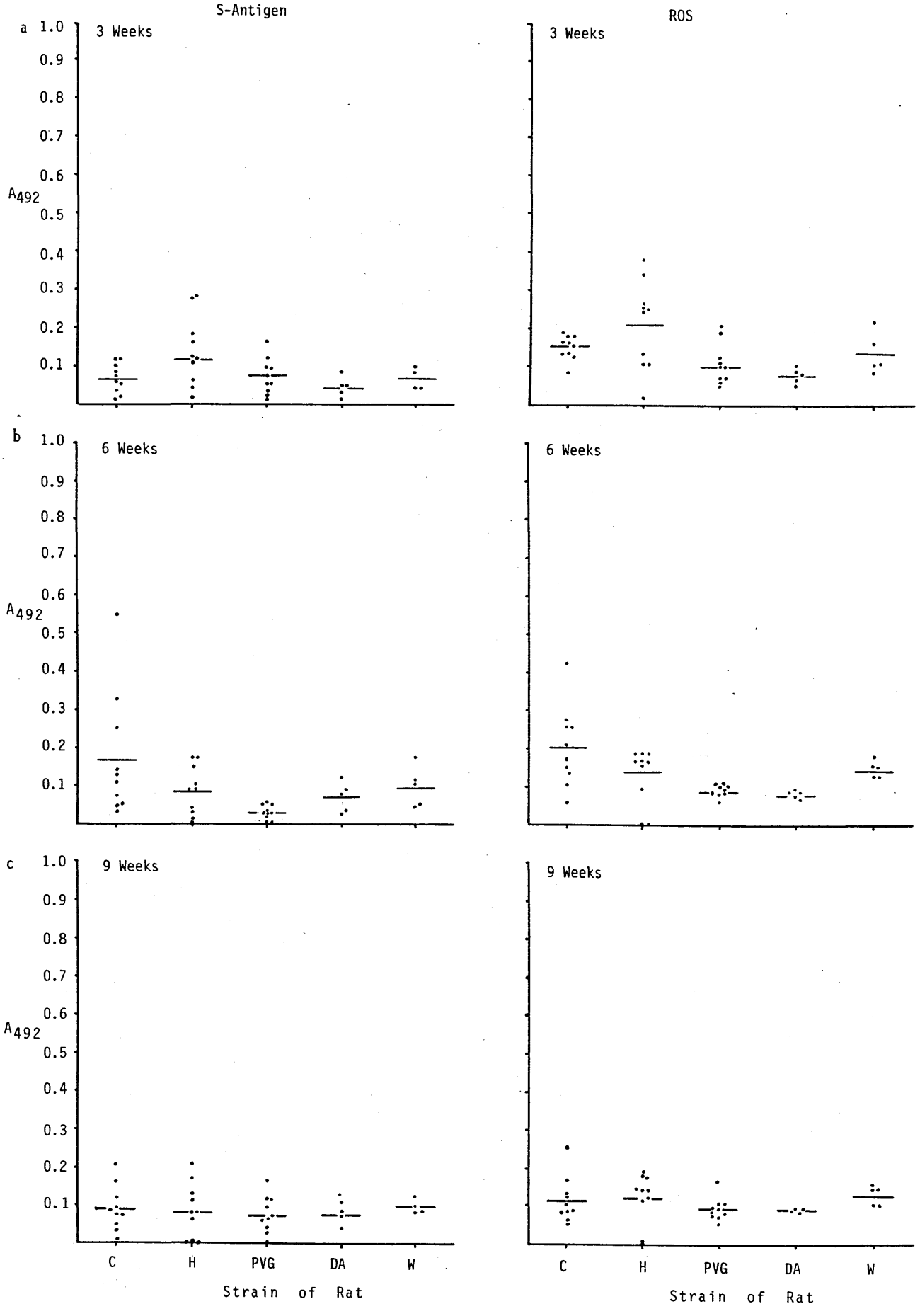
C: Campbell RCS rats

H: Hunter RCS rats

PVG: Control rats

DA: Control rats

W: Wistar control rats


S-Antigen
ROS
a
3 Weeks
3 Weeks
b
6 Weeks
6 Weeks
c
9 Weeks
9 Weeks
A492
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
C
H
PVG
DA
W
Strain of Rat
Strain of Rat

4.5). Figures 4.3a,b,c shows the ELISA results of these individual Campbell rats, C1, C4, C5 and C8, which demonstrated reactions to S-antigen, significantly above the controls and above the apparently unresponsive littermates, at the age of 6 weeks. These rats also demonstrated raised reactions to ROS, although lower than those to S-antigen (Figure 4.3b). Readings are shown for these rats, to both antigens, at 3, 6 and 9 weeks. The three apparently positive sera, resulted in a raised mean response to S-antigen, for the Campbell rat sera, at 6 weeks (Figures 4.2a and 4.3b). Two separate means are included in each of Figures 4.3a,b,c. In addition to the mean responses to both antigens at each week, for all 10 Campbell rats, means are shown which do not include the results of the apparently positive Campbell sera, from rats C1, C4, C5 and C8, since these appear to have a significant effect on the mean response to S-antigen at 6 weeks (Figures 4.3a,b,c; Tables 4.1 to 4.6).

None of the Campbell RCS rats were significantly positive to either antigen at the age of 3 weeks (Figure 4.3a). The positive responses of rats C1, C4 and C8, evident at 6 weeks, and at both 1:20 and 1:80 dilutions (Figures 4.3b, 4.4b and 4.5b), became less significant at a dilution of 1:160 (Figure 4.3b). At the age of 9 weeks, the responses of rats C1 and C4, had fallen to levels similar to those of the unresponsive littermates, and only the sera of rat C8 remained raised, albeit at a lower level (Figures 4.3c, 4.4c and 4.5c). At the age of 9 weeks, the fourth positive Campbell (C5) serum was detected. At 9 weeks rats C5 and C8 demonstrated similar responses (Figure 4.3c). The serum of rat C5, generated a higher reaction than that of rat C8, at a 1:80 dilution, as rat C5 gave an A492 reading of 0.208, and C8 gave a reading of 0.163

(Figure 4.3c; Table 4.3, Appendix). The results are discussed in Section 4.1.9).

4.1.6 Analysis of ELISA Responses of Hunter RCS Rats

The mean responses of the Hunter RCS rats, to S-antigen, at 3, 6 and 9 weeks were very close together, suggesting that there was no significant change in titre over the period of time studied (Figure 4.2b). There was also little indication of a significant change in responses to ROS with time (Figure 4.2b). Overall, the mean readings for the Hunter rats appeared similar to those of the Wistars (Figure 4.2e), being lower than those of the Campbells (Figure 4.2a), and higher than those of the pigmented control rats (Figures 4.2c,d).

Generally, the readings given by the rat sera, at the 1:20 dilution (Figure 4.4), to both antigens, demonstrated greater scatter than the readings obtained at the 1:80 dilution (Figure 4.5). At the age of 3 weeks, however, the readings given by the Hunter rats to both S-antigen and ROS, were more widespread than those of the other strains, including the Campbells (Figure 4.5a). At 3 weeks, the sera (diluted 1:20) of 3 rats, H1, H6 and H9, gave ELISA readings to S-antigen close to 0.5 and 0.6 A492 (Figure 4.4a; Table 4.7). The readings obtained for the sera of rats H6 and H9, at the dilution of 1:80, remained above those of the apparently unresponsive littermates (ie. close to 0.3 A492) (Figure 4.5a; Table 4.7, Appendix). This suggested that these rats may have had a positive response to S-antigen at 3 weeks. Rat H6 also demonstrated a reading to ROS of over 0.3 A492, at a serum dilution of 1:80 (Figure 4.5a). In addition, rat H10 at 3 weeks only, indicated a response to ROS of over 0.55 A492, at 1:20 dilution, and over 0.3

at a 1:80 dilution (Figures 4.4a; 4.5a; Table 4.10). Rat H10 was not significantly reactive to S-antigen in the ELISA, at any stage.

At 6 weeks, only one Hunter rat, H8, gave a response to S-antigen above those of the littermates, at the 1:20 dilution (Figure 4.4b; Table 4.8, Appendix). This response may have indicated the presence of very low affinity or cross reacting antibodies, since the serum reaction was no longer significantly different from those of the littermates at the 1:80 dilution (Figure 4.5b; Table 4.8). At 9 weeks, rat H3 indicated a response to S-antigen of over 0.59 A492, at a serum dilution of 1:20 (Figure 4.4c). However, this may not have been a true specific response since the serum did not indicate a significant reaction at the 1:80 dilution (Figure 4.5c). No significant reactions with the ROS were observed for the Hunter rats at 6 or 9 weeks (Figures 4.4b,c and 4.5b,c).

4.1.7 Reaction of RCS and Control Rat Sera in Tissue Sections

Indirect immunofluorescence tests on frozen sections of rat eye (Section 2.4.5) were performed on some of the RCS and PVG control sera obtained from animals at the ages of 3, 6 and 9 weeks. The fluorescence results (Table 4.31) were quantified by means of a Leitz MPV Compact Photometer. The indirect immunofluorescence test is generally considered to be of a lower sensitivity in comparison to the indirect immunoperoxidase reaction. Undiluted serum was applied to the tissue sections in order to increase the binding of specific antibody which may be present in low concentrations.

The immunofluorescence results were not found to be very reproducible during testing of undiluted, potentially autoimmune

Table 4.31 Quantitative IFAT with rat sera readings over the photoreceptor cell bodies (ONL)

		Relative fluorescence units		
Rat No.		Age 3 Weeks	Age 6 Weeks	Age 9 Weeks
RCS C2	X	1.22	20.66	
	SD	0.77	4.16	nd
	n	2	7	
RCS C3	X	6.38	8.4	7.87
	SD	0.18	0.81	2.24
	n	6	6	7
RCS C4	X	3.03		ROS 5.6 (RIS 16.7)
	SD	0.45	nd	7.7
	n	5		3
RCS C5	X	10.23		10.35
	SD	1.32	nd	0.65
	n	6		4
PVG2	X	4.67	10.53	33.02
	SD	3.2	6.18	6.95
	n	6	6	6
PVG3	X	4.57	6.44	
	SD	2.36	1.15	nd
	n	6	6	
PVG5	X	0.88	3.94	5.3
	SD	0.86	0.38	1.46
	n	4	5	6

Controls		ROS	RIS
S2.4.C5 McAb to S-antigen	X	20.45	15.71
	SD	1.38	2.07
	n	3	6
Irrelevant McAb (to V. cholerae)	X	3.31	3.2
	SD	0.80	0.9
	n	6	6

X : Mean
SD: Standard deviation (n-1)
n : Number of readings
nd: Not determined

sera. Intense fluorescence, with all undiluted rat sera, was obtained in the choroid of the sections of rat eye. The choroid is a highly vascular tissue, and thus contained a high concentration of rat Ig, with which the FITC conjugated rabbit anti-rat IgG(H+L) (Section 2.1.16) could react. Apart from the choroid, the highest readings were obtained over the inner nuclear layer (INL). No significant reactions were observed in the photoreceptor ROS layer. Table 4.31 shows the readings obtained over the INL, when frozen sections of rat eye were incubated with Campbell RCS and PVG control sera, followed by the FITC conjugate. The highest reading was obtained for PVG control rat 2, at the age of 9 weeks. This animal was not significantly positive to S-antigen or ROS in the ELISA assays (Tables 4.15 and 4.18).

The use of undiluted serum, not only increases the chances of the binding of relatively low affinity autoantibodies, but greatly increases the opportunity for nonspecific interaction of irrelevant antibodies. The irrelevant antibodies include those directed to tissue antigens, which may give rise to a background reaction in the section. Other unwanted antibody interactions involve crossreactions through the antibody binding sites, the binding to fc receptors, and nonspecific electrostatic or hydrophobic attractions of irrelevant antibodies (Section 8.4.1). The high reading obtained for PVG rat 2, at 9 weeks, probably represents a nonspecific binding of irrelevant antibody.

The immunofluorescence technique, however, was found to give satisfactory results with monoclonal antibody S2.4.C5, raised against S-antigen (Chapter 6). Table 4.31 also shows the readings obtained for this monoclonal antibody, and an irrelevant monoclonal

Figure 4.6 Detection of Autoantibody to Photoreceptor Antigens in RCS Rat Sera

a) Immunoperoxidase reaction in the photoreceptor cell layer, with 1:40 dilution of Campbell RCS Rat C4 serum.

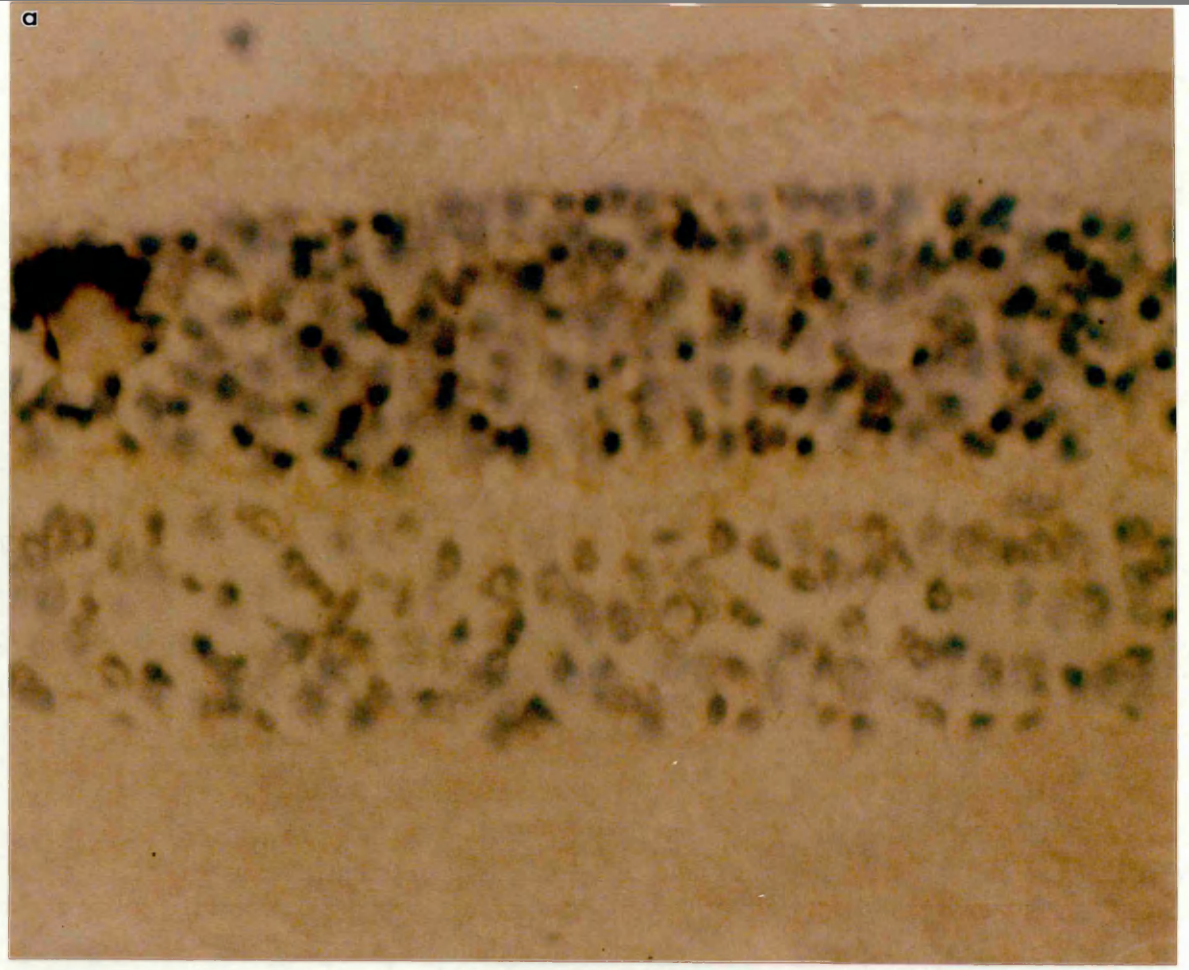
b) Absence of a peroxidase reaction with a 1:40 dilution of the serum from Campbell RCS Rat C1.

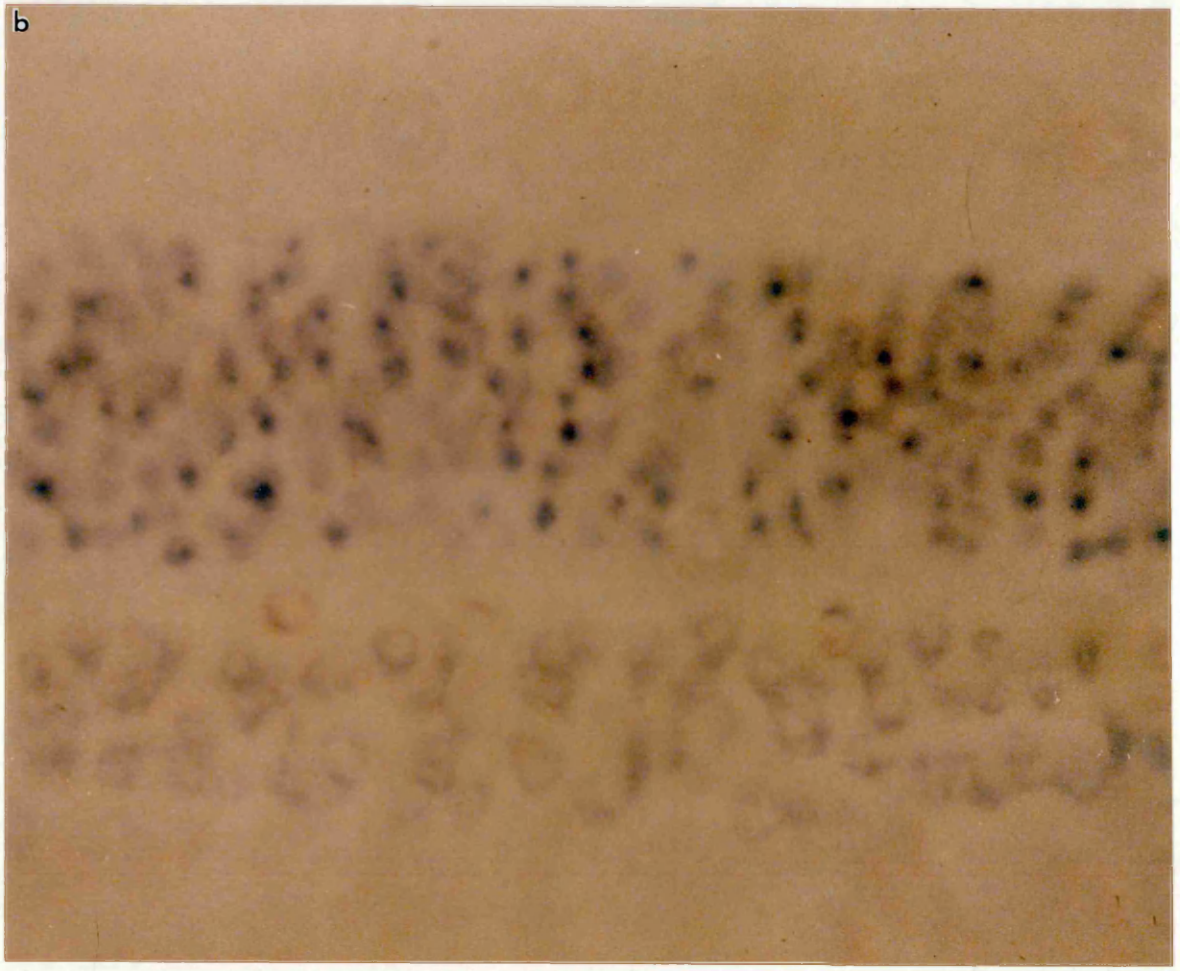
Ethanol fixed, dewaxed sections of albino rat retina were utilised for the immunoperoxidase experiments. The sera of Campbell Rats C4 and C1 were identified as positive to S-antigen in the ELISA, but in this less sensitive immunochemistry test, only the C4 serum appeared positive (a) (Section 4.1.7). The pattern of staining produced by the C4 serum was similar to that obtained for monoclonal or polyclonal anti- S-antigen antibodies, albeit less intense (Figure 7.1).

KEY

1. Photoreceptor outer segment layer
2. Photoreceptor inner segment layer
3. Photoreceptor nuclei (outer nuclear layer)
4. Outer plexiform layer (synapses)
5. Nuclei of second order neurons (inner nuclear layer)
6. Inner plexiform layer (synapses)

Photographed at Magnifications X 400


a
1
2
3
4
5
6


b
1
2
3
4
5
6

to Vibrio cholerae LPS used as a control. The antibodies were applied in the form of cell-free culture supernatant. These antibodies gave more reproducible results between assays on different sections, compared with the study involving undiluted rat sera. This is probably because there was a much lower concentration of antibody present in the hybridoma supernatant, and also the monoclonal antibodies used were very specific to S-antigen, or to the LPS. Also, no other rat Ig, which might give rise to a nonspecific reaction with the tissue section, was present in the supernatant.

In this study, the immunoperoxidase technique (Section 4.1.2.2) offered greater sensitivity, and reproducibility in comparison to the immunofluorescence procedure. The four Campbell (C1, C4, C5 and C8) and one Wistar (W4) rat sera which demonstrated positive reactions to S-antigen in ELISA, at 6 and/or 9 weeks (Sections 4.1.4-5), were tested for their binding in dewaxed sections of paraffin embedded rat eye in indirect immunoperoxidase experiments (Sections 2.4.3-4). Only one rat, RCS C4 (6 weeks), gave any indication of a reaction at a dilution of 1:40 (Figure 4.6a). The ROS and photoreceptor synapses were stained in a similar pattern to that obtained with monoclonal antibody S2.4.C5, to S-antigen (Figure 7.1a,b). Figure 4.6b shows the lack of reaction of 1:40 dilution of serum, from Campbell rat C1. Dilutions below 1:40 resulted in a general background reaction which rendered interpretation of any specific staining impossible.

4.1.8 Immunoblotting Results

The sera of four Campbell RCS (C1, C4, C5 and C8) and one Wistar (W4) rat which indicated positive responses to S-antigen in the

ELISA at the age of 6 and/or 9 weeks (Sections 4.1.4-5), were subjected to immunoblotting experiments (Section 2.3.9). Two of the four Campbell rat sera (C4 and C8) were shown to react with S-antigen in the immunoblots (Figure 4.7). The sera of the Campbell rat, designated C8, reacted with at least two additional lower molecular weight bands present in the crude soluble retinal extract (Figure 4.7c). The sera of the Campbell rat designated C4, recognised one of these lower molecular weight bands (Figure 4.7a). These bands had molecular weights of approximately 35K and 40K. The 40K antigen was possibly the same protein recognised by monoclonal antibody R1.2.D2 (Figure 6.8). The 40k antigen was not recognised by antibodies to S-antigen. The 35K band could represent a breakdown product of the S-antigen, or a subunit of a larger retinal protein, eg. transducin. It is interestring that Gregerson and Abrahams (1983) reported that the sera of some uveitis patients recognise a 35K antigen. The reaction for the Wistar rat (W4) (Figure 4.8b), appeared similar to that of C4 (Figure 4.7a). The sera of rats C1 and C5, did not give rise to a significant reaction in the immunoblots, at the serum dilutions employed during these experiments. Figures 4.8c,d show the lack of a reaction for control sera obtained from rats W1 and PVG1, which gave no indication of a reaction to S-antigen or ROS in the ELISA assays.

The results obtained for the sera of rats C4, C8 and W4 were demonstrated to be reproducible; identical patterns of reaction were obtained for three separate immunoblotting experiments subject to the same conditions. There was no indication, in any of the immunoblots with the rat sera, for a reaction with the marker proteins.

Figure 4.7 Specificity of ELISA Positive RCS Rat Sera in Immunoblots

Immunoblot of sera from 6 week old Campbell RCS rats C4 and C8 demonstrating a response to S-antigen. The sera was diluted 1:80, and incubated with the nitrocellulose strips for 3 hours. Rabbit anti-rat IgG(H+L) and I^{125} protein A were used in the detection of the reaction.

The two sera specifically recognised S-antigen and a 40K antigen. The 35K antigen recognised by rat C8, might represent a breakdown product of S-antigen (Section 4.1.8).

a) Autoradiograph of blot with serum from Campbell rat C4

c) Autoradiograph of blot with serum from Campbell rat C8

b) and d) Amido black stained blots

Lanes

St: Low molecular weight markers

S: Purified S-antigen

C: Crude retinal extract

R: Bovine ROS

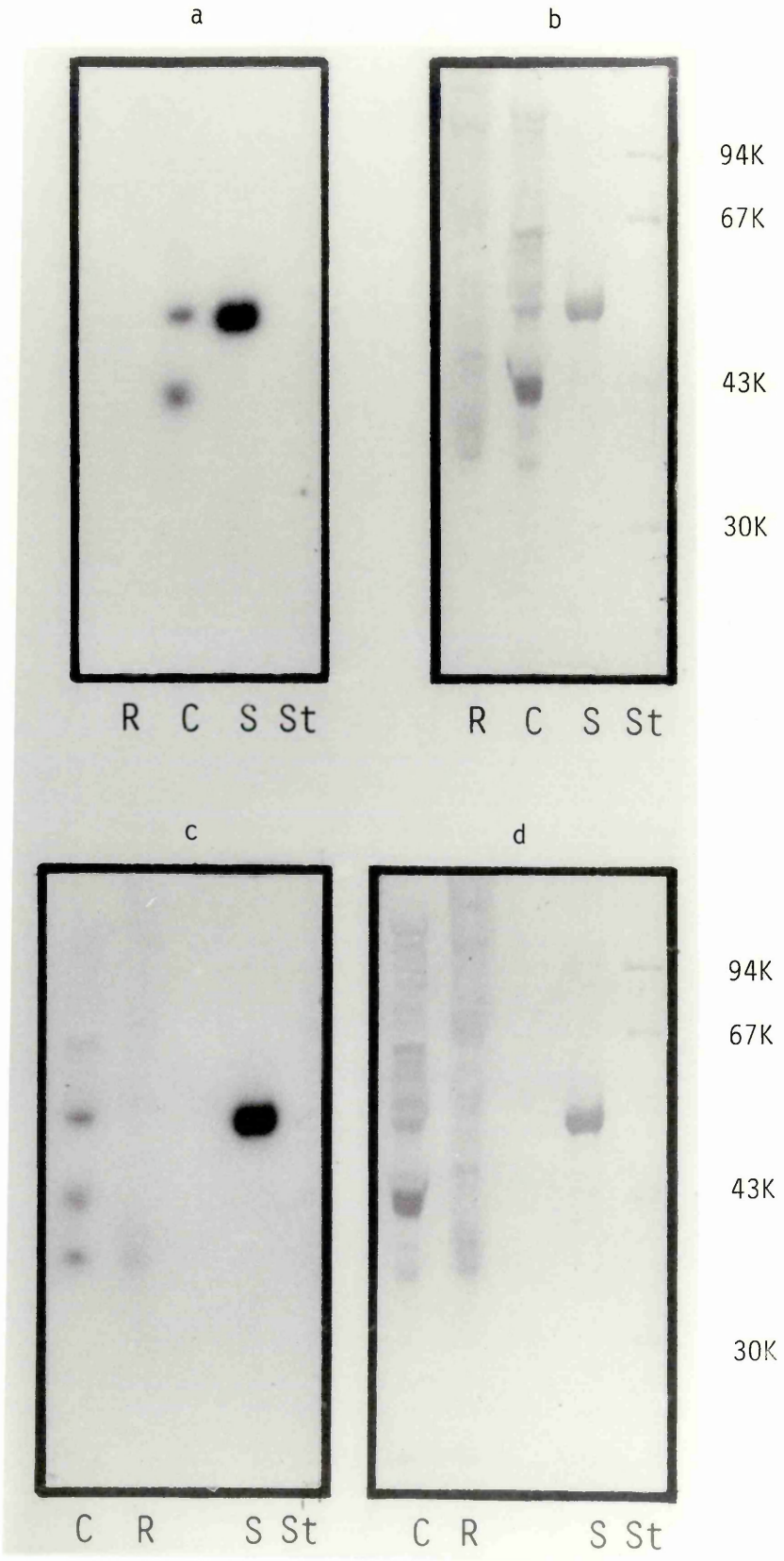

a
b
94K
67K
43K
30K
R C S St
R C S St
c
d
94K
67K
43K
30K
C R S St
C R S St

Figure 4.8 Immunoblotting Results for the Sera of two Wistar and one PVG Rat, against Retinal Antigens

Immunoblot of sera obtained from a 6 week old Wistar rat W4, demonstrating a response to S-antigen in the ELISA above those of the littermates. Blots with sera of Wistar rat W1, and PVG rat 1 which gave no indication of a positive response to retinal antigens, were included as controls. The sera was diluted 1:80 and incubated with the nitrocellulose strips for 3 hours. Rabbit anti-rat IgG(H+L) and I^{125} protein A were used in the detection of the reaction.

The sera from rat W4, specifically recognised S-antigen and a 40K antigen (Section 4.1.8). The pattern of response was similar to that obtained for the Campbell RCS rats C4 and C8 (Figure 4.7).

a) Amido black stained blot

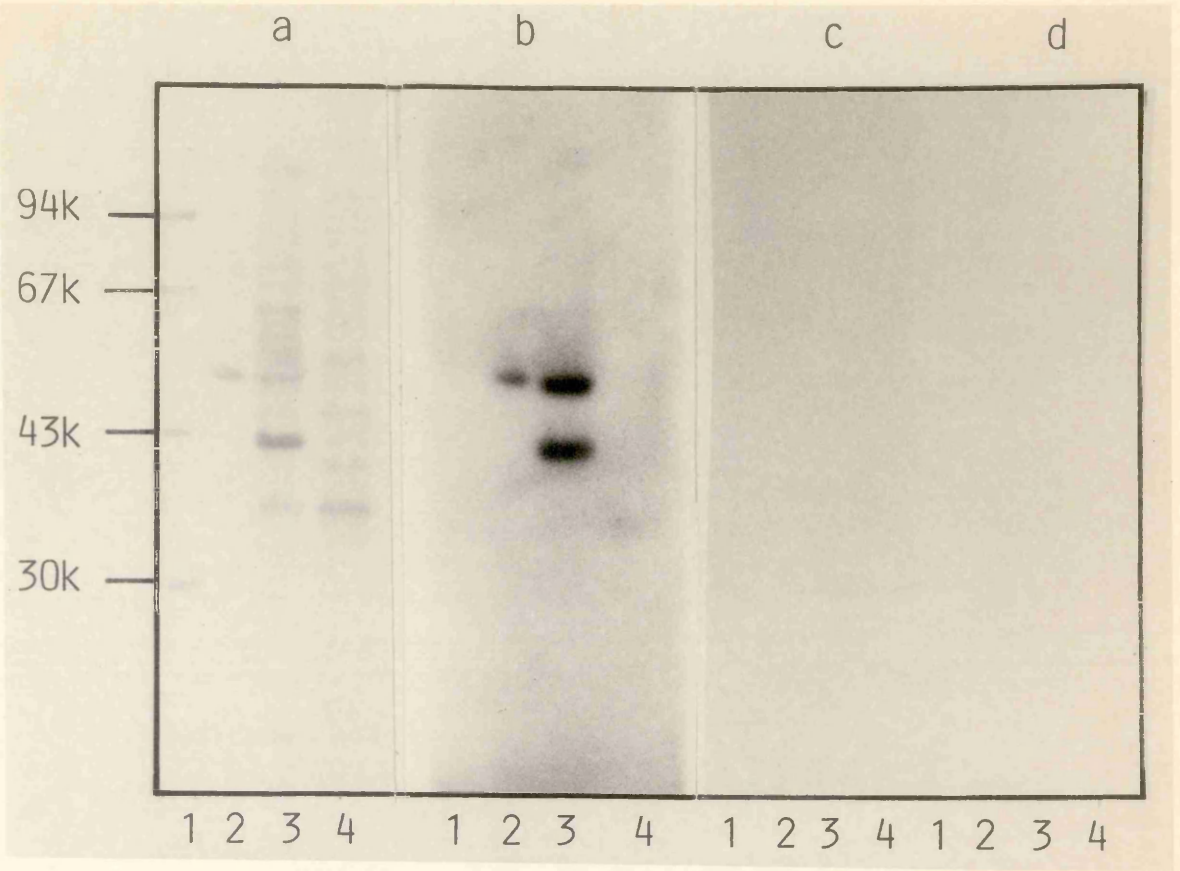
b) Autoradiograph of blot with Wistar rat W4 sera

c) Autoradiograph of blot with Wistar rat W1 sera

d) Autoradiograph of blot with PVG rat PVG1 sera

Lanes

1. Low molecular weight markers
2. Purified S-antigen
3. Crude retinal extract
4. Bovine ROS


a
b
c
d
94k
67k
43k
30k
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4

4.1.9 Discussion

The data presented above, describes the results of a study in which circulating anti-retinal autoantibodies, were sought in RCS and control rat sera. There was no significant change with time for the pigmented control rats in the mean ELISA responses to S-antigen and ROS (Figure 4.2c,d). Significantly raised titres to S-antigen or ROS were not found in the individual sera obtained from the pigmented control strains of rat (Section 4.1.4). Considering the mean responses of the various strains of rat to both antigens, there was no striking difference between the RCS and control strains of rat. However, the Campbell rats demonstrated a raised mean response to S-antigen, at the age of 6 weeks. This was attributed to three Campbell rats, C1, C4 and C8, which were identified as exhibiting a positive serum response to S-antigen at this time (Figure 4.3b). Only rat C8, remained positive at the age of 9 weeks, albeit at a lower level (Figure 4.3c). A fourth Campbell rat (C5) demonstrated a reaction above those of the littermates, at the age of 9 weeks (Figure 4.3c). It was interesting that two Wistar rats, W2 and W4, also indicated raised serum responses to S-antigen at the age of 6 weeks (Table 4.26, Appendix). On dilution beyond 1:40, however, many of the Campbell, Hunter and Wistar rat sera which were apparently positive at a dilution 1:20, gave readings which were no longer significantly raised above the unresponsive littermates. However, such sera may generate a significant positive reponse in immunoblots, for example, rat W4 (Figure 4.8b). The successful blotting of a serum which is only weakly positive in the ELISA could suggest that there were sufficient antibodies of high enough avidity, which remained bound to the antigens during the washing procedures. None of the Wistar rats were significantly positive to the ROS antigen.

The faint reaction of serum from rat C4 with the photoreceptor synapses, further suggests the possiblity of the reaction with S-antigen, since the protein is also located in this region (Figure 7.1a,b). The specificities of the serum reactions observed in the ELISA to S-antigen, were confirmed in immunoblotting experiments for two of the four positive Campbell sera and Wistar serum (W4). Campbell rats C4 and C8, and Wistar rat, W4, specifically identified S-antigen in protein A immunoblots, plus a 40K antigen within the crude retinal extract (Figures 4.7a,c and 4.8b). S-antigen is known to be highly antigenic (Wacker et al., 1977; Faure, 1980), and if the immune system is exposed to the retinal antigens, the most antigenic components may tend to dominate the immune response. The 40K antigen might also be one such antigenic component, and may be the same antigen to which monoclonal antibody R1.2.D2, has specificity (Section 6.3).

Antibodies specific to the insoluble ROS proteins, which consist of 85% opsin (Heitzmann, 1972), were less readily demonstrated in the immunoblots. The immunoblotting was possibly influenced by the amount of protein in each band. S-antigen and the 40K protein did form two of the main bands on the nitrocellulose strip. Perhaps at higher protein densities, other relevant auto-antigens could be identified. The majority of the RCS rat antibodies (from rats C1, C4 and C8), with specificity to the insoluble ROS proteins, may be directed against conformational determinants which are disrupted in SDS-PAGE and binding to the nitrocellulose paper. Barlow et al. (1986) suggested that most antibodies are directed to conformational (or discontinuous) determinants. It is also possible that the antibodies to the ROS antigens were of lower affinity and

avidity than those to S-antigen, and were more easily removed by the Tween wash buffer during the immunoblotting procedure. Human antibodies to the ROS insoluble antigens were similarly difficult to demonstrate in immunoblots (Section 4.2.7).

The pathophysiological changes in the RCS rat retina, which may result in a mild stimulation of the immune system, are yet to be determined. Clearly the autosensitisation to the retinal antigens was not great, and involved only certain individuals. Raised levels of serum antibody to retinal antigens, in particular S-antigen, could be detected more frequently in the Campbell than in Hunter RCS rats. The raised responses detected in two Wistar rats, were of similar magnitude to those of the Hunter RCS rats.

The positive responses to S-antigen observed in the sera of certain Campbell and albino Wistar rats may reflect secondary immunological responses occurring after light induced damage within the albino retinae. The higher responses of the Campbell rats over the Wistars, might suggest that the genetic retinal dystrophy predisposed the Campbell rats to further light induced damage. It is possible that light caused further disruption in the blood retinal barrier in some pink-eyed animals, thus increasing the possibility of stimulating the immune system. The light absorbing, melanin containing pigment granules, protect the eye against excess illumination. This may explain why the Campbell rats showed stronger reactions than the Hunter rats. Pigmentation has been found to delay the progress of degeneration by 7 to 10 days, in the posterior retina (Yates et al., 1974; La Vail and Battelle, 1975).

The results obtained for the RCS and control rat sera, have

together helped to build up a picture of the potentially autoimmune status of RCS and perhaps albino animals. The role of the immune response genes is an important consideration in studies of this kind, and genetic factors are likely to cause small variations in the levels of autoantibodies produced by individuals and by different strains of rat (Roitt et al., 1985). Since the levels of autoantibody detected in the RCS rats, were relatively low, and were found in less than 50% of the animals, the humoral immune response at least, probably has little effect on the progress of the retinal dystrophy. If, however, serum autoantibody was sequestered in soluble immune complexes, then possibly a greater proportion of rats were sensitised than could be detected by serum analysis. Hybridoma technology allows the investigation of B cell specificity, in vitro, where the potential complications of immune complexes and background reactions may be difficult eliminate (Section 5.2).

4.2 STUDIES ON HUMAN RETINAL AUTOIMMUNITY

This section describes the results obtained from serological assays of sera from RP and uveitis patients, and healthy volunteers. The sera were screened by the ELISA against S-antigen or solubilised rod outer segment membranes. The specificities of the positive responses were further assessed in immunoblotting experiments.

This study was undertaken in order to provide information on the sera obtained from RP and uveitis patients, whose peripheral B lymphocytes were transformed in related study (Section 5.3). The numbers of patients involved were thus small, and a detailed investigation of the potential retinal autoimmune responses of RP and uveitis was beyond the scope of this project.

4.2.1 Experimental Approach

4.2.1.1 The subjects of study

Eleven patients from two heterogeneous groups were investigated (Table 4.32). Retinitis pigmentosa patients were selected, since there is evidence that in some sub-groups of RP, retinal autoimmunity may be detected (Brinkman et al., 1980; Newsome and Nussenblatt, 1984; Chant et al., 1985) (Section 1.4.4.3). Three of the five RP patients had the autosomal dominant form of the disease, and two had advanced disease with no known affected relatives. Patients with uveitis, a group of inflammatory diseases of the retina and uveal tract, were also studied. There is a growing number of reports suggesting ocular autoimmunity in uveitis patients (Section 1.4.4). Eleven control subjects included healthy volunteers, for example unaffected family members, and laboratory

Table 4.32 Details of the human subjects involved in the serum study

Controls				Patients				
Initial	Sex	Age	Transformations*	Initial	Disease	Sex	Age	Transformations
AC	F	40	-	AA	ADRP	F	50	T
BD	M	30	T	CM	Uveitis	F	-	T
BM	M	24	-	EN	Uveitis	F	-	-
CD	M	30	-	GA	Uveitis	M	-	T
HD	M	-	T	GG	RP	M	70	T
JF	M	40	T	HM	RP	M	55	T
KM	F	24	T	HS	Uveitis	F	40	T3
LR	F	24	-	IM	ADRP	M	40	T
MM	M	30	-	KM	ADRP	M	17	T
OM	F	40	T	MK	Uveitis	M	15	T
SG	F	26	-	UV	Uveitis	-	-	-

* Section 5.2

T=Transformation

T3=Three transformations

workers (Table 4.32). The laboratory workers included people handling retinal antigens in order to ascertain the passive sensitisation to the antigens in question. The approximate ages of the subjects were recorded (Table 4.32), since the diseases are progressive with time and also, age may play a role in the immune responsiveness in general.

4.2.1.2 Choice of assays

The ELISA assay was selected for the same reasons as described for the assay of the rat sera (Section 4.1.2.2), and there was no alteration in protocol other than the starting dilution of sera, and the use of a rabbit anti-human peroxidase conjugate (Section 2.1.16). Variation between plates was assessed by control positive rat antisera and preimmune sera as described in Section 4.1.2.2, and found to be minimal.

The sera obtained from the eleven uveitis and RP patients and the eleven healthy controls were screened against bovine retinal ROS and S-antigen in ELISA (Sections 4.2.3-5). All samples were screened against the same preparations of antigens and tested under identical conditions (Section 3.8). Doubling dilutions of the serum samples, from 1:10 to 1:1280, were made on the plate. The samples were incubated for 4 hours at room temperature to allow sufficient time for the antibodies of lower affinity and avidity to react with the antigens (Mason and Williams, 1980). The ELISA reaction was developed in the usual manner.

Those sera identified as positive to either or both antigens were tested for their ability to bind to ocular antigens in sections of rat eye, in immunoperoxidase experiments (Section 2.4.4). The

indirect immunofluorescence assay was not used since it was found to give unreliable results in the study with the rat sera (Section 4.1.7).

The specificities of the positive ELISA titres were examined in immunoblotting experiments (Sections 2.3.9 and 4.2.7). Bovine retinal S-antigen, crude soluble retinal antigens, and the ROS proteins were electophoretically separated in SDS 10% acrylamide gels and transferred to nitrocellulose paper. After blocking, the strips of nitrocellulose paper were incubated with the human serum samples, diluted 1:80 in the same buffer for four hours, to allow sufficient time for the reaction of antibodies of lower affinity or avidity (Mason and Williams, 1980). The reaction was visualised by the ^{125}I protein A method, with the use of the intermediate rabbit anti-human IgG,M,A (Section 2.3.7.6).

4.2.2 Methods of Human ELISA Data presentation

The ELISA results obtained for the patients and control sera are recorded in Tables 4.33 to 4.36, in the Appendix, as the mean of two assays, with individual standard deviations. The titres are displayed graphically in Figures 4.9a,b to 4.12a,b. The figures only show the standard deviations for those serum samples which demonstrated serum responses significantly above the control range.

Table 4.37 examines in three different ways, the responses of human subjects, demonstrating significant reactions in the ELISA, to retinal antigens. The results for these individuals were compared with the pooled results of the normal control subjects.

4.2.3 Normal Range of Reactions to S-Antigen and ROS

Figure 4.9 Data obtained from the serial dilution of Eight Control Human Sera against S-Antigen and ROS in ELISA Assays

a) Reactions of 8 control sera to S-antigen in ELISA

b) Reaction of 8 control sera to ROS in ELISA

Serial dilutions of sera obtained from eight healthy volunteers were tested in the ELISA assay against (a) 1µg/ml of S-antigen, and (b) 5µg/ml ROS. The results were obtained in the form of absorbance readings at A492. The results are recorded as the mean of two assays. The standard deviations, together with the means are recorded in Tables 4.33 to 4.36, in the Appendix.

The range of readings covered by the control sera provided a criterian, for the comparison with the patients sera, above which suggested a positive response.

KEY

◣ Response of control subject AC

◺ Response of control subject BD

■ Response of control subject BM

△ Response of control subject CD

● Response of control subject HD

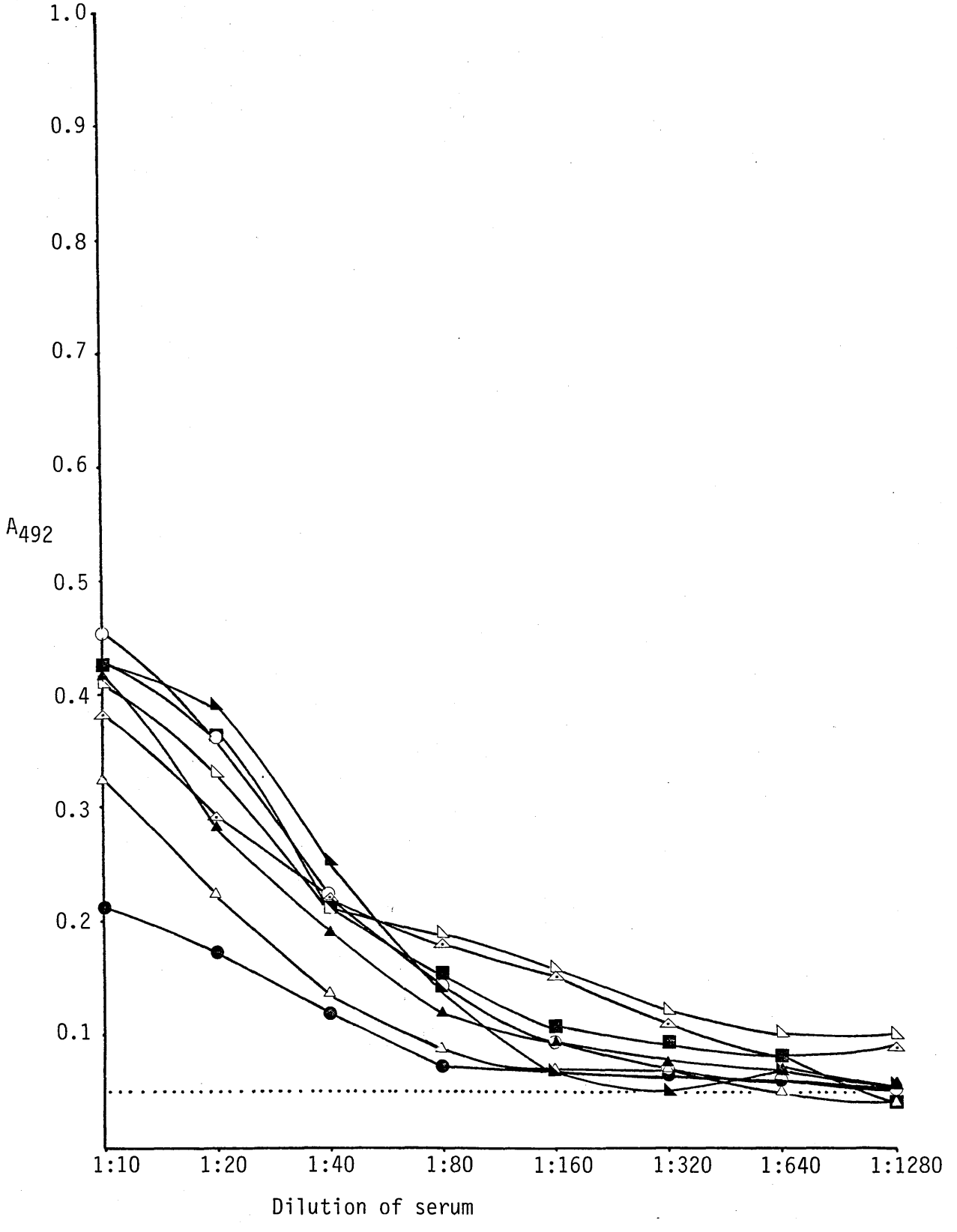
▲ Response of control subject MM

⛰ Response of control subject OM

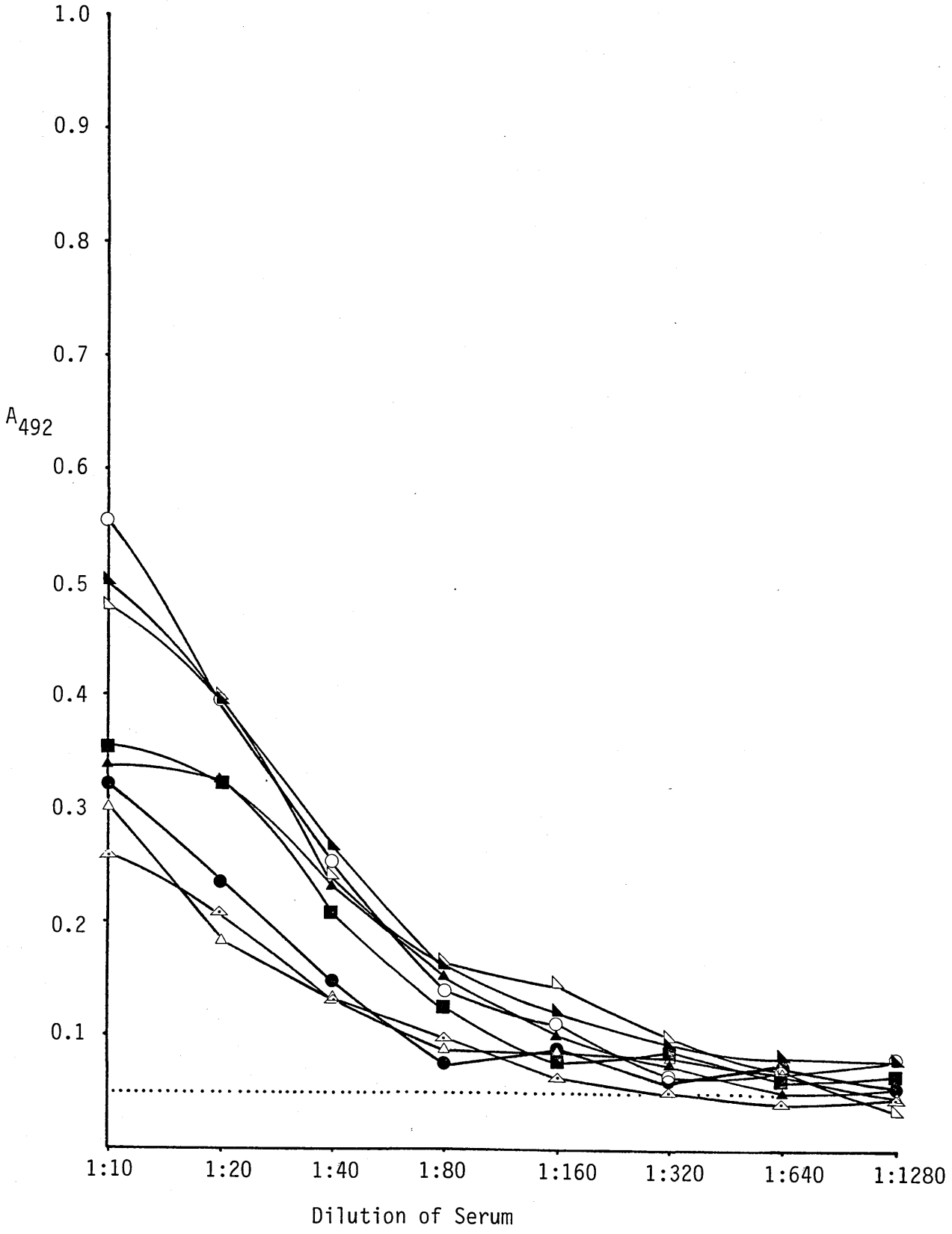
○ Response of control subject SG

··· Omission of primary antibody

(a)


1.0
0.9
0.8
0.7
0.6
A492
0.5
0.4
0.3
0.2
0.1
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution of serum

(b)


1.0
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0.5
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0.3
0.2
0.1
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution of Serum

The normal range of readings to the antigens was obtained from the 'titres' of the healthy controls (Figures 4.9a,b). The eight normal control sera, obtained from people who were not involved with retinal antigens, generated readings in the ELISA assays to S-antigen and ROS, which were fairly close together. Sera lying in the upper range of the responses against one antigen, tended also to lie in the upper range of responses to the other antigen. There was a similar, but opposite tendency, with the sera lying in the lower range of responses.

For S-antigen, the ELISA readings for the eight control sera ranged between 0.21 and 0.45 absorbance units at 492nm (A492) at a dilution of 1:10, and decreased to approximately 0.04 to 0.1 A492 at a dilution of 1:1280 (Figure 4.9a). The responses of the healthy controls to the ROS antigen varied between 0.26 to 0.55 A492 at a serum dilution of 1:10, to 0.04 to 0.08 A492 at a dilution of 1:1280 (Figure 4.9b). A template of the normal range of responses to each antigen was constructed for overlay on the graphs of the patients' serum responses to the retinal antigens.

4.2.4 Responses of the five RP and six Uveitis Patients

The titres of all five RP patients to both S-antigen and ROS fell within the normal range of responses (Figures 4.10a,b). Two out of six patients with uveitis (patients HS and UV) demonstrated a significant response to S-antigen, their titres falling outwith the range observed for the healthy controls (Figure 4.11a). Two serum samples were taken from patient HS at approximately one year apart. The titres of the two samples remained similar, with perhaps a slight change in the response to S-antigen in the second sample. The positive serum reaction observed for this patient has been

Figure 4.10 Data obtained from the serial dilution of Sera from Five RP Patients against S-Antigen and ROS in ELISA Assays

a) Reactions of 5 RP sera to S-antigen in ELISA

b) Reaction of 5 RP sera to ROS in ELISA

Serial dilutions of sera obtained from five RP patients were tested in the ELISA assay against (a) 1µg/ml of S-antigen and (b)µ5 g/ml ROS. The results were obtained in the form of absorbance readings at A492. The results are recorded as the mean of two assays. The standard deviations, together with the means, are recorded in Tables 4.33 to 4.36, in the Appendix.

The sera from these five RP patients to (a) S-antigen and (b) ROS yielded reactions within the normal range (Sections 4.2.3-4).

KEY

◬ Response of RP patient AA

▲ Response of RP patient IM

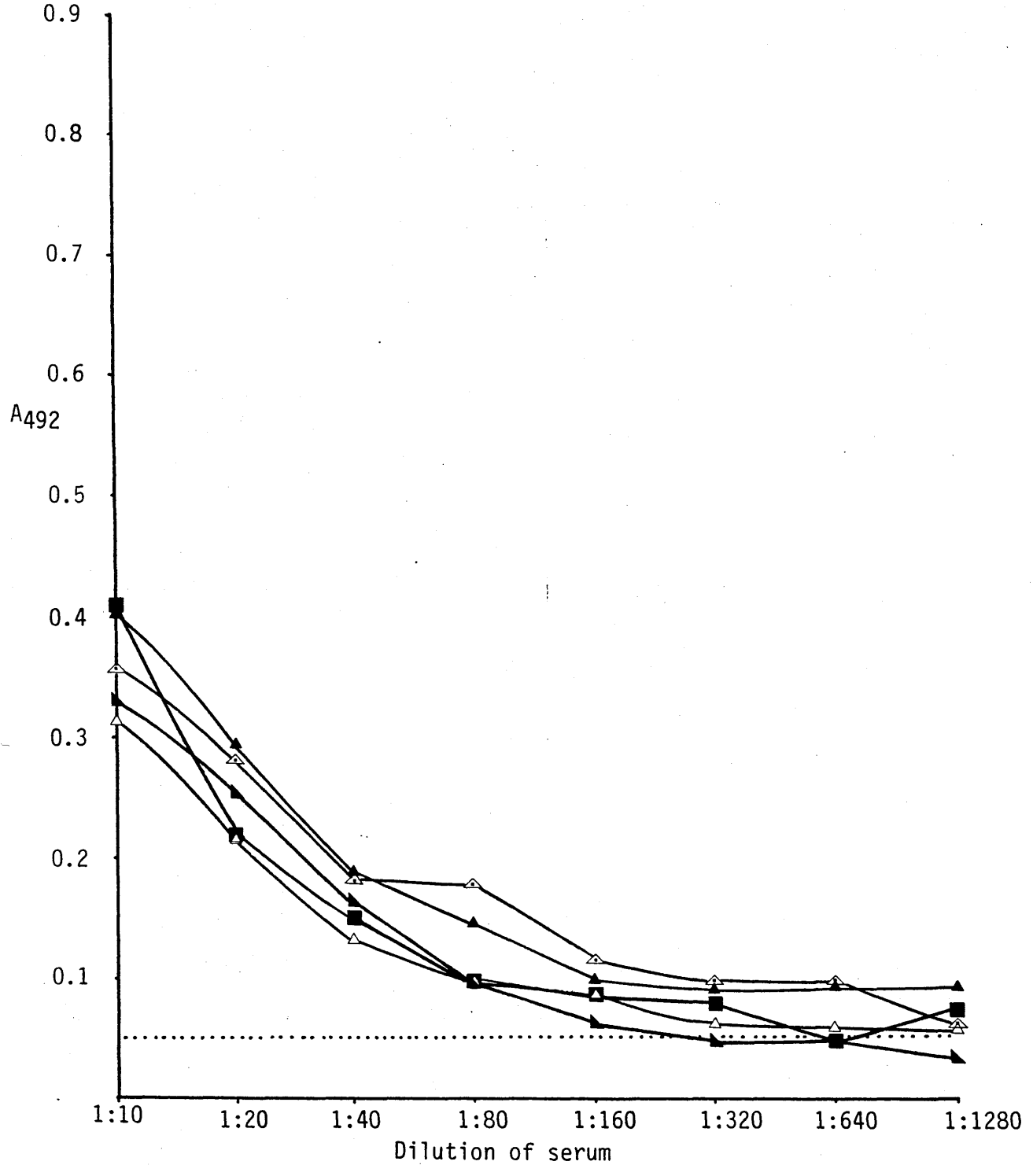
△ Response of RP patient KM

■ Response of RP patient GG

◣ Response of RP patient HM

... Omission of primary antibody

(a)


0.9
0.8
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0.6
A492
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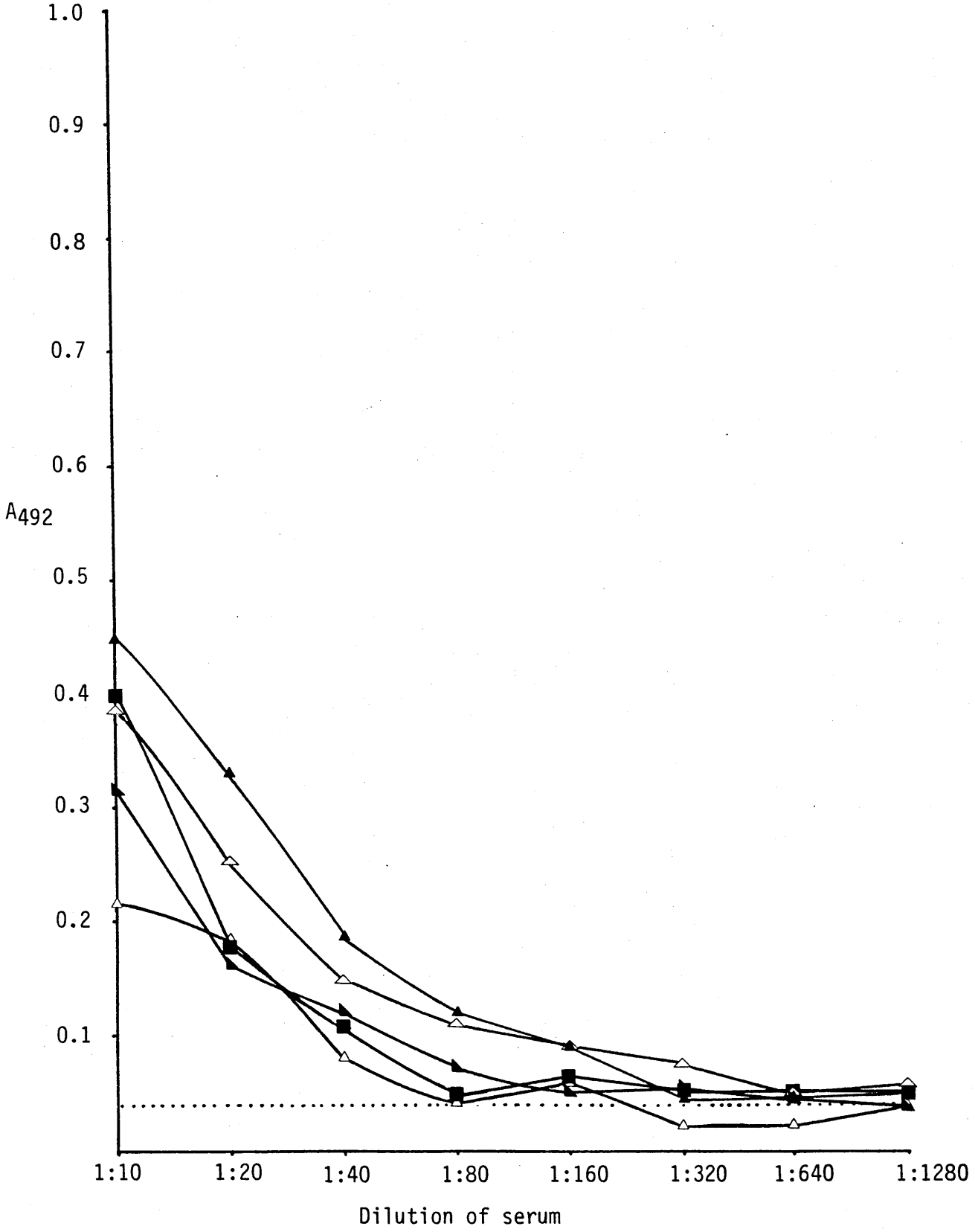
0.4
0.3
0.2
0.1
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution of serum

(b)


1.0
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A492
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0.3
0.2
0.1
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution of serum

Figure 4.11 Data obtained from the serial dilution of Sera from Six Uveitis Patients against S-Antigen and ROS in ELISA Assays

a) Reactions of 6 uveitis sera to S-antigen in ELISA

b) Reaction of 6 uveitis sera to ROS in ELISA

Serial dilutions of sera obtained from six uveitis patients were tested in the ELISA assay against (a) 1µg/ml of S-antigen and (b) 5µg/ml ROS. The results were obtained in the form of absorbance readings at A492. The results are recorded as the mean of two assays (Tables 4.33 to 4.36, Appendix). The standard deviations are only shown for the positive sera. The sera from two of the uveitis patients (HS and UV) demonstrated significantly raised serum responses to (a) S-antigen, compared with the normal range (Figure 4.9a). Two different serum samples were obtained from patient HS. Three uveitis patients (EN, GA and MK) indicated raised serum antibody to the ROS antigen (b). The results are described in Section 4.2.4.

KEY

▲ Response of uveitis patient CM

□ Response of uveitis patient EN

■ Response of uveitis patient GA

▼ Response of uveitis patient HS1

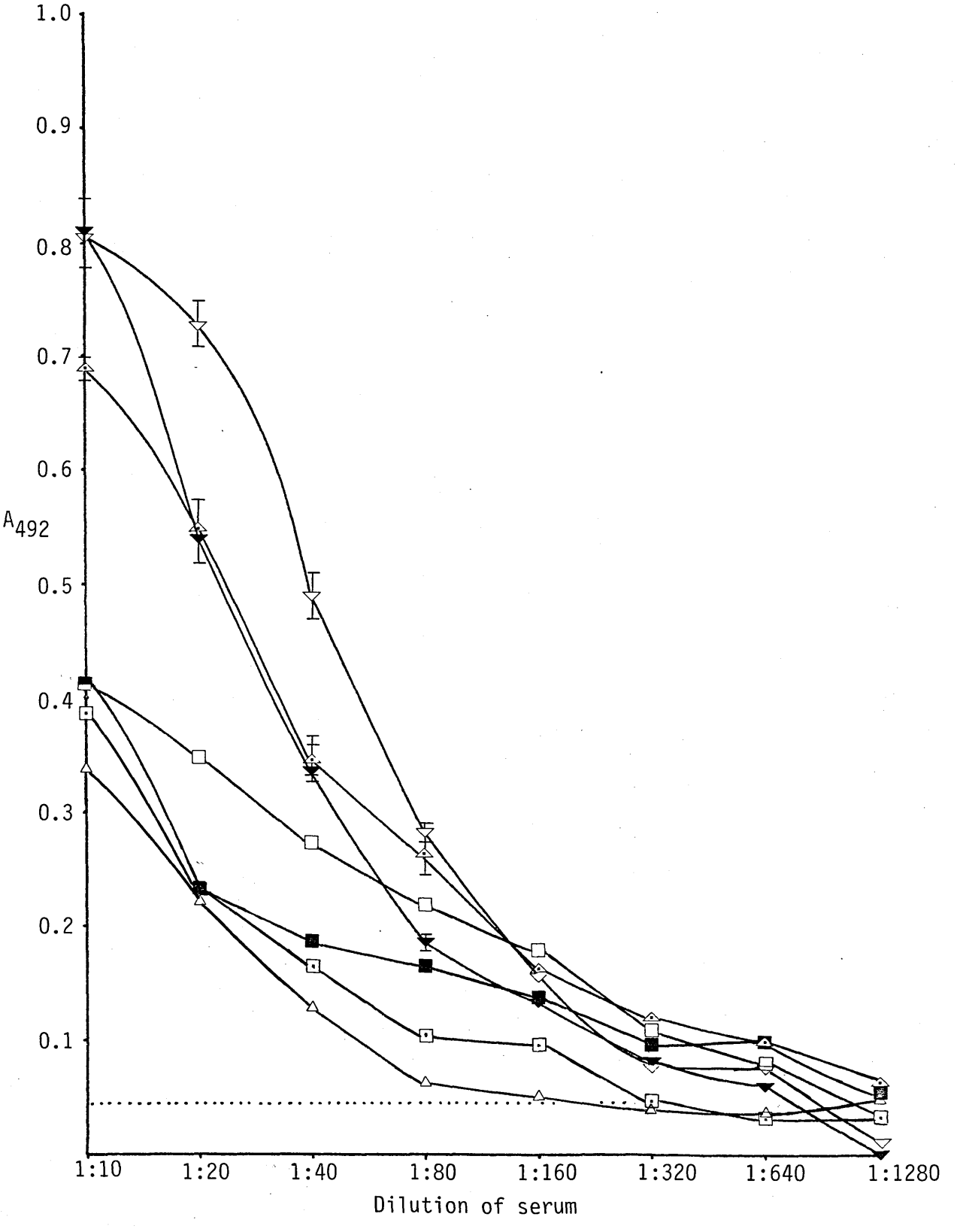
▽ Response of uveitis patient HS2

⊡ Response of uveitis patient MK

◬ Response of uveitis patient UV

... Omission of primary antibody

(a)


1.0
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A492
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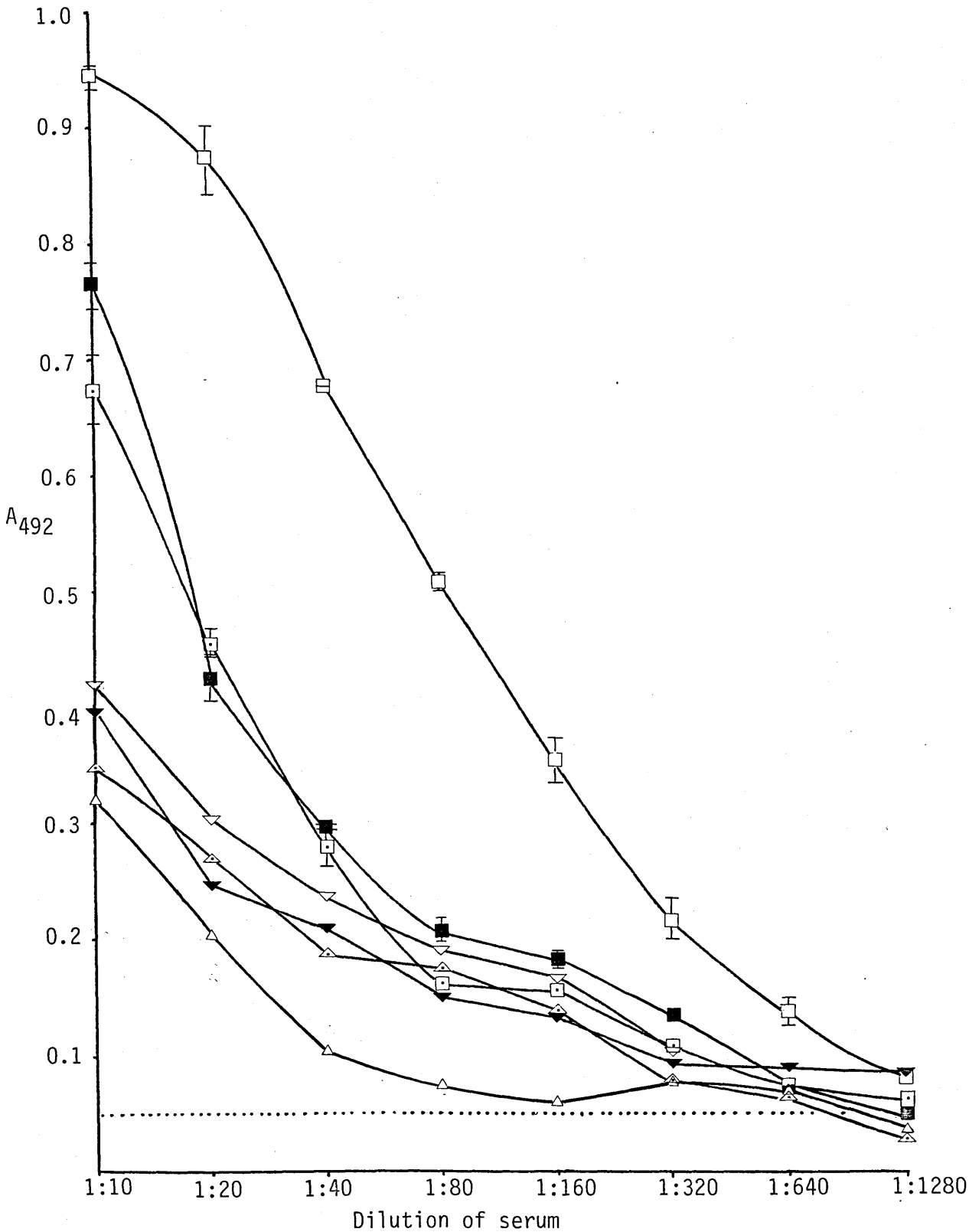
0.3
0.2
0.1
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution of serum

(b)


1.0
0.9
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0.7
0.6
A_{492}
0.5
0.4
0.3
0.2
0.1
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution of serum

confirmed in another laboratory (Forrester, 1986; personal communication). Neither of these two patients gave a response to the solubilised bovine ROS in the ELISA (Figure 4.11b). Patient CM did not demonstrate a significant response to either antigen in the ELISA. The remaining three of the uveitis patients (EN, GA and MK) demonstrated significant titres to the bovine ROS (Figure 11b). These sera were not, however, positive in the ELISA to S-antigen (Figure 11a). The strongest response was indicated by patient EN. The titres of patients GA and MK, lay close to one another. The titres of the positive individuals (Table 4.37), were determined by use of a transparent template of the normal range of responses (Figures 4.9a,b), layed over the graphs of the patients' ELISA readings. The titre was taken to be that dilution at which the patient's response came within the normal range (Table 4.37). Table 4.37 also shows the ELISA A492 readings for the positive individuals given by serum dilutions of 1:20 and 1:80. The five patients gave titres or readings (at 1:20) to either S-antigen or ROS, above the control values, although the rank order of the patients' responses varied with each method of presentation (Table 4.37). The responses were less marked at the 1:80 dilution, especially for S-antigen.

Thus, none of the RP patients tested, showed a significant serum response to S-antigen or solublised ROS, and five of the six uveitis patients tested, showed a raised response to either antigen, but not to both.

4.2.5 Responses of Laboratory Workers using S-Antigen

The sera of three users of retinal antigens were tested for their activity to S-antigen and ROS. One of the three users (LR)

Table 4.37 ELISA Positive Human Serum Responses*

Positive Subjects	Condition	S-antigen Titre	S-antigen A492 1:20	S-antigen A492 1:80	ROS Titre	ROS A492 1:20	ROS A492 1:80
HS1	uveitis	1:80	0.544	0.188	NA+	0.246	0.150
HS2	uveitis	1:160	0.733	0.284	NA	0.310	0.191
UV	uveitis	1:160	0.549	0.265	NA	0.272	0.163
MK	uveitis	NA	0.235	0.103	1:80	0.458	0.163
EN	uveitis	NA	0.349	0.221	1:1280	0.876	0.509
GA	uveitis	NA	0.233	0.167	1:40	0.426	0.209
LR1	user of	1:120	0.668	0.253	1:1280	0.678	0.254
LR2	S-antigen	1:320	0.681	0.293	1:1280	0.806	0.386
Mean of 8 controls		NA	0.303	0.135	NA	0.308	0.125
Range of 8 controls		NA	0.172 to 0.394	0.072 to 0.187	NA	0.398 to 0.178	0.166 to 0.076
SD (n-1), n = 8			0.076	0.041		0.090	0.034

* mean of two assays

+ NA = not applicable

Figure 4.12 ELISA Reactions of Sera from Three Users of Retinal Antigens against S-Antigen and ROS in the ELISA

a) Reactions of the 3 sera to S-antigen in ELISA

b) Reaction of the 3 sera to ROS in ELISA

Serial dilutions of sera obtained from 3 users of retinal antigens, were tested in the ELISA assay against (a) 1µg/ml of S-antigen and (b) 5µg/ml ROS. The results were obtained in the form of absorbance readings at A492. The results are recorded as the mean of two assays. The standard deviations are only shown for the positive sera. The standard deviations, together with the means, are recorded in Tables 4.33 to 4.36, in the Appendix. Two samples were obtained from subject LR on different occasions.

The results suggest that only one user of S-antigen, LR, demonstrated a significant response to (a) S-antigen and (b) ROS (Section 4.2.5).

KEY

⏢ Response of subject JV

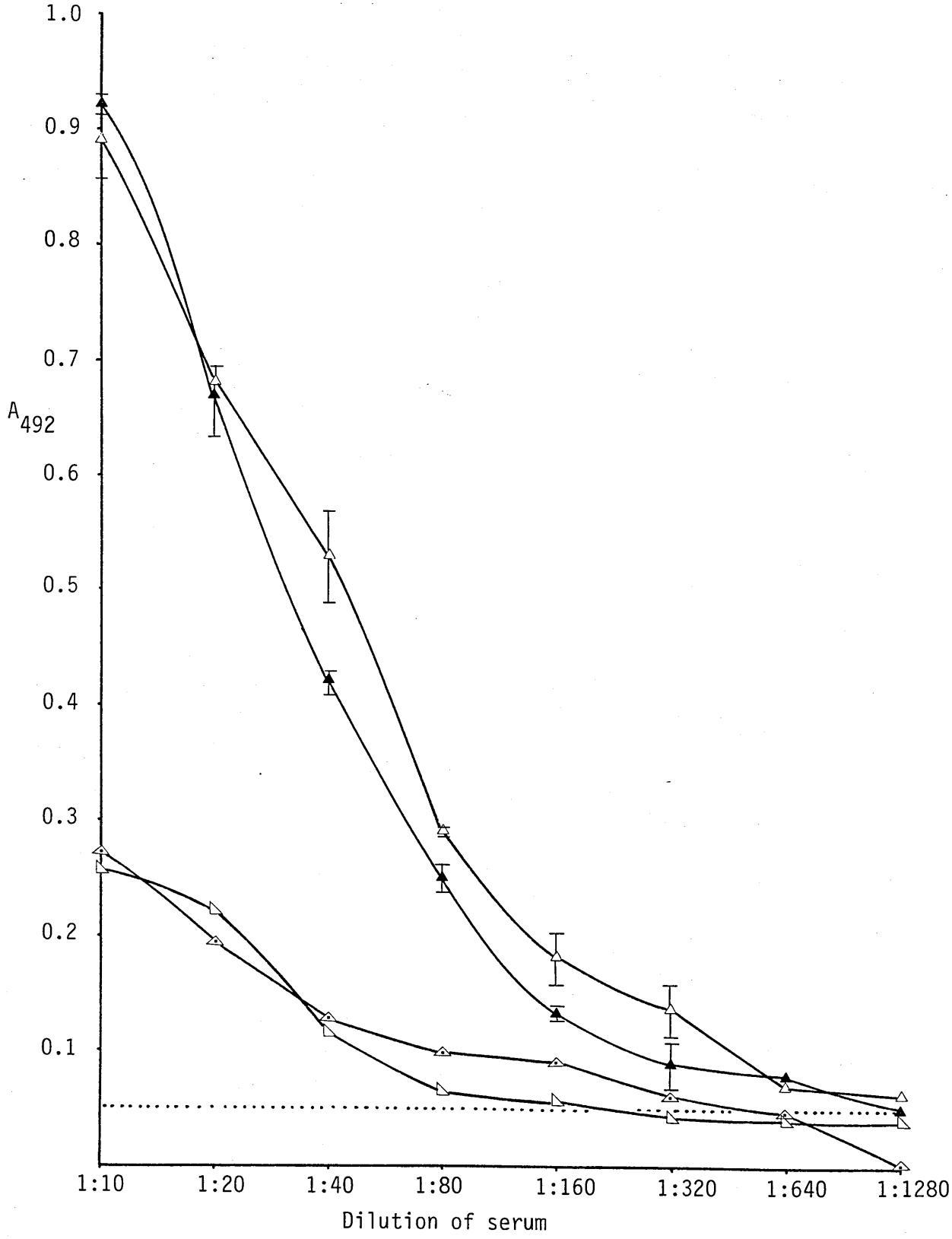
◺ Response of subject KM

▲ Response of subject LR1

△ Response of subject LR2

··· Omission of primary antibody

(a)


1.0
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A492
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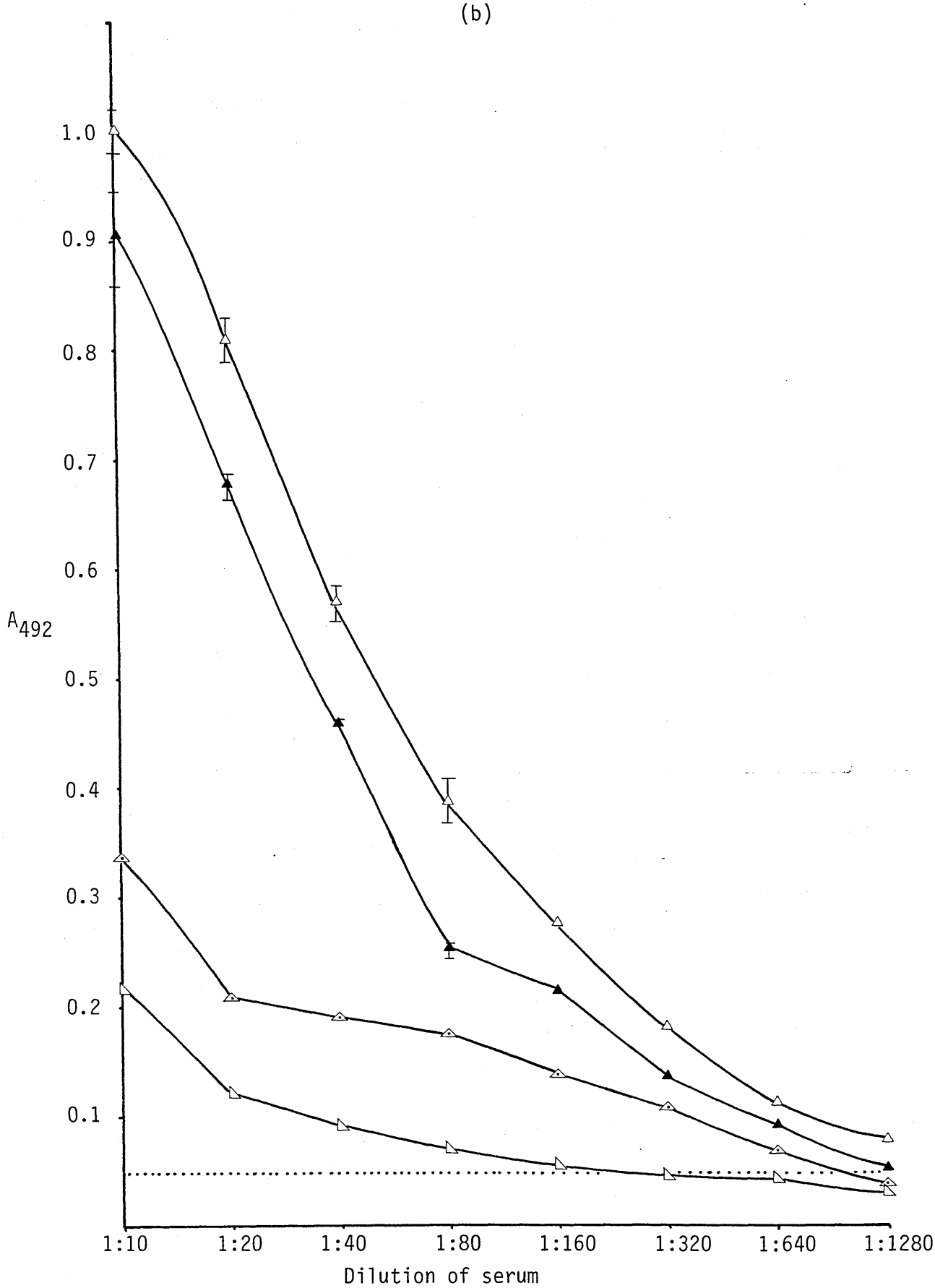
0.3
0.2
0.1
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution of serum

(b)


1.0
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0.6
A_{492}
0.5
0.4
0.3
0.2
0.1
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution of serum

demonstrated a significantly positive titre to both antigens, whereas the titres of the remaining two fell within the normal range (Figure 4.12a,b). Two serum samples were taken from subject LR at approximately 18 months apart. The titres of both samples to each antigen remained similar, although the ROS response in the second sample (LR2) was slightly increased (Figure 4.12b) (Table 4.37). The responses observed in subject LR, probably resulted from a passive sensitisation to these very antigenic substances.

4.2.6 Specificity of the Human Reaction to Retinal Antigens

The specificities of the positive reactions observed for the patients' sera, were indicated in part, by a reproducible reaction to one antigen but not the other (Section 4.2.4). Two human serum samples were titred against the BSA in order to assess for interference of the blocking agent in the ELISA assays to retinal antigens. All sera were tested at a dilution of 1:10 on plates treated with the BSA blocking agent only, and the readings all fell within a range of 0.00 to 0.05 A492. There was no indication of a reaction for any of the sera with the BSA. These results indicate that with the sera of normal subjects, as well as the patients, there was a greater tendency for reactions with the bovine retinal antigens than with the BSA, employed as a blocking agent. It may be worth noting that most of the people involved in this study had been exposed to dietary bovine antigens.

4.2.7 Specificities of ELISA Positive Sera in Immunoblotting Experiments

The specificities of the serum responses, previously demonstrated to be positive to the S-antigen or ROS in ELISA (Sections 4.2.4-5), were determined by protein A immunoblotting experiments (Sections 2.3.9 and 4.2.1.2).

Figure 4.13 Reaction of Sera from Healthy Subjects in Immunoblots against Retinal Antigens

Sera from healthy subjects, not demonstrating significant responses to S-antigen or ROS in the ELISA assays, were used as negative controls in immunoblotting experiments involving human sera. The I^{125}Protein A detection system was employed, with the intermediate incubation with rabbit anti-human IgG+ IgM + IgA.

No significant reaction was observed with the resolved retinal antigens (Section 4.2.7). Nonspecific reactions were obtained with one of the protein standards, and also with the dye front.

KEY

a) Amido black stained immunoblot

b) Reaction of sera from subject JF

c) Reaction of sera from subject SG

d) Reaction of sera from subject CD

LANES

1. Low molecular weight standards
2. Purified bovine S-antigen
3. Bovine crude retinal extract
4. Bovine ROS

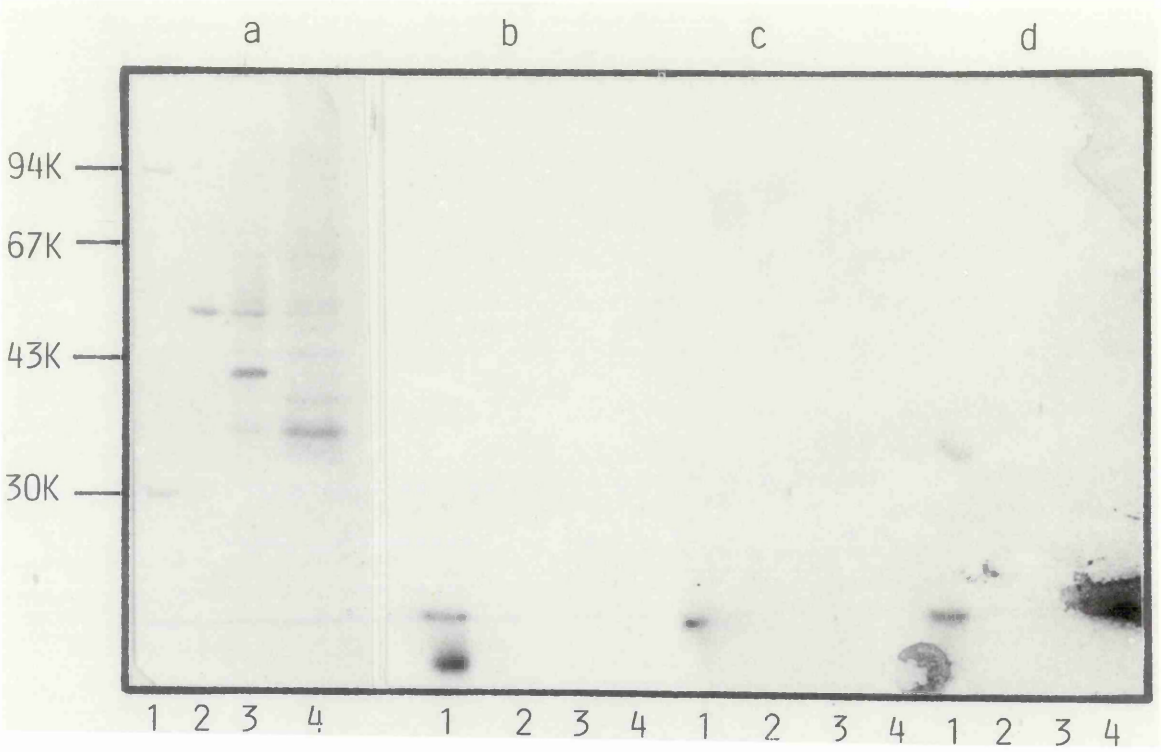

a
b
c
d
94K
67K
43K
30K
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4

Figure 4.14 Specificity in Immunoblots of Sera from Subjects positive in the ELISA to Retinal Antigens

The specificity of sera from subjects demonstrating significant responses to S-antigen and/or ROS in the ELISA assays, was investigated in immunoblotting experiments. The I^{125}Protein A detection system was employed, with the intermediate incubation with rabbit anti-human IgG, IgM and IgA.

The pattern of reaction was similar in all the immunoblots, regardless of the specificity of the sera in ELISA (Sections 4.2.4 and 4.2.7).

KEY

a) Reaction of sera from uveitis patient EN

b) Reaction of sera from uveitis patient HS

c) Reaction of sera from uveitis patient UV

e) Reaction of sera from uveitis patient GA

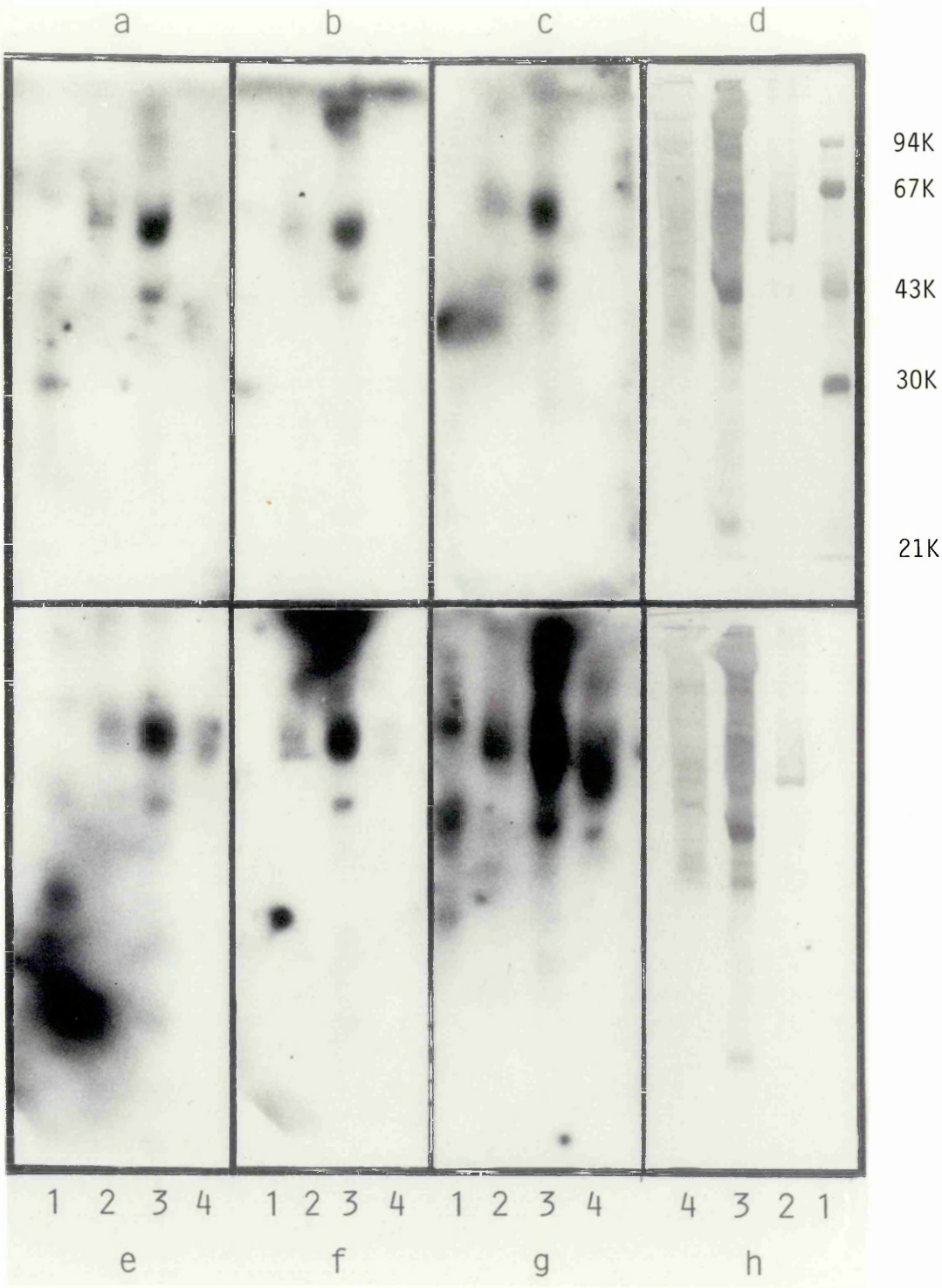
f) Reaction of sera from uveitis patient MK

g) Reaction of sera from subject LR
(user of retinal antigens)

d) and h) Amido black stained immunoblots

LANES

1. Pharmacia low molecular weight stards
2. Purified bovine S-antigen
3. Bovine crude retinal extract
4. Bovine ROS


a
b
c
d
94K
67K
43K
30K
21K
1 2 3 4
1 2 3 4
1 2 3 4
4 3 2 1
e
f
g
h

There was little indication of a specific reaction of the control sera to the retinal antigens (Figure 4.13). There was a nonspecific reaction in the control blots, with one of the low molecular weight standard proteins, and with the dye front, which was a fairly common phenomenon in the immunoblots. The human sera, positive to S-antigen and/or ROS in the ELISA, all demonstrated very similar patterns of reactions in the immunoblots (Figure 4.14). In each case intense reactions with S-antigen were identified, and also less intense reactions a 40K antigen, present within the crude retinal extract. In most blots shown in Figure 4.14, a reaction with the 30K protein standard can be seen, which ranges from weak to strong in the individual blots.

4.2.8 Immunocytochemical Tests of Human Sera in Sections of Rat Eye

The sera of individuals who displayed a positive reaction in the ELISA to either S-antigen or ROS (Sections 4.2.4-5) were tested for reactivity to the retina in sections of rat eye. An indirect peroxidase immunocytochemical staining procedure was employed (Section 2.4.4).

The sera of the healthy controls were utilised for the assessment of a positive reaction above the background. The serum samples were diluted 1:20, 1:40, and 1:80 and tested in triplicate. At serum dilutions below 1:80 the general high background reaction, which could not be eliminated by blocking with undiluted normal rabbit serum, rendered it impossible to distinguish a specific reaction with the photoreceptors, other retinal cells or the choroid. The control, comprising identical conditions, except for the omission of the primary antibody, indicated that the background reaction was

due to the nonspecific adsorption of the human immunoglobulins onto the tissue sections when low dilutions were employed.

At dilutions of 1:80, no obvious positive reactions above the reduced background, could be detected in any of the sera previously assessed as positive to either S-antigen or ROS in the ELISA assays. The sera of only one subject, LR, a user of bovine and rat retinal antigens, gave possible positive staining with the rat photoreceptor ROS, above the general background reaction, at all dilutions tested.

4.2.9 Discussion

In this limited study, none of the five RP patients gave any indication of a response to S-antigen or to ROS, in the ELISA (Figures 4.10a,b). Five of the six uveitis patients (Section 4.2.4), however, demonstrated a response to either S-antigen or ROS (Figures 11a,b; Table 3.37). The ELISA data obtained for the human subjects, provided supplementary information for a related study (Section 5.3).

RP is a progressive disease. It is possible that one or all of them had become sensitised to their retinal antigens in the past, and later humoral immune response subsided during the regulation of the immune system (Roitt et al., 1985). It is also possible that the anti-retinal antibodies were unavailable for assay, being sequestered in soluble immune complexes with shed antigen. Alternatively, the titres of the autoantibodies were too low to detect, even in the sensitive ELISA. It is more likely that the failure to detect the antibodies in the ELISA, even at only a 1:10 dilution, was related to the affinity and specificity of the

antibodies. Since the ELISA procedure involves washing steps, with a mild detergent, antibodies of low affinity and specificity are discouraged from binding. The sera were only screened against S-antigen and ROS, and perhaps the sera of the patients contain antibodies to other retinal antigens, such as the 40K antigen recognised by the sera of some uveitis patients and some Campbell rat RCS rats in immunoblots (Sections 4.2.7 and 4.1.8, respectively).

It is evident that some uveitis patients may become sensitised to their retinal antigens and produce a humoral immune response to them. It is of interest that the two patients, UV and HS, generated high reactions to S-antigen in the ELISA, but not to ROS, and that patient EN, preferentially responded to ROS, but not to S-antigen. Patients GA and MK, also demonstrated elevated responses to ROS, although lower than that of patient EN. Patients GA and MK, were apparently unresponsive to S-antigen. Evidently the autoantibodies produced by these patients, are recognising epitopes of the bovine antigens, which are shared by those of humans. Possibly the sera from some of the patients employed in this study, were only able to recognise human retinal antigens. If the immune response is related to the stage of the disease, it may be that the initial sensitisation is to the most antigenic components, including S-antigen. Later antibodies might be produced against the less antigenic components, perhaps with the regulation of the humoral immune response to other antigens, like S-antigen.

The sera demonstrating a positive response in the ELISA to S-antigen and/or ROS, were tested in an immunoperoxidase procedure in sections of rat eye. No positive reactions could be detected in

any of the patients sera tested (Section 4.2.8). Higher background reactions were obtained with the human sera, compared with the rat sera (Section 4.1.7). The humans were likely to have had a more complex repertoire of antibodies and possibly autoantibodies, since they have been exposed to a much wider range of external antigens. The antibodies would differ in their intrinsic abilities to crossreact with irrelevant antigens in tissue sections. Given the relative insensitivity of the immunoperoxidase technique, especially in comparison with the ELISA (Polak and Van Noorden, 1984), any specific antibodies may not have been detected at the dilutions of 1:80, or may have been obscured by the high general background present at lower dilutions. In addition, the reading of the ELISA reactions was aided by the use of the Titertek Multiskan whereas the immunoperoxidase reactions were interpreted by eye. Similar problems were encountered when retinal autoimmune responses in rats were investigated by this technique (Section 4.1.7). Human retina would provide a more suitable set of antigens than the rat tissue employed in the immunoperoxidase experiments.

The sera indicating significant responses to the retinal antigens in the ELISA assays were further studied in immunoblots (Section 4.2.7). An interesting pattern of responses was obtained with the positive human sera. The reactions of all of the uveitis patients' sera tested in the immunoblots, gave similar results (Figure 4.14) irrespective of the specificities in the ELISA assays (Section 4.2.4). All the sera were capable of recognising S-antigen and an antigen with a molecular weight in the region of 40K. The 40K antigen may be the same component recognised by some Campbell rat sera (Section 4.1.8). The autoantibodies reacting in the ELISA to ROS, were not readily demonstrated in the immunoblots, presumably

due to denaturation of the protein epitopes (Section 4.1.9).

Thus, uveitis patients who responded in the ELISA assays, to either S-antigen or ROS could be identified. Although no positive serum responses were detected for the RP patients, only five subjects were tested, which is not representative of this heterogeneous disease. However, the data presented in Section 5.2 suggests that at least some uveitis patients might have become sensitised to their retinal antigens, although a larger study of controls may indicate that perhaps only patients EN and HS were significantly positive to ROS and S-antigen, respectively. It would be of interest to determine the relationship between the immune responses and the various subclasses and also stages of the diseases. Further investigations into the nature of auto-sensitisation in these patients would yield useful information on the aetiology of these heterogeneous groups of diseases.

CHAPTER FIVE

STUDIES ON AUTOIMMUNITY TO RETINAL ANTIGENS USING TEMPORARILY STABILISED B LYMPHOCYTES IN CULTURE

Chapter 5

STUDIES ON AUTOIMMUNITY TO RETINAL ANTIGENS USING TEMPORARILY STABILISED B LYMPHOCYTES IN CULTURE

5.1 INTRODUCTION

This chapter describes the potential humoral autoimmune responses of the rat retinal dystrophic model and human subjects to retinal antigens. The investigations involved the use of techniques allowing the long term culture of B lymphocytes, and the testing of the cell culture supernatants for the secretion of specific antibody directed against retinal S-antigen or the solubilised rod outer segment (ROS) membranes.

The rat system, described in Section 5.2, involved the temporary stabilisation of the splenic B lymphocytes, by fusion with a rat myeloma cell line. The human transformation system, described in Section 5.3, involved the isolation of B lymphocytes from peripheral blood. These were temporarily stabilised by transformation with Epstein Barr virus (EBV). Transformed B cells can be further stabilised by fusion with a human lymphoblastoid cell line, followed by cloning. The freshly isolated human peripheral blood B lymphocytes are not generally fused, for technical reasons (Section 5.3.1).

5.2 IMMORTALISATION OF RAT B LYMPHOCYTES

5.2.1 Rationale of Study by Rat Hybridoma Generation

The autoimmune response of the rats with retinal degeneration was further investigated by the stabilisation of their splenic lymphocytes after fusion with a rat myeloma cell line (Galfre *et*

al., 1979) (Section 2.6.6). Primary clones of hybrid cells secreting antibody of interest, can be further stabilised, or immortalised, by cloning (Section 2.6.9). The immortalised cells can be grown indefinitely in culture, secreting large amounts of monoclonal antibody for experimental purposes. Hybridoma technology has been used to generate autoantibodies from non-immunised animals with established autoimmune conditions in order to examine further the mechanisms of autoimmunity (Kani et al., 1985; Lewis et al., 1981). The potential complications of serum studies, where antibody may be sequestered as soluble immune complexes, and where background reactions are hard to eliminate are thus avoided.

5.2.2 The Hybridoma Study

The rats employed in this study, which gave rise to successful fusions in terms of the presence of at least 12 primary clones, did not demonstrate a significant serum response to S-antigen or ROS in the ELISA assays. The rats employed for the hybridoma studies were not those utilised for the serum studies (Section 4.1), although the animals were obtained from the same breeding stocks.

The splenic lymphocytes of four Hunter RCS, three Campbell RCS rats and three PVG control rats were fused with the rat myeloma cell line Y3.Ag1.2.3 (Galfre et al., 1979) (Section 2.1.18), and the cells seeded into four 24-well plates per fusion (Section 2.6.6.3). Of the 96 wells available, 4 wells were reserved for the unfused Y3.Ag1.2.3 and spleen cell controls. Thus, 92 wells for each fusion were seeded with the cells, and of these wells 0 to over 90%, per experiment, yielded hybrids after 2 to 3 weeks. Generally, better fusion frequencies were obtained with Y3 cells, grown in a spinner flask, than those grown in plastic flasks (Section 2.1.19). It was

also important not to allow the cells to become crowded, or the medium to become too acidic, in the few days prior to fusion.

5.2.3 Strategy for the Assay of Fusions

The emerging hybrids were fed weekly, and the supernatants were assayed when the cells had became approximately 80% confluent. The hybrids were assayed in duplicate. The fusions were screened against solubilised bovine ROS and S-antigen on ELISA plates coated with the same concentrations of antigen as described for the serum study (Section 4.1.2.2). The ELISA plates were incubated with the cell culture supernatants of the primary fusion wells, for 4 hours at room temperature or overnight at 4°C. It has been suggested that a longer incubation period may be necessary for antibodies of lower affinity, or when the antibody is present in very small quantities (Mason and Williams, 1980).

To avoid the detection of false positive clones, it was necessary to change the cell culture supernatants of the fusion at least twice before assay. Experience with immunised rats has shown that the residual unfused spleen cells are capable of secreting antibody in culture for several days. Primary growths which were negative, were reassayed until the wells became overgrown. Those wells remaining negative at this stage were abandoned.

5.2.4 Choice of ELISA Categories

The ELISA reactions were categorised in order to clarify the results and facilitate a comparison between fusions. The categories were chosen to cover a range of 0.2 absorbance units at 492nm (A492). This range was selected in order to allow for the element of variability present when assaying cell culture supernatants.

Variability is inevitable, since it is impossible to standardise the system, such that each primary culture has only one cell line, i.e. monoclonal. Each potential cell line will grow and secrete antibody at a different rate. Furthermore, the ELISA readings for different cells may be affected, to different extents, by dilution of the antibody when the cells are fed. Even a monoclonal cell line can produce an ELISA reading of 1.0 A492 on one day and 1.4 A492 on the following day. Thus, re-assay in addition to assay in duplicate, is important.

The ELISA plates utilised for the screening of fusions were coated with the same stocks of S-antigen and ROS, and treated under identical conditions (Section 3.8). Although positive and negative reaction controls were included on the ELISA plates, variability between plates was not assessed by means of titration of the controls, as in the rat serum studies (Section 4.1.2.2). The reproducibility of the ELISA assay systems has been described (Sections 3.8.5 and 3.8.6) and, although minor variation between plates is inevitable, the choice of ELISA categories described below were considered to allow for this.

5.2.5 The ELISA Results

The ELISA absorbance readings at 492nm obtained for the primary hybrid cultures to both antigens, are tabulated (Tables 5.1 to 5.8). The data is presented in terms of the number of primary hybrid growths falling into the categories of absorbance at 492nm. The readings obtained from confluent primary hybridomas are recorded as the mean of two assays (Tables 5.1 to 5.8). The results are graphically displayed in the form of histograms (Figures 5.1 to 5.8).

TABLE 5.1 ELISA readings of confluent primary hybridoma clones from the fusion for rat Hunter 1

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.00-0.20	60	67.4	84	94.4
2	0.21-0.40	19	21.3	4	4.5
3	0.41-0.60	5	5.6	1	1.1
4	0.61-0.80	2	2.25	0	0
5	0.81-1.00	1	1.1	0	0
6	1.01-1.20	0	0	0	0
7	1.21-0.40	0	0	0	0
8	1.41-1.60	1	1.1	0	0
9	1.61-1.80	1	1.1	0	0
	TOTAL	89	100.00	89	100.00

TABLE 5.2 ELISA readings of confluent primary hybridoma clones from the fusion for rat Hunter 2

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.00-0.20	47	78.33	53	88.33
2	0.21-0.40	11	18.33	7	11.67
3	0.41-0.60	1	1.67	0	0
4	0.61-0.80	1	1.67	0	0
	TOTAL	60	100.00	60	100.00

TABLE 5.3 ELISA readings of confluent primary hybridoma clones from the fusion for rat Hunter 3

Category	A492 Grouping	S-antigen NO. Wells	%	ROS No. Wells	%
1	0.00-0.20	24	77.4	26	83.9
2	0.21-0.40	6	19.4	5	16.1
3	0.41-0.60	1	3.2	0	0
	TOTAL	31	100.0	31	100.0

TABLE 5.4 ELISA readings of confluent primary hybridoma clones from the fusion for rat Hunter 4

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.00-0.20	36	97.3	36	97.3
2	0.21-0.40	1	2.7	1	2.7
	TOTAL	37	100.0	37	100.0

TABLE 5.5 ELISA readings of confluent primary hybridoma clones from the fusion for rat Campbell 1

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.00-0.20	23	71.9	22	68.75
2	0.21-0.40	8	25.0	8	25.0
3	0.41-0.60	1	3.1	2	6.25
	TOTAL	32	100.0	32	100.0

TABLE 5.6 ELISA readings of confluent primary hybridoma clones from the fusion for rat PVG 1

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.00-0.20	28	93.3	30	100.0
2	0.21-0.40	2	6.7	0	0.0
	TOTAL	30	100.0	30	100.0

TABLE 5.7 ELISA readings of confluent primary hybridoma clones from the fusion for rat PVG 2

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.00-0.20	10	83.3	8	66.7
2	0.21-0.40	2	16.7	4	33.3
	TOTAL	12	100.0	12	100.0

TABLE 5.8 ELISA readings of confluent primary hybridoma clones from the fusion for rat PVG 3

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.00-0.20	19	100.0	19	100.0
	TOTAL	19	100.0	19	100.0

TABLE 5.9 Pooled ELISA readings of confluent primary hybridoma clones from fusions for five RCS and three PVG rats

a) RCS Rats

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.00-0.20	190	76.3	221	88.8
2	0.21-0.40	45	18.1	25	10.0
3	0.41-0.60	8	3.2	3	1.2
4	0.61-0.80	3	1.2	0	0
5	0.81-1.00	1	0.4	0	0
6	1.01-1.20	0	0	0	0
7	1.21-1.40	0	0	0	0
8	1.41-1.60	1	0.4	0	0
9	1.61-1.80	1	0.4	0	0
	TOTAL	249	100.0	249	100.0

b) PVG Rats

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.00-0.20	57	93.4	57	93.4
2	0.21-0.40	4	6.6	4	6.6
	TOTAL	61	100.0	61	100.0

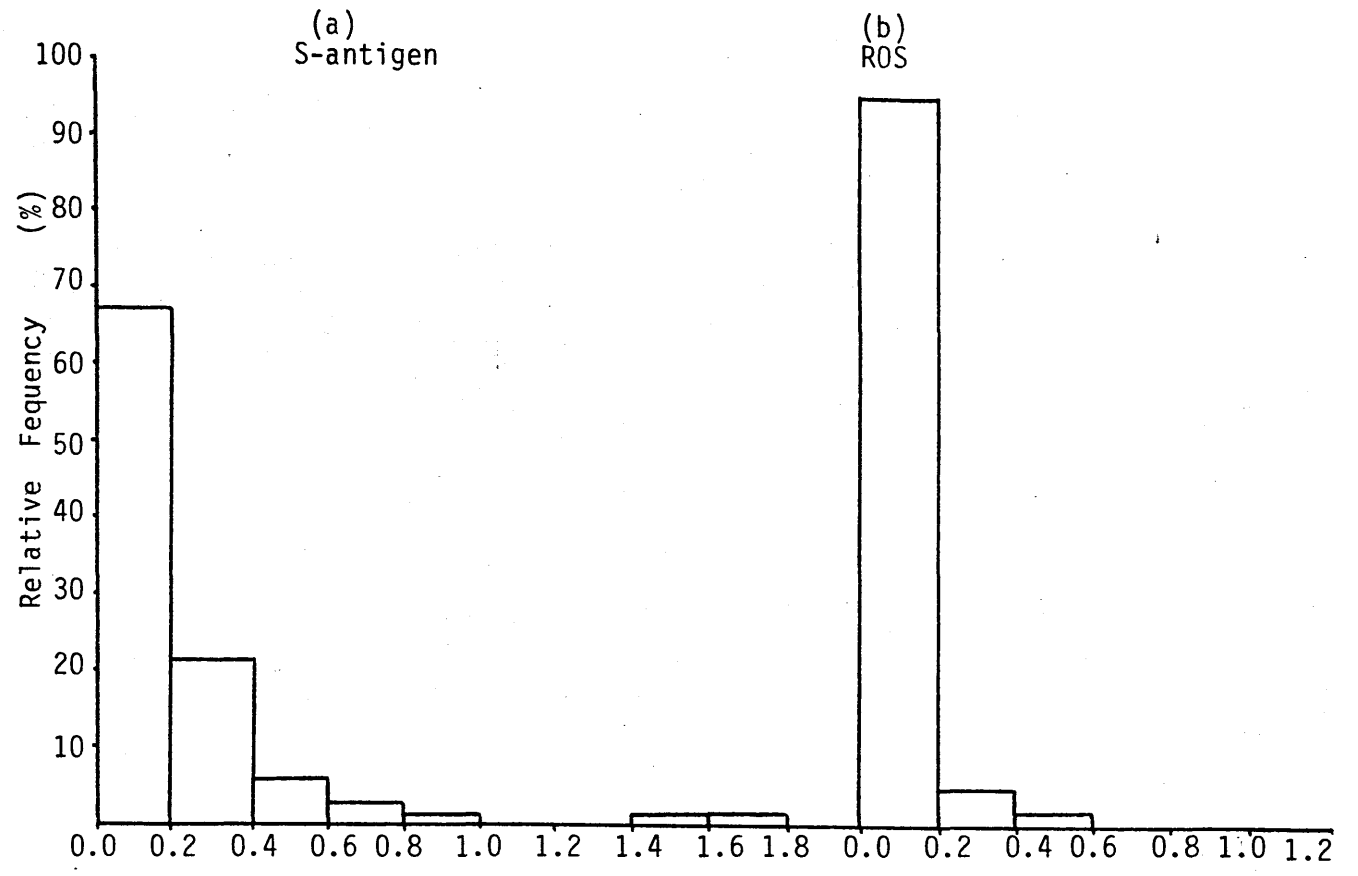
Figure 5.1 ELISA Assay of Clones from Fusion with Hunter Rat 1

The primary hybridoma clones generated from the fusion of RCS rat splenic lymphocytes, with cells of the myeloma Y3.Ag1.2.3 line, were assayed by ELISA.

The histograms show the relative frequencies of confluent primary clones, falling into defined categories of absorbance at 492nm (A492), after assay of the supernatants on ELISA plates coated with (a) 1µg/ml S-antigen and (b) 5µg/ml ROS.

N = number of primary clones assayed

Figure 5.2 ELISA Assay of Clones from Fusion with Hunter Rat 2



Hunter RCS Rat 2

N=60

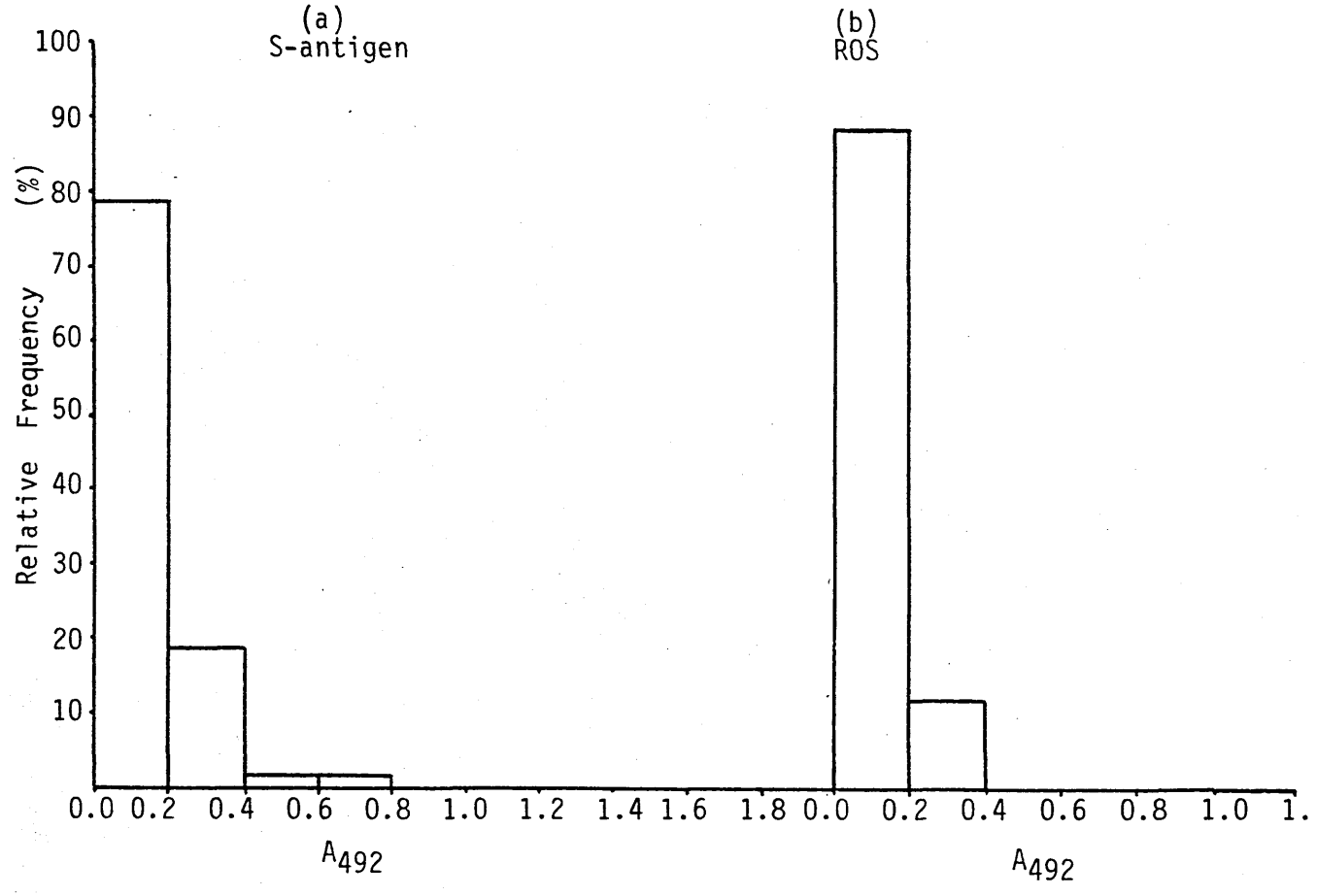


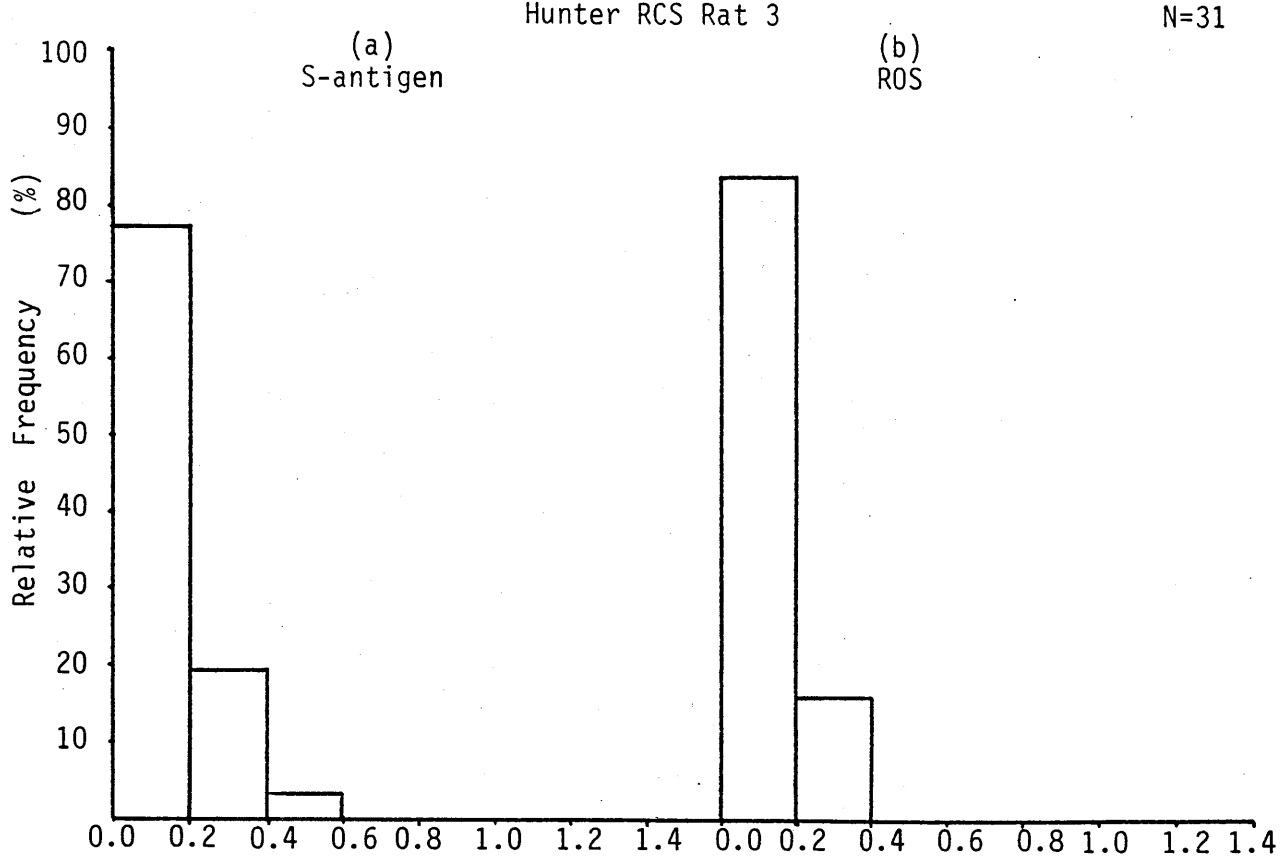
Figure 5.3 ELISA Assay of Clones from Fusion with Hunter Rat 3

The primary hybridoma clones generated from the fusion of RCS rat splenic lymphocytes, with cells of the myeloma Y3.Ag1.2.3 line, were assayed by ELISA.

The histograms show the relative frequencies of confluent primary clones, falling into defined categories of absorbance at 492nm (A492), after assay of the supernatants on ELISA plates coated with (a) 1µg/ml S-antigen and (b) 5µg/ml ROS.

N = number of primary clones assayed

Figure 5.4 ELISA Assay of Clones from Fusion with Hunter Rat 4


Hunter RCS Rat 3
N=31
(a)
S-antigen
(b)
ROS
Relative Frequency (%)
100
90
80
70
60
50
40
30
20
10
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4

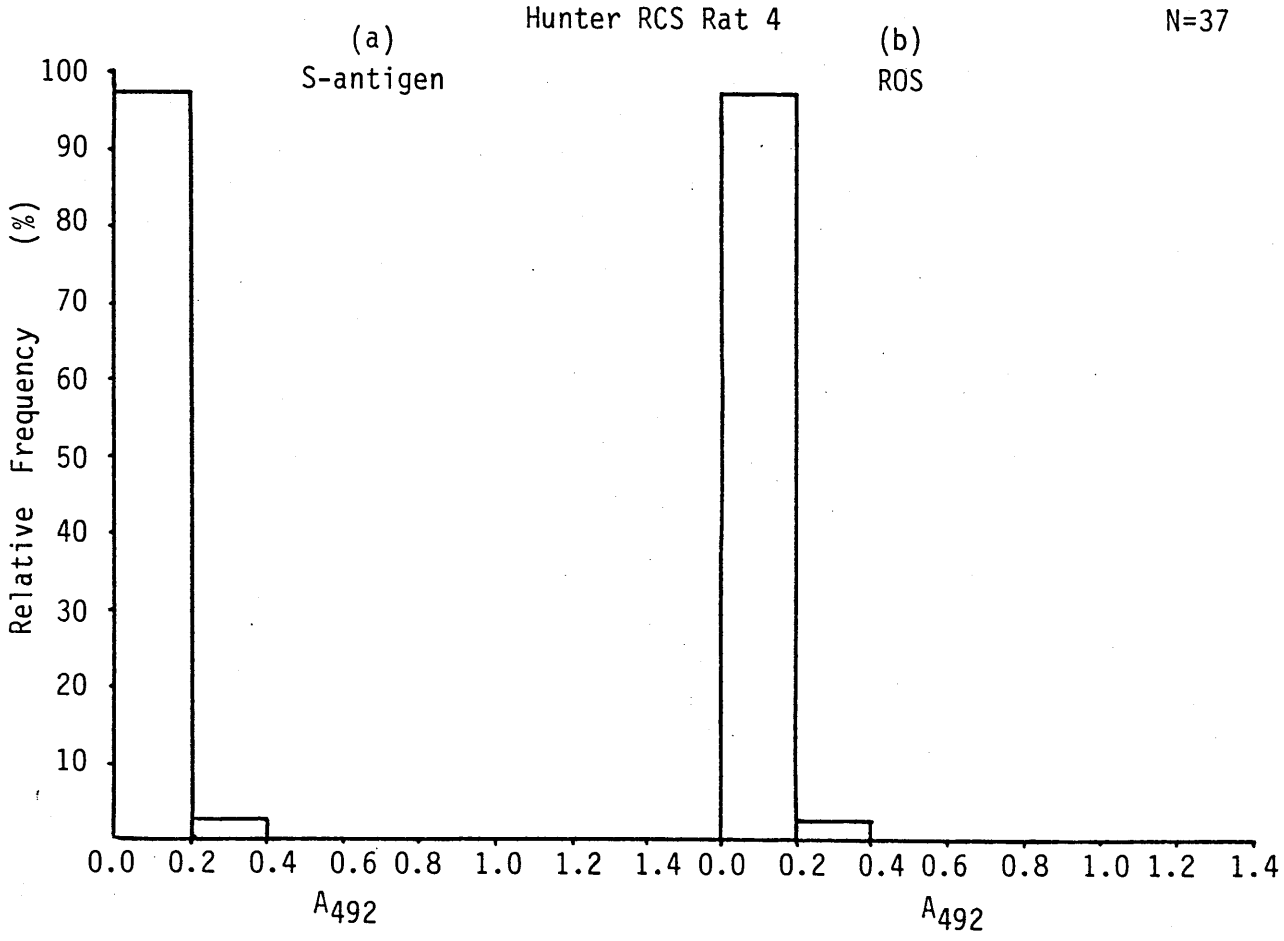

Hunter RCS Rat 4
N=37
(a)
S-antigen
(b)
ROS
Relative Frequency (%)
100
90
80
70
60
50
40
30
20
10
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
A492
A492

Figure 5.5 ELISA Assay of Clones from Fusion with Campbell Rat 1

The primary hybridoma clones generated from the fusion of rat splenic lymphocytes, with cells of the myeloma Y3.Ag1.2.3 line, were assayed by ELISA.

The histograms show the relative frequencies of confluent primary clones, falling into defined categories of absorbance at 492nm (A492), after assay of the supernatants on ELISA plates coated with (a) 1µg/ml S-antigen and (b) 5µg/ml ROS.

N = number of primary clones assayed

Figure 5.6 ELISA Assay of Clones from Fusion with PVG Rat 1

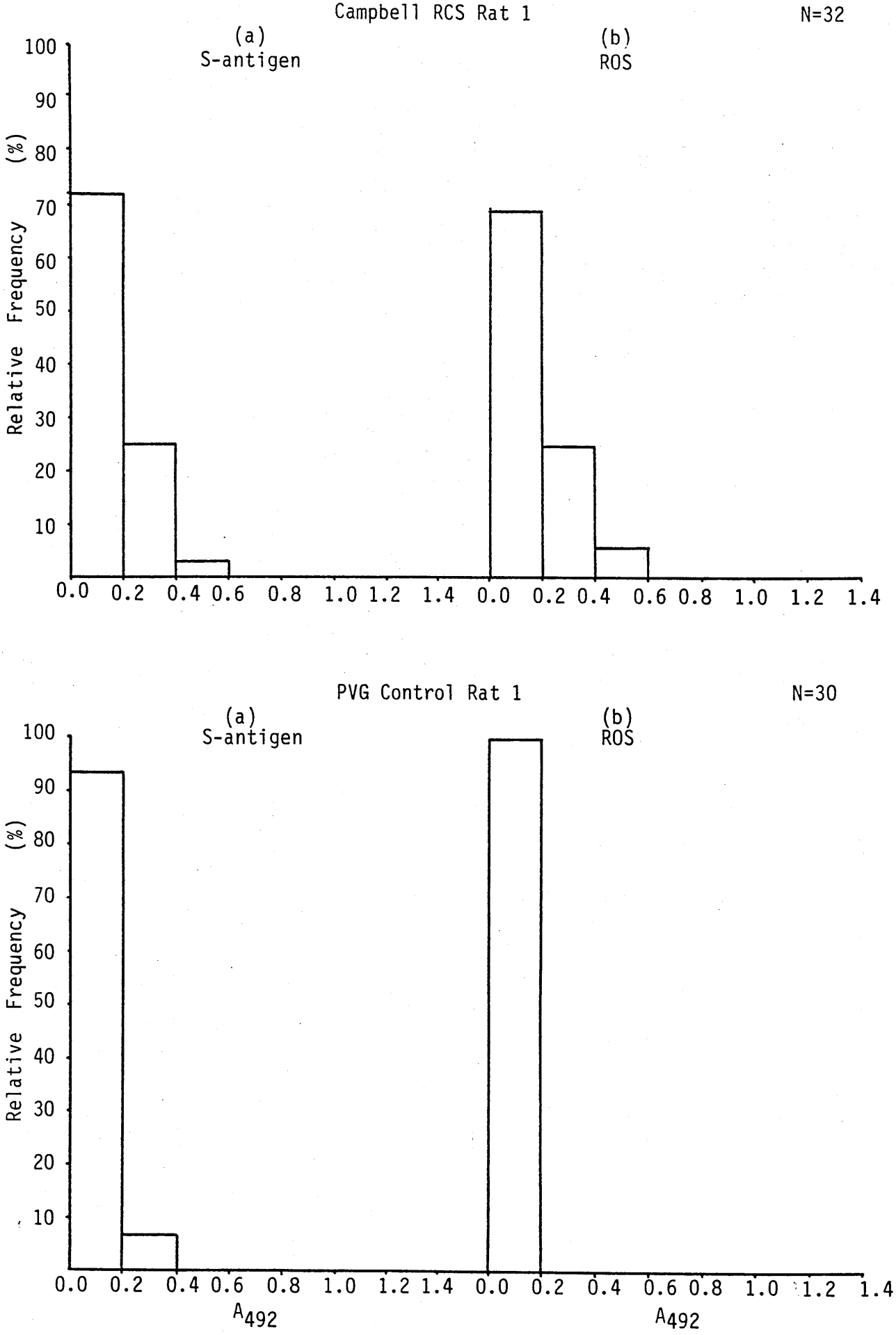

Campbell RCS Rat 1
N=32
(a)
S-antigen
(b)
ROS
Relative Frequency (%)
100
90
80
70
60
50
40
30
20
10
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
PVG Control Rat 1
N=30
(a)
S-antigen
(b)
ROS
Relative Frequency (%)
100
90
80
70
60
50
40
30
20
10
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
A492
A492

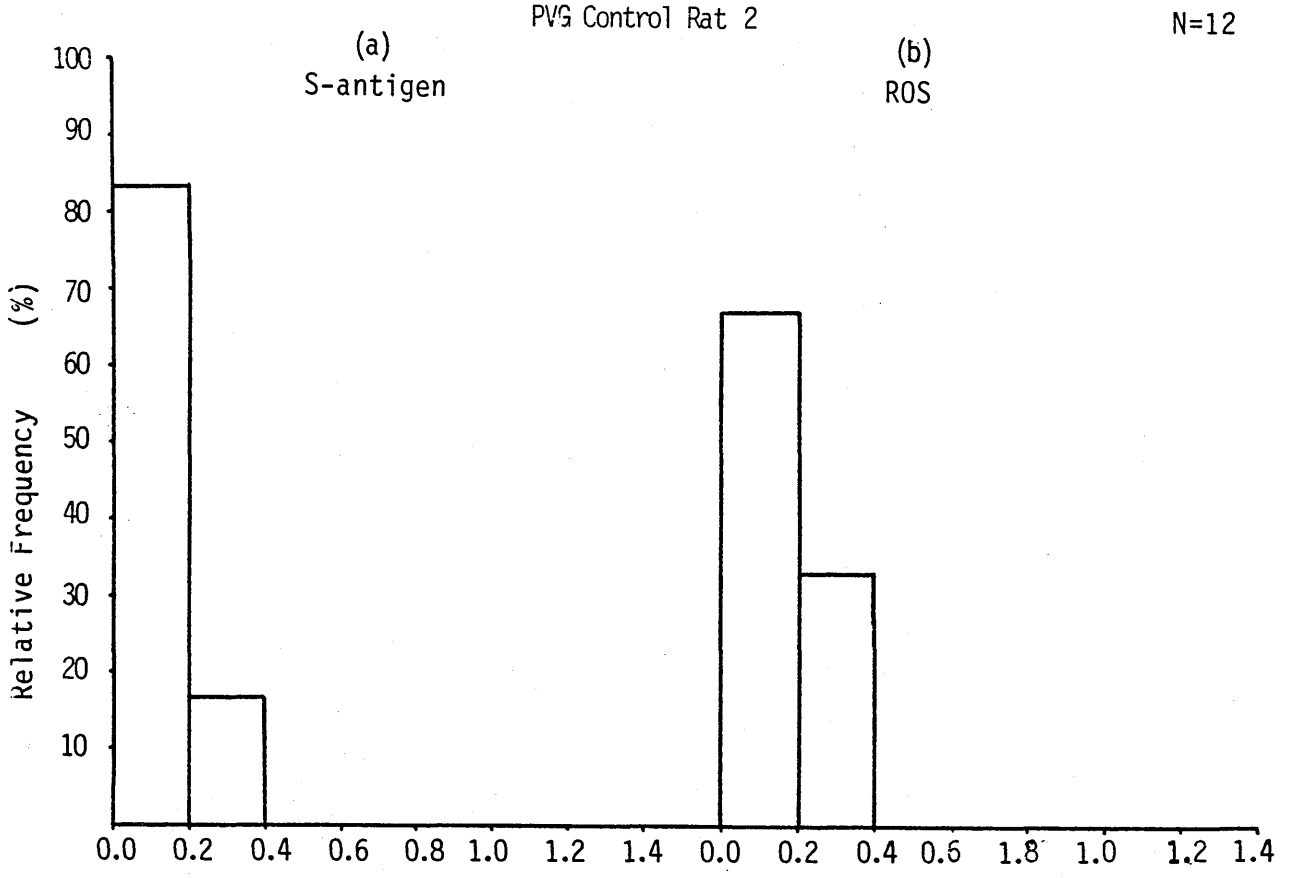
Figure 5.7 ELISA Assay of Clones from Fusion with PVG Rat 2

The primary hybridoma clones generated from the fusion of PVG rat splenic lymphocytes, with cells of the myeloma Y3.Ag1.2.3 line, were assayed by ELISA.

The histograms show the relative frequencies of confluent primary clones, falling into defined categories of absorbance at 492nm (A492), after assay of the supernatants on ELISA plates coated with (a) 1µg/ml S-antigen and (b) 5µg/ml ROS.

N = number of primary clones assayed

Figure 5.8 ELISA Assay of Clones from Fusion with PVG Rat 3


PVG Control Rat 2
N=12
(a)
S-antigen
(b)
ROS
Relative Frequency (%)
100
90
80
70
60
50
40
30
20
10
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 0.0 0.2 0.4 0.6 1.8 1.0 1.2 1.4

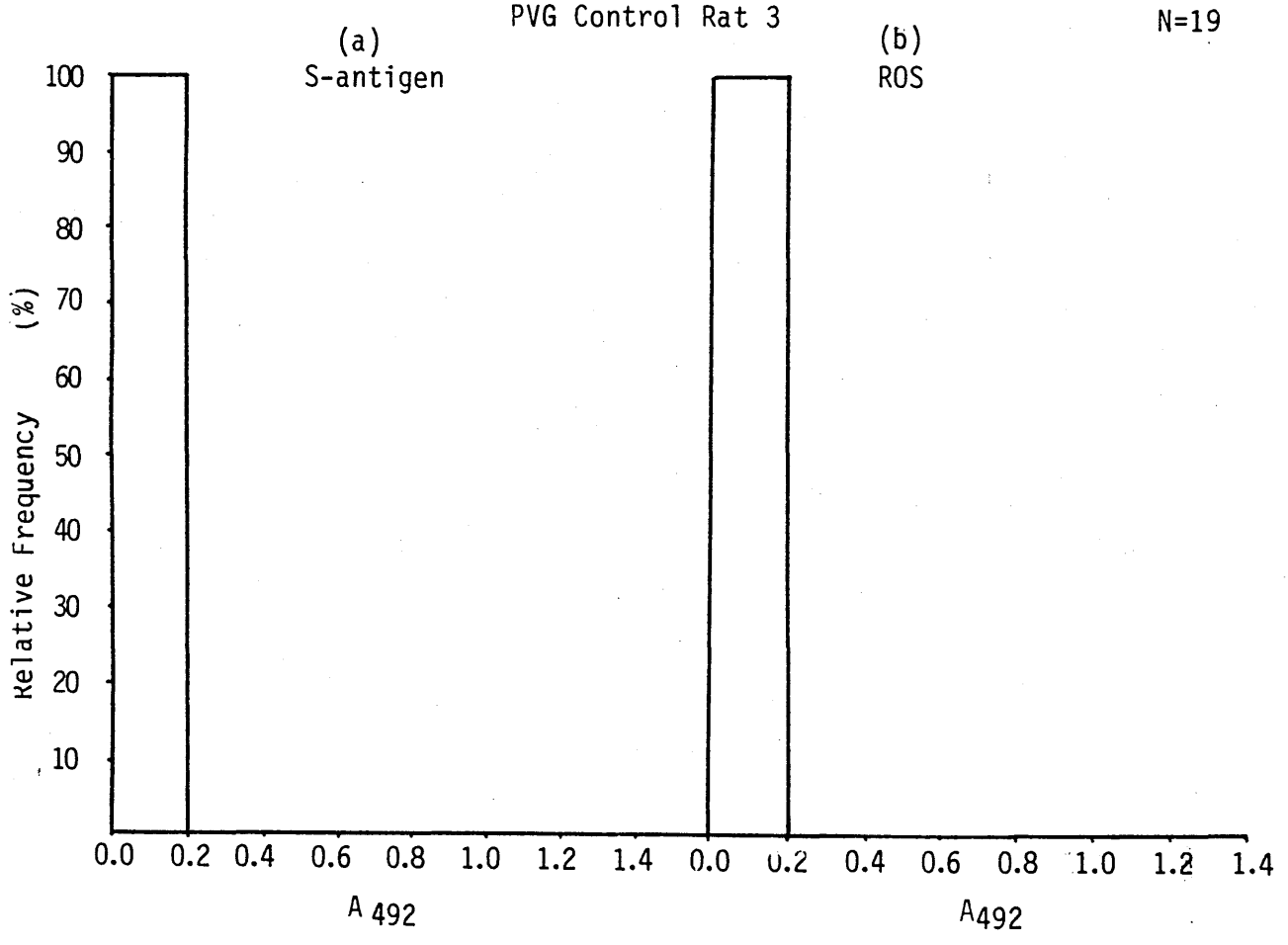

PVG Control Rat 3
N=19
(a)
S-antigen
(b)
ROS
Relative Frequency (%)
100
90
80
70
60
50
40
30
20
10
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
A 492
A492

Figure 5.9 Pooled Results from the Assay of primary Clones derived from Fusions with RCS and PVG Rats

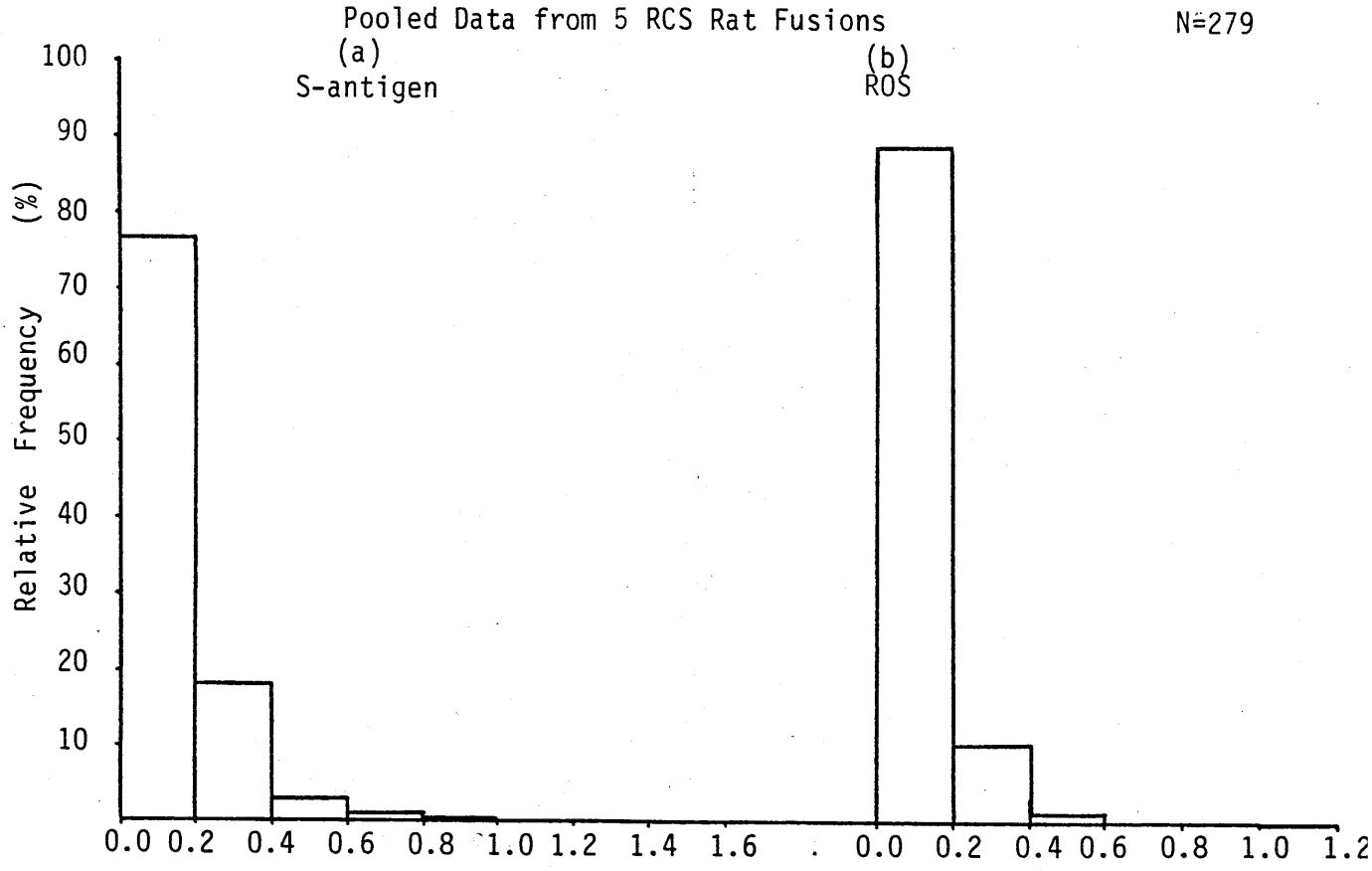
a) Pooled data for RCS rats

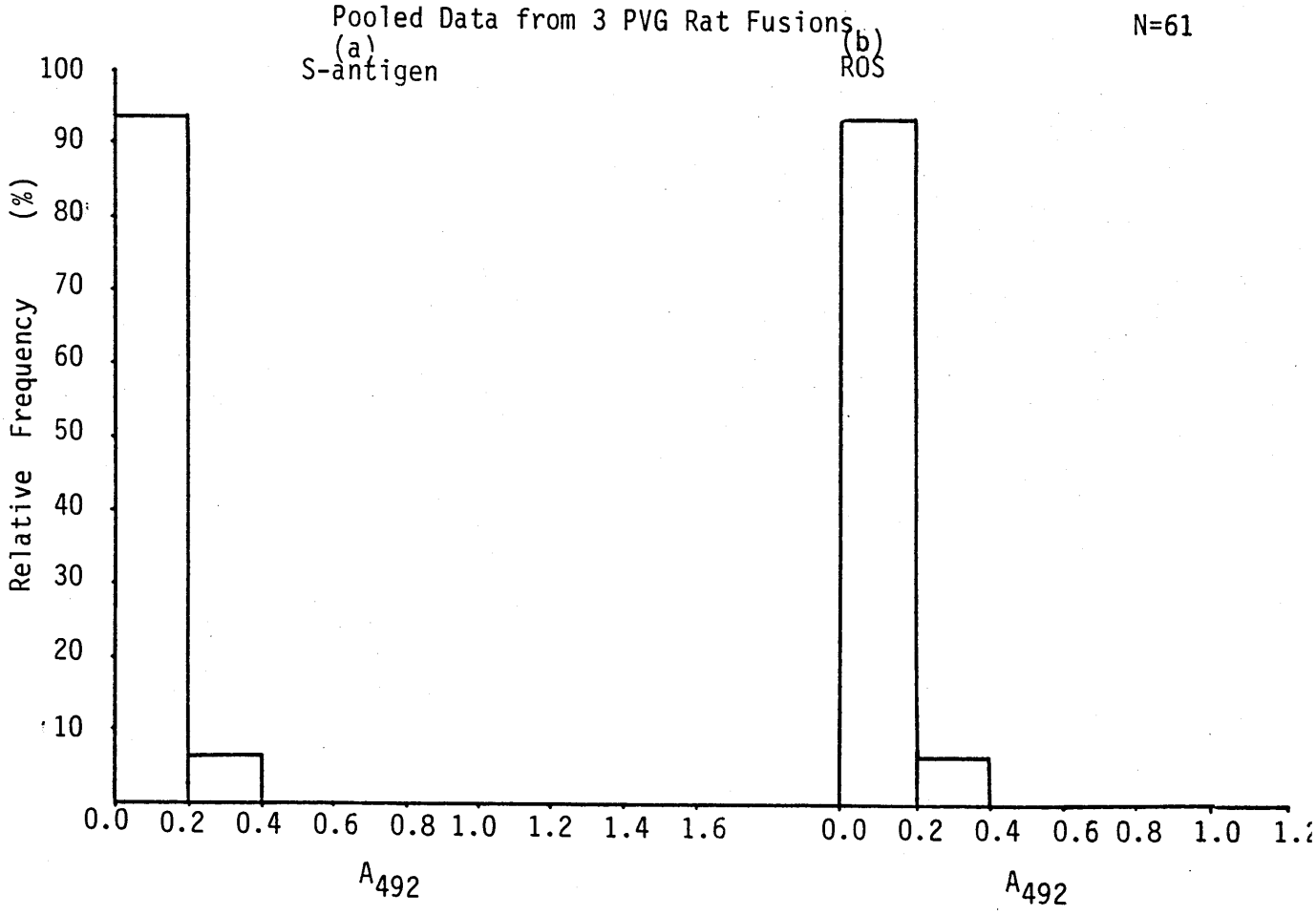
b) Pooled data for PVG rats

The primary hybridoma clones generated from fusions of rat splenic lymphocytes, with cells of the myeloma Y3.Ag1.2.3 line, were assayed in ELISA plates coated with (a) 1μg/ml S-antigen and (b) 5μg/ml ROS.

The histograms show the relative frequencies of confluent primary clones, obtained from five individual fusions, falling into defined categories of absorbance at 492nm (A492), after assay of the supernatants by ELISA.

N = number of primary clones assayed


Pooled Data from 5 RCS Rat Fusions
N=279
(a)
S-antigen
(b)
ROS
Relative Frequency (%)
100
90
80
70
60
50
40
30
20
10
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6
0.0 0.2 0.4 0.6 0.8 1.0 1.2


Pooled Data from 3 PVG Rat Fusions
N=61
(a)
S-antigen
(b)
ROS
Relative Frequency (%)
100
90
80
70
60
50
40
30
20
10
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6
0.0 0.2 0.4 0.6 0.8 1.0 1.2
A492
A492

Negative controls, in the form of supernatants of the unfused Y3 or spleen cells, gave readings between 0.05 and 0.09 A492 in the ELISA assays. Readings below 0.4 A492, may reflect the binding of low affinity crossreacting antibody. In fusions derived from an immunised animal, a reading of 0.4 A492 was not considered to be significantly positive. Readings above 0.4 A492, were considered to indicate a positive response in fusions derived from potentially autoimmune animals.

Fourteen primary clones, positive to S-antigen, were detected in four out of five fusions with the RCS rats (Figures 5.1 to 5.5). No such positives were obtained from the fusions with the healthy PVG control rats (Figures 5.6 to 5.8). The fusion designated H1 was responsible for 10 of these positive primary clones, and generated three positive clones with readings above 0.8 A492 (Figure 5.1). Responses of this magnitude would be considered positive, even in a fusion from a hyperimmunised animal.

Primary clones, indicating positive reponses to the ROS antigen, were detected in only two of the five RCS rat fusions. Two of these clones were from a fusion with a Campbell rat (Figure 5.5). A third clone, positive to ROS, was derived from the Hunter rat, H1 (Figure 5.1). The positive reactions were not of a very high magnitude, falling within Category 3 (0.4 to 0.6 A492). No positives were obtained from the PVG control rats (Figures 5.6 to 5.8).

5.2.6 Established Monoclonal Autoantibodies

Three primary clones, derived from fusions with the splenic lymphocytes of RCS rats, were expanded and stabilised by

subcloning, to generate monoclonal antibodies (Kohler and Milstein, 1975) (Section 2.6.9). Two monoclonal autoantibodies were derived from the fusion with a nine week old animal designated H1 (Figure 5.1). These clones were selected because their supernatants gave the strongest reaction in the ELISA, and the cells grew actively in flasks. The third monoclonal antibody was obtained from a rat sacrificed at the age of 10 weeks (Rat H2) (Figure 5.2), and was selected for its interesting cross reactivity with the S-antigen and ROS antigens in the ELISA, as well as its good growth rate. The data generated for these monoclonal antibodies is presented in Section 6.3.

The considerable cost involved in the stabilisation of monoclonal antibodies, and the limitation of time, prevented more of the clones from being studied further at this time, and several potentially interesting primary clones remain stored in liquid nitrogen (Section 2.6.8).

5.2.7 Discussion

The reactivities to retinal antigens in ELISA of the primary clones of hybridomas, obtained from fusions with dystrophic RCS rats and PVG controls, are summarised in Table 5.9. Fourteen primary growths positive to S-antigen were detected in four RCS rats (fusions with Hunter Rats 1, 2 and 3, and Campbell Rat 1) and only three clones reactive against ROS were detected, generated in fusions from Hunter 1 (H1) and Campbell 1 (C1).

The total number of primary fusion wells with growth is an important consideration, when comparing the results obtained from the RCS and control fusions. Fusion H1, which generated three

positive potential hybridoma cell lines, reactive with S-antigen in the ELISA above 0.8 A492, had yielded a total of 89 wells with primary growth (Table 5.1). One of the control fusions produced hybridomas in only 12 wells (Table 5.7). Clearly, the total number of wells producing hybrids will affect the chances of detecting those cells secreting the antibody of interest.

There appears to have been an interesting difference in the number of positive clones detected against the two antigens. The apparent 'dominance' of S-antigen in the anti-retinal response is also reflected in the rat serum studies (Section 4.1.3). The potential anti-retinal autoimmune reponses apparent in some RCS rats, and the possible dominance of S-antigen in the stimulation, are discussed in Sections 4.1.9 and 8.4).

The RCS rats, used in the fusion study, did not demonstrate a serum response in the ELISA to S-antigen or ROS significantly above the healthy control PVG and DA rats. Perhaps, an RCS rat demonstrating a significantly positive titre to either antigen would yield a greater frequency of positive clones, although experience here with immunised DA rats suggested that the detection of the positive clones need not reflect the serum titre. Immunisation, however, increases the frequency of high affinity and high specificity antibodies, and also increases the chances of generating IgG antibodies, by the matration of the immune response (see Roitt et al., 1985).

Fusions with splenic lymphocytes are thought to involve the actively dividing plasma cells. This may explain why the fusion frequencies were relatively poor with some of the rats, notably the

PVG controls. This might also indicate that the RCS rats, in which positive clones were identified (Section 5.2.5), were in the process of developing a serum response to the retinal antigens (Section 8.4.5).

Thus, primary clones positive to S-antigen could be detected in some dystrophic rats, although the possibility remains that positives could be detected in healthy animals, if enough fusions were undertaken. Immortalised, fully cloned rat hybridomas, secreting unlimited amounts of monoclonal anti-retinal autoantibody (Chapter 6), may aid the identification and characterisation of relevant autoantigens.

5.3 TEMPORARY STABILISATION OF HUMAN B LYMPHOCYTES

5.3.1 Rationale to the Transformation Study

The following section describes the in vitro responses of human B lymphocytes, transformed by the Epstein-Barr virus (EBV), to bovine retinal antigens in ELISA assays. The transformation methodology was similar to that of Kozbor and Roder (1981, 1983). Fusions with a human lymphoblastoid cell line (KR-4) were not made with the freshly isolated peripheral blood lymphocytes, since there would be only a very small number of circulating B cells with the required specificity, available for fusion. The transformation procedure allowed expansion of the number of antigen specific B cells, which could then be fused. In addition, the frequency of fusion between B cells and the KR-4 cells is approximately 10^{-6}. Thus it would be more difficult to obtain a clone of the required specificity from a fusion with non-transformed (and non-expanded B cells). EBV is generally considered to be a polyclonal activator of B lymphocytes (Rosen et al., 1977) and also considerably lengthens their in vitro life span (Miller and Lipton, 1973). In this way, transformation experiments can facilitate the investigation of human circulating B lymphocyte specificity.

This pilot study involved a relatively small number of human subjects, and may form a basis for future work.

5.3.2 Human Subjects

Uveitis and RP patients were selected for the transformation studies, since there is evidence that patients of certain sub-groups, are capable of responding immunologically to retinal antigens (Section 1.4). Five RP patients (three autosomal dominant

and two advanced simplex) and three uveitis patients (with associated vasculitis) were employed (Table 4.32). Five healthy volunteers, with no known eye disease, were used as controls for the study (Table 4.32). The sera from all of the people involved in the ELISA study were screened against S-antigen and ROS (Section 4.2). Not all of the people involved in the serum study (Section 4.2) were employed in the transformation experiments.

5.3.3 Choice of Transformation Protocol

It is generally considered preferable to remove or suppress the T cell activity of in vitro cultured EBV transformed B lymphocytes, since the cytotoxic T lymphocytes are capable of anti-viral activity, especially when the donor has been exposed to the EB virus (90% of the adult population) (Campbell, 1984). Even if clinical mononucleosis has failed to develop, the immune system is capable of reacting against the virus.

There are two principal experimental strategies for the T cell problem. Firstly, the T cells may be removed by rosetting with AET-treated sheep red blood cells, and separated from the B cells on a Percoll gradient (Kaplan and Clark, 1974) (Section 2.7.3.3). Alternatively, the activity of the T cells can be inhibited by the selective action of the drug, Cyclosporin A (CsA) (Powles et al., 1980; reviewed by Shevach, 1985) (Section 2.7.5). CsA has been found to markedly facilitate the growth of EBV transformed cells derived form EBV immune donors (Bird et al., 1981). These two procedures were compared in some of the transformation experiments, where the blood samples were divided into three portions. The T cells of one portion were removed by the rosetting method, and those of the second suppressed by CsA. The remaining cells received

neither treatment. Thus the relative merits of the two approaches to the solution of the potential T cell problem could be assessed with respect to their effect on the growth of the transformed cells and the activity of the transformants in terms of the number of positives detected (Section 5.3.9).

The transformed cells were seeded into 96-well plates (Section 2.7.4). Generally the inside 60 wells of one 96-well plate, were used per 20ml blood sample. The rat study employed 24-well plates (Section 5.2.2). The frequency of cell transformation was always very much higher than the frequency of fusion obtained with the rat hybridoma system. Thus the use of 96-well plates determined that there were fewer clones of transformed cells per cell culture well than there would be if 24-well plates were used. This is an important consideration in the human transformation, and human and mouse hybridoma systems, where the cells may cease to secrete antibody or become overgrown by cells which do not secrete the antibody of interest (Campbell, 1984).

5.3.4 Growth of primary Transformed Cells and Strategy for Assay

Transformed cells could be detected in the 96-well culture plates 18 hours after infection with the EBV. Generally, many human (and sometimes sheep) red blood cells (RBCs) were present among the cells, due to imperfect separation on the Ficoll and Percoll gradients (Sections 2.7.2 and 2.7.3.3). The wells were usually clear of the RBCs after a few days, and the transformed cells were more clearly visible. The transformed cells were gregarious nature tending to form clusters (Figure 2.3).

Three weeks after transformation, the wells contained numerous

transformed cells (transformants), although the number in each well varied. The cells were usually ready for assay at this time. The plates were screened on ELISA plates coated with S-antigen or ROS at 1µg and 5µg/ml, respectively. The small amount of supernatant within each well, did not permit the plates to be assayed in duplicate in addition to assay against the two antigens. Only approximately 150µl was available for assay against the two antigens. Sometimes, if two plates of transformed cells were obtained from one patient, one plate was assayed against S-antigen and the other plate assayed against ROS. A few days later, the plates were assayed against the alternative antigen.

Due to the variation in growth between wells, it was often necessary to re-assay the plates. Similar problems of standardisation of the assaying was encountered in the transformation system, to those in the rat hybridoma system (Section 5.2.4). If no further positive wells could be identified at the second assay, at 4 weeks, the plates were discarded. Those wells in which clones had not developed by this time, were not likely to become sufficiently established in flasks, even if they were positive.

5.3.5 Choice of ELISA Categories

Smaller categories of ELISA data were selected for the human transformation system, compared with the rat hybridoma system (Section 5.2.4). The reason for this was that the human transformed lymphocytes secrete 10 to 100 fold less antibody than do rodent hybridomas (Kozbor and Roder, 1983).

In comparison of ELISA results obtained from transformations

performed at intervals of several months, there were the inevitable minor variations due to the use of different batches of microtitre plates and rabbit anti-human peroxidase conjugate. Such variations were unimportant in the screening of fusions from hyperimmunised rats, since strongly positive ELISA readings were expected. The readings obtained from fusions with autoimmune rats or from transformations of human B lymphocytes, were not expected to be so high.

ELISA readings of below 0.2 A492 were considered to be insignificant, representing non-specific reactions of the primary antibody, or of the conjugated second antibody. Readings below 0.3 A492 might also fall into this category. Reactions above 0.3 A492 were considered to be significantly positive in the human transformation system. The ELISA readings were categorised in intervals of 0.1 A492 units, from 0.2 A492. The ELISA results obtained from the assay of the transformations are presented in Tables 5.10 to 5.17. The results are presented in terms of the number (and percentage) of wells generating ELISA readings, falling into the defined categories of A492 values. The results are graphically displayed in the form of histograms (Figures 5.10 to 5.17).

5.3.6 ELISA Reactions of primary Transformed B Lymphocytes from Control Subjects

The results obtained from the assay of primary growths of control B lymphocytes are given in Tables 5.10a,b,c to 5.11a,b, and Figures 5.10a,b,c to 5.11a,b. None of the control sera gave significant responses in the ELISA assays against S-antigen or ROS (Figures 4.9a,b and 4.12a.b). Only one potentially positive primary clone

was identified in the five control transformations, giving a value between 0.3 and 0.4 A492, in the ELISA against S-antigen (Figure 5.10). This clone, however, was negative upon reassay after 6 days. It is possible that this apparently weak positive result represented a false reaction, since the assays could not be made in duplicate (Section 5.3.4). Spurious reactions were occasionally observed in the ELISA assays where rabbit anti-human peroxidase conjugates were involved. Such phenomena were rare in the rat system. These false, weak, positive reactions might be a property of the rabbit anti-human conjugates, since they have been observed where complete medium or RPMI was used in place of the cell culture supernatant.

Transformations derived from control subjects, JF and KM were users of S-antigen, and generated a higher proportion of primary clones, falling within the second Category (A492 0.2 to 0.3) (Tables 5.11a,b and Figures 5.11a,b), compared with the other contol transformations. This is not likely to be significant, and probably reflects variations in the batch of conjugate, since the transformations of JF and KM were performed at the same time, and those of the others were performed several months later.

5.3.7 ELISA of primary Transformed B Lymphocytes from RP Patients

B cell specificity to S-antigen and ROS were investigated in five RP patients. Patients IM and KM were father and son, with the autosomal dominant form of the disease. No positive reactions were detected in the transformation derived from patient IM (Table 5.12a and Figure 5.12a). The transformation derived from patient KM, however, yielded 2 primary clones reactive in the ELISA assays against both S-antigen and ROS, giving readings between 0.3 and 0.4

TABLE 5.10a ELISA readings of the three week old CsA treated transformants from control subject BD

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.21	81	95.3	85	100.0
2	0.21-0.30	4	4.7	0	0
	TOTAL	85	100.0	85	100.0

TABLE 5.10b* ELISA readings of the three week old CsA treated transformants from control subject HD

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.21	56	93.3	60	100.0
2	0.21-0.30	3	5.0	0	0
3	0.31-0.40	1	1.7	0	0
	TOTAL	60	100.0	60	100.0

* Assays repeated 1 week later yielded readings in category 1 only.

TABLE 5.10c ELISA readings of the three week old transformants from control subject OM

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	64	100	58	90.6
2	0.21-0.30	0	0	6	9.4
	TOTAL	64	100.0	64	100.0

TABLE 5.11a ELISA readings of the three week old transformants from control subject JV: user of S-antigen

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	54	90.0	46	76.7
2	0.21-0.30	6	10.0	14	23.3
	TOTAL	60	100.0	60	100.0

TABLE 5.11b ELISA readings of the three week old transformants from control subject KM: user of S-antigen

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	54	90.0	44	73.3
2	0.21-0.30	6	10.0	16	26.7
	TOTAL	60	100.0	60	100.0

TABLE 5.12a ELISA readings of the three week old transformants from RP Patient IM

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	69	98.6	70	100.0
2	0.21-0.30	1	1.4	0	0
	TOTAL	70	100.0	70	100.0

TABLE 5.12b ELISA readings of the three week old transformants from RP patient KM

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	78	97.5	76	95.0
2	0.21-0.30	0	0	2	2.5
3	0.31-0.40	2*	2.5	2*	2.5
	TOTAL	80	100.0	80	100.0

* Two wells were positive to both S-antigen and ROS

TABLE 5.13a ELISA readings of the three week old transformants from RP patient AA

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	49	81.6	45	75.0
2	0.21-0.30	7	11.7	7	11.7
3	0.31-0.40	2	3.3	2	3.3
4	0.41-0.50	0	0	2	3.3
5	0.51-0.60	1	1.7	3+	5
6	0.61-0.70	0	0	0	0
7	0.71-0.80	1*	1.7	0	0
8-9	0.81-1.00	0	0	0	0
10	1.01-1.10	0	0	1**	1.7
	TOTAL	60	100.0	60	100.0

TABLE 5.13b ELISA readings of the four week old transformants from RP patient AA

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	50	83.3	45	75.0
2	0.21-0.30	4	6.7	5	8.3
3	0.31-0.40	4	6.7	6	10.0
4	0.41-0.50	0	0	2	3.3
5	0.51-0.60	0	0	0	0
6	0.61-0.70	0	0	1+	1.7
7	0.71-0.80	0	0	0	0
8	0.81-0.90	2*	3.3	0	0
9-11	0.91-1.20	0	0	0	0
12	1.21-1.30	0	0	1**	1.7
	TOTAL	60	100.0	60	100.0

+,*,** Indicate wells positive at both 3 and 4 weeks

TABLE 5.14a ELISA readings of the three week old transformants from RP patient GG

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	36	90.0	39	97.5
2	0.21-0.30	3	7.5	1	2.5
3	0.31-0.40	0	0	0	0
4	0.41-0.50	1	2.5	0	0
	TOTAL	40	100.0	40	100.0

TABLE 5.14b ELISA readings of the three week old CsA treated transformants from RP patient GG

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	18	75.0	19	79.2
2	0.21-0.30	6	25.0	4	16.7
3	0.31-0.40	0	0	1	4.1
	TOTAL	24	100.0	24	100.0

TABLE 5.14c ELISA readings of three week old transformants from RP patient GG. T Cell activity not removed

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	17	58.3	19	79.2
2	0.21-0.30	7	29.2	4	16.7
3	0.31-0.40	3	12.5	1	4.1
	TOTAL	24	100.0	24	100.0

TABLE 5.15a ELISA readings of the three week old transformants from RP patient HM

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	57	95.0	47	78.3
2	0.21-0.30	3	5.0	9	15.0
3	0.31-0.40	0	0	4	6.7
	TOTAL	60	100.0	60	100.0

TABLE 5.15b ELISA readings of the three week old CsA treated transformants from RP patient HM

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	46	76.7	57	95.0
2	0.21-0.30	12	20.0	3	5.0
3	0.31-0.40	2	3.3	0	0
	TOTAL	60	100.0	60	100.0

TABLE 5.15c ELISA readings of three week old transformants from RP patient HM. T Cell activity not removed

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	39	65.0	40	66.7
2	0.21-0.30	21	35.0	17	28.3
3	0.31-0.40	0	0	3	5.0
	TOTAL	60	100.0	60	100.0

Figure 5.10 ELISA Results of primary Clones of Transformed Cells obtained from three Healthy Controls

The primary clones of transformed cells, derived from transformations of PBL isolated from healthy control subjects, were assayed in ELISA plates coated with 1μg/ml S-antigen and 5μg/ml ROS.

The histograms show the relative frequencies of primary transformants, falling into defined categories of absorbance at 492nm (A492), after assay of the supernatants by ELISA. Clones demonstrating reactions in the ELISA below Category 3, were not taken as positive.

a) ELISA of transformation derived from control subject BD

b) ELISA of transformation derived from control subject HD

c) ELISA of transformation derived from control subject OM

N = number of primary clones assayed

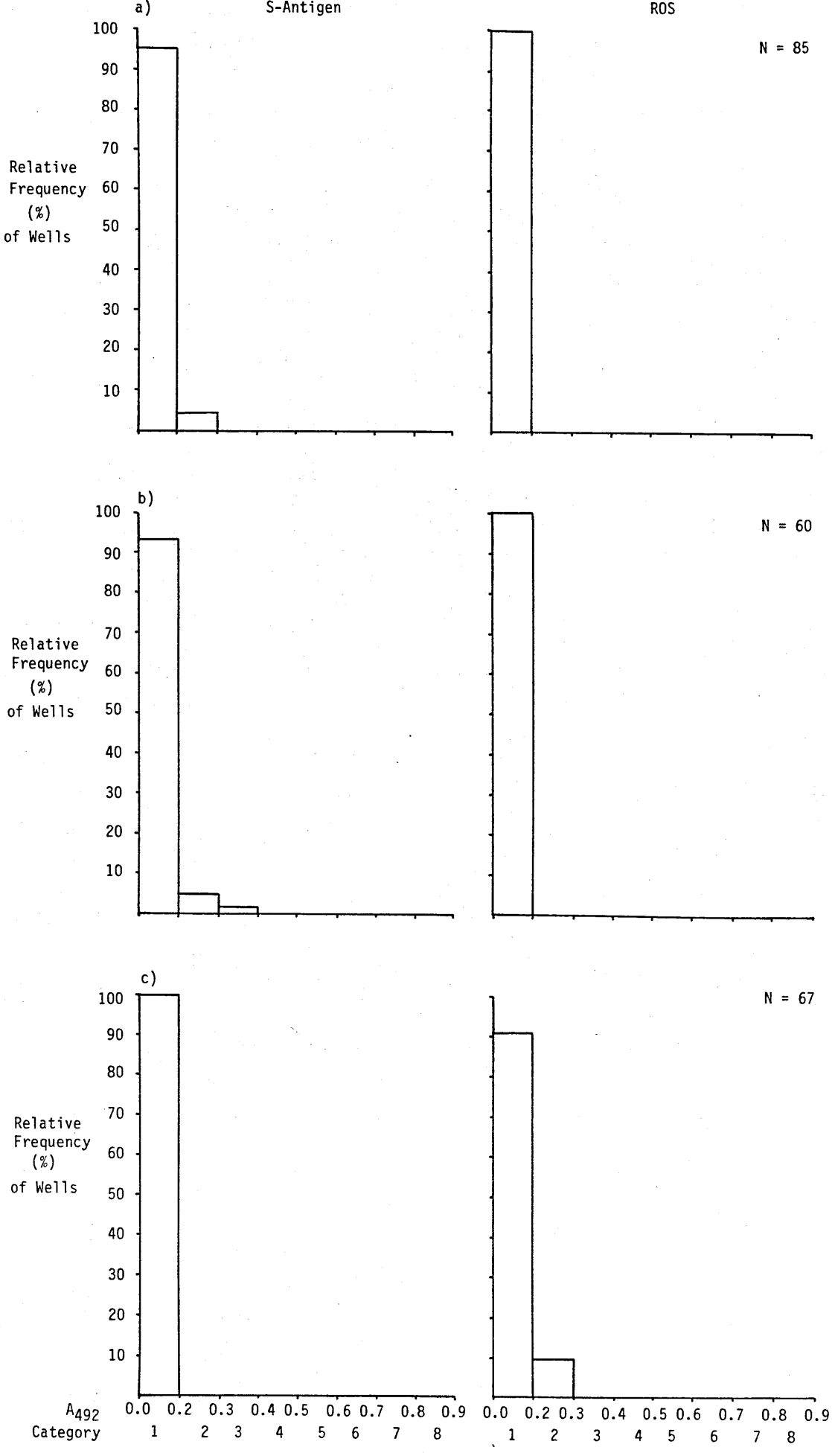

a)
S-Antigen
ROS
N = 85
b)
N = 60
c)
N = 67
Relative Frequency (%) of Wells
100
90
80
70
60
50
40
30
20
10
A_{492} Category
0.0 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
1 2 3 4 5 6 7 8

Figure 5.11 ELISA Results of primary Clones of Transformed Cells obtained from two users of S-Antigen

The primary clones of transformed cells, derived from transformations of PBL isolated from subjects who employed S-antigen, were assayed in ELISA plates coated with 1μg/ml S-antigen and 5μg/ml ROS.

The histograms show the relative frequencies of primary transformants, falling into defined categories of absorbance at 492nm (A492), after assay of the supernatants by ELISA. Clones demonstrating reactions in the ELISA below Category 3, were not taken as positive.

a) ELISA of transformation derived from subject JF

b) ELISA of transformation derived from subject KM

N = number of primary clones assayed

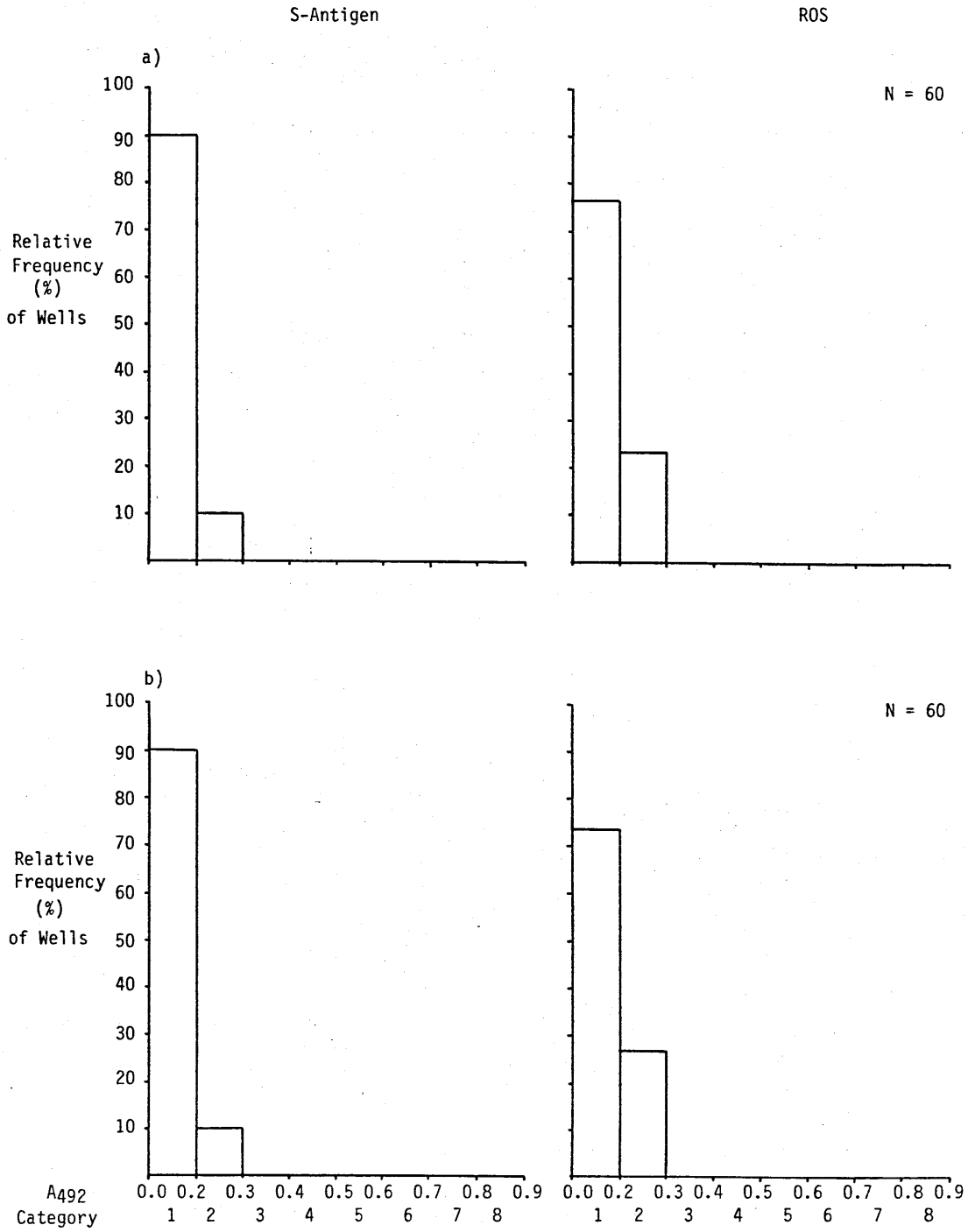

S-Antigen
ROS
a)
b)
N = 60
N = 60
Relative Frequency (%) of Wells
100
90
80
70
60
50
40
30
20
10
A492 Category
0.0 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
1 2 3 4 5 6 7 8

Figure 5.12 ELISA Results of primary Clones of Transformed Cells obtained from two Related Patients with ADRP

The primary clones of transformed cells, derived from transformations of PBL isolated from two related patients with ADRP, were assayed in ELISA plates coated with 1µg/ml S-antigen and 5µg/ml ROS.

The histograms show the relative frequencies of primary transformants, falling into defined categories of absorbance at 492nm (A492), after assay of the supernatants by ELISA. Clones demonstrating reactions in the ELISA within Category 3 and above, were selected as positives.

No significant positive clones were identified upon assay of the transformation derived from patient IM. The transformation obtained from patient KM, however, yielded 2 clones positive to both S-antigen and ROS, within Category 3.

a) ELISA of transformation derived from patient IM (father)

b) ELISA of transformation derived from patient KM (son)

N = number of primary clones assayed

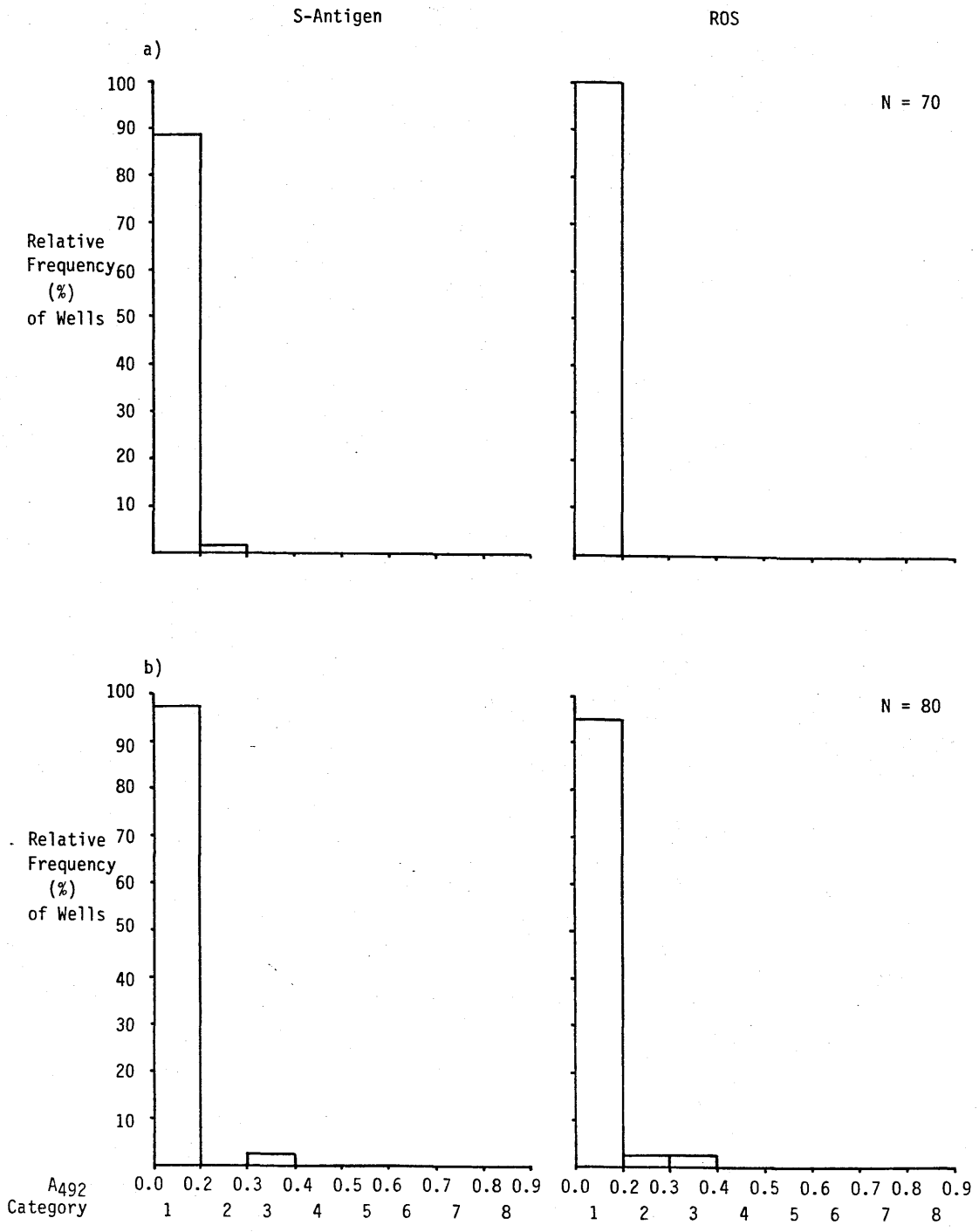

S-Antigen
ROS
a)
N = 70
Relative Frequency (%) of Wells
100
90
80
70
60
50
40
30
20
10
b)
N = 80
Relative Frequency (%) of Wells
A492
Category
0.0 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
1 2 3 4 5 6 7 8

Figure 5.13 ELISA Results for two Assays of primary Clones of Transformed Cells obtained from ADRP Patient AA

The primary clones of transformed cells, derived from transformations of PBL isolated from ADRP patient AA, were assayed in ELISA plates coated with 1μg/ml S-antigen and 5μg/ml ROS. The 96 well plate containing the transformed cells was assayed at (a) 3 and (b) 4 weeks post transformation.

The histograms show the relative frequencies of primary transformants, falling into defined categories of absorbance at 492nm (A492), after assay of the supernatants by ELISA. Clones demonstrating reactions in the ELISA within Category 3 and above, were selected as positives.

Three of the positive clones, detected at 3 weeks, with readings above 0.51 A492, were still positive at 4 weeks (see Tables 5.13a,b). At 4 weeks, one additional clone was identified as relatively strongly positive to S-antigen, falling within Category 8 (Table 5.13b).

a) ELISA of transformation after 3 weeks

b) ELISA of transformation after 4 weeks

N = number of primary clones assayed

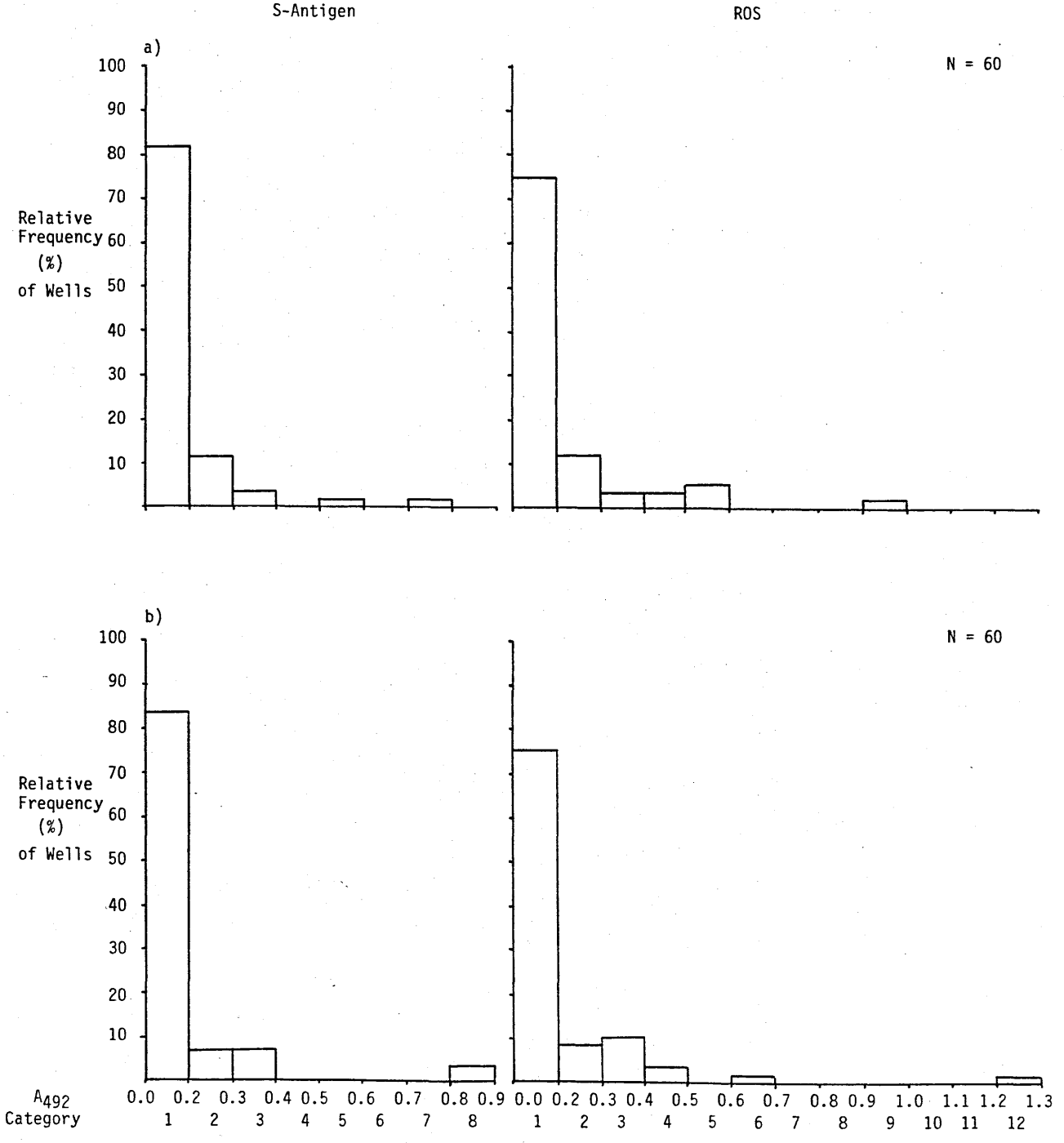

S-Antigen
ROS
a)
N = 60
100
90
80
70
60
50
40
30
20
10
Relative Frequency (%) of Wells
b)
N = 60
100
90
80
70
60
50
40
30
20
10
Relative Frequency (%) of Wells
A492 Category
0.0 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
1 2 3 4 5 6 7 8
0.0 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1 1.2 1.3
1 2 3 4 5 6 7 8 9 10 11 12

Figure 5.14 ELISA Results for the Assays of primary Clones of Transformed Cells obtained from RP Patient GG

The primary clones of transformed cells, derived from the transformation of PBL isolated from RP (symplex) patient GG, were assayed in ELISA plates coated with 1µg/ml S-antigen and 5µg/ml ROS. In this transformation, the effects of T cell rosetting (a) and CsA (b) treatments, on cell growth and positivity in two groups, were compared with cells receiving no such treatment (c).

The histograms show the relative frequencies of primary transformants, falling into defined categories of absorbance at 492nm (A492), after assay of the supernatants by ELISA. Clones demonstrating reactions in the ELISA within Category 3 and above, were selected as positives.

Positive clones, to at least one of the antigens, could be detected in all groups of the transformed cells. No striking difference was observed, in this experiment, between the three different treatments. See also Tables 5.14a,b,c, and text (Section 5.3.9).

a) ELISA of transformation involving T cell rosetting

b) ELISA of transformation involving the drug CsA

c) ELISA of transformation involving no such treatments

N = number of primary clones assayed

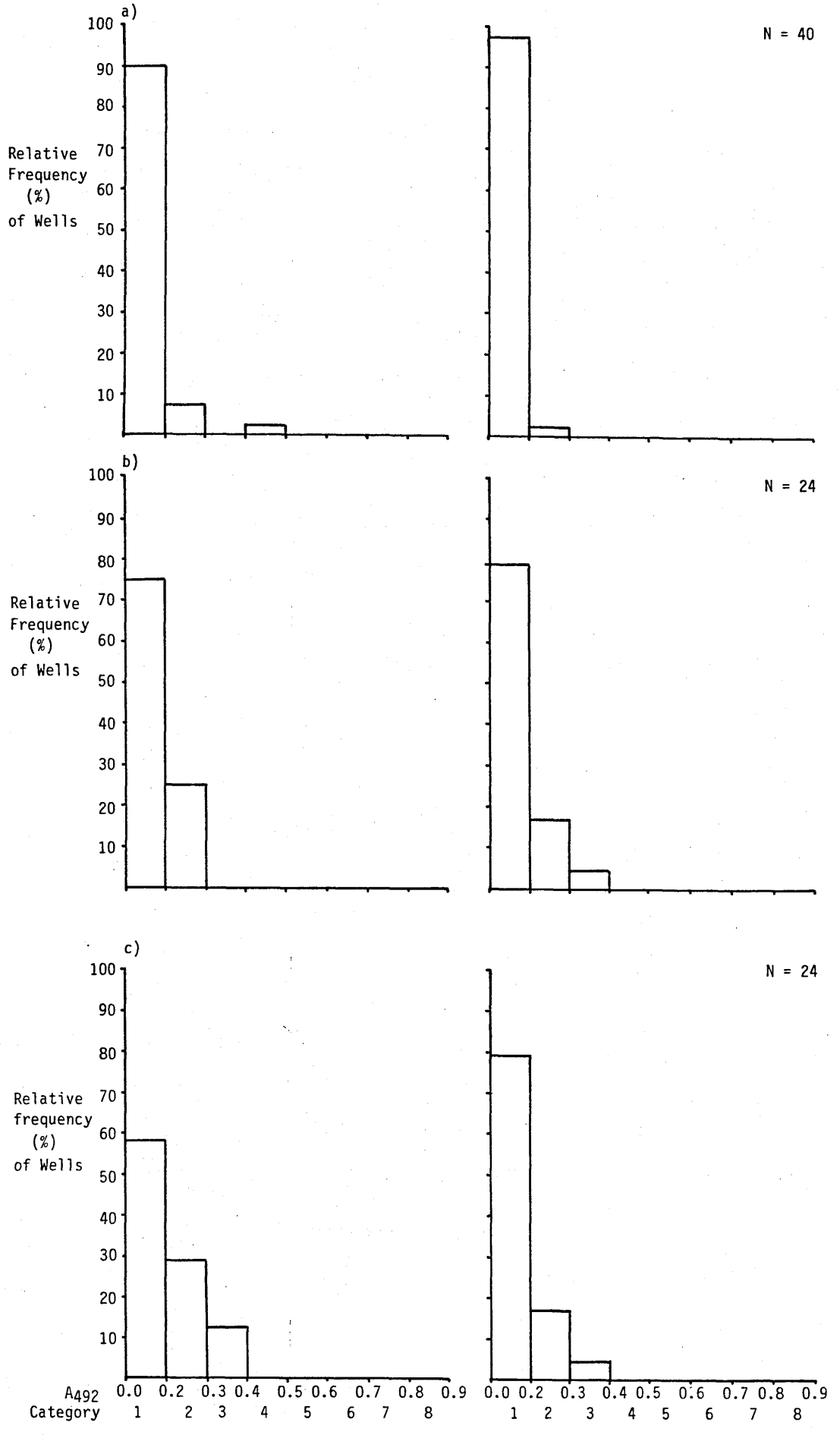

a)
b)
c)
N = 40
N = 24
N = 24
Relative Frequency (%) of Wells
Relative Frequency (%) of Wells
Relative frequency (%) of Wells
100
90
80
70
60
50
40
30
20
10
A492 Category
0.0 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
1 2 3 4 5 6 7 8

Figure 5.15 ELISA Results for the Assays of primary Clones of Transformed Cells obtained from RP Patient HM

The primary clones of transformed cells, derived from the transformation of PBL isolated from RP (symplex) patient HM, were assayed in ELISA plates coated with 1µg/ml S-antigen and 5µg/ml ROS. In this transformation, the effects of T cell rosetting (a) and CsA (b) treatments, on cell growth and positivity in two groups, were compared with cells receiving no such treatment (c).

The histograms show the relative frequencies of primary transformants, falling into defined categories of absorbance at 492nm (A492), after assay of the supernatants by ELISA. Clones demonstrating reactions in the ELISA within Category 3 and above, were selected as positives.

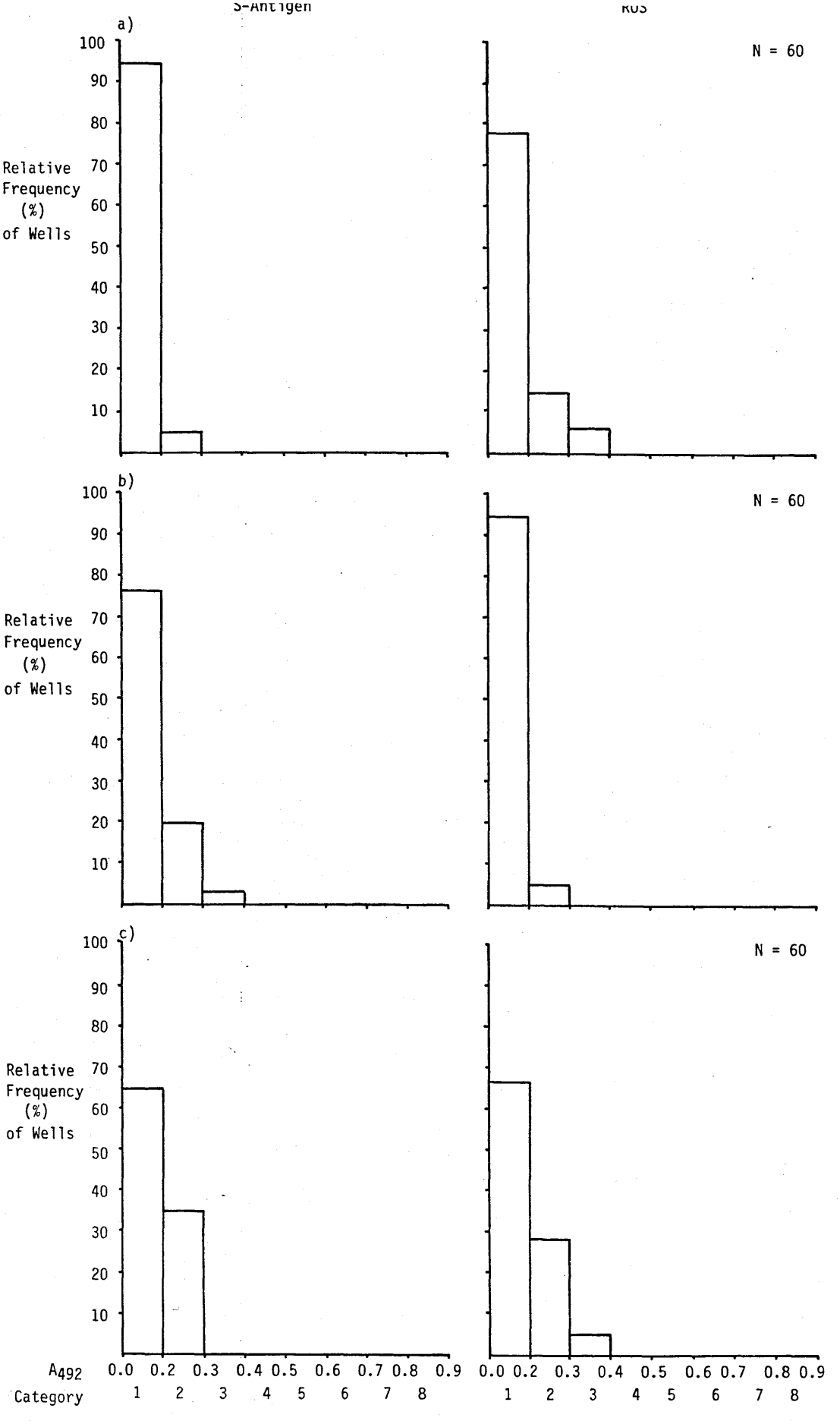
Positive clones, to at least one of the antigens, could be detected in all groups of the transformed cells. No striking difference was observed, in this experiment, between the three different treatments. See also Tables 5.15a,b,c, and text (Section 5.3.9).

a) ELISA of transformation involving T cell rosetting

b) ELISA of transformation involving the drug CsA

c) ELISA of transformation involving no such treatments

N = number of primary clones assayed


S-Antigen
RUS
a)
b)
c)
N = 60
N = 60
N = 60
Relative Frequency (%) of Wells
Relative Frequency (%) of Wells
Relative Frequency (%) of Wells
100
90
80
70
60
50
40
30
20
10
A492
0.0 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
0.0 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
Category
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8

A492 (Table 5.12b and Figure 5.12b). These were likely to be true readings since they were the only 2 significant positives out of 80 wells, and were positive to both antigens. Possibly they were low affinity antibodies, crossreacting with S-antigen and a major insoluble ROS antigen, such as opsin. Alternatively, there may have been two or more clones involved, one secreting antibody to S-antigen and one or two other clones secreting antibodies to ROS antigens.

These potentially very interesting clones were expanded into 24-well plates, but no further positive reactions could be obtained from them, although the cells were growing well. This may have been the result of dilution of the antibody in the larger volumes involved in the 24-well plate. The failure to detect the antibodies could also indicate that the cells were channelling more energy into growth than into antibody synthesis. Another likely possibility is the overgrowth by non-secreting cells, or cells secreting irrelevant antibody. Early cloning can prevent this problem in the rodent hybridoma system. However the transformed cells tend not to tolerate cloning (Kozbor and Roder, 1982).

The transformation derived from patient AA, generated several potentially interesting clones (Figures 5.13a,b and Tables 5.13a,b). A few of these primary clones, generated reactions greater than those of the transformation obtained from patient KM (Table 5.12b). Three clones were identified yielding readings over 0.8 A492. These reactions would be considered positive, even in a fusion derived from a hyperimmunised individual. The readings obtained from the assay of this transformation were not likely to have resulted from spurious ELISA plate phenomena, since there were

several positive wells giving stronger reactions than those seen in nonspecific reactions occasionally obtained with complete medium alone.

Three weeks after transformation, many of the apparently positive cells, derived from patient AA, were not ready for expansion. Thus, the transformation plates were re-assayed after a further 7 days (Table 5.13b and Figure 5.13b). Three of the relatively strong positive wells remained at similar A492 levels, with one primary clone, positive to ROS, moving from Category 5 to Category 6. Some clones, originally identified as positive, were no longer so, and some new positives were obtained. All positive clones were expanded. Few primary clones remained positive in the 25ml flasks. Five clones were expanded into 250ml flasks and fused with the human lymphoblastoid KR-4 cell line (Kozbor et al., 1982) (Sections 2.1.18 and 2.7.8). The cell culture supernatants were no longer positive at this time, but were fused in the hope of detecting a positive reaction after the partial cloning at fusion (Section 2.7.8.2). Assay of the fused cells did not suggest that there were any positive clones. In view of the problem of overgrowth by non-secreter cells, subsequent fusions were made with smaller numbers of cells, before the primary clones were expanded from in 25ml flasks.

Relatively weak positives to S-antigen and ROS (0.31 to 0.40 A492), could be detected in transformations derived from RP (advanced simplex form) patients GG and HM (Figures 5.14 and 5.15, respectively), who did not give any indication of significant serum responses to these antigens (Figures 4.10a,b). In these two transformations, the T cell rosetting and Cyclosporin A methods

were compared (Section 5.3.9).

5.3.8 ELISA Reactions of primary Transformed B Lymphocytes from Uveitis Patients

Transformations were performed with PBL from only three patients with uveitis. Two of these patients demonstrated significant serum responses to the antigens in question, at the time of transformation. Patient MK demonstrated a positive response to ROS, but not to S-antigen (Figure 4.11b), and patient HS indicated a positive serum reponse to S-antigen, but was unreactive to ROS (Figure 4.11a,b). No positive clones could be detected in the assays of the transformed PBLs obtained from patient MK (Figure 5.16b). Patient CM gave no indication of a serum response in the ELISA assays (Figures 4.11a,b), and no positives were obtained upon transformation of the PBL (Figure 5.16a). Only patient HS gave rise to primary clones of transformed cells which gave readings above 0.3 A492 in the ELISA assays to ROS and S-antigen (see also Section 5.3.8.1). It is interesting, that although patient HS demonstrated a significantly positive serum response to S-antigen, but not to ROS, clones positive to ROS could be identified upon transformation (Figure 5.17).

5.3.8.1 Comparison of transformations performed at different intervals

Three transformations were performed with PBLs isolated from patient HS (Tables 5.17a,b,c and Figure 5.17a,b,c). The three transformations were derived from 40ml blood samples, thus 120 wells were available for assay after 3 to 4 weeks. Only the rosetting method was employed. The second transformation was performed approximately 8 months after the first, and the third was performed about 12 months after the first transformation. There was

TABLE 5.16a ELISA readings of the three week old transformants from uveitis patient CM

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	118	98.3	120	100.0
2	0.21-0.30	2	1.7	0	0
	TOTAL	120	100.0	120	100.0

TABLE 5.16b ELISA readings of the three week old transformants from uveitis patient MK

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	59	98.3	60	100.0
2	0.21-0.30	1*	1.7	0	0
	TOTAL	60	100.0	60	100.0

* Assays repeated 1 week later yielded the same results; the same cells gave rise to the reading category 2.

TABLE 5.17a ELISA readings of the three week old transformants from uveitis patient HS; transformation 1

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	88	73.3	106	88.3
2	0.21-0.30	12	10.0	11	9.2
3	0.31-0.40	11	9.2	2	1.7
4	0.41-0.50	5	4.2	0	0
5	0.51-0.60	2	1.7	0	0
6	0.61-0.70	1	0.8	0	0
7	0.71-0.80	0	0	0	0
8	0.81-0.90	1	0.8	0	0
9	0.91-1.00	0	0	1	0.8
	TOTAL	120	100.0	120	100.0

TABLE 5.17b* ELISA readings of the three week old transformants from uveitis patient HS; transformation 2

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	118	98.3	119	99.2
2	0.21-0.30	2	1.7	1	0.8
	TOTAL	120	100.0	120	100.0

* Similar results were obtained upon re-assay after 6 days.

TABLE 5.17c ELISA readings of the three week old transformants from uveitis patient HS; transformation 3

Category	A492 Grouping	S-antigen No. Wells	%	ROS No. Wells	%
1	0.0 -0.20	39	65.0	107	89.2
2	0.21-0.30	13	21.6	11	9.2
3	0.31-0.40	4	6.7	0	0
4	0.41-0.50	0	0	1	0.8
5	0.51-0.60	4	6.7	1	0.8
	TOTAL	60	100.0	120	100.0

Figure 5.16 ELISA Results for the Assays of primary Clones of Transformed Cells obtained from two Uveitis Patients

The primary clones of transformed cells, derived from transformations of PBL isolated from two patients with uveitis, were assayed in ELISA plates coated with 1µg/ml S-antigen and 5µg/ml ROS.

The histograms show the relative frequencies of primary transformants, falling into defined categories of absorbance at 492nm (A492), after assay of the supernatants by ELISA. Clones demonstrating reactions in the ELISA within Category 3 and above, were selected as positives.

No significantly positive clones could be identified in the transformation derived from patient CM (a). The clone, indicating a positive reaction to S-antigen (Category 3), obtained from the transformation of patient MK, yielded the same reading, in the ELISA, one week later (b). No other positives could be identified.

a) ELISA of transformation derived from patient CM

b) ELISA of transformation derived from patient MK

N = number of primary clones assayed

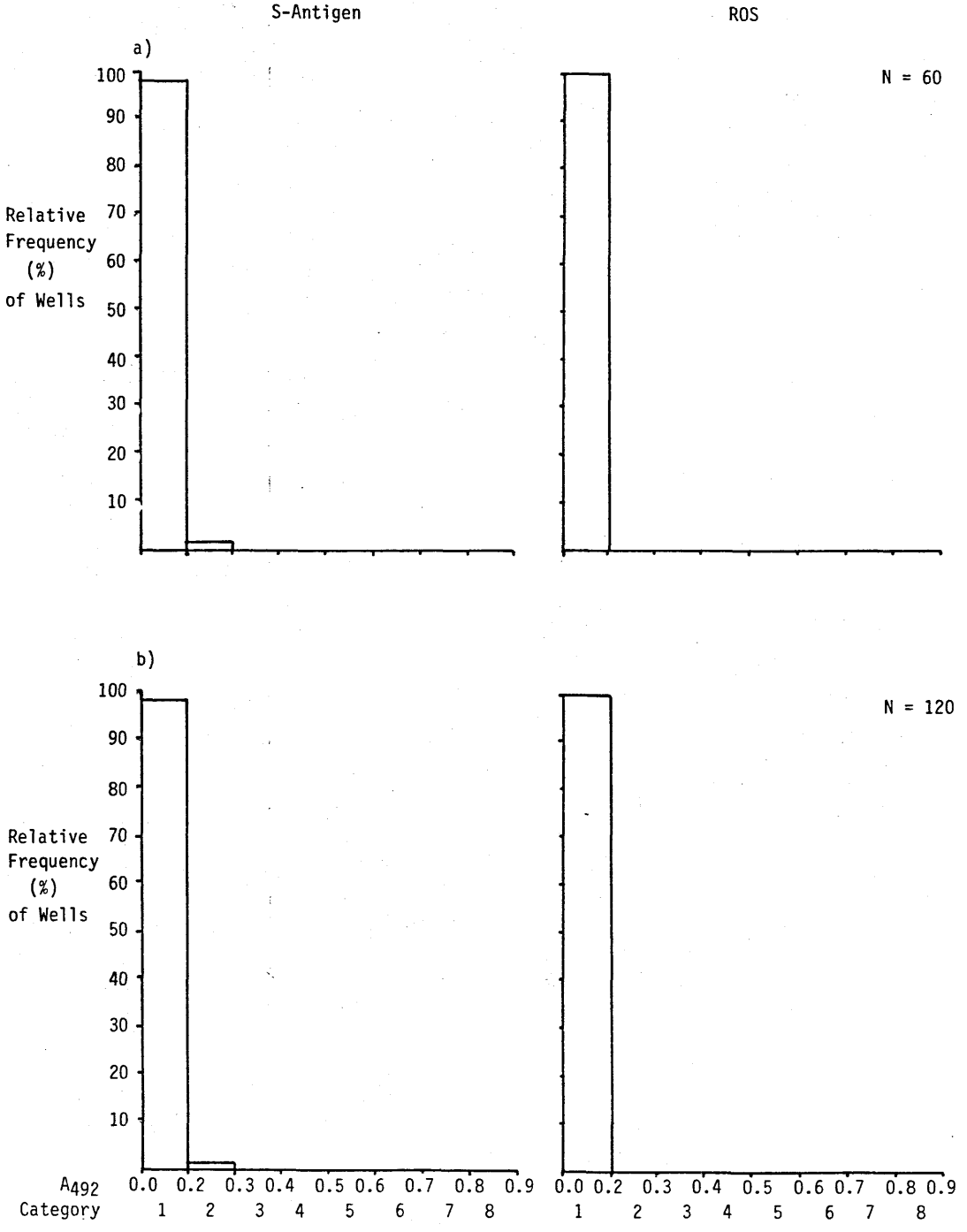

S-Antigen
ROS
a)
N = 60
Relative Frequency (%) of Wells
100
90
80
70
60
50
40
30
20
10
b)
N = 120
Relative Frequency (%) of Wells
100
90
80
70
60
50
40
30
20
10
A492
0.0 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
Category
1 2 3 4 5 6 7 8
0.0 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
1 2 3 4 5 6 7 8

Figure 5.17 ELISA Assays of three separate Transformations derived from Uveitis Patient HS

The primary clones of transformed cells, derived from the transformation of PBL isolated from uveitis patient HS, on three different occasions, were assayed in ELISA plates coated with 1μg/ml S-antigen and 5μg/ml ROS. Approximately 8 months after the first transformation (a), a second transformation (b) was performed. The third transformation (c) was performed about 12 months after the first transformation.

The histograms show the relative frequencies of primary transformants, falling into defined categories of absorbance at 492nm (A492), after assay of the supernatants by ELISA. Clones demonstrating reactions in the ELISA within Category 3 and above, were selected as positives.

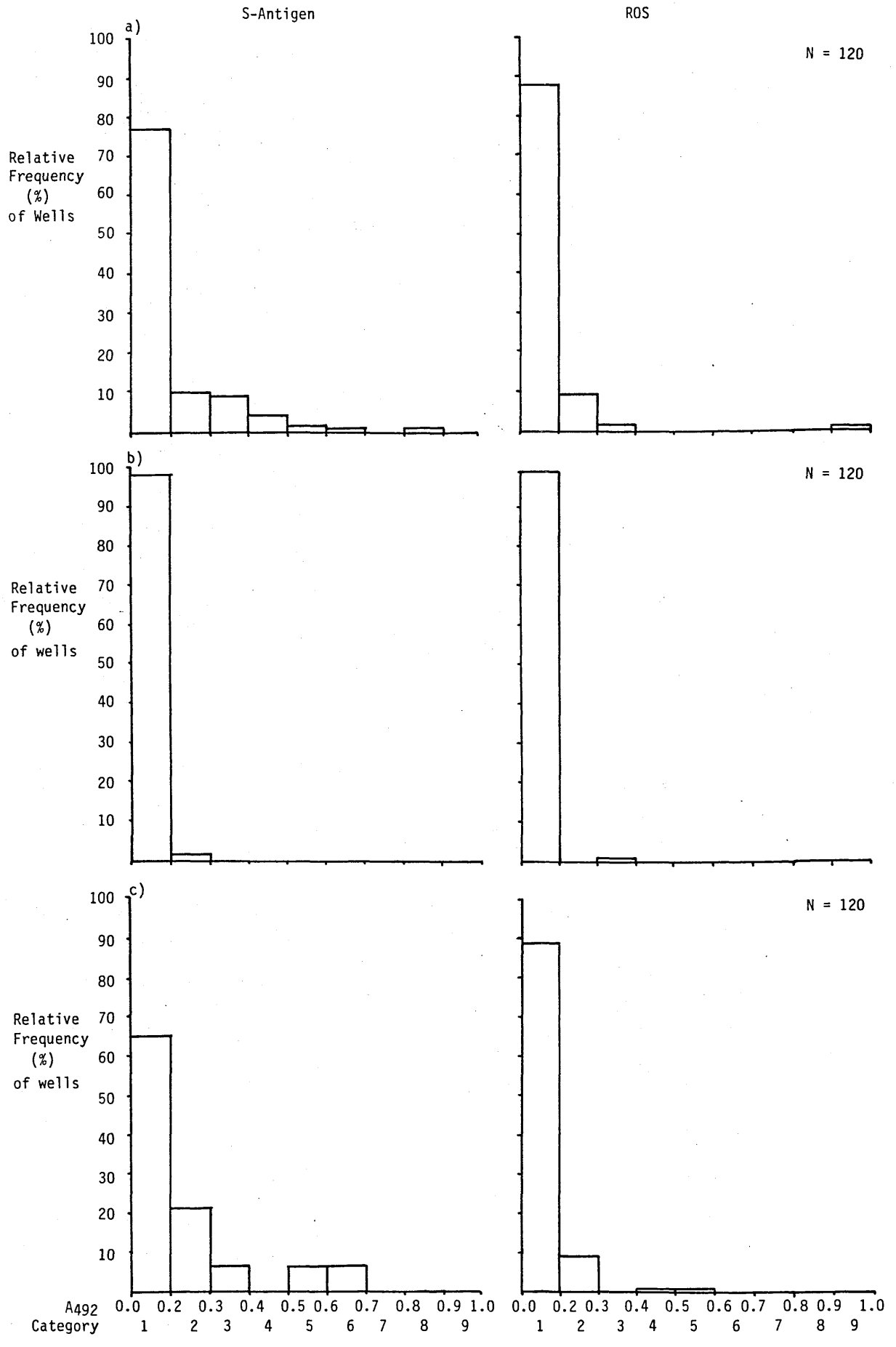
Clones positive to both antigens, could be detected the first and third transformations. The frequencies of positives obtained to each antigen were similar. No positives were detected in the second transformation, even upon re-assay. See also Tables 5.17a,b,c, and text (Section 5.3.8.1).

a) ELISA of transformation 1

b) ELISA of transformation 2

c) ELISA of transformation 3

N = number of primary clones assayed


S-Antigen
ROS
a)
b)
c)
N = 120
N = 120
N = 120
Relative Frequency (%) of Wells
Relative Frequency (%) of wells
Relative Frequency (%) of wells
100
90
80
70
60
50
40
30
20
10
A492
Category
0.0 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
1 2 3 4 5 6 7 8 9

a clear difference between the number of positives obtained from the second transformation, and the two other transformations (Tables 5.17a,b,c and Figures 5.17a,b,c). At least three clones, obtained from the first transformation, generated readings which would have been considered positive in a fusion from a hyperimmunised animal (Table 5.17a and Figure 5.17a). In this transformation the majority of the strongest positives were reactive in the ELISA to S-antigen. Two clones were identified which gave reading of greater than 0.51 A492 in the ELISA against ROS (Tables 5.17a,c and Figure 5.17a,c). The clone with strongest reaction obtained from transformations derived from patient HS, was to ROS, with a reading in the region of 0.91 to 1.0 A492 (Table 5.17a). This is interesting since no significant serum reaction to ROS, could be detected in the ELISA.

The second transformation did not yield any significant positives to either antigen. No significant positives were obtained upon re-assay of the transformation, 6 days later. The third transformation, performed about one year after the first, yielded 8 positives to S-antigen (out of 60 wells), and 2 positives to ROS (out of 120). The frequency of positives obtained in the assay against ROS (Table 5.17c), was similar to that obtained in the first transformation (Table 5.17a). The frequency of clones positive to S-antigen was also similar in the first and third transformations, although the proportion of these clones generating readings above 0.5 A492 was greater in the third transformation. The range of positive reactions obtained in the first transformation was from 0.31 to 1.0 A492 (Table 5.17a), and the range in the third transformation was from 0.31 to 0.6 A492 (Table 5.17c).

The larger number of readings above 0.3 A492, detected in the first and third transformations, compared to the second transformation, is not likely to reflect minor differences in batches of antigen, conjugate or ELISA plates.

5.3.9 Comparison of Transformation Procedures

The PBL, isolated from RP patients GG and HM, were divided into three portions in order to assess the effect of the T lymphocytes on the growth and positivity of the EBV transformed cells. Figures 5.14a and 5.15a show the results obtained from the assay of the transformed cells from which the T cells were removed by rosetting (Section 2.7.3.3). Figures 5.14b and 5.15b show the ELISA results for the transformations treated with CsA, which specifically inhibits the activity of T cell (Powles et al., 1980; reviewed by Shevach, 1985). Figures 5.14c and 5.15c show the results obtained from the assay of the transformations in which the T cells were not removed or inhibited.

No significant difference was observed in the growth rates of the cells in the three groups described above, and the results obtained for the assay of these three groups were similar. The transformation, derived from patient GG, generated 4 primary clones positive to S-antigen. Three of these clones were obtained from the group of cells in which the activity of the T cells was not inhibited or removed. (Table 5.14c). The third positive clone was identified within the group where the T cells were removed by rosetting (Table 5.14a). The readings obtained from the latter clone, gave a reaction to S-antigen between 0.41 and 0.50 A492, in the ELISA (Table 5.14a). Two primary clones generated readings to

ROS, in the ELISA, above 0.31 A492 (Table 5.14c).

The transformation derived from patient HM, yielded only 2 clones generating readings in the ELISA to S-antigen above 0.31 A492. These were obtained from the CsA treated transformed cells (Table 5.15b). Assay of the transformation against ROS, indicated that there were 7 clones giving readings above 0.31 A492 (Tables 5.15a,c). No clones were identified in the transformation from patient HM, which generated ELISA reactions, to either antigen, above 0.4 A492.

In transformations derived from subjects not described in this study, similar growth rates were obtained with the rosetting and CsA treatments, and poorer growth was apparent after the first week in the wells where T cell activity was not removed or inhibited. After the third week, the transformed cells which received no anti-T lymphocyte treatment, were growing actively. By this time, although the T cells probably inactivated several of the early virally infected cells, the remaining transformed cells appeared to recover and grow. In conclusion, the subversive influence of the T cells (cytotoxic rather than helper T cells) was apparent in some samples and not in others, probably reflecting the immunological status of the donor, to EBV or viruses in general, at the time of transformation at least. The drug CsA was found to be as effective in the removal of T cell activity, as the rosetting method, with the advantage of shortening the transformation protocol by about 5 hours (Sections 2.7.5). The drug was not found to have any detrimental effects on cell growth or on the frequency of positives detected, in patients GG and HM, at least.

5.3.10 Problems in Stabilisation of Transformed Lymphocytes

Primary transformed cells within the wells of the 96-well plates, which generated reactions above 0.3 A492, in th ELISA assays to S-antigen and/or ROS, were expanded into the wells of 24-well plates. The supernatants of these expansions were re-assayed a few days later, when the cells had become established. Often the cells seemed to cease to secrete antibody, especially if the original reaction was in the region of 0.3 to 0.4 A492. A few expanded primary clones remained positive, after growth in 25ml flasks. These were the cells which tended to generate readings in the upper range, eg. of 0.7 A492 and above, in the original assay of the transformation plate. The readings obtained in subsequent assay after expansion of these cells in the wells of 24-well plates and 25ml flasks, were always much lower, eg. 0.3 to 0.5 A492.

Transformed cells are only temporarily stabilised, and although some expanded clones could be cultured for over two months, many clones a few clones failed to expand into 24-well plates, and others regressed after expansion into 25ml flasks.

Two clones, expanded from the first transformation derived from uveitis patient HS, remained positive (at 0.4 A492) in the ELISA to S-antigen, while growing in 250ml flasks. These cells were fused with the human lymphoblastoid cell line, KR-4 (Section 2.7.8), and although good growth was obtained, no clones generated a reaction above 0.3 A492 in the ELISA assay. Fusions performed with transformants grown in 25ml flasks were also unsuccessful. The greatest problems encountered in the stabilisation of cells secreting the required antibody, included the overgrowth by non-producers, and the inherent low rate of antibody production

(Kozbor and Roder, 1982; Sikora and Neville, 1982) (Section 5.3.10). These factors made it difficult to detect antibody in the supernatants of cells grown in 25ml flasks. Expansion into 25ml flasks was required in order to provide sufficient cells for the purpose of stabilisation by fusion, and subsequent cloning.

5.3.11 Discussion

Transformations of the PBL, isolated from blood samples obtained from certain patients with RP and uveitis, demonstrated positive clones in ELISA assays against S-antigen and ROS. One apparent weak, positive primary clone, was identified in one of the five control transformations (Figure 5.10b). However, this clone was not positive upon re-assay, and the original result might have arisen from a spurious ELISA plate reaction.

It is possible that significantly positive clones of transformed B lymphocytes, could be detected in transformations derived from healthy individuals, if a larger number of experiments were performed. It is the relative frequencies of such clones, in transformations from healthy controls, which should be compared with those from patients with ocular disease. However, Winger et al. (1983) have shown that without prior immunisation of the subject, it is possible to identify primary clones of transformed human B lymphocytes, with specificities to a variety of antigens.

The removal of the T cells by rosetting or their suppression by CsA in the transformations derived from RP patients GG and HM, did not give rise to an improved growth or an increase in the number of positive primary clones (Section 5.3.9). The removal or suppression

of the T cells, in transformations not described in this study, has resulted in improved growth. Bird et al. (1981), demonstrated that the addition of CsA to EBV infected B lymphocytes, enhanced their growth. The effect of the T cells on the transformed B lymphocytes is likely to vary with the immunological status of the patient to EBV and to viruses in general.

The majority of circulating B cells are thought to be memory cells (Roitt et al., 1985). It has been suggested that the majority of B cells involved in transformation, are the memory cells (Volkman and Weetman, 1986). Thus, the detection of specific clones, using this methodology, may suggest that the patients who did not give any indication for a positive serum response to the retinal antigens in the ELISA, might have become sensitised to them in the past. The most obvious example in this study, is given by the ADRP patient AA (Section 5.3.7). Conversely, recent evidence suggests that EBV may selectively transform B lymphocytes which have already begun to secrete immunoglobulin in vivo, but which have not yet developed into plasma cells (Steel et al., 1986). Perhaps, during a certain phase of the progressive retinal disease, the blood-retinal barrier was disrupted, and the immune system of the patient became exposed to the retinal antigens. Newly stimulated antigen specific B cells, which are just beginning to secrete antibody, become available for transformation by EBV (Steel et al., 1986). Similarly, uveitis diseases might also undergo phases of activity and regression. This could be reflected in immunological assays, where immunological factors are involved in the disease. The ocular inflammation observed in uveitis, might occasionally give rise to the specific sensitisation of the immune system to certain ocular antigens, at the more acute phases of the disease.

Uveitis patient HS demonstrated a relatively strong response to S-antigen in the ELISA, but the response to ROS was within the normal range. Several primary clones, generating positive reactions with both antigens, could be identified in two of the three transformations (Figures 5.17a,c). This may indicate that the patient was in the process of developing a humoral immune response to ROS insoluble antigens. The second of the three transformations derived from patient HS, did not generate any significantly positive primary clones to S-antigen or ROS, in the ELISA assay (Figure 5.17b). If memory cells are involved in the transformations, then positives would be expected from the second transformation, since they were obtained in the first transformation. However, if it is the newly stimulated B cells which are transformed, then the detection of positive clones will probably depend on the present phase of the disease, and the physiological state of the patient. There may also be a sampling error, in a statistical sense, and that fewer B lymphocytes, specific to retinal antigens, or transformable by EBV (Steel et al., 1986) were present within that particular 40ml blood sample.

In this study, only antibodies specific to S-antigen and ROS insoluble antigens, were sought. There is no reason to believe that no other retinal proteins are involved; indeed the immunoblotting experiments with certain patients sera suggest that a 40K antigen may be important (Section 4.2.7). The possible involvement of the T lymphocytes was not investigated in this study. T cell mediated immune reactions may be of importance in certain groups of patients with RP and uveitis (Sections 1.4.3.5, and 1.4.4.4). The presence of humoral antibody may merely represent a by-product of the T

lymphocyte mediated responses, which might be pathologically more significant.

Thus, circulating B lymphocytes, with specificity to retinal antigens, could be identified in certain uveitis and RP patients by transformation technology. However, the stage of maturation of those B lymphocytes with specificity for retinal antigens, which are transformable by EBV, remains to be determined.

CHAPTER SIX

ESTABLISHMENT AND CHARACTERISATION OF MONOCLONAL ANTIBODIES

Chapter 6

ESTABLISHMENT AND CHARACTERISATION OF MONOCLONAL ANTIBODIES

The development and characterisation of monoclonal antibodies derived from fusions with hyperimmunised and potentially autoimmune rats are described in this chapter. The specificities and immunoglobulin classes of a panel of five monoclonal antibodies are summarised in Table 6.1.

6.1 THE ESTABLISHMENT OF MONOCLONAL ANTIBODIES

6.1.1 Frequency of Positive Clones

The fusions were screened against 1µg/ml of S-antigen and 5µg/ml of solubilised rod outer segment membranes in ELISA assays (Section 3.8.3-6). Absorbance readings of 0.8 or greater, at 492nm were taken to indicate a significant positive reaction of hybridoma supernatants, from an immunised animal. The frequency of positive primary clones obtained from fusions with rats hyperimmunised with S-antigen or ROS antigen ranged from 0 to close to 100%.

The results for the screening of fusions with the potentially autoiummune RCS rats are described in Section 5.2.5. For these fusions, readings of 0.4 and greater were taken as significantly positive (Section 5.2.5).

6.1.2 Expansion and selection of Positive Hybrids

Primary fusion wells identified as positive in the ELISA to either S-antigen or ROS, were expanded into 25ml flasks, when the wells had become confluent. The growth rate and general appearance of the hybrids varied considerably between fusions, and even within one

fusion plate. Some primary clones would grow rapidly and expand readily in flasks. Such cells tended to have smooth membranes. Clones exibiting poorer growth, generally had less smooth membranes. These cells tended to regress after their initial appearance, although after feeding with spleen cells from non-immunised rats (Section 2.6.9.1) in complete medium, some such clones would improve dramatically. Only those potential clones demonstrating stable growth and antibody production were selected for further characterisation.

Due to the considerable cost involved in the establishment of monoclonal antibodies, it was necessary to identify those antibodies with the greatest potential, by preliminary experiments with the supernatants from the expanded, but as yet uncloned hybridomas, for example, immunoblotting (Section 2.3.9) and immunocytochemistry (Section 2.4). The primary uncloned cells, not selected for the present study, were stored frozen in liquid nitrogen (Section 2.6.8).

6.1.3 Subcloning to achieve Monoclonality

The hybrids were subcloned by limiting dilution, as described in Section 2.6.9. Generally, less than 100% growth was obtained when the cells were cloned at one cell per well; similarly, less than 50% and less than 25% of wells had hybrids growing in them after subcloning at 0.5 and 0.25 cells per well, respectively. At the first subcloning, not all of the supernatants from wells with growth were positive in the ELISA, especially when the original fusion had been highly successful in terms of the number of wells with growth. In these cases, there was probably an irrelevant clone sharing the same well as the clone of interest. Positive clones

were expanded from the wells seeded at 0.25 cells per well, since these wells were more likely to contain the offspring descended from a single cell. All subsequent subcloning gave rise to 100% of positive clones. Often it was only necessary to assay those wells with cells cloned at 0.25 and 0.5 cells per well. After subcloning three times at 0.25 cells per well, the hybrids were considered to be monoclonal, and could be grown in bulk culture for the production of large quantities of antibody for experimental purposes (Section 2.6.10).

6.1.4 Established Monoclonal Antibodies

Two monoclonal antibodies were established from hyperimmunised DA rats (Table 6.1). Monoclonal antibody S2.4.C5, detected against bovine S-antigen in ELISA, was obtained from a fusion with a rat immunised with this protein. Antibody R1.2.D2, detected in the ELISA against ROS, was derived from a rat immunised against bovine rod outer segment membranes (Section 2.6.1). Time did not permit the antibody R1.2.D2, to be cloned more than once. Thus R1.2.D2 is not referred to as a monoclonal antibody, although it could have been regarded as one under less stringent criteria.

Three monoclonal autoantibodies were stabilised from fusions with the splenic lymphocytes of RCS rats (Table 6.1). Two monoclonal antibodies were derived from the nine week old animal designated H9; monoclonal antibody, H9.1.C2 was reactive against S-antigen, and antibody H9.5.A4 was reactive to ROS in ELISA. The third monoclonal antibody H10.1.D2, was obtained from a rat sacrificed at the age of 10 weeks (H10), and was reactive against both S-antigen and ROS.

TABLE 6.1 Class and specificity of monoclonal antibodies

Monoclonal Antibody	Parent Rat Strain	Immunising Antigen	Class	Specificity
S2.4.C5	DA	S-antigen	IgG1	S-antigen
R1.2D2	DA	ROS	IgM	39K
H9.1.C2	RCS Hunter	-	IgM	S-antigen
H9.5.A4	RCS Hunter	-	IgM	ROS protein
H10.1.D3	RCS Hunter	-	IgM	Opsin/S-ag

6.2 ISOTYPE OF MONOCLONAL ANTIBODIES

Tenfold concentrates of the monoclonal antibodies, ammonium sulphate precipitated from cell free culture supernatant and dialysed against PBS (Section 2.2.4.1), were assayed with isotype specific rabbit antisera in Ouchterlony immunodiffusion experiments (Section 2.6.11). Immune complexes precipitated where the monoclonal antibody came in contact with the appropriate isotype specific antisera (Section 2.1.16). An immunoprecipitate formed when rat monoclonal antibody S2.4.C5, with specificity to S-antigen, reacted with rabbit anti-rat IgG (gamma chain) and sheep anti-rat IgG1. This monoclonal antibody did not form a precipitin band with rabbit anti-rat IgM, goat anti-rat IgG 2a or 2c, or rabbit anti-rat IgG 2b. Monoclonal antibody R1.2.D2 with specificity to ROS, and also the three autoantibodies (Section 6.1.4) formed precipitin bands with rabbit anti-rat IgM, but not with rabbit anti-rat IgG (gamma chain) or any of the other antisera to rat IgG subclasses. Table 6.1 shows the classes of the monoclonal antibodies obtained during the course of this work.

6.3 SPECIFICITY OF MONOCLONAL ANTIBODIES

6.3.1 ELISA Titres of Monoclonal Antibodies

The specificities of the monoclonal antibodies in ELISA were determined by titrating of the cell culture supernatants against the S-antigen and washed ROS antigen (Figures 6.1 to 6.6). Assay controls included the titration of the monoclonal antibodies on plates coated with BSA only, and also the titration of irrelevant IgG and IgM monoclonal antibodies on plates coated with the two antigens.

6.3.1.1 Titration of monoclonals from immunised rats

Monoclonal antibody S2.4.C5, demonstrated a titre to S-antigen of more than 1000, which suggested that it might be used at high dilutions in certain experiments. Figure 6.1 shows the titres given by monoclonal antibody S2.4.C5, to S-antigen, at time intervals from 30 seconds to 4 hours. The short period required for binding, suggests that the antibody is likely to be of relatively high affinity (Mason and Williams, 1980). Figure 6.2 shows the decrease in the ELISA readings when the concentration of either S-antigen or S2.4.C5 was reduced, while the other parameter remained constant.

Antibody R1.2.D2, demonstrated a titre to bovine ROS in the ELISA assay (Figure 6.3). The antibody was unreactive to S-antigen and BSA in the ELISA, generating readings similar to that of the background reaction given by the omission of the primary antibody step, shown by the dotted line in Figure 6.3.

6.3.1.2 Titration of monoclonal autoantibodies

The RCS rat monoclonal autoantibodies were of apparently low affinity, with the reaction falling rapidly even at low dilutions. A higher antibody concentration was apparently required to drive the reaction forward, in comparison with the apparently high affinity IgG monoclonal antibody S2.4.C5 (Figure 6.1). Monoclonal antibody H9.1.C2 exhibited a low titre to S-antigen and was unreactive against the washed ROS (Figure 6.4). Monoclonal antibody H9.5.A4 reacted with ROS in ELISA, but not with S-antigen, and also demonstrated a relatively low titre (Figure 6.5). The third monoclonal autoantibody, H10.1.D3, showed a weak response to both antigens, although at a level above the background and above the reading given by the omission of the primary antibody (Figure 6.6).

Figure 6.1 Reactions of serial dilutions of MAb S2.4.C5 against 1µg/ml of S-Antigen in the ELISA

The monoclonal antibody, in the form of hybridoma supernatant, was titrated on the ELISA plate at different time intervals, in order to measure the effect of dilution and incubation time on the reaction. The result for the 4 hour incubation is the mean of 5 assays. The readings for all other periods of MAb incubation were obtained from single assays.

KEY

○ 4 hour incubation

● 60 minute incubation

△ 45 minute incubation

▲ 30 minute incubation

□ 15 minute incubation

■ 10 minute incubation

◺ 5 minute incubation

◣ 1 minute incubation

▽ 30 second incubation

▼ 4 hour incubation with BSA alone

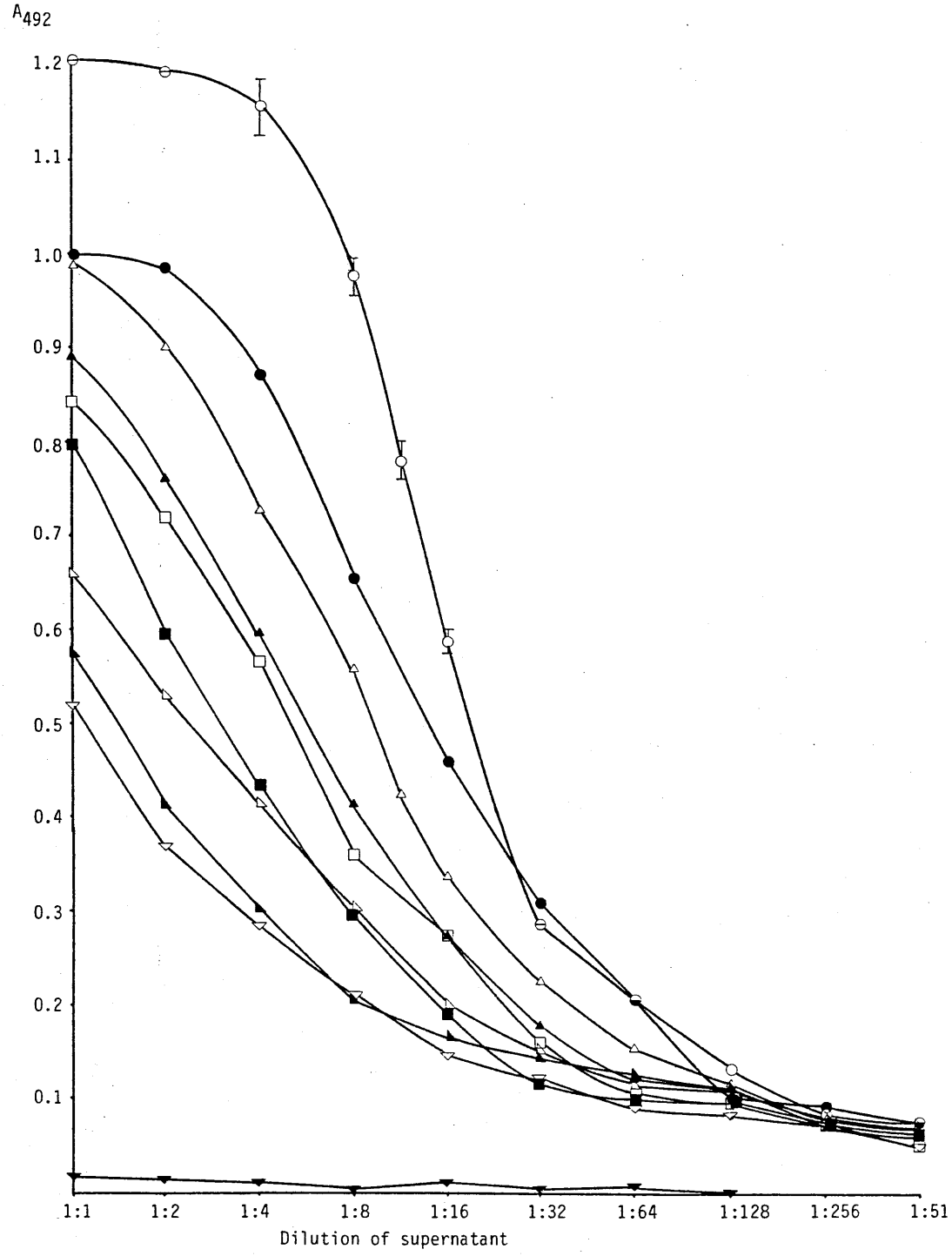

A_{492}
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:51
Dilution of supernatant

Figure 6.2 Chequerboard Experiment with MAb S2.4.C5 and S-Antigen

The effects on the ELISA reaction of variation in concentration of MAb S2.4.C5, in the form of hybridoma supernatant, and S-antigen, was determined by means of a chequerboard experiment (Section 3.8.4). The incubation time with the monoclonal antibody was 2 hours.

KEY

O dilution of MAb S2.4.C5

● concentration of S-antigen

... background reaction; no antibody

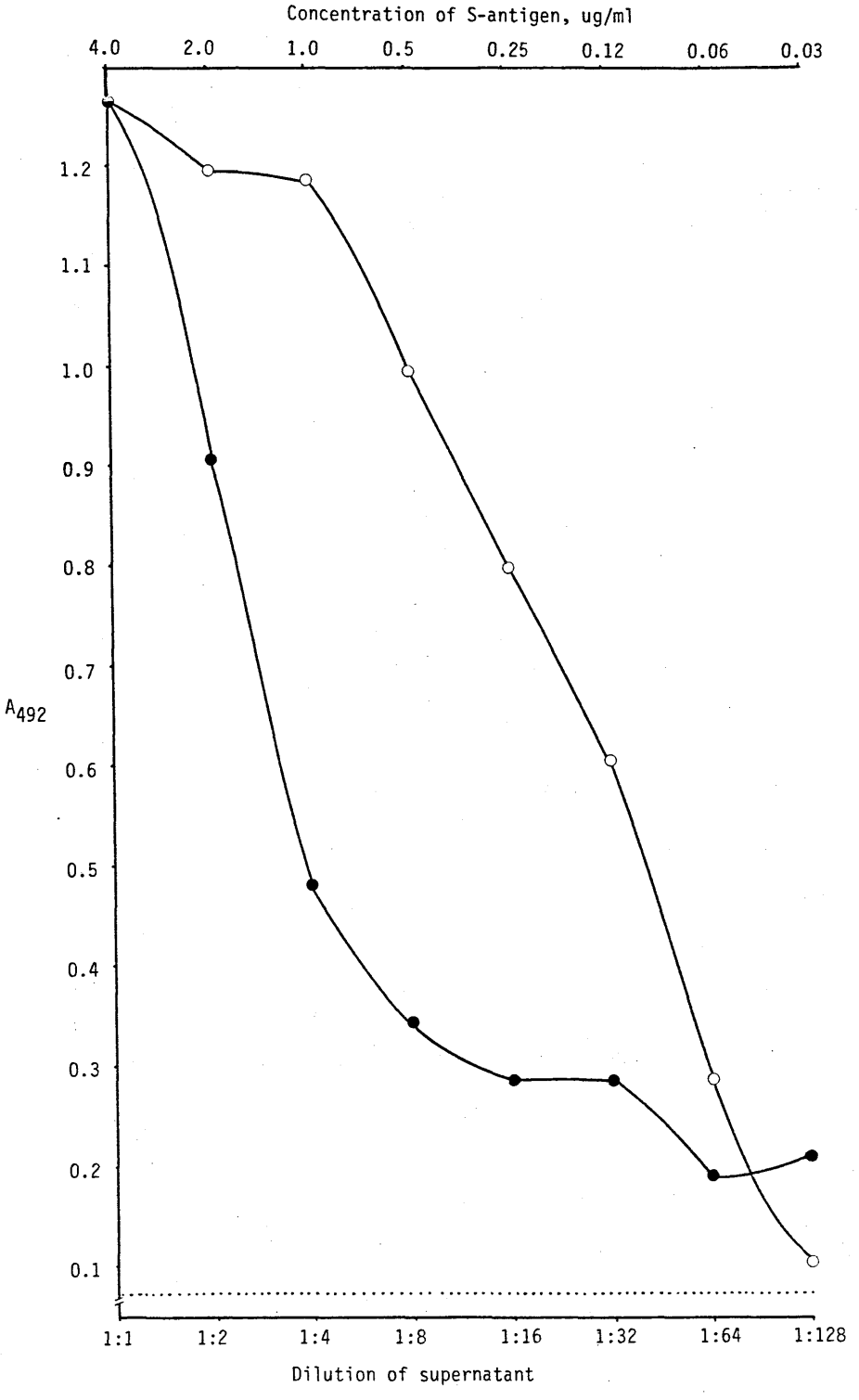

Concentration of S-antigen, ug/ml
4.0
2.0
1.0
0.5
0.25
0.12
0.06
0.03
1.2
1.1
1.0
0.9
0.8
0.7
A492
0.6
0.5
0.4
0.3
0.2
0.1
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
Dilution of supernatant

Figure 6.3 Reaction of Antibody R1.2.D2 to Retinal Antigens in ELISA

The antibody was applied to ELISA plates coated with 5ug/ml of ROS, as cell-free hybridoma supernatant. The titration against ROS was the mean of 5 assays. The incubation time for the primary antibody was 4 hours.

KEY

□ titration of R1.2.D2 against ROS

··· background reaction; no antibody

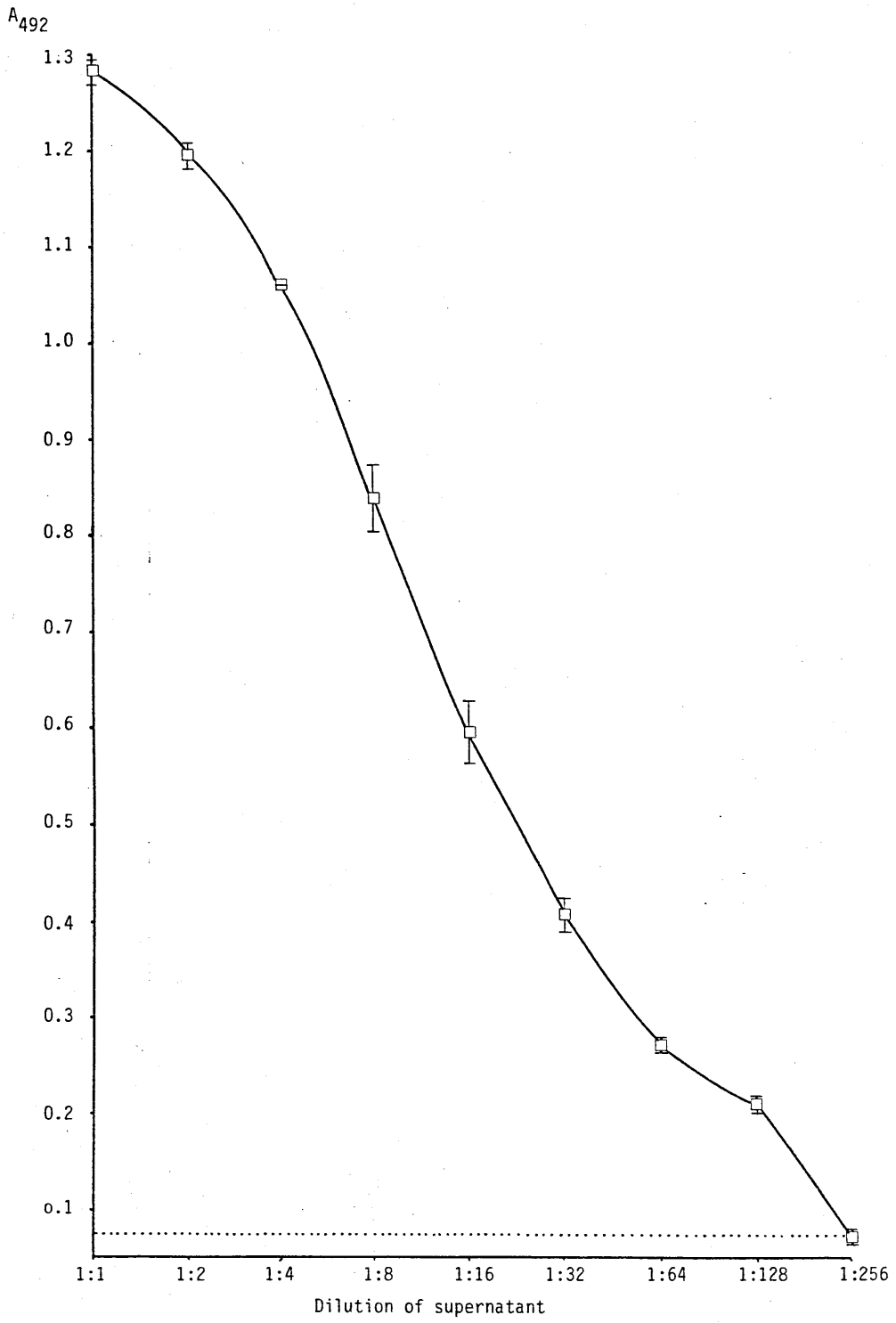

A_{492}
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
Dilution of supernatant

Figure 6.4 Reaction of RCS Rat MAb H9.1.C2 to S-Antigen in ELISA

MAb H9.1.C2 was applied to the ELISA plates as hybridoma supernatant, from which serial dilutions were made. A 10 fold ammonium sulphate concentrate of the antibody was also applied to the plate. The incubation time for the monoclonal antibody was 4 hours.

The monoclonal autoantibody was reactive to bovine S-antigen (1µg/ml) but was unreactive to ROS (5µg/ml). Only a small increase in the intensity of reaction was obtained with the 10 fold concentrate of antibody.

KEY

● titration of H9.1.C2 against S-antigen

○ titration of H9.1.C2 against ROS

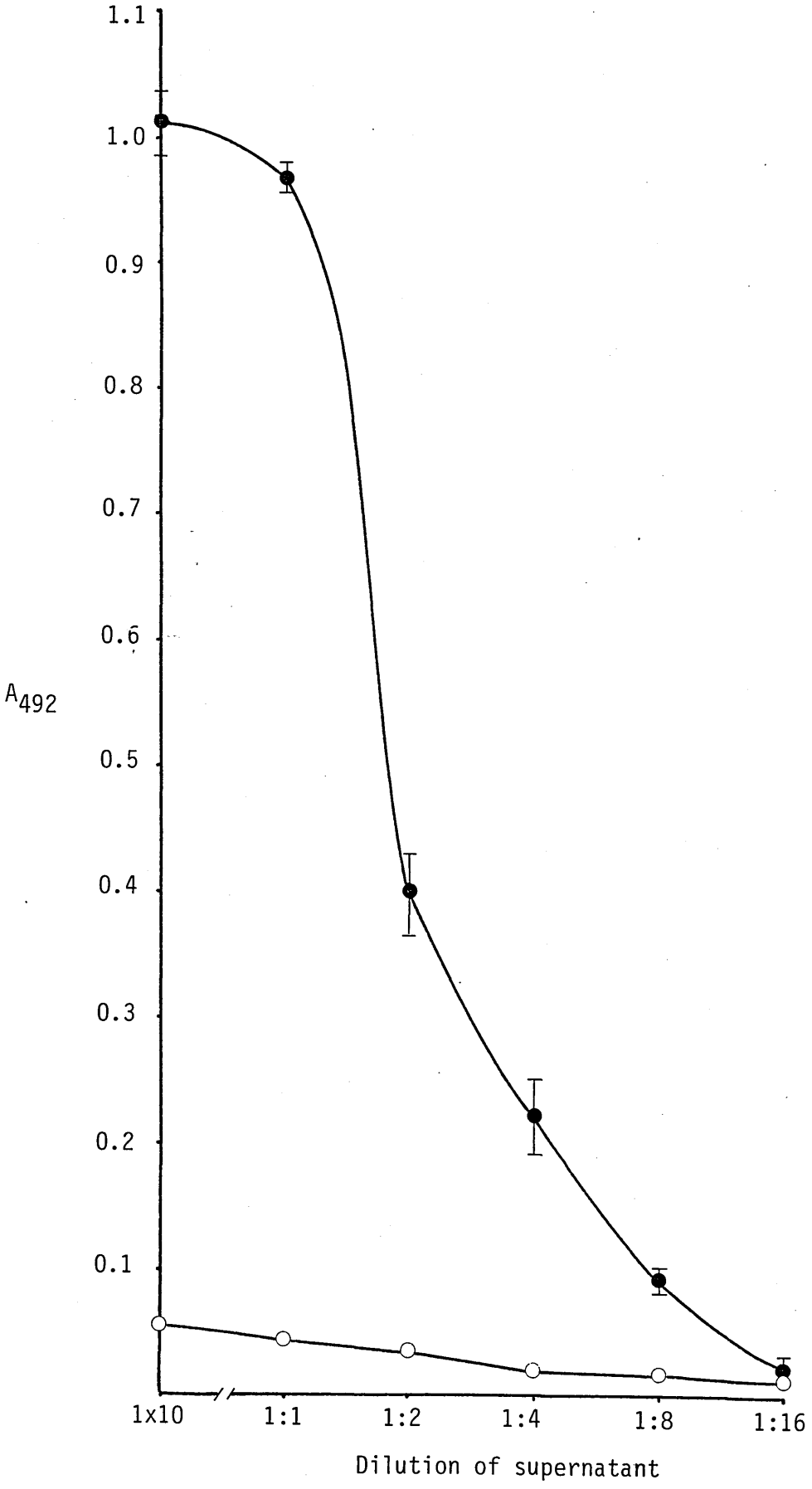

1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A492
1x10
1:1
1:2
1:4
1:8
1:16
Dilution of supernatant

Figure 6.5 Reaction of RCS Rat MAb H9.5.A4 to ROS Antigens in ELISA

MAb H9.5.A4 was applied to the ELISA plates as hybridoma supernatant, from which serial dilutions were made. A 10 fold ammonium sulphate concentrate of the antibody was also applied to the plate. The incubation time for the monoclonal antibody was 4 hours.

The monoclonal autoantibody was reactive to bovine ROS (5µg/ml) but was unreactive to S-antigen (1µg/ml). Only a small increase in the intensity of reaction was obtained with the 10 fold concentrate of antibody.

KEY

□ titration of H9.5.A4 against ROS

■ titration of H9.5.A4 against S-antigen

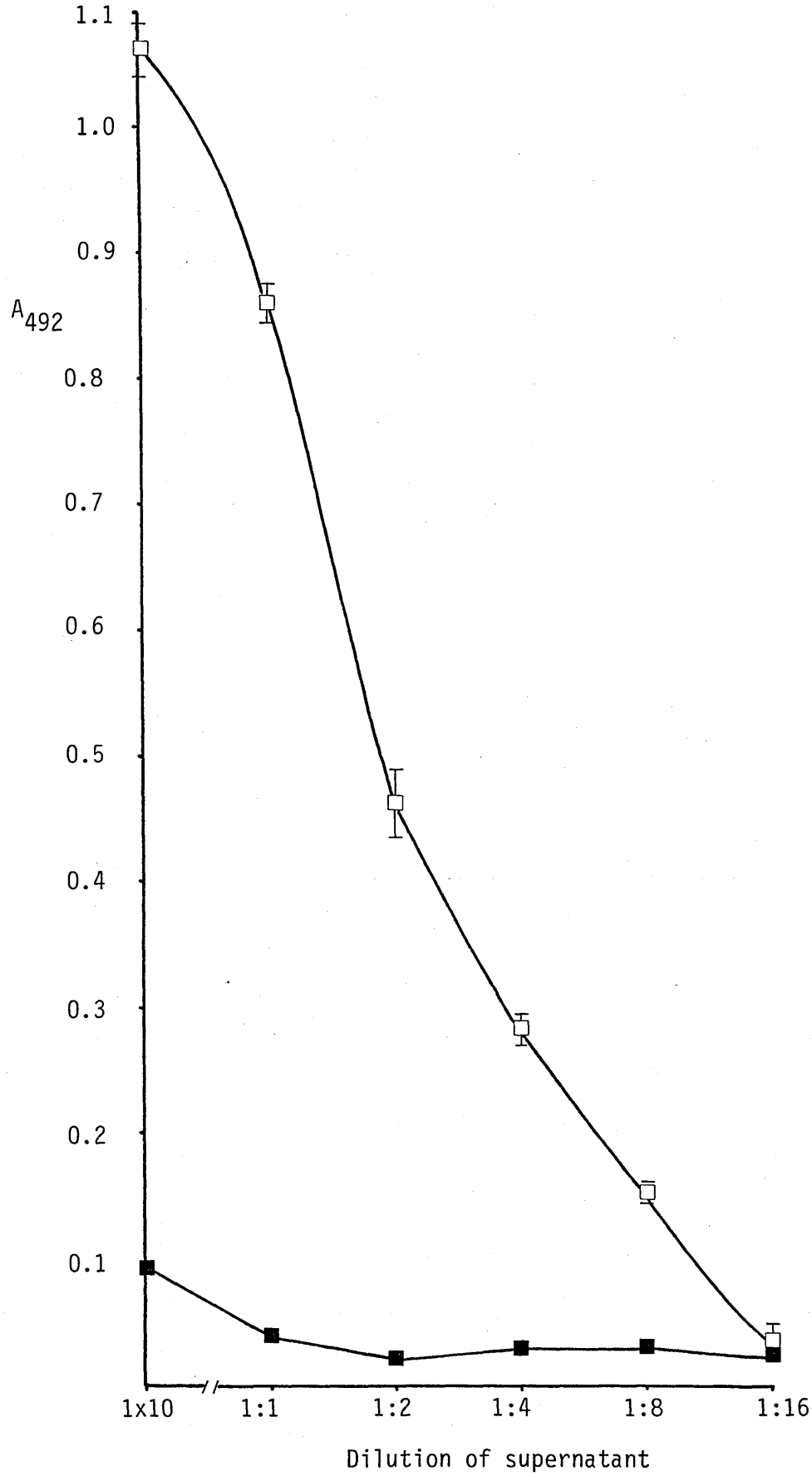

1.1
1.0
0.9
A_{492}
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1x10
1:1
1:2
1:4
1:8
1:16
Dilution of supernatant

Figure 6.6 Reaction of RCS Rat MAb H10.1.D3 Retinal Antigens in ELISA

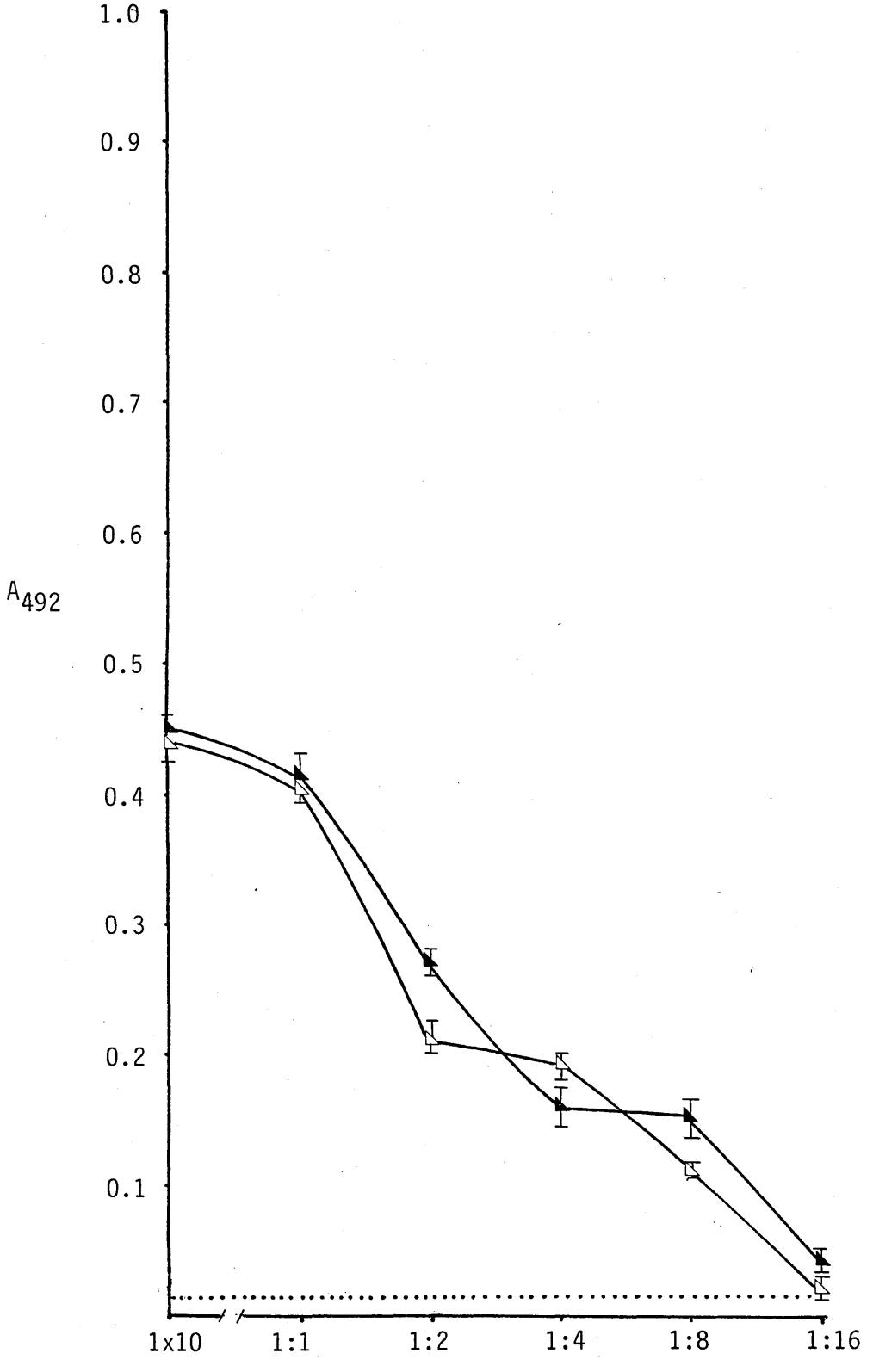
MAb H10.1.D3 was applied to the ELISA plates as hybridoma supernatant, from which serial dilutions were made. A 10 fold ammonium sulphate concentrate of the antibody was also applied to the plate. The incubation time for the monoclonal antibody was 4 hours.

The monoclonal autoantibody demonstrated a relatively weak titre to bovine S-antigen (1µg/ml) and ROS (5µg/ml). Only a small increase in the intensity of reaction was obtained with the 10 fold concentrate of antibody.

KEY

◺ titration of H10.1.D3 against S-antigen

◣ titration of H10.1.D3 against ROS


1.0
0.9
0.8
0.7
0.6
A492
0.5
0.4
0.3
0.2
0.1
1x10
1:1
1:2
1:4
1:8
1:16
Dilution of supernatant

Figures 6.4 to 6.6 include the reactions of the monoclonal autoantibodies in the form of 10 fold concentrates of the hybridoma supernatant. The tenfold increase in the concentration of the antibodies H9.1.C2 and H10.1.D3, resulted in only a relatively small rise in the ELISA reaction (Figures 6.4 and 6.6, respectively). A tenfold concentration of monoclonal antibody R1.2.D2, resulted in a more significant increase in the ELISA reading.

6.3.2 Specificity of the Monoclonal Antibodies in Immunoblots

6.3.2.1 High titre monoclonal antibodies

The specificity of the monoclonal antibodies was investigated in immunoblotting experiments (Batteiger et al., 1982) (Section 2.3.9). When antibody S2.4.C5, from a hyperimmunised animal (Section 2.6.1) was tested in ^{125}I protein A blots, against electrophoretically resolved proteins from complex protein mixtures in addition to purified S-antigen and crude retinal antigens, it was found to be very specific to S-antigen (Figures 6.7 and 6.11a,b).

Figure 6.8b shows a peroxidase enzyme blot (Sections 2.3.9.6,7) for antibody R1.2.D2. The antibody reacted against a band of apparent molecular weight 37K to 39K, present within the crude extract of the retina. In this experiment there was a relatively high background reaction with the other proteins on the nitrocellulose strip. The antibody was apparently unreactive against the opsin protein of the ROS track, and unreactive to the S-antigen. The antibody did, however, react with a band in the region of 39K, in the crude retinal extract. The intense reaction with the 30K protein standard (carbonic anhydrase) which represented the

Figure 6.7 Specificity of MAb S2.4.C5 in Immunoblots against Retinal Antigens

The ^{125}I protein A detection system was employed with the omission of the intermediate rabbit anti-rat IgG, since the protein A was capable of binding to this rat IgG1 MAb. The MAb was applied as the hybridoma supernatant.

a) Amido Black stained blot

b) Autoradiograph of blot with S2.4.C5

LANES

1. Pharmacia low molecular weight standard proteins
2. Bovine S-antigen
3. Crude retinal extract
4. Bovine ROS membrane proteins

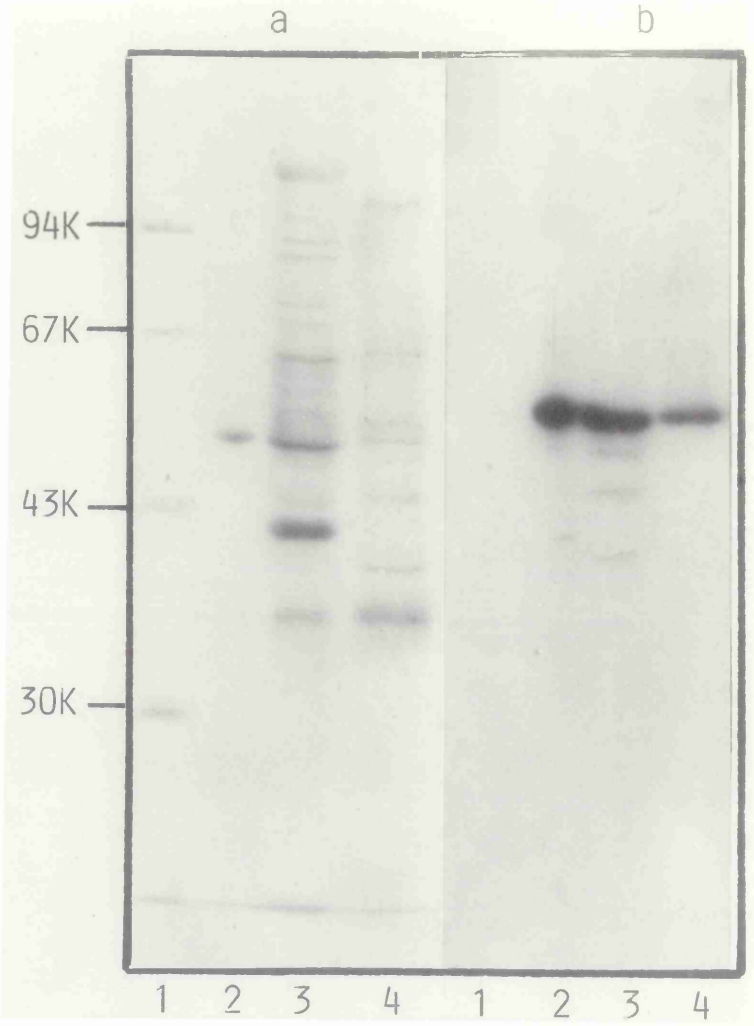

a
b
94K
67K
43K
30K
1 2 3 4 1 2 3 4

Figure 6.8 Specificity of Antibody R1.2.D2 in Immunoblots against Retinal Antigens

The enzyme detection system was employed with the peroxidase conjugated rabbit anti-rat IgG(H+L), and o-dianisidine as a substrate. The antibody was applied as the hybridoma supernatant. An enzyme blot of MAb S2.4.C5 was included for comparison.

There was a relatively high background with the retinal antigens, although the R1.2.D2 antibody appeared to identify with a 39K antigen in the crude retinal extract. The reaction with the 30K standard protein was a common feature in the immunoblots, and was attributed to a nonspecific binding of the rabbit anti-rat antibody.

a) Amido Black stained nitrocellulose strip

b) Enzyme blot with R1.2.D2

c) Enzyme blot with S2.4.C5

LANES

1. Pharmacia low molecular weight standard proteins
2. Bovine S-antigen
3. Crude retinal extract
4. Bovine ROS membrane proteins

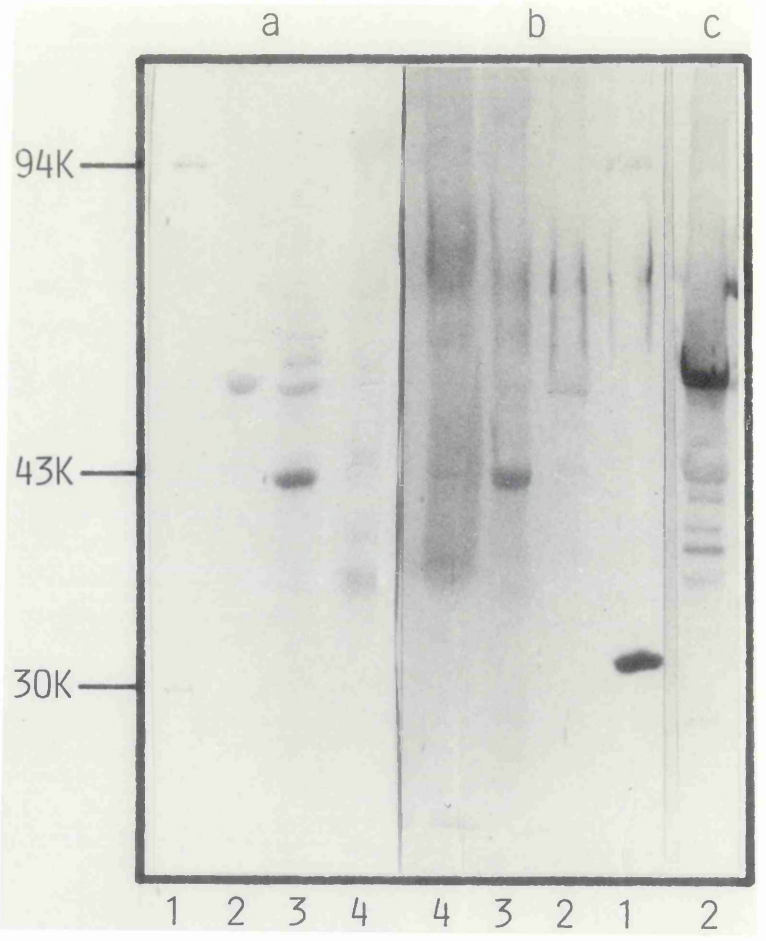

a
b
c
94K
43K
30K
1 2 3 4 4 3 2 1 2

Figure 6.9 Specificity of RCS Rat MAbs in Immunoblots with Retinal Antigens

The I protein A detection system was employed with the use of the intermediate rabbit anti-rat IgG(H+L), since the protein A did not readily bind to rat IgM. The MAbs were applied as hybridoma supernatants. MAb H10.1.D3 appeared to react against S-antigen and opsin. MAb H9.5.A4 was reactive against S-antigen only.

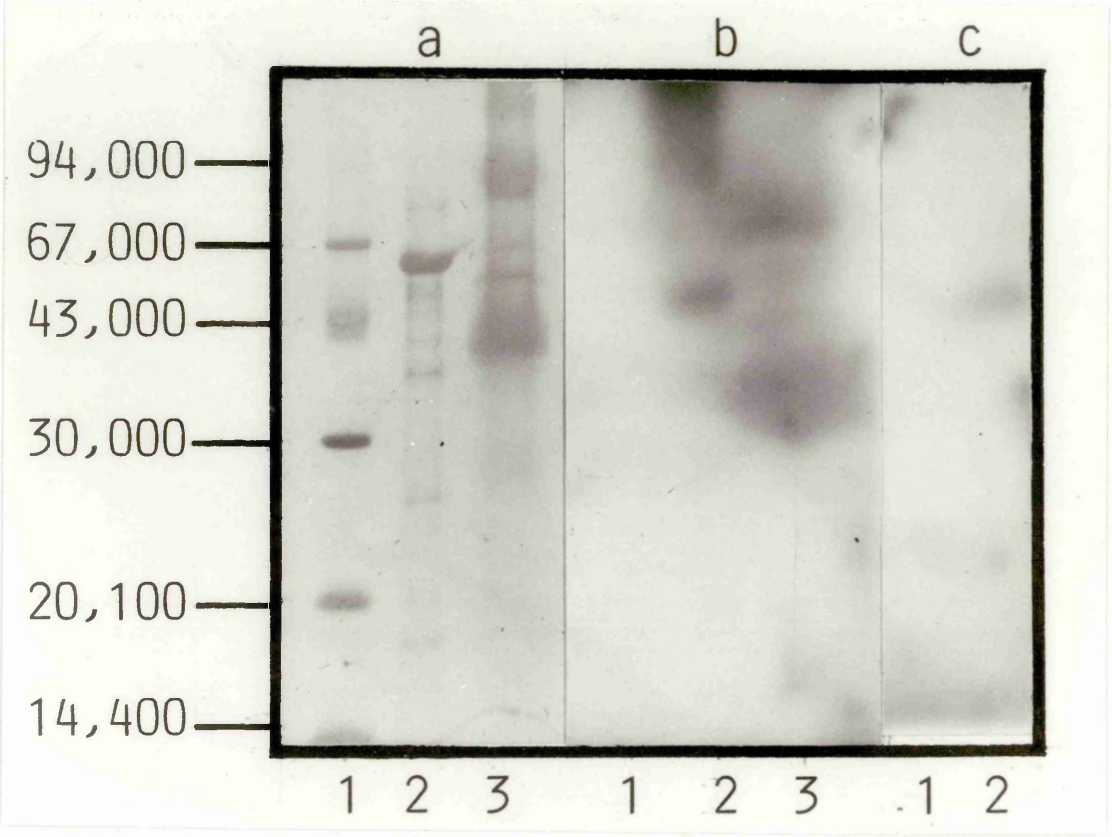
a) Amido Black stained transfer

b) Autoradiograph of blot with H10.1.D3

c) Autoradiograph of blot with H9.1.C2

LANES

1. Pharmacia low molecular weight standard proteins
2. Bovine S-antigen
3. Bovine ROS membrane proteins


a
b
c
94,000
67,000
43,000
30,000
20,100
14,400
1 2 3 1 2 3 1 2

nonspecific reaction of the rabbit anti-rat sera with this protein, was a relatively common feature in the enzyme and protein A blots.

Figure 6.8c shows the intense reaction of monoclonal antibody S2.4.C5, with S-antigen, for comparison with the enzyme blot with antibody R1.2.D2. The stained bands below and above the S-antigen band, almost certainly represent breakdown products of S-antigen and micro-aggregates of S-antigen and its breakdown products. These reactive bands were not detected in the Amido black (Section 2.3.9.2) stained nitrocellulose strips, due to the relative insensitivity of this stain.

6.3.2.2 Monoclonal autoantibodies

The reactions of the monoclonal autoantibodies in ^{125}I protein A immunoblotting experiments (Sections 2.3.9.6,7) were not as intense as that of the S2.4.C5 antibody. Antibody H9.1.C2 appeared to identify S-antigen (Figure 6.9c) and antibody H10.1.D2 cross-blotted with both opsin and S-antigen (Figure 6.9b). The observed crossreactivity may have arisen from the presence of similar carbohydrate residues on S-antigen and opsin, and the epitope recognised by the antibody may have been carbohydrate (Section 8.4.5). H10.1.D2 also recognised a higher molecular weight band in the ROS which probably represented an aggregate of opsin. Monoclonal antibody H9.5.A4 did not react with any of the material on the nitrocellulose paper. Presumably the relevant epitopes were denatured upon SDS-PAGE (Section 2.4.2) and binding to the nitrocellulose paper.

6.3.3 Immunoblotting Controls

The omission controls for the ^{125}I protein A blots of retinal

antigens, are described in Section 3.8.1 (Figure 3.9, Legend). The negative control immunoblots were exposed to X-ray film for the same length of time as the blots with the monoclonals. Normal rat serum used as a negative control, resulted in a negligible reaction. Irrelevant monoclonal antibodies, also employed as negative controls, did not give rise to a detectable reaction. In some enzyme and protein A blots, a nonspecific reaction with the 30K (carbonic anhydrase), and occasionally the 14.4K (α-lactalbumin), molecular weight standard was observed (Figures 6.8b and 4.13, respectively). This could be attributed to the nonspecific reaction of the second antibody, when the primary antibody was omitted.

6.3.3.1 Irrelevant antigens

In addition to being tested against crude retinal antigens, the antisera and monoclonal antibodies, with the only exception being antibody R1.2.D2, were immunoblotted against soluble extracts from various rat tissues (prepared as for the crude retinal extract; Section 3.2.1). This experiment provided a variety of proteins and other antigens for the investigation of the specificity of the antibodies. The samples were not treated with mercaptoethanol in the sample buffer (Section 2.2.5.5) in order to reduce disruption of native epitopes. The ^{125}I protein A detection system was used (Section 2.3.9.6,7).

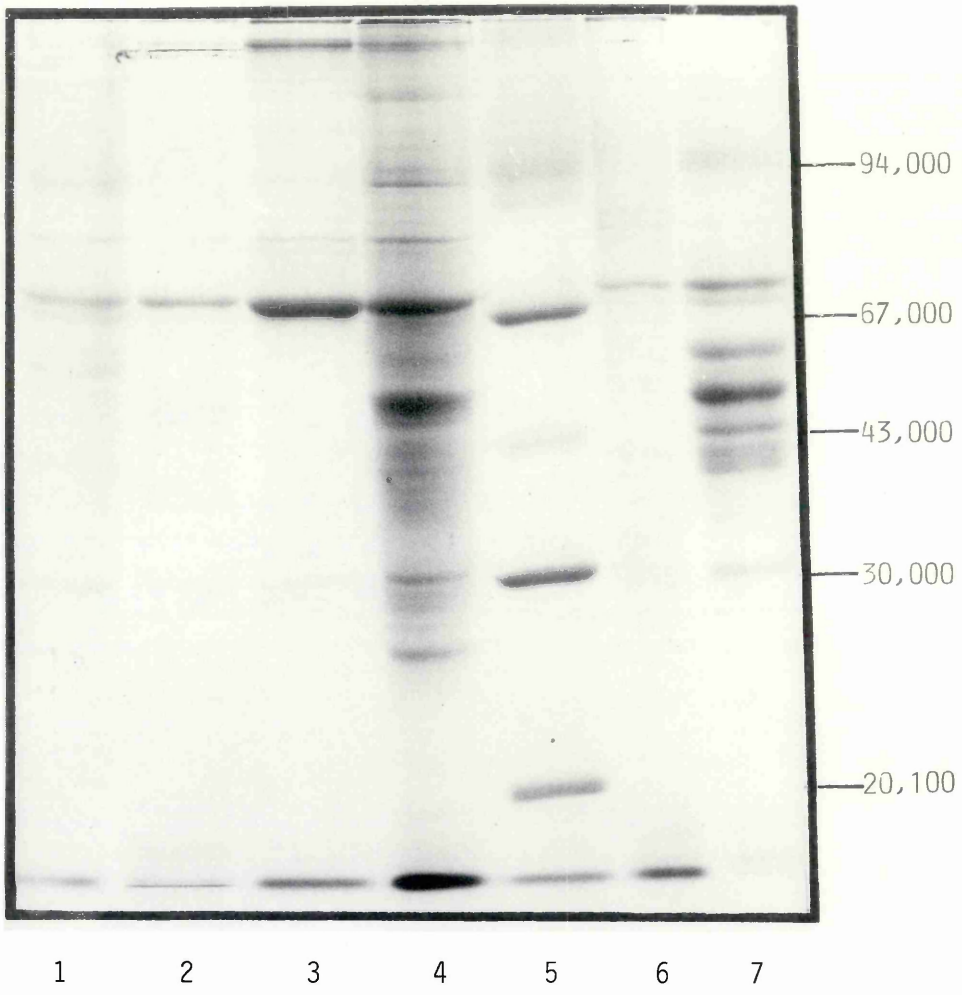
Figure 6.10 shows a Coomassie Blue stained gel of the resolved rat tissue antigens used in the specificity tests. All of the monoclonal antibodies and antisera immunoblotted against these irrelevant antigens appeared to give rise to intense reactions with certain high molecular weight components. Figure 6.11a shows this

Figure 6.10 Coomassie Blue stained SDS Gel of Soluble Extracts obtained from several Rat Tissues

Soluble extracts were taken from several rat tissues in order to provide a wide variety of irrelevant antigens to test the specificity of the monoclonal antibodies in immunoblots (Figures 6.11a,b). The samples were not treated with mercaptoethanol, in order to preserve a greater number of native eptitopes.

LANES

1. Crude soluble extract of brain
2. Crude soluble extract of heart
3. Crude soluble extract of lung
4. Crude soluble extract of liver
5. Pharmacia low molecular weight standards
6. Crude soluble extract of muscle
7. Crude soluble extract of spleen


94,000
67,000
43,000
30,000
20,100
1
2
3
4
5
6
7

reaction for an immunoblot with antibody S2.4.C5. The apparent crossreactions observed were attributed to the reaction of the second antibody, rabbit anti-rat IgG(H+L) (Section 2.1.16), with the rat immunoglobulins present in the crude tissue extracts. This was shown by the omission of the primary antibody (Figure 6.11b). The protein A did not significantly react with this rat tissue immunoglobulin, although it is able to bind to the rat IgG subclasses, with varying efficiency (Rousseaux et al., 1981; Nilsson et al., 1982). It was possible to use the ^{125}I protein A blotting technique, with the rat IgG1 monoclonal antibody, S2.4.C5, without the aid of the rabbit anti-rat second antibody (Figure 6.7).

No reactions, other than those obtained when only the rabbit anti-rat IgG was used, were obtained with any of the monoclonal or polyclonal antibodies tested.

The specificity of monoclonal antibody S2.4.C5 to S-antigen, was further demonstrated when it was immunoblotted against a solubilised extract of eye melanoma, Hela chromatin and Vibrio cholerae LPS. In this experiment, involving labelled protein A, the rabbit anti-rat IgG(H+L) antibody was not used. Monoclonal antibody S2.4.C5, gave rise to no nonspecific reaction with these irrelevant antigens.

Thus, the monoclonal antibodies produced during the course of this work were not found to crossreact with any of the irrelevant antigens tested.

Figure 6.11a Specificity of MAb S2.4.C5 in Immunoblots against irrelevant Rat Antigens

The MAb, in the form of hybridoma supernatant, was incubated with nitrocellulose strips bearing resolved crude antigens extracted from several rat tissues, which had not been treated with mercaptoethanol. The ^{125}I protein A detection system was employed, with the inclusion of the intermediate rabbit anti-rat IgG(H+L).

Intense reactions were observed with certain high molecular weight bands, which did not form major bands in the Coomassie Blue stained gel (Figure 6.10). These reactions were attributed to the reaction of the rabbit anti-rat IgG(H+L) with the endogenous rat immunoglobulin (Figure 6.11b). No additional reactions were obtained.

LANES

1. Purified bovine S-antigen
2. Crude soluble extract of brain
3. Crude soluble extract of heart
4. Crude soluble extract of lung
5. Low molecular weight standards
6. Crude soluble extract of liver
7. Crude soluble extract of spleen
8. Crude soluble extract of muscle
9. Crude soluble extract of muscle

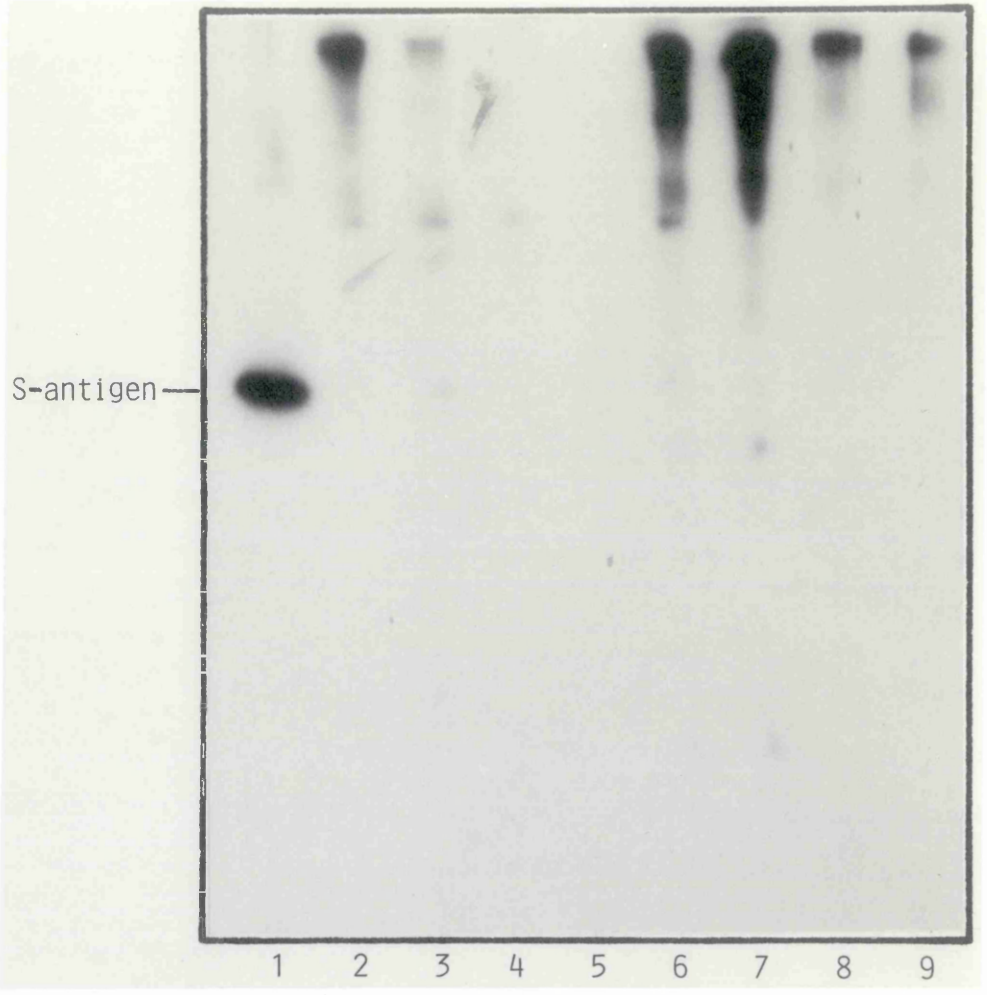

S-antigen
1
2
3
4
5
6
7
8
9

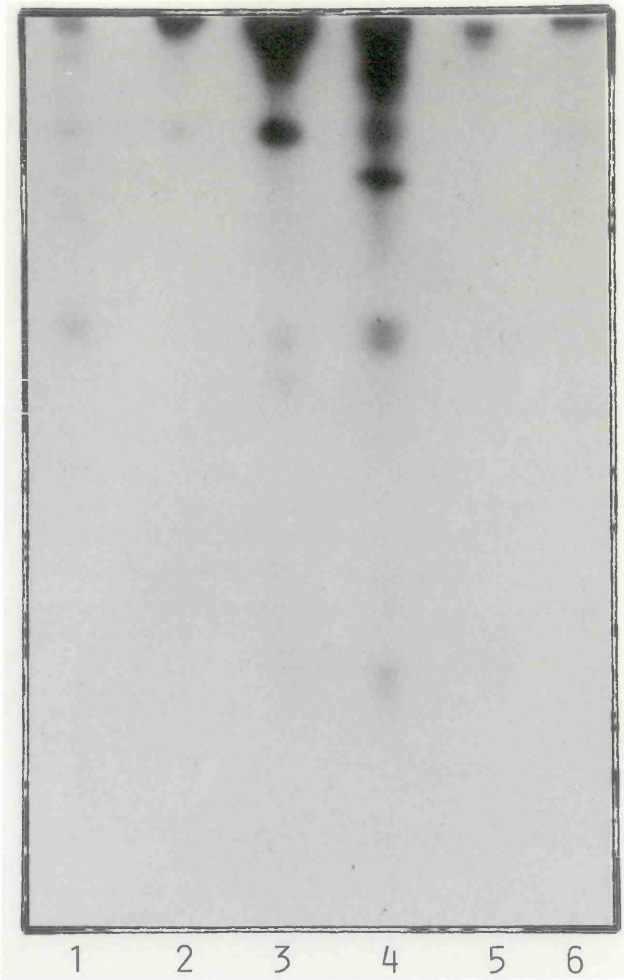
Figure 6.11b Reactivity of Rabbit Anti-rat IgG(H+L) in Protein A Immunoblots against complex Mixtures of Rat Antigens

The rabbit anti-rat IgG(H+L) was incubated with nitrocellulose strips bearing resolved crude antigens extracted from several rat tissues, which had not been treated with mercaptoethanol.

Intense reactions were observed with the same high molecular weight bands, as observed in immunoblots with the monoclonal antibodies (Figure 6.11a). These reactions were attributed to the reaction of the rabbit anti-rat IgG(H+L) with the endogenous rat immunoglobulin. No additional reactions were obtained.

LANES

1. Crude soluble extract of brain
2. Crude soluble extract of heart
3. Crude soluble extract of liver
4. Crude soluble extract of spleen
5. Crude soluble extract of lung
6. Crude soluble extract of muscle


1
2
3
4
5
6

6.4 IMMUNOBLOTTING OF S-ANTIGEN FRAGMENTS WITH MONOCLONAL ANTIBODY S2.4.C5

Immunoblotting experiments were performed with S2.4.C5 against enzymatic digests of S-antigen in order to identify the smallest fragment bearing the S2.4.C5 epitope. Enzymatic digestion was carried out according to the procedure of Cleveland et al. (1977) (Section 2.3.8). The enzymes employed included trypsin, chymotrypsin and Staphylococcus aureus V8 protease (Section 2.1.6). Trypsin cleaves peptides on the carboxyl side of lysine or arginine, and chymotrypsin cleaves on the carboxyl side of aromatic amino acids (eg. tryptophan). Staphylococcus V8 protease cleaves specifically glutamyl, and sometimes aspartyl peptide bonds.

The resolved fragments of S-antigen were electrophoretically transferred to nitrocellulose paper and immunoblotted with monoclonal antibody S2.4.C5 and ^{125}I labelled protein A (Batteiger et al., 1982) (Section 2.3.9). Although the nitrocellulose strips containing the resolved fragments of S-antigen were blocked with 0.5% Tween 20 in the Tris buffer (Section 2.3.9.6), the detergent in the wash buffer was reduced to 0.05% in order to reduce the leaching of protein from the nitrocellulose paper. The loss of protein, in the presence of high Tween concentrations, was shown by the amido black staining of nitrocellulose strips (Section 2.3.9.2), washed with either 0.5% and 0.05% Tween.

The protein A detection system was selected, since by increasing the exposure time of the blot to film, those fragments present in smaller concentrations could be more readily visualised. The denser bands required shorter periods of exposure.

6.4.1 Molecular Weights of Silver stained Peptides of S-Antigen

Figures 6.12A,B show two silver stains of the peptide map obtained for the digestion of S-antigen with trypsin, chymotrypsin and V8 protease. The staining method of Wray et al. (1981) was employed, with 5 minutes longer allowed for the incubation with the silver stain for this 15% gel (Section 2.3.6.2). The Cleveland method of digestion with the three enzymes (Section 2.3.8), was found to generate reproducible patterns of bands and was not significantly affected by the enzyme concentration or time allowed for the digestion. In some gels, a few of the bands or part of the bands were apparently missing (Figures 6.12A,B). This represents an artifact of silver staining.

6.4.2 Molecular Weights of the smallest Fragments bearing the S2.4.C5 Epitope

Reproducible patterns of bands were obtained on the autoradiographs of the Cleveland ^{125}I protein A blots for monoclonal antibody S2.4.C5 (Figure 6.13A). Inspection of several experimental results indicated that all detectable bands bore the S2.4.C5 epitope; no unreactive bands could be identified.

The Rf values were calculated for the Pharmacia low molecular weight standard protein markers, from the gel shown in Figure 6.12A, and a standard curve of molecular weight versus mobility, was plotted (Figure 6.14). The pattern of bands on the autoradiographs, obtained from the ^{125}I protein A blots with monoclonal antibody S2.4.C5, were compared with those on the original blot, stained with Amido black (Figures 6.13A,B). All bands visible on the nitrocellulose paper corresponded to a band on

Figure 6.12 Silver stained SDS 15% Acrylamide Gel of Enzymatic Digests of S-Antigen

Fragments of S-antigen were generated by digestion in SDS acrylamide gels with trypsin, chymotrypsin and V8 protease employing the method of Cleveland et al., (1977), and subjected to sensitive silver staining. Two gels (A and B) are shown since there was an artifactual absence of staining in some regions of the gel.

A wide range of peptides were obtained, the smallest of which were approximately 9.2K to 9.4K.

LANES

V8: Digest with V8 protease (LHS: 50ng enzyme; RHS: 5ng enzyme)

T: Trypsin (LHS: 50ng enzyme; RHS: 5n enzyme)

Ch: Chymotrypsin (LHS: 50ng enzyme; RHS: 5ng enzyme)

LMW: Pharmacia low molecular weight standards

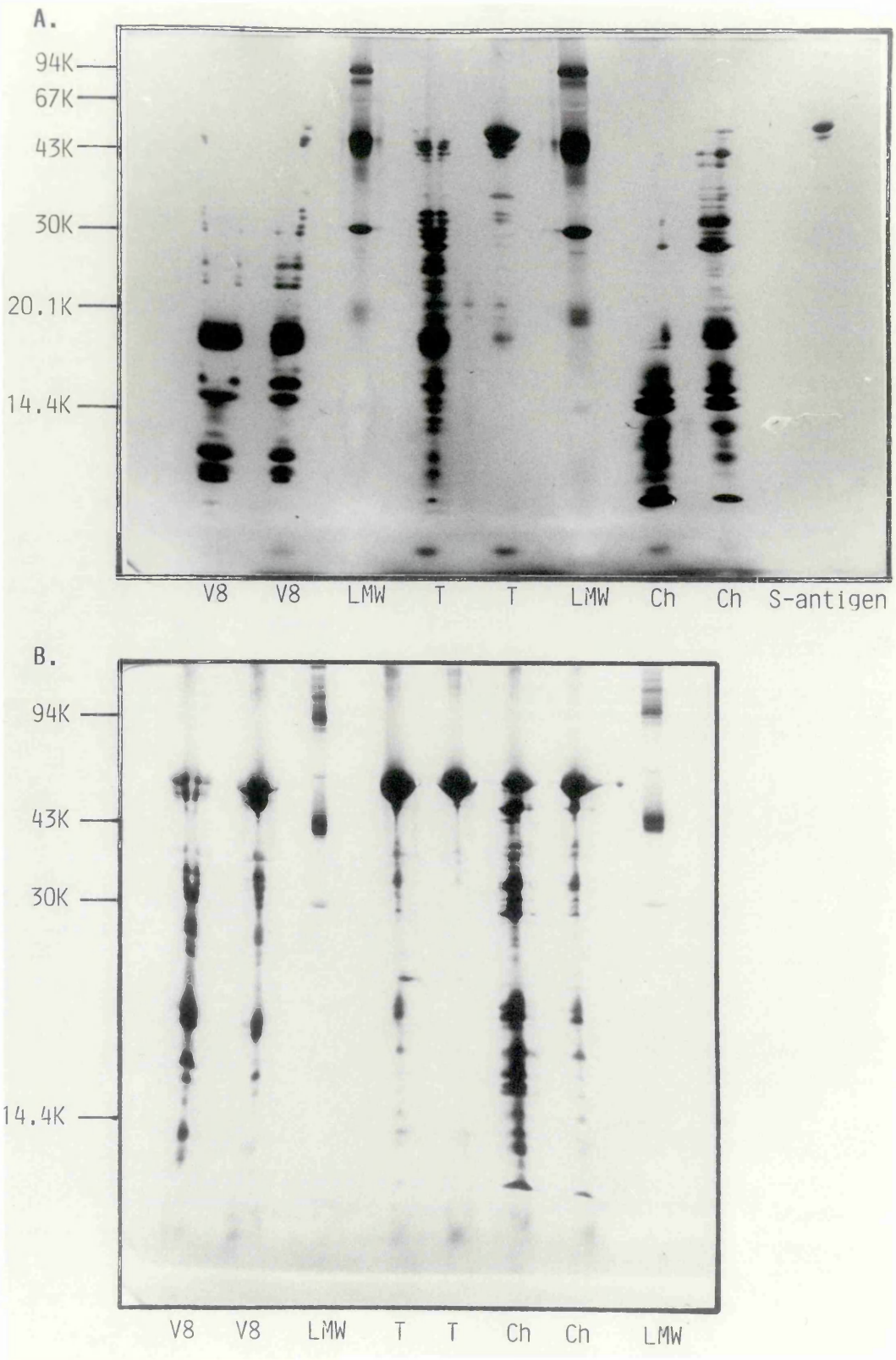

A.
94K
67K
43K
30K
20.1K
14.4K
V8
V8
LMW
T
T
LMW
Ch
Ch
S-antigen
B.
94K
43K
30K
14.4K
V8
V8
LMW
T
T
Ch
Ch
LMW

Figure 6.13 Immunoblot of MAb S2.4.C5 against S-Antigen Fragments

A) Autoradiograph of protein A immunoblot with MAb S2.4.C5

B) Amido black stain of the immunoblot

Fragments of S-antigen were generated by digestion in SDS acrylamide gels with trypsin, chymotrypsin and V8 protease employing the method of Cleveland et al., (1977). The peptides were electrophoretically transferred to nitrocellulose paper and subsequently immunoblotted with MAb S2.4.C5, using the ^{125}I protein A technique.

The MAb appeared to react with all fragments of S-antigen detected on the nitrocellulose paper, the smallest of which were approximately 9.0K to 9.4K (arrows).

LANES

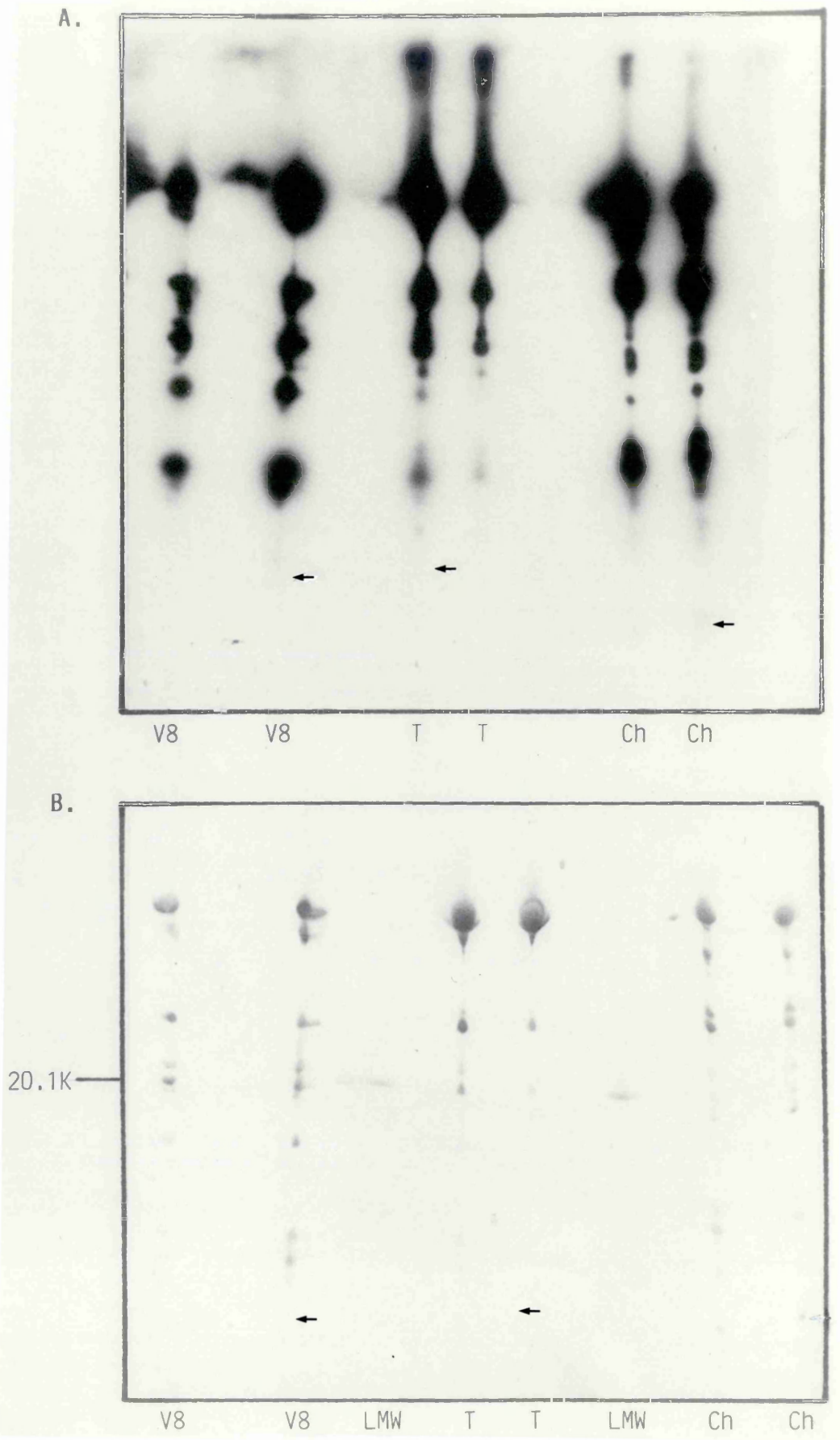
V8: Digest with V8 protease (LHS: 50ng enzyme; RHS: 5ng enzyme)

T: Trypsin (LHS: 50ng enzyme; RHS: 5n enzyme)

Ch: Chymotrypsin (LHS: 50ng enzyme; RHS: 5ng enzyme)

LMW: Pharmacia low molecular weight standards

← Smallest peptides obtained; reactive with MAb S2.4.C5.


A.
V8
V8
T
T
Ch
Ch
B.
20.1K
V8
V8
LMW
T
T
LMW
Ch
Ch

the autoradiograph. The bands visible on the nitrocellulose paper were compared with those of the silver stained Cleveland gels (Figures 6.12A,B). From this comparison, it was possible to identify the smallest detectable fragments bearing the S2.4.C5 epitope, obtained by this method. The comparison also suggested that there were no obvious unreactive bands.

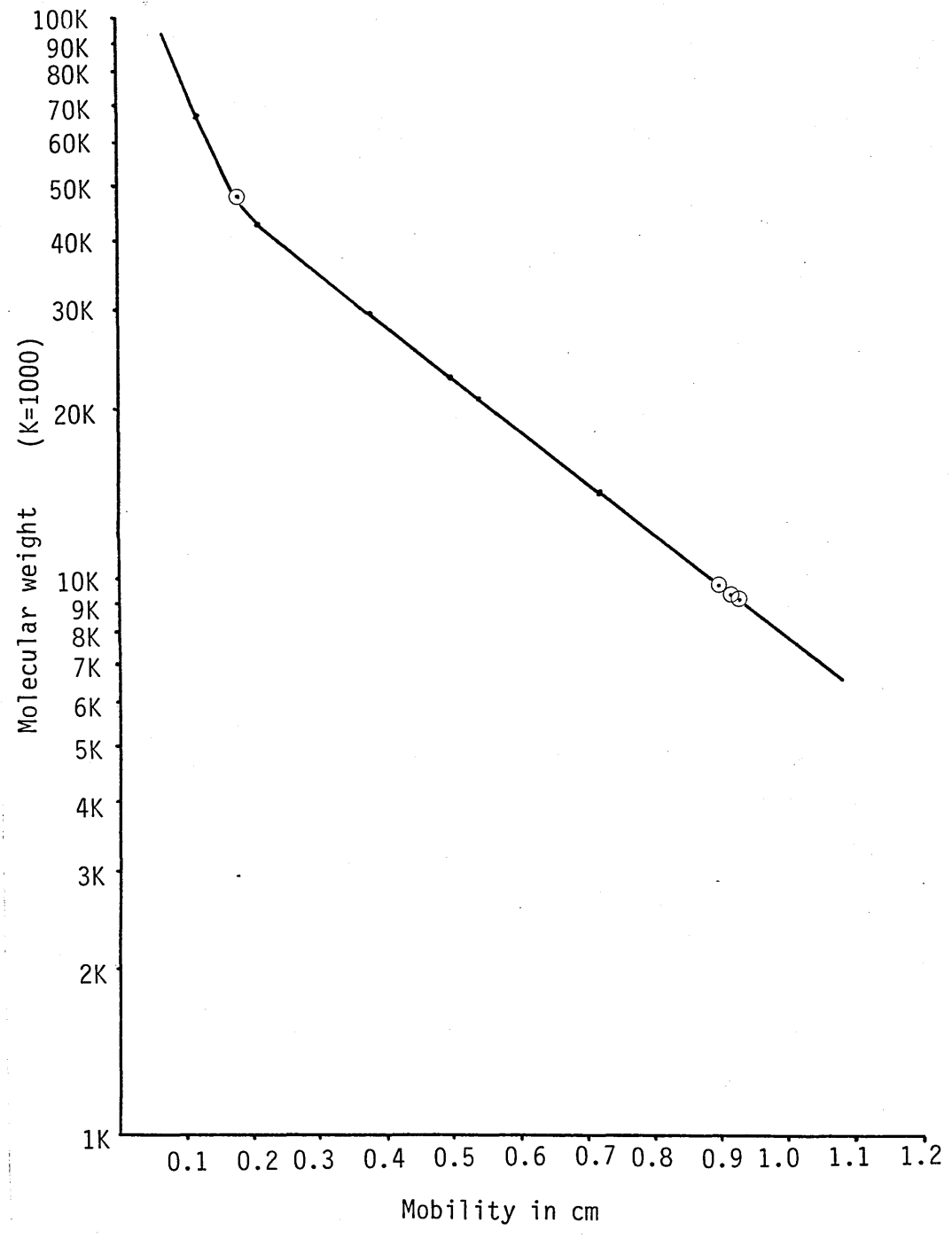
The smallest fragments of S-antigen, produced by digestion with the three enzymes, which gave detectable reactions in the blots with S2.4.C5, are indicated by arrows in Figure 6.13A,B. The apparent molecular weight of the smallest fragment bearing the S2.4.C5 epitope, in the trypsin digest, was calculated using the standard curve shown in Figure 6.14, to be approximately 9.3K (Figures 6.12, 6.13). The smallest fragment produced by digestion with V8 protease was apparently 9.2K to 9.4K (Figures 6.12, 6.13 and 6.14). The smallest peptide of S-antigen shown in the chymotrypsin digest in Figure 6.12, had a molecular weight in the region of 9.6K. However, in the Cleveland blot, a fragment of less than 9K could be detected in the chymotrypsin digest. Fragments smaller than these could not be detected in the silver stained gels, and could not be generated upon increasing the concentration of the enzymes, or by increasing the time or temperature for the digestion. Smaller fragments could not be produced by the digestion of S-antigen with mixtures of the three enzymes, in every combination.

The apparent absence of unreactive bands might imply that the S2.4.C5 epitope was unaffected by cleavage by the enzymes, and that the cleavage sites did not form a significant part of the S2.4.C5 epitope. However, a peptide of 9K indicates the presence of about 50 amino acids. It seems unlikely that the cleavage sites for the

Figure 6.14 Standard Curve for Molecular Weight determination of the smallest S-Antigen Fragments bearing the S2.4.C5 Epitope

The molecular weights of the smallest epitopes bearing the S2.4.C5 epitope were estimated from a standard curve of the mobility versus molecular weight of the Pharmacia low molecular protein markers. Since the smallest marker protein was 14K, the curve was lengthened by extrapolation.

The molecular weights of the smallest S-antigen peptides obtained by enzymatic digestion, were in the region of 9.4K to 9.2K, and are indicated by circles on the standard curve. The molecular weight of S-antigen was also determined by this method; giving a molecular weight of 48K (indicated by a circle on the standard curve).


100K
90K
80K
70K
60K
50K
40K
30K
20K
10K
9K
8K
7K
6K
5K
4K
3K
2K
1K
Molecular weight (K=1000)
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1 1.2
Mobility in cm

three enzymes were absent in this region. It is possible that smaller peptides travelled past the end of the gel during electrophoresis. In addition, the very small peptides are likely to travel through the nitrocellulose paper during electro-transfer. It is also possible that complete digestion was not obtained within the gel, under the conditions employed for the digestion (Section 2.3.8.2).

6.4.3 Comparison with Rat Polyclonal Antisera

Three rat polyclonal antisera were blotted against the S-antigen fragments for a comparison with the monoclonal antibody (Figures 6.15a,b,c). The smallest bands giving rise to a detectable reaction in the ^{125}I protein A blots were apparently of a larger molecular weight than those identified in the blots with monoclonal antibody, S2.4.C5 (Section 6.4.2). Although the antisera employed may not have recognised the epitope presented on the 9.2K to 9.7K fragments, it is possible that this was a reflection of the dilution (1:200) of the antisera.

Cleveland blotting experiments with antisera can yield useful information, which cannot be obtained from a monoclonal antibody alone. Antisera from different individuals contain their own repertoire of antibodies, with differing isotypes, specificities and affinities. The use of polyclonal antisera could aid the identification of peptides containing the dominant antigenic determinants. Antisera raised in different species are likely to recognise a different pattern of determinants. Cleveland blotting experiments with the rat and human autoimmune sera could yield important information on the epitopes of S-antigen involved in the induction of the humoral immune respone observed in certain

Figure 6.15 Reaction of Rat Polyclonal Antisera to S-Antigen in Cleveland Blots

a) Autoradiograph of immunoblot with rat antiserum S1

b) Autoradiograph of immunoblot with rat antiserum S2

c) Autoradiograph of immunoblot with rat antiserum S3

Three rat polyclonal antisera raised against S-antigen were tested for their reactivity in Cleveland immunoblots, with fragments of S-antigen generated by digestion in SDS acrylamide gels with 50ng of trypsin, chymotrypsin and V8 protease. The ^{125}I protein A detection system was employed with the second antibody, rabbit anti-rat IgG(H+L).

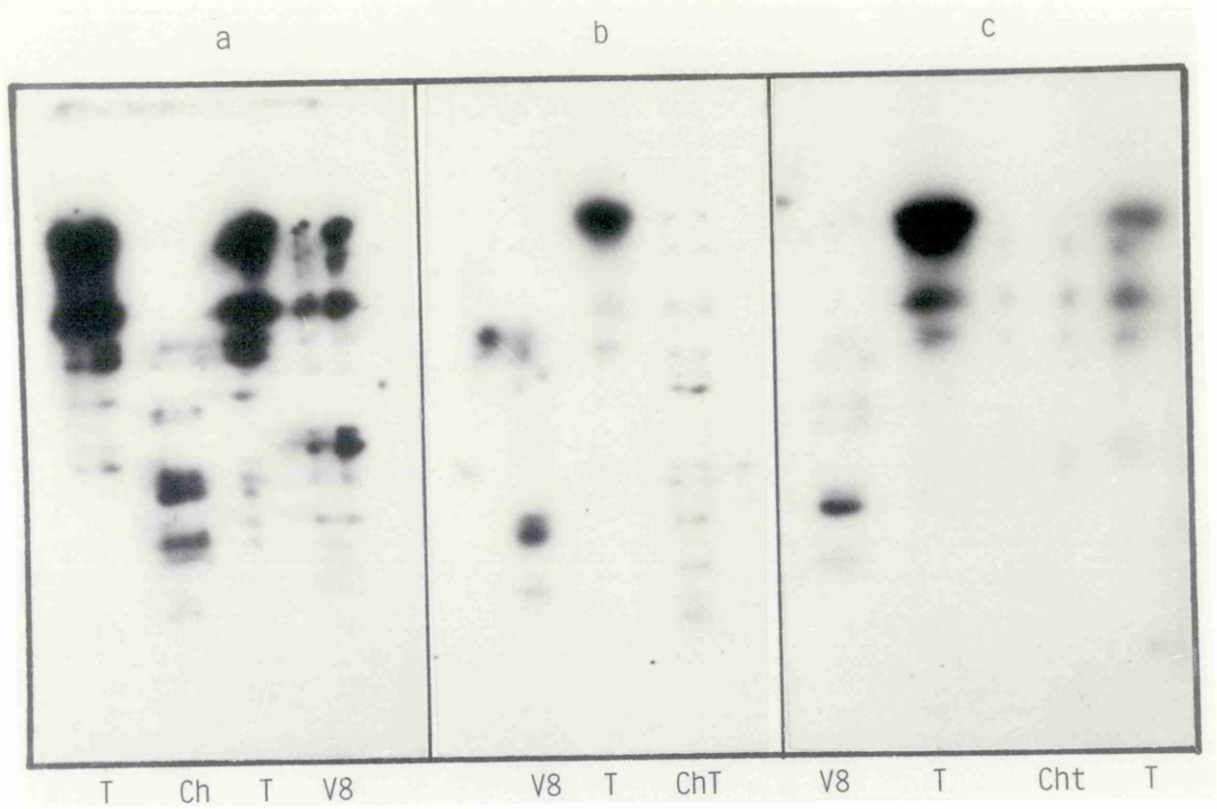
The polyclonal antisera, at a 1:400 dilution, appeared to react with all fragments of S-antigen, visible on the nitrocellulose paper, the smallest of which was approximately 9.2K to 9.7K.

LANES

V8: Digest with V8 protease (50ng enzyme)

T: Trypsin (50ng enzyme)

Ch: Chymotrypsin (50ng enzyme)


a
b
c
T
Ch
T
V8
V8
T
ChT
V8
T
Cht
T

individuals (Chapter 4). In addition, certain autoantibodies may be found to identify minor epitopes of antigens not 'seen' by hyperimmune sera, where the antibodies tend to react with the dominant determinants.

6.5 PURIFICATION OF MONOCLONAL ANTIBODY S2.4.C5

Purified rat IgG1 monoclonal antibody S2.4.C5 was required for experiments investigating its effect on the experimental autoimmune uveitis (EAU) in rodents (Section 6.6).

6.5.1 Production of Monoclonal Antibody S2.4.C5 in Serum-free Media

The hybrids secreting monoclonal antibody S2.4.C5 were grown in complete medium containing 20% foetal calf serum (FCS), and were weaned through 10% to 5% FCS (Section 2.2.7.1). A volume of 600ml containing 10 to 10 cells per ml, was centrifuged at 200g to pellet the cells. The cell pellets were resuspended and pooled before washing twice with RPMI (Section 2.1.20). Finally, the cells were resupended in RPMI and placed in a spinner culture flask (Section 2.6.10) in a total volume of 300 ml of RPMI, supplemented with glutamine (Section 2.1.20). The volume of RPMI was half that of the original volume since the hybrids secrete much lower quantities of antibody in the absence of FCS, and the cells could only be grown in RPMI without FCS for short periods of time.

After 48 hours, the cell culture supernatant was harvested and the antibody precipitated by the addition of ammonium sulphate (adjusted to pH 7.2 with ammonia), and dialysed against PBS (Section 2.2.4.1) using a similar method for the precipitation of the soluble retinal proteins (Section 3.2.1). Only a minute pellet

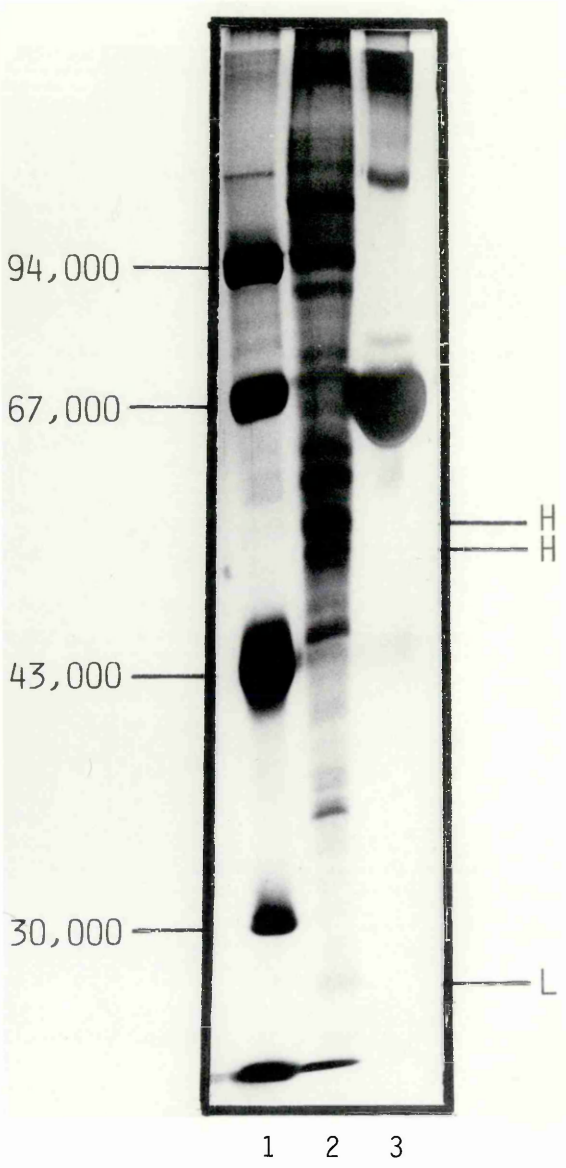
Figure 6.16 Silver stained SDS Gel of a Preparation of MAb S2.4.C5 from Serum-Free RPMI

MAb S2.4.C5 was produced by hybrids grown in serum free RPMI, ammonium sulphate concentrated, dialysed, and subject to SDS-PAGE with silver staining as a criterion for purity.

The results indicate that the monoclonal antibody produced by this method was not pure. Some of the contaminating bands may represent partially re-associated immunoglobulin chains (Section 6.5). The bands which are likely represent the heavy and light chains are indicated with H (molecular weights of 47.8K and 55K) and L (27K), respectively. There may be more than one variation of each chain (Section 6.5).

LANES

1. Pharmacia low molecular weight standards
2. MAb S2.4.C5, produced in RPMI
3. BSA


94,000
67,000
43,000
30,000
H
H
L
1
2
3

of precipitated protein was obtained when compared with that obtained for monoclonal antibody produced in the presence of FCS. The vast majority of precipitated protein obtained from the latter procedure was derived from the FCS.

Figure 6.16 shows a silver stained SDS polyacrylamide gel of RPMI produced S2.4.C5. Although the majority of the BSA from the FCS, was absent, there was a large number of contaminating bands. Some of these may represent proteins released by dying hybrids, although others could represent the partially re-associated heavy and light IgG monoclonal antibody. At least two bands, one of 47.8K and another of 55K, could represent the heavy chain of the rat IgG1, each expressing a differing extent of glycosylation. Only one band was visible in the region of 27K, indicating that there may be only one light chain type. The presence of the large number of contaminating bands indicated that, although this method of obtaining BSA free antibody may be suitable for some purposes, the preparation was unsuitable for the in vivo studies (Section 6.6).

6.5.2 Purification of S2.4.C5 by Ion-exchange Chromatography

The QAE-Sephadex anion exchanger (Section 2.1.3), frequently utilised for the purification of serum IgG, was employed. Monoclonal antibody S2.4.C5, was produced in medium in which the FCS was reduced to 5%. The column gel was equilibrated with 0.1M Tris/HCl, pH 6.5, and the concentrated antibody was dialysed against the same buffer. At this pH, the BSA bound to the gel and the IgG monoclonal passed through. The elution of the monoclonal antibody was followed by measuring the total protein of the column fractions by reading the absorbance at 280nm and by assay in ELISA plates coated with 1µg/ml of S-antigen (Sections 2.3.1 and 3.8.3)

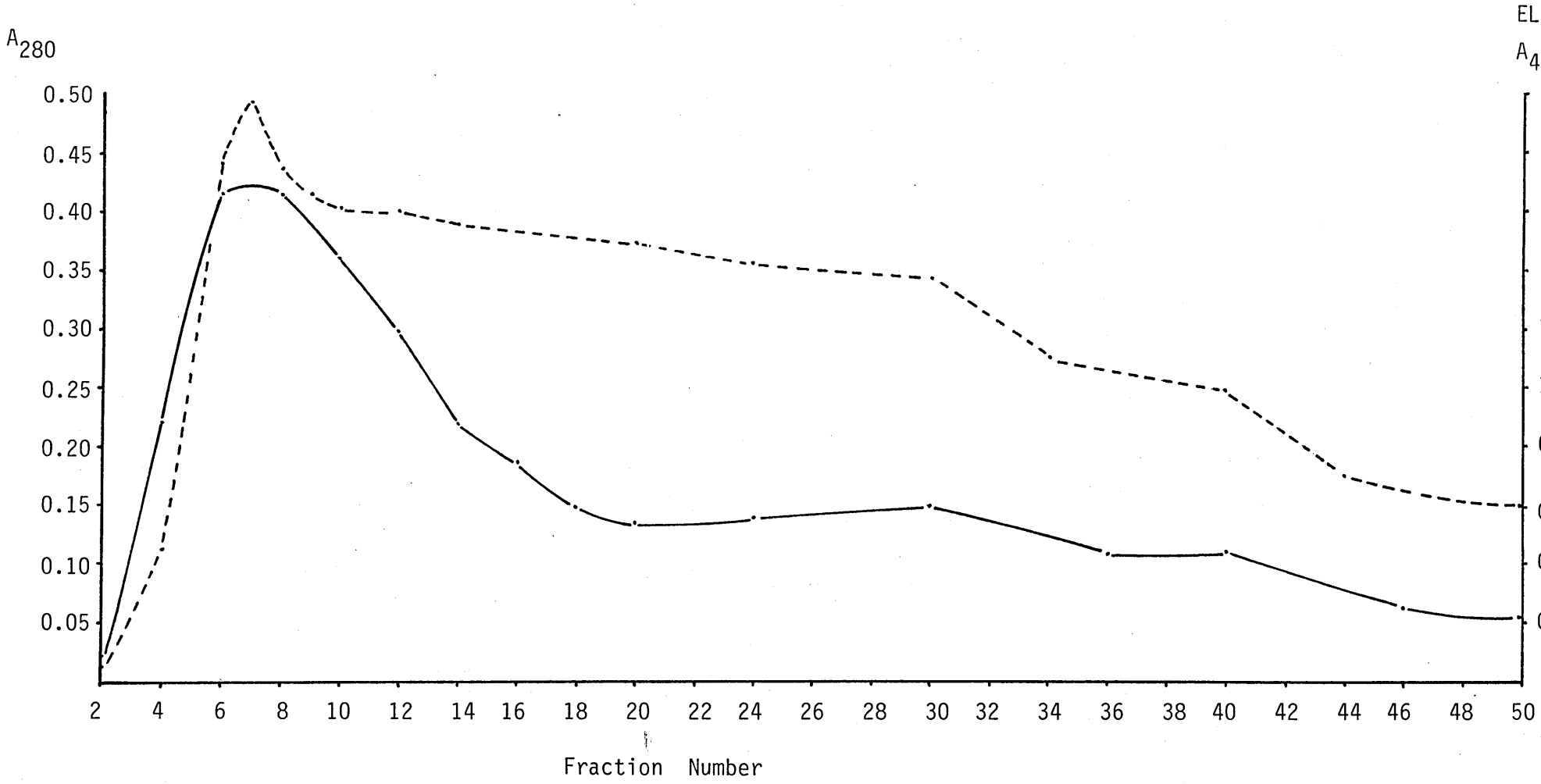
Figure 6.17 Purification of MAb S2.4.C5 on QAE-Sephadex

MAb S2.4.C5 was purified by ion exchange chromatography on a QAE-Sephadex column (Section 6.5.2). The absorbance of the column fractions were measured at 280nm, to give an indication of protein concentration. The column fractions were assayed in the ELISA against 1µg/ml S-antigen.

KEY

--- ELISA activity with S-antigen

— protein concentration (A280)


A280
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50
Fraction Number

(Figure 6.17). The purity of the fractions and the relative concentration of monoclonal antibody and other proteins was assessed by SDS-PAGE followed by silver staining (Sections 2.3.4,6) (Figure 6.18).

From the elution profile for the purification of this monoclonal antibody, it can be seen that no sharp peak was obtained for S2.4.C5 (Figure 6.17). The antibody could be detected throughout the column fractions both in the ELISA (Figure 6.17) and upon silver staining of SDS gels of samples of the fractions (Figure 6.18). Two bands were identified, one of 50K and the other, 55K. Either, or both bands may represent the IgG1 heavy (gamma) chain; the second band possessing a different amount of carbohydrate. A single band in the region of 29K was likely to represent the light chain. Very little BSA could be detected in the column fractions in the silver stained gel. The major contaminants consisted of a 105K and a 148K band. The 105K band could represent two re-associated heavy chains, and the 148K band might be the whole IgG monoclonal antibody (Figure 6.18). Although 2-mercaptoethanol was present in the sample buffer (Section 2.2.5.5), and the samples were boiled, immunoglobulins are prone to re-association upon cooling, even in the presence of the mercaptoethanol.

Pooled column fractions were solvent precipitated with 66.6% (v/v) ethanol, and the protein pellets were collected by centrifugation after an incubation at -20°C, for 48 hours. The protein was lyophilised, dissolved in the Tris buffer, pH 6.5, and re-purified through a smaller QAE column in order to remove most of the remaining BSA. The monoclonal antibody was again concentrated and lyophilised before finally being dissolved in distilled water, to

Figure 6.18 Silver stained SDS Gel of MAb, S2.4.C5, Purified on a QAE-Sephadex Column

MAb S2.4.C5 was concentrated from hybridoma supernatant containing FCS dialysed, and purified on a QAE-Sephadex column. The column fractions were subjected to SDS-PAGE, with silver staining as a criterian for purity.

The results indicate that the monoclonal antibody produced by this method was not pure. Some of the contaminating bands might represent partially re-associated immunoglobulin chains (Section 6.5). The bands which are likely represent the heavy and light chains are indicated with an H (molecular weight of 50K and 55K) and L (29K), respectively. There may be more than one variation of each chain (Section 6.5).

LANES

1 - 13. Samples from column fractions

S: Partially purified S-antigen (48K)

LMW: Pharmacia low molecular weight standards

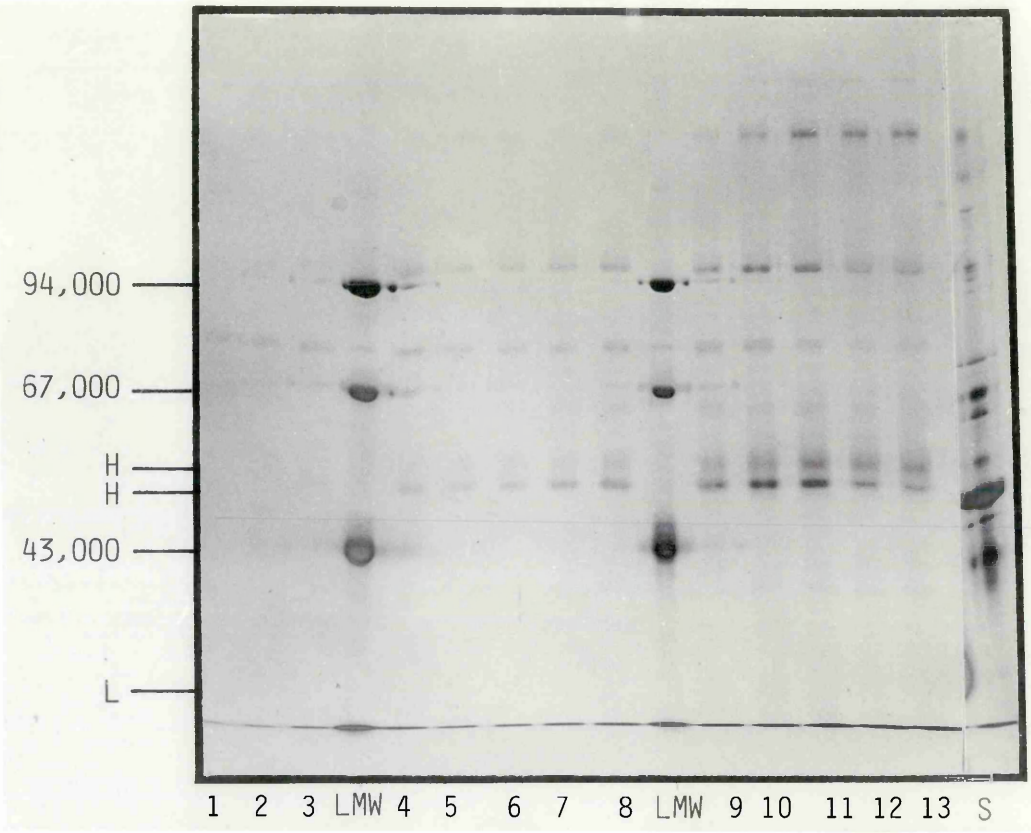

94,000
67,000
H
H
43,000
L
1 2 3 LMW 4 5 6 7 8 LMW 9 10 11 12 13 S

Figure 6.19 Silver stained SDS Gel of concentrated Purified S2.4.C5

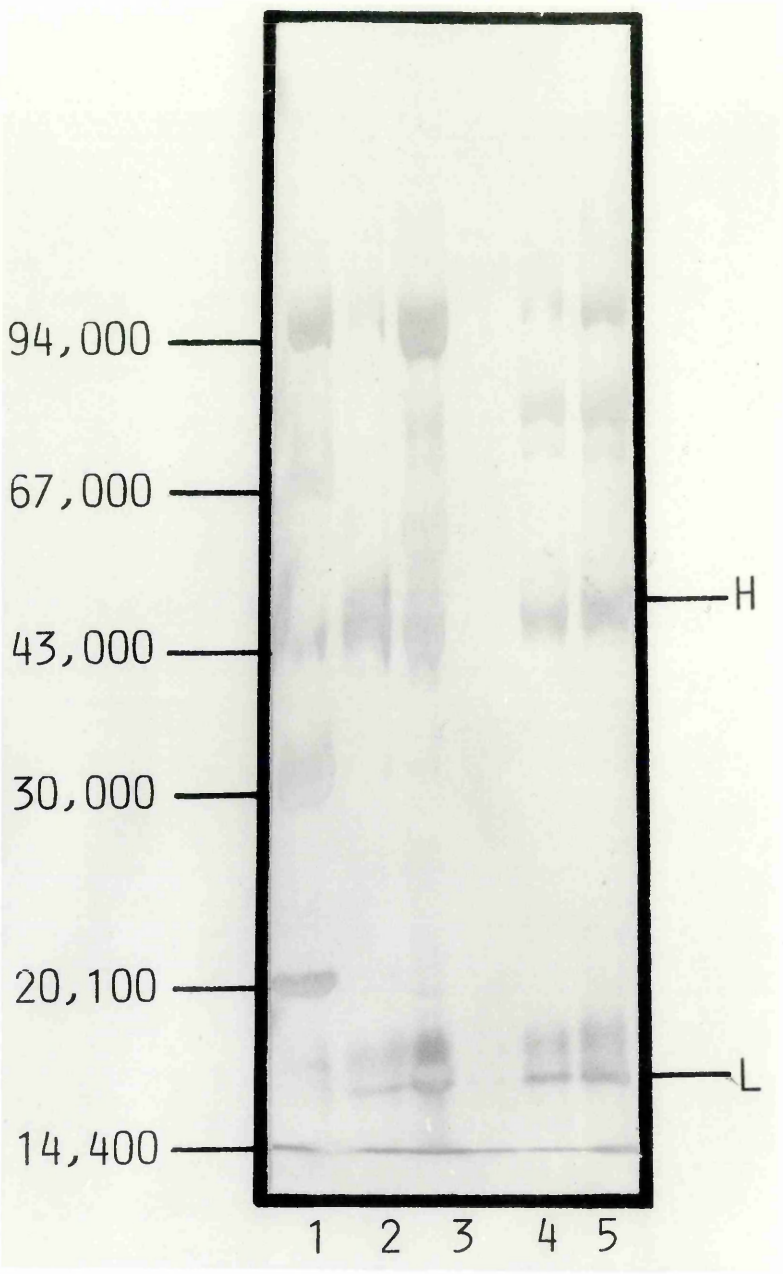
Pooled fractions containing MAb S2.4.C5, after ion-exchange chromatography on a QAE-Sephadex column, were lyophilised and subjected to SDS-PAGE with silver staining.

The band in the region of 50K is likely to represent the heavy (H) chain. Bands which may be light (L) chain are indicated in the region of 18K. There may be more than one variation of each chain (Section 6.5).

LANES

1. Pharmacia low molecular weight standards

2 to 5. Pooled column fractions of S2.4.C5


94,000
67,000
43,000
30,000
20,100
14,400
H
L
1 2 3 4 5

give a final protein concentration of 1mg/ml. The antibody was dialysed against distilled water, mixed 1:1 with glycerol and stored at -20°C before its injection into experimental animals.

Figure 6.19 shows a silver stained SDS polyacrylamide gel of QAE-Sephadex purified, and lyophilised S2.4.C5. The streaking of the bands in the gel probably resulted from the presence of a large amount of Tris after lyophilisation and before its removal by dialysis. BSA was virtually absent, and the major contaminating bands were at 72K and 100K. The 72K band could represent an associated heavy and light chain, and the 100K band could indicate the presence of two associated heavy chains (Figure 6.19). In addition, there appeared to be a single heavy chain, with an apparent molecular weight of 48K. There were two bands, one at 17K and the other at approximately 18K, which are unlikely to be light chains, since light chains usually have molecular weight in the region of 25K.

There appeared to be two heavy chains in some preparations of the antibody (Figure 6.16 and 6.18) and possibly only one in others (Figure 6.19). The second apparent band of the heavy chain could represent contaminating proteins in the preparation. The absence of a second band, in some gels, might indicate that the two forms of the heavy chain, exhibiting differing elution properties during the purification. Alternatively, it may represent an artifact of the silver staining. Internal labelling of the monoclonal antibody with 35S methionine followed by immunoprecipitation, might have helped to answer this question.

Figure 6.20 shows a chequerboard experiment (Section 3.8.4) with

Figure 6.20 Chequerboard Experiment with Purified MAb S2.4.C5 and S-Antigen

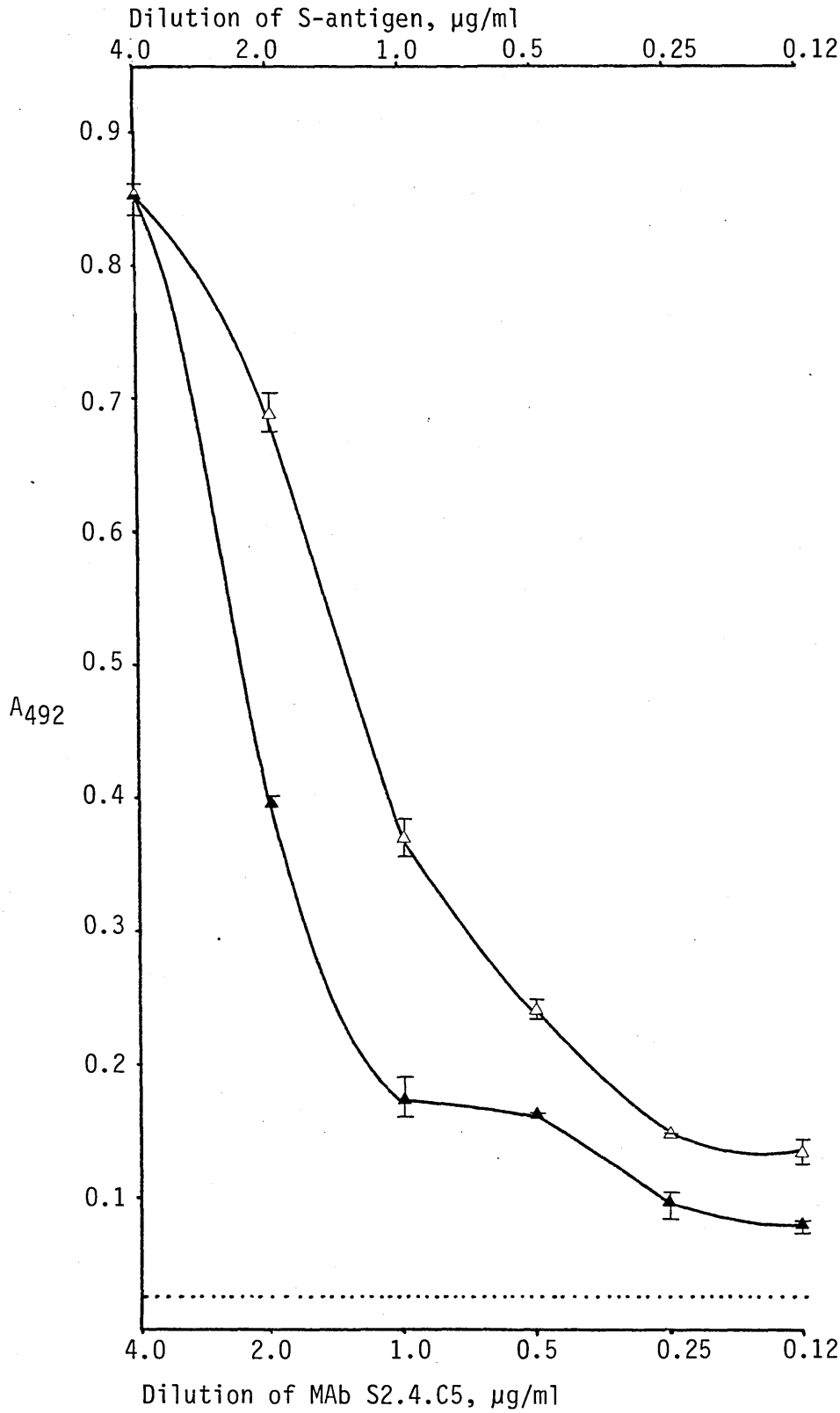
The effects of variation in concentration of MAb S2.4.C5, purified on a QAE-Sephadex column, and S-antigen, in the ELISA reaction was determined by means of a chequerboard experiment (Section 3.8.4). The incubation time for the monoclonal antibody was 4 hours.

KEY

△ dilution of S2.4.C5

▲ concentration of S-antigen

••• background reaction; no antibody


Dilution of S-antigen, µg/ml
4.0
2.0
1.0
0.5
0.25
0.12
0.9
0.8
0.7
0.6
0.5
A492
0.4
0.3
0.2
0.1
4.0
2.0
1.0
0.5
0.25
0.12
Dilution of MAb S2.4.C5, µg/ml

the purified monoclonal antibody S2.4.C5, and S-antigen. The relatively high ELISA reaction of the antibody, present in microgram quantities, suggests that there was no serious loss of activity after the purification and concentration procedures.

6.6 EFFECT OF MONOCLONAL ANTIBODY S2.4.C5 ON EAU

Preliminary studies were made on the effect of monoclonal antibody S2.4.C5, on the development of S-antigen induced experimental autoimmune uveoretinitis (EAU) in guinea pigs and rats. Nine Duncan-Hartley guinea pigs were injected subcutaneously, with 100µg of S-antigen, purified on a DEAE-Sephacel column (Section 3.2), mixed 1:1 with complete Freund's adjuvant (Section 2.6.1). Each animal was injected in both hind foot pads. Five of the guinea pigs received an intraperitoneal boost of 1mg of the monoclonal antibody, purified as described in Section 6.5.2. Six Lewis rats were injected with 50µg of S-antigen in the foot pads. Three of these animals simultaneously received an intraperitoneal injection of 1mg of monoclonal antibody S2.4.C5. Two of the six rats received an intraperitoneal injection of 1mg of affinity purified rat IgG, in addition to the foot pad injection of S-antigen, and served as controls. The remaining two animals which received only the S-antigen, also served as controls.

The progress of the ocular inflammation was assessed by ophthalmoscopical examination of the eyes. Ultrastructural investigations of the ocular histopathology were to be carried out at a later date.

Ophthalmoscopical examination revealed that all five guinea pigs, injected the monoclonal antibody simultaneously with S-antigen,

developed more severe inflammation in comparison with the guinea pigs injected with S-antigen alone. In contrast, the disease in the rats appeared to be inhibited by the monoclonal antibody. The rats receiving S-antigen alone, developed extensive inflammation after 13 to 18 days. The control rats receiving rat IgG and S-antigen appeared to develop even more severe disease. However, one of the rats which received the monoclonal antibody and S-antigen, did not develop clinical EAU, and the remaining two exhibited a delayed onset of the inflammation, appearing after 13 to 18 days, and the inflammation was markedly attenuated and the eyes recovered spontaneously.

Further work is required to establish these findings, and to determine the mechanisms by which monoclonal antibody S2.4.C5 exascerbates or inhibits EAU in different species of laboratory animals (see Section 8.2.2). These studies are presently in progress.

6.7 DISCUSSION

The specificities and classes of five monoclonal antibodies to retinal antigens are summarised in Table 6.1. Two monoclonal antibodies derived from hyperimmunised rats have been characterised. In addition, three monoclonal autoantibodies were derived from two RCS rats.

Monoclonal antibody S2.4.C5, an IgG1, derived from a hyperimmunised rat, was specific for S-antigen in the ELISA (Figure 6.1) and immunoblots (Figures 6.7 and 6.11a,b) when tested against a variety of irrelevant antigens. The apparently high affinity and avidity of antibody S2.4.C5, indicated by its behaviour in the ELISA (Figures

6.1 and 6.2), was probably related to the class of the monoclonal. IgG antibodies tend be of higher affinity than those of the IgM class, occurring at a later stage in the maturation of the immune response (Roitt et al., 1985).

The smallest fragment of S-antigen bearing the S2.4.C5 epitope, detectable in Cleveland blots, was identified in the region of 9-9.4K (Section 6.4.2). The monoclonal antibody was probably capable of recognising the epitope in smaller fragments of S-antigen, however, smaller fragments could not be generated in the Cleveland experiments. The epitope recognised by antibody S2.4.C5, is unlikely to have a three dimensional conformation, since no unreactive bands could be detected in the Cleveland blots. Three dimensional determinants, especially in the smaller peptide fragments, would probably be denatured under the conditions of SDS-PAGE and binding to the nitrocellulose paper.

The apparent high specificity and affinity of monoclonal antibody S2.4.C5, suggested that it might be suitable for the immunocytochemical localisation of S-antigen at the electron microscopical level (Polak and Van Noorden, 1984) (Section 7.6).

Monoclonal antibody S2.4.C5, was purified by ion exchange chromatography on a QAE-Sephadex column (Section 6.5.2) in order to obtain a relatively pure preparation to inject into guinea pigs. The purification method was not found to be ideal for this monoclonal antibody. Monoclonal antibodies have unique properties, and there is no standard purification method suitable for all. The preparation of antibody S2.4.C5 was at least 50% pure, and some of the contaminating bands, evident after SDS-PAGE (Figures 6.18 and

6.19), may represent partially re-associated heavy and light chains. The clinical results of preliminary experiments investigating the effect of the monoclonal antibody on S-antigen induced EAU, suggest that S2.4.C5 exacerbates the inflammation in guinea pigs, and virtually prevents the disease in rats (Section 6.6). Further work is required in order to confirm these findings, and to uncover the mechanisms by which the monoclonal antibody affects the disease progress (Section 8.2.2).

R1.2.D2, an IgM monoclonal antibody, was reactive against ROS in ELISA assays (Figure 6.3), and in immunoblots against an antigen present in the crude retinal extract, with an apparent molecular weight of 37K to 39K (Figure 6.8). The antibody did not demonstrate a specific reaction with opsin, and may have specificity for a subunit of transducin (Shichi et al., 1984) or an ROS rim protein (Molday et al., 1986).

The three IgM monoclonal autoantibodies derived from RCS rats (Section 5.16), demonsrated lower titres compared with antibodies obtained from immunised rats (Section 6.3.1). Antibody H9.1.C2 was reactive to S-antigen in ELISA (Figure 6.4) and immunoblots (Figure 6.9c). Antibody H9.5.A4 was reactive with the ROS antigen in the ELISA (Figure 6.5), but was unreactive in immunoblots. The third monoclonal autoantibody H10.1.D3, was crossreactive against the ROS and the S-antigen in both the ELISA assays (Figure 6.6) and in immunoblots (Figure 6.9b) raising the possibility of shared carbohydrate or protein sequences between S-antigen and opsin. The crossreactivity may also result form dual secificity of the monoclonal antibody (Ghosh and Campbell, 1986) (Section 8.4.5).

The protein A blots with the three monoclonal autoantibodies, required a longer exposure time of the blot to the X-ray film, compared with antibody S2.4.C5. This probably indicates that there was a greater proportion of bound antibody in the blots with antibody S2.4.C5, compared with the autoantibodies. There may have been less autoantibody bound, due to their lower relative affinity. The autoantibodies were probably raised to more native forms of the antigens, whereas the monoclonal antibody S2.4.C5 was raised in a rat hyperimmunised with S-antigen denatured within the complete Freund's adjuvant. Thus the monoclonal autoantibodies derived from the RCS rats possibly demonstrated a reduced affinity for the denatured antigens.

The lack of reactivity with monoclonal antibody H9.5.A4 in the immunoblots, and the relatively weak reactivity shown by the other two autoantibodies, may also be an indication of the relatively low affinity of the antibodies for the appropriate antigenic determinants.

The monoclonal antibodies described in this chapter, provide useful tools in the study of the nature and physiology of retinal antigens. Monoclonal antibody S2.4.C5, was useful in the localistion of S-antigen at the ultrastructural level (Sectoin 7.6), and antibody R1.2.D2 was used to localise the 39K antigen at the electron microscopical level (Section 7.7). Further work is required in order to identify the physiological role of this antigen.

CHAPTER SEVEN

LIGHT AND ELECTRON IMMUNOCYTOCHEMICAL LOCALISATION OF RETINAL ANTIGENS BY MEANS OF MONOCLONAL ANTIBODIES

Chapter 7

LIGHT AND ELECTRON IMMUNOCYTOCHEMICAL LOCALISATION OF RETINAL ANTIGENS WITH MONOCLONAL ANTIBODY REAGENTS

7.1 INTRODUCTION

In this chapter, the subcellular localisation of retinal S-antigen and a 39K photoreceptor antigen, by means of specific monoclonal antibodies (Chapter 6) is described.

Retinal S-antigen has been localised at the light microscopical level using conventional polyclonal antisera and mouse monoclonal antibodies (reviewed in Chapter 1). The protein has also been localised electron microscopically in Lowicryl embedded tissue using polyclonal antisera (McKechnie et al., 1986).

In this study retinal S-antigen was localised at the ultrastructural level in the photoreceptor rod cells of Lowicryl embedded sections of human and swine retinae, and also in LR White embedded human retina. The electron immunocytochemical localisation of the protein involved the specific rat IgG1 monoclonal antibody, S2.4.C5, produced during the course of this work (Chapter 6). The detection of S-antigen by the monoclonal antibody was visualised by means of electron-dense gold probes. Results for the detection of S-antigen by means of rat antisera to the protein, are included for comparison.

An additional retinal antigen, of apparent molecular weight 39K (Figure 6.8) was also localised at the electron microscopical level, using the IgM monoclonal antibody R1.2.D2. The antigen was localised

in the human and pig retinae, employing the same techniques as for the localisation of the S-antigen.

7.2 LOCALISATION OF S-ANTIGEN AND THE 39K ANTIGEN AT THE LIGHT MICROSCOPICAL LEVEL

7.2.1 Localisation of S-Antigen in Rat Retina

7.2.1.1 Effect of method of fixation

The photoreceptors of rat retina mainly consist of rods, since the rat is a nocturnal animal. Thus, although the photoreceptor cell layer appeared grossly stained by light microscopy, and the rods were clearly involved, it was not possible to ascertain whether the cone cells were also stained.

Monoclonal antibody S2.4.C5 reacted intensely with the photoreceptor cell layer of rat retina, fixed in 70% ethanol (Section 2.4.1) (Figure 7.1a). The distribution of staining was similar to that of the rat polyclonal antisera, raised against S-antigen (Section 2.6.1) (Figure 7.1b). The monoclonal antibody offered the advantage of greater specificity and the absence of a background reaction with other retinal cells, compared with the polyclonal antisera (Figures 7.1a,b). Although the entire photoreceptor cell was labelled, the intensity of reaction, in the ethanol-fixed tissue, was greater in the ROS than in the inner segments. The reaction in the perinuclear region was of comparable intensity to that of the ROS. In these sections, the reaction in the photoreceptor synapses appeared more intense than the staining in the ROS. While these results indicate the presence of S-antigen throughout the photoreceptor cell, this pattern was thought to arise partially from the method of tissue fixation, since gluteraldehyde fixed sections demonstrate a different pattern, under identical experimental conditions (Figure

Figure 7.1 Localisation of S-Antigen within Rod Photoreceptors

a) Localisation with Rat MAb S2.4.C5

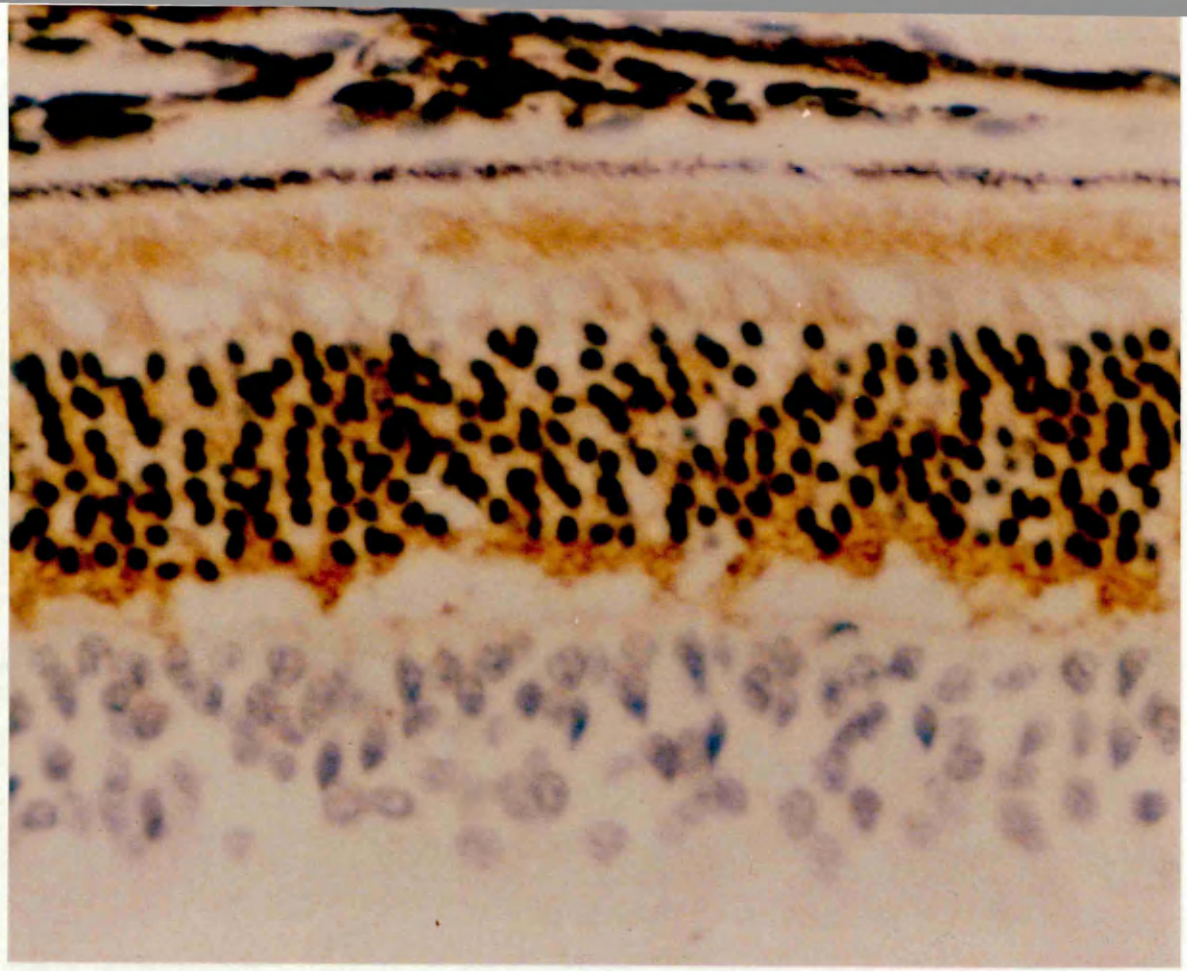
b) Localisation with rat polyclonal antisera

S-antigen was localised in dewaxed sections of alcohol fixed rat eye, using an immunoperoxidase technique. The reaction was present throughout the photoreceptor cell layer, which consists almost entirely of rods in the rat. The inner segment regions were less intensely stained compared to the rest of the cell. The pattern of labelling was similar for the MAb and the antisera, although the antisera gave rise to some background reaction with other retinal neurons.

KEY

1. Choroid (pigmented and vascular)
2. Retinal pigment epithelium
3. Photoreceptor outer segment layer
4. Photoreceptor inner segment layer
5. Photoreceptor nuclei (outer nuclear layer)
6. Outer plexiform layer (synapses)
7. Nuclei of second order neurons (inner nuclear layer)
8. Inner plexiform layer (synapses)

Photographed at magnification x 400


1
2
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7
8

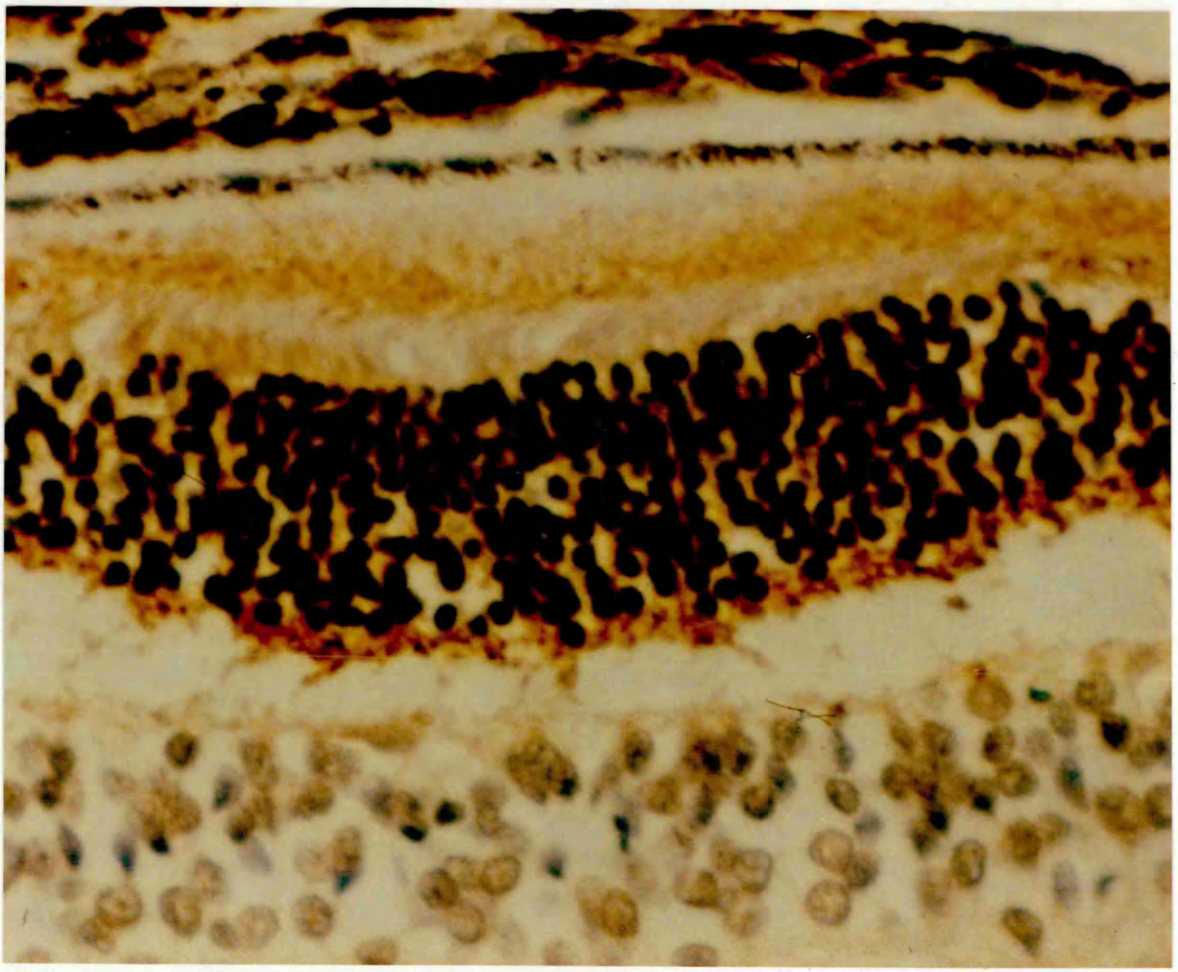

1
2
3
4
5
6
7

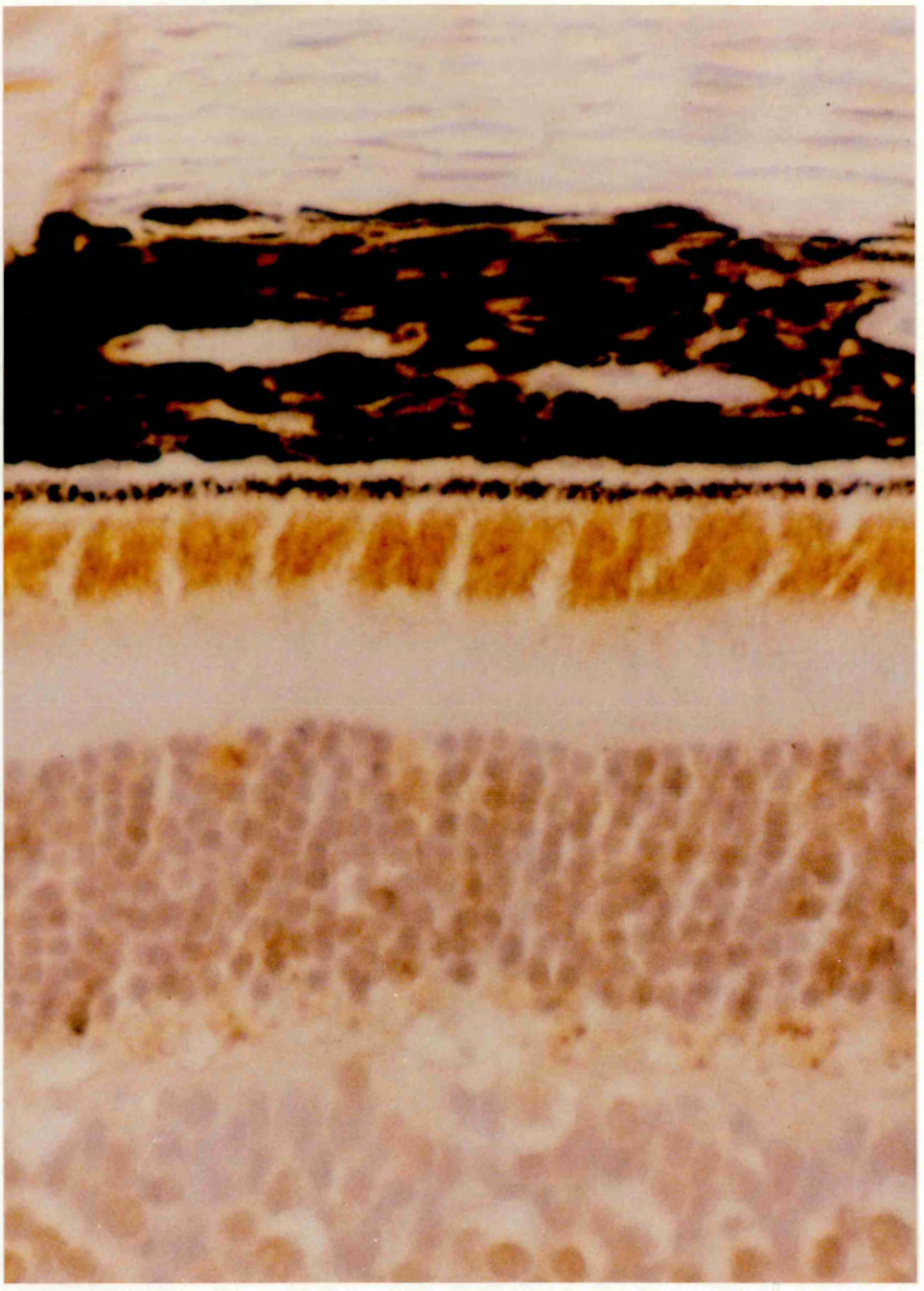
Figure 7.2 Immunoperoxidase Localisation of S-Antigen in dewaxed Sections of Rat Eye fixed in 4% Gluteraldehyde

S-antigen was localised, by means of MAb S2.4.C5 and an immunoperoxidase procedure, in dewaxed sections of gluteraldehyde (4%) fixed rat eye. The reaction was mainly confined to the ROS, with a light staining of the apical regions of the inner segments. Staining was also present around the nuclei and in the photorecptor synapses. There was a light background reaction with other retinal neurons.

KEY

1. Sclera
2. Choroid (pigmented and vascular)
3. Retinal pigment epithelium
4. Photoreceptor outer segment layer
5. Photoreceptor inner segment layer
6. Photoreceptor nuclei (outer nuclear layer)
7. Outer plexiform layer (synapses)
8. Nuclei of second order neurons (inner nuclear layer)

Photographed at magnification x 400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7.2).

In the gluteraldehyde fixed retina (Section 2.4.1), the ROS were intensely stained with monoclonal antibody S2.4.C5. The ellipsoid region of the inner segments, immediately below the ROS, appeared lightly stained. Staining was also present within the perinuclear area and in the region of the photoreceptor synapses. In the gluteraldehyde fixed sections, there was a slight background reaction with some of the nuclei of the inner nuclear layer (INL) and of the photoreceptors. There may also be some nonspecific deposition of the peroxidase substrate (DAB, Section 2.4.4) in the region of the melanin pigment within the choroid. The reaction of the monoclonal antibody in gluteraldehyde fixed retina could not be compared with polyclonal antiserum since sera gave rise to a high background reaction over the entire section, due to the presence of a large number of antibodies of differing specificities.

That monoclonal antibody S2.4.C5, was capable of reacting in sections fixed in 4% gluteraldehyde (Figure 7.2), suggested that the antibody may be suitable for electron immunocytochemistry (Section 7.6), where there may be altered antigenicity due to fixation and EM processing.

7.2.1.2 Localisation in light and dark adapted rat retinae

Preliminary studies on the light versus dark localisation of S-antigen at the light microscopical level, in ethanol fixed rat eyes (Section 2.4.1), indicate that there may be a difference in the intensity of staining in the rod outer and inner segments (Figures 7.3a,b). In immunoperoxidase experiments with monoclonal antibody S2.4.C5 and polyclonal antisera to S-antigen, on sections of (at

Figure 7.3 Localisation of the S2.4.C5 Epitope of S-Antigen under conditions of Light and Dark Adaptation

a) Immunolocalisation with S2.4.C5 in light adapted retina

b) Immunolocalisation with S2.4.C5 in dark adapted retina

Light and dark adapted, ethanol fixed, dewaxed sections of albino rat eyes were treated with MAb S2.4.C5 in an immunoperoxidase technique.

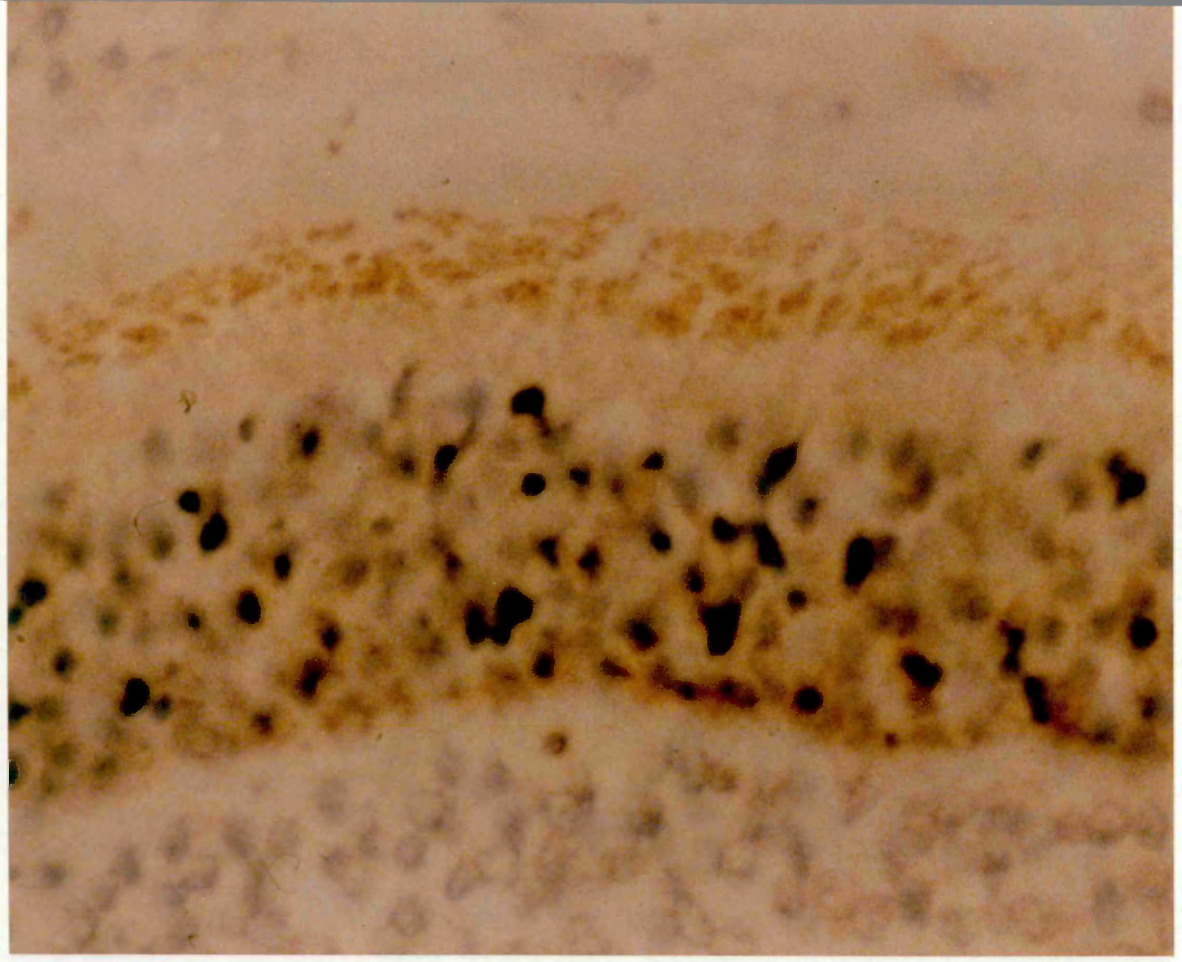
In the light adapted rat retina (a), the epitope of S-antigen recognised by MAb S2.4.C5 appeared to be localised primarily in the ROS, with a light reaction around the nuclei and in the synapses. The inner segment regions were less intensely stained compared to the rest of the cell.

The S2.4.C5 epitope in the dark adapted retina (b) appeared to be distributed throughout photoreceptor cell, where the reaction was of fairly uniform intensity.

KEY

1. Photoreceptor outer segment layer
2. Photoreceptor inner segment layer
3. Photoreceptor nuclei (outer nuclear layer)
4. Outer plexiform layer (synapses)
5. Nuclei of second order neurons (inner nuclear layer)

Photographed at magnification x 400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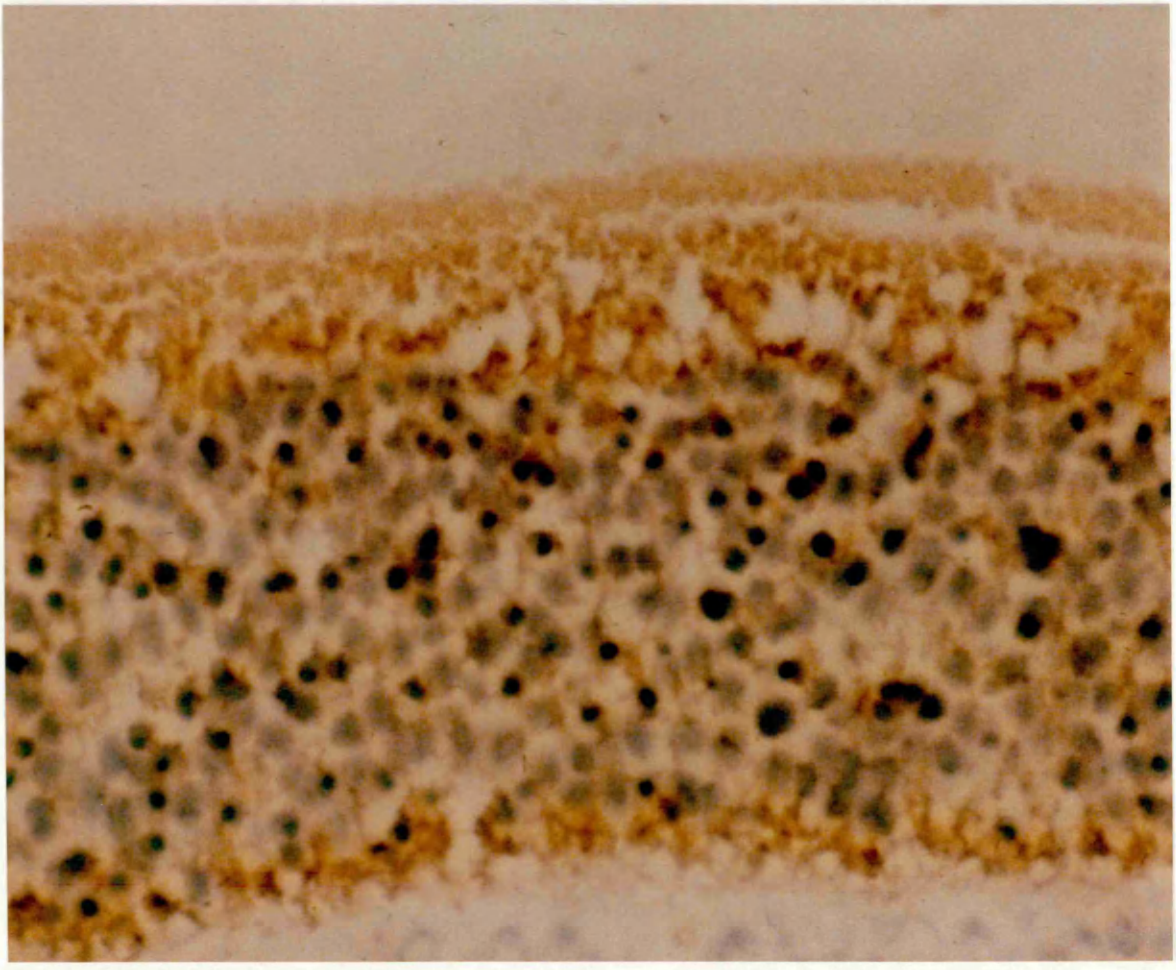
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least 2 hours) light adapted rat retina, the pattern of staining (Figure 7.3a) was similar to that seen in Figures 7.1a,b. The pattern of staining of light adapted retinae was found to be consistent in all specimens examined.

In the dark adapted specimen (12 hours darkness, with dissection and fixation procedures carried out in dim red light), the reaction was much more intense in the inner segments (Figure 7.3b), than in the light adapted specimens (Figure 7.3a). The reaction was, perhaps, stronger in the inner segments than in the outer segments, in the dark adapted retina (Figure 7.3b). These initial observations require confirmation in additional specimens of dark adapted retinae. It would be useful to compare these results with similar experiments on gluteraldehyde fixed tissue, in view of the effect of fixation method (Section 7.2.1.1).

7.2.2 Localisation of the 39K Antigen at the Light Microscope level

The rat IgM antibody R1.2.D2, obtained from a fusion with a rat immunised with bovine ROS (Section 2.6.1), was tested at the light microscopical level in sections of alcohol fixed rat eye (Section 2.4.1), in an immunoperoxidase procedure (Section 2.4.4). The antibody reacted intensely with the photoreceptor outer segments, sparing the rest of the cell (Figure 7.4) and all other cells of the retina. The reaction certainly involved the rod photoreceptors (Section 7.2.1.1), although it was not possible to determine staining of the cones at the level of the light microscope. The pattern of reaction was similar to that given by polyclonal antisera raised in rats against bovine ROS (Figure 7.5) (Section 2.6.1). This antisera reacted with bovine opsin in immunoblots (Section 2.3.9.6). The antibody R1.2.D2, applied as hybridoma supernatant, did not

Figure 7.4 Immunoperoxidase Localisation of a 39K Retinal Antigen within Rod Photoreceptors by means of a Rat MAb

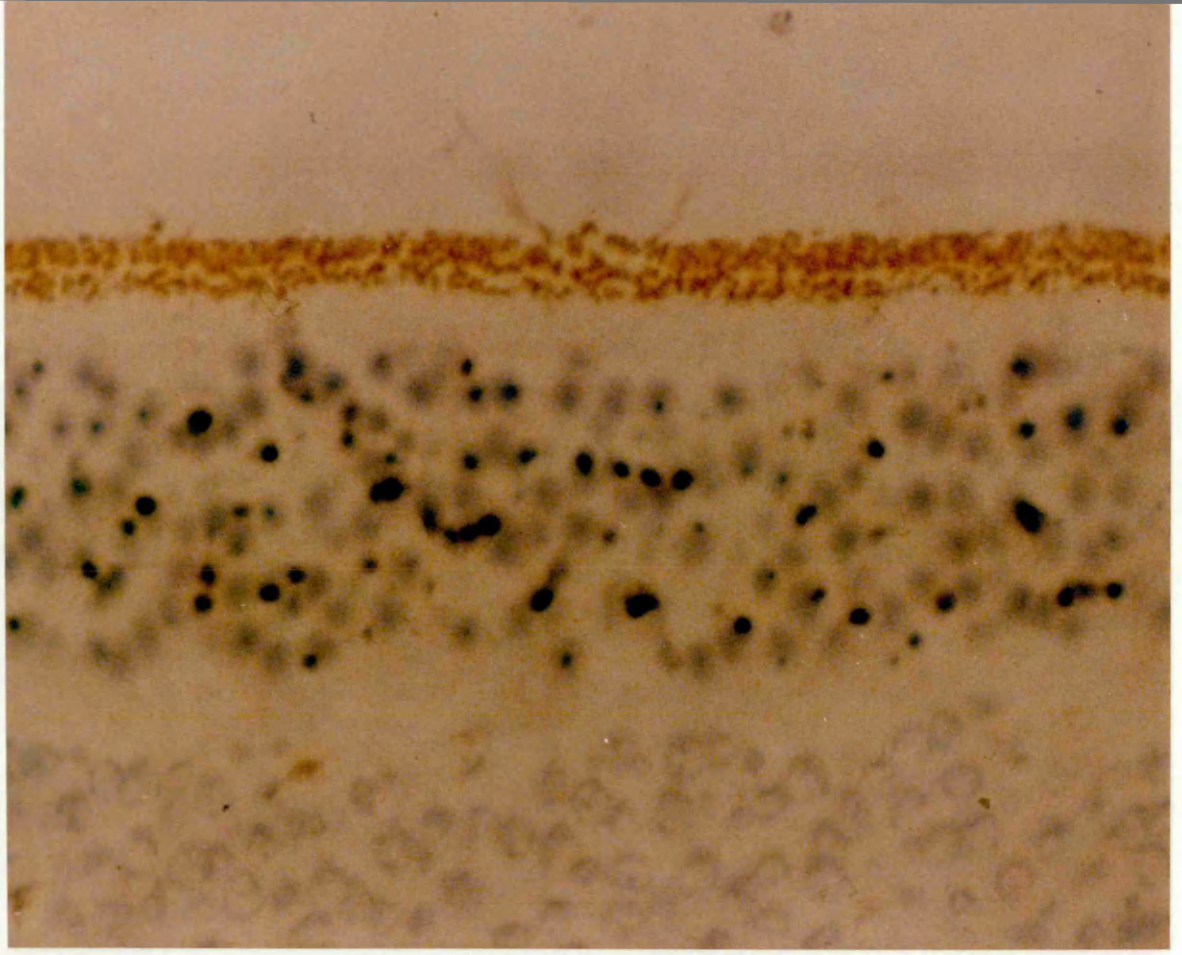
The 39K antigen was localised with MAb R1.2.D2, in dewaxed sections of ethanol fixed rat eye, using an immunoperoxidase technique. The antigen appeared to be confined to the photoreceptor outer segments. Photographed at magnification x 400

KEY

1. Choroid (pigmented and vascular)
2. Retinal pigment epithelium
3. Photoreceptor outer segment layer
4. Photoreceptor inner segment layer
5. Photoreceptor nuclei (outer nuclear layer)
6. Outer plexiform layer (synapses)
7. Nuclei of second order neurons (inner nuclear layer)
8. Inner plexiform layer (synapses)

Figure 7.5 Immunoperoxidase Localisation of Opsin with Polyclonal Antiserum

Opsin was localised in rat photoreceptors of ethanol fixed tissue, using antiserum raised in rats against bovine ROS membrane proteins. The peroxidase reaction was mainly confined to the ROS, with a faint reaction in other regions of the retina, which may be nonspecific. This pattern of staining provided a useful comparison for the R1.2.D2 MAb (Figure 7.4). Photographed at magnification x 400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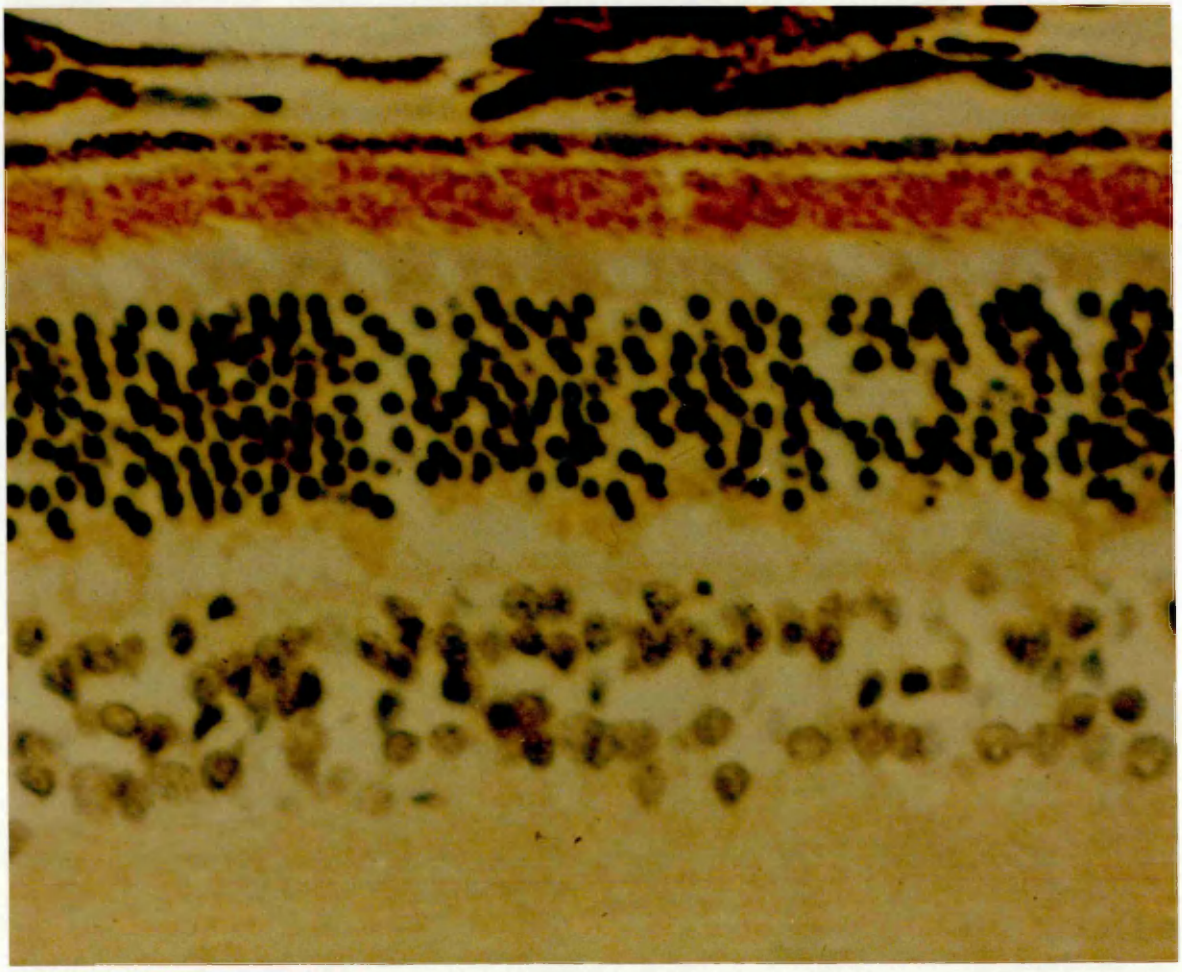
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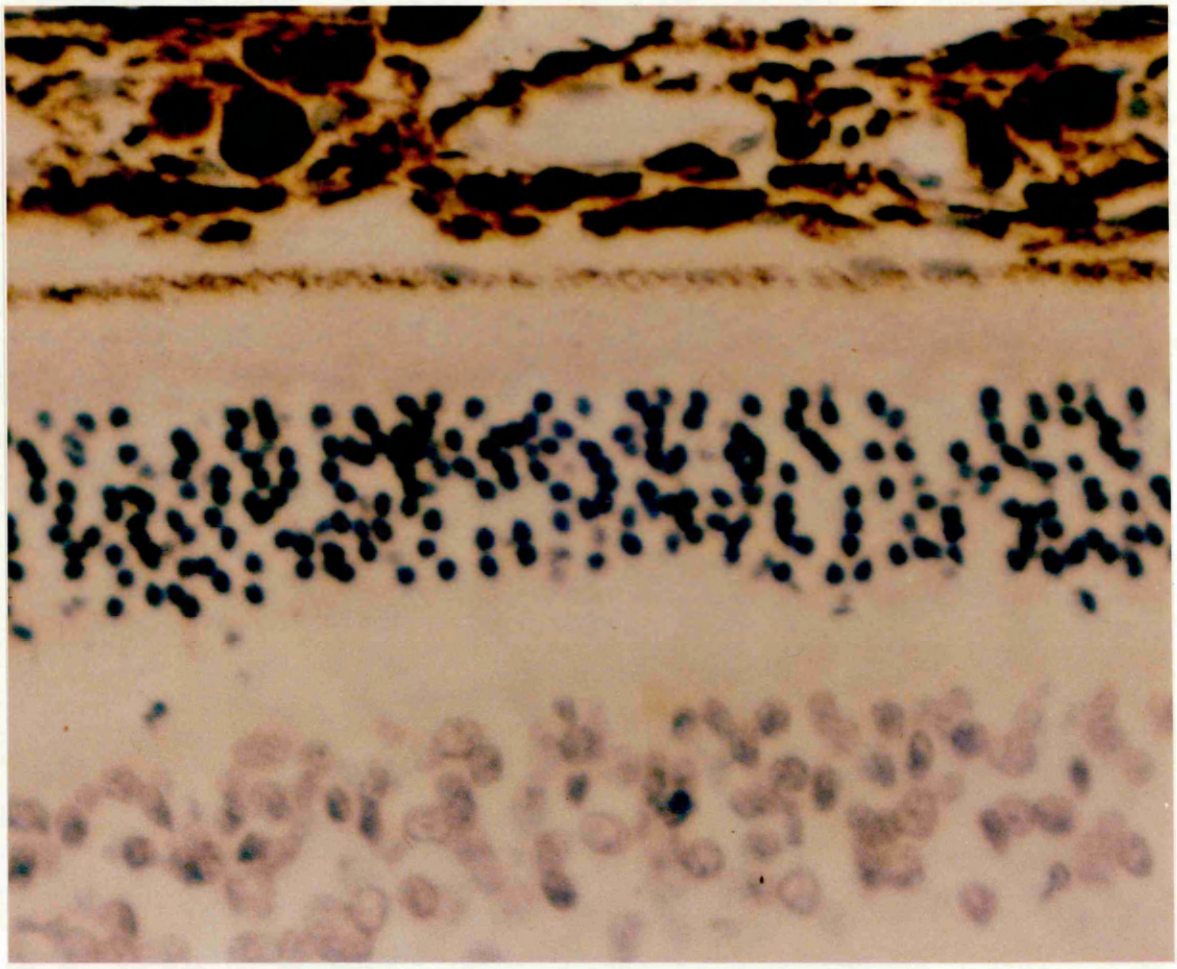
Figure 7.6 Immunoperoxidase Experimental Control

Lack of peroxidase reaction in dewaxed sections of ethanol fixed rat eye, when an irrelevant rat IgG MAb is used in place of rat anti-retinal antibody.

KEY

1. Choroid (pigmented and vascular)
2. Retinal pigment epithelium
3. Photoreceptor outer and inner segments
4. Photoreceptor nuclei (outer nuclear layer)
5. Outer plexiform layer (synapses)
6. Nuclei of second order neurons (inner nuclear layer)

Photographed at magnification x 400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present any significant background reaction (Figure 7.4). The antisera, however, gave rise to some background reaction with the inner (IPL) and outer plexiform layers (OPL) (Figure 7.5). This may be due to the presence of antibodies to contaminating retinal antigens within the immunising ROS antigen, or to the crossreaction of low affinity antibodies.

Immunoperoxidase experiments with antibody R1.2.D2, in light and dark adapted retinae, gave rise to similar staining reactions, indicating that the epitope recognised by this antibody was not differentially localised or exposed in the presence or absence of light, under the conditions employed. This antibody was not tested on gluteraldehyde fixed paraffin embedded sections.

7.2.3 Immunoperoxidase Experimental Control

A negative control, for the immunoperoxidase reaction, involving the use of an irrelevant rat IgG monoclonal antibody to Vibrio cholerae, is shown in Figure 7.6. The control shows that there was little tendency for the peroxidase conjugated rabbit anti-rat IgG(H+L) to bind non-specifically with the tissue. The control also showed that the peroxidase DAB substrate (Section 2.4.4), which has the potential to become deposited on the melanin granules, did not interfere with the localisation of the antigens.

7.3 PREPARATION OF TISSUE SPECIMENS

7.3.1 Embedding Media

Three different embedding media were investigated. Araldite resin was investigated since it offers a greater morphological preservation, and therefore the presence of plasma membrane staining might be more readily determined. Lightly fixed (Section 2.5.1.1)

specimens of rat retina were embedded in Araldite (Section 2.5.1.3). The immunostaining of the araldite embedded sections was too light for meaningful interpretation, probably partially due to antigen masking within this hydrophobic resin.

Lowicryl K4M and LR White (Section 2.1.8.3) resins are more frequently utilised for electron immunocytochemistry, because of their hydrophilic nature.

7.3.2 Lowicryl Embedded Material

The Lowicryl K4M (Section 2.1.8.3) embedded specimens of human and swine retinae were the generous gift of Dr N. McKechnie, Institute of Ophthalmology, London. The human tissue was obtained from the eye of a 50 year old male whose eye was enucleated as part of an exenteration procedure for sebaceous carcinoma of the eyelids and conjunctiva. The globe was not affected by the tumour. The eye was immediately dissected and small pieces of retina were removed and fixed in 1% gluteraldehyde (Section 2.1.8.1) in PBS, pH7.2 (Section 2.2.4.1) for one hour. The pig retina was fixed in the same manner.

The fixed tissues were dehydrated through a graded series of dimethylformaldehyde and water at -4°C, and infiltrated with the Lowicryl resin at the same temperature. Polymerisation was achieved by exposure to long wavelength ultraviolet light Sylvana (F475/BLB or Philips TDL/6W/05), as described by Ambruster et al. (1982), with minor modifications.

7.3.3 LR White Embedded Human Retina

Human retina was also processed for EM histochemistry. Blocks of chorioretinal tissue were excised from the posterior pole of the eye

of a 71 year old female, removed due to the presence of a malignant melanoma of the anterior choroid. The tissue was fixed in 1% gluteraldehyde in cacodylate buffer (Section 2.2.8.2) for 2 hours at room temperature, washed with PBS and ammonium chloride, dehydrated in 70% ethanol and embedded in LR White, hard grade (Section 2.1.8.3). Paraffin embedded sections of the same area of tissue utilised for immuno-electron microscopy, showed no significant abnormality.

7.4 MORPHOLOGICAL PRESERVATION OF TISSUE SPECIMENS

The preservation of ultrastructure was similar in the Lowicryl and LR White embedded specimens. The ultrastructural preservation of the outer segment disc membranes was satisfactory, although those of the pig and LR White embedded human retinae appeared somewhat disorganised, presumably due to a less immediate fixation. The identification of the intracellular structures of the inner segments were, less certain, although mitochondrial cristae, endoplasmic reticulum, centrioles, synaptic vesicles and cell junctions could often be distinguished. The layer of retinal pigment epithelial cells (the RPE) was retained in the LR White embedded human retina, which allowed the identification of S-antigen within the phagosomes of the RPE cells (Section 7.6.3) (Figures 7.15 and 7.16).

7.5 STAINING METHODS

The visualisation of the reaction of the antibodies with S-antigen was achieved by two methods; gold adsorbed protein A and gold adsorbed goat or rabbit anti-rat IgG(H+L) were employed (Section 2.5.3). The protein A technique was essentially that of Roth (1974), and the immunogold localisation method was similar to the method of De May (1963). Both commercial (Janssen) goat anti-rat

IgG-gold and protein A-gold (10nm and 5nm gold particle size) (Section 2.1.16), and the rabbit anti-rat IgG conjugated to 10nm gold particles by the method of Roth for the conjugation of protein A with gold (Roth, 1974) were utilised (Section 2.5.2). There was some variation in the background reactions between preparations of the gold reagent. In the protein A-gold technique, best results were obtained when a second antibody, rabbit anti-rat IgG(H+L) was used, although protein A does bind to the S2.4.C5 antibody as demonstrated in immunoblots (Figure 6.7). The immunogold staining experiments were selected for the quantification of the labelling of S-antigen.

7.6 ULTRASTRUCTURAL LOCALISATION OF S-ANTIGEN

7.6.1 Specific labelling of the Rod Cell

The distribution of S-antigen at the electron microscopical level was similar to that observed in light microscopy, where the protein was localised throughout the photoreceptor cells in rat retina (Figures 7.1a,b). The immunostaining methods involving gold conjugated protein A or immunogold gave rise to similar results. The Lowicryl embedded sections of human and pig tissue, were stained with immunogold, and the LR White embedded sections of human retina were treated with protein A-gold.

Monoclonal antibody S2.4.C5, and antiserum raised in rats against bovine S-antigen, labelled the rod outer segment disc membranes of human (Figures 7.7, 7.8a and 7.9) and porcine retinae (Figure 7.11). Irrelevant rat IgG and IgM monoclonal antibodies, employed as negative controls did not give rise to any significant staining (eg. Figures 7.10 and 7.12). There was no indication of specific labelling of the rod cell membrane in either the Lowicryl or LR White embedded tissue, even in regions where the plasma membrane

Figure 7.7 Electron Microscopical Localisation of S-Antigen within a Human Rod Photoreceptor Cell

Electron micrograph of a rod cell in a Lowicryl embedded section of human retina treated with MAb S2.4.C5, in an immunogold cytochemistry experiment.

The disc membranes of the rod cell were specifically decorated. The connecting cilium was also labelled. The labelling in the inner segment was less intense, relative to the disc membranes. Gold label tended not to be present on the mitochondria or the Lowicryl resin.

KEY

ROS: Photoreceptor rod outer segment

RIS: Photoreceptor rod inner segment

cc: Region of connecting cilium

m: Mitochondrion in ellipsoid region of inner segment

← ROS disc membranes

Magnification x 34,000

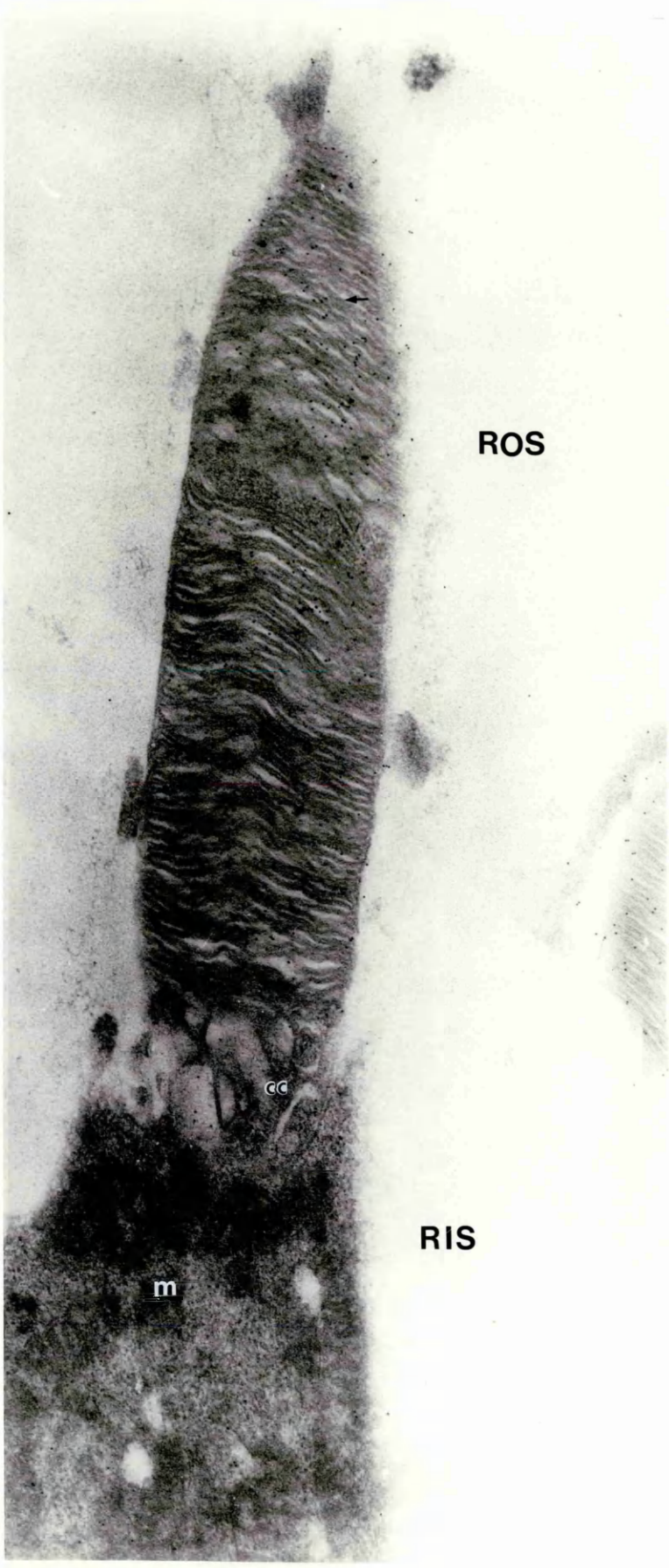
ROS
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Figure 7.8 Ultrastructural Localisation of S-Antigen within Lowicryl embedded Sections of Human Rod Cells by means of MAb S2.4.C5 and a Gold Probe

a) Electron micrograph of an immunostained rod outer segment, showing specific decoration of the disc membranes. Labelling of the ROS plasma membrane was less readily determined.

Magnification x 43,000

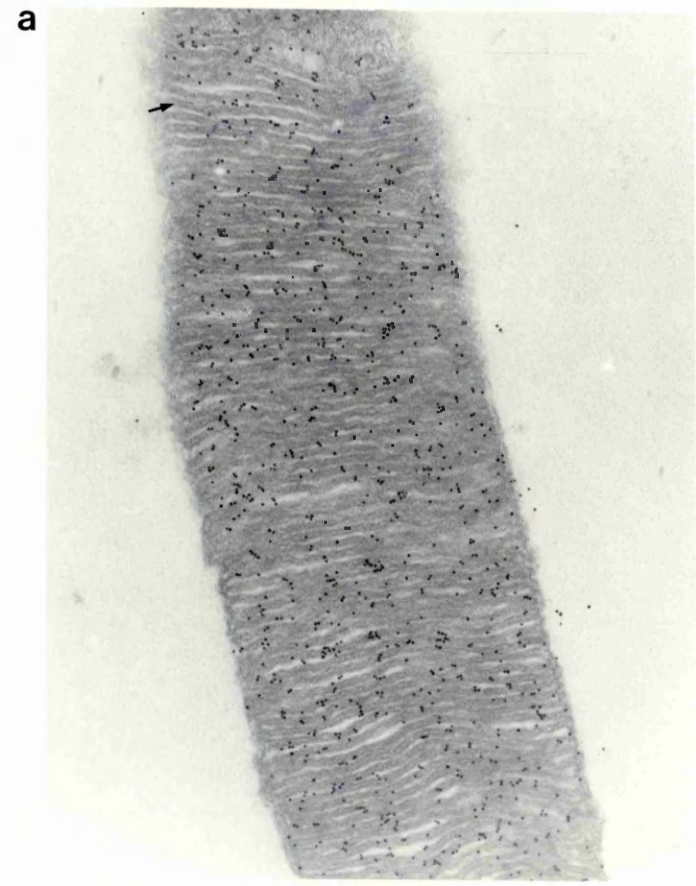
KEY

m: Mitochondrion in ellipsoid region of inner segment

← ROS disc membranes

b) Electron micrograph of an immunostained rod inner segment. The inner segment was less intensely labelled than the ROS. Gold label tended not to be present on the mitochondria.

Magnification x 44,000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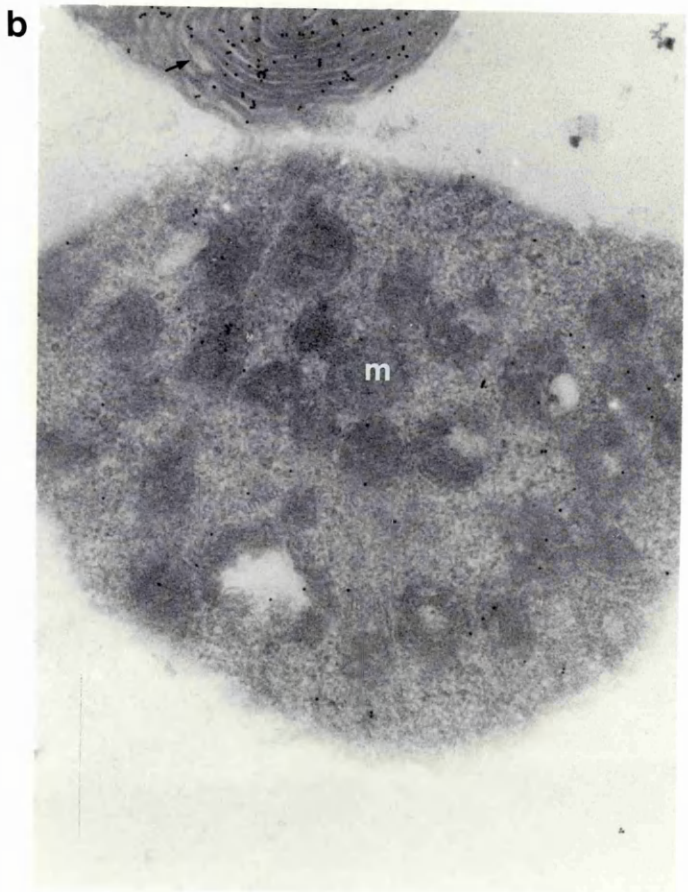

b
m

Figure 7.9 Localisation of S-Antigen on the ROS Disc Membranes of LR White embedded Human Retina

Electron micrograph of an apical region of human ROS, proximal to the RPE, showing specific rod disc membrane decoration, after immunocytochemistry with MAb S2.4.C5 and the protein A-gold probe. The plasma membrane of this ROS appeared unlabelled.

Magnification x 43,000

Figure 7.10 Immunocytochemical Negative Control on LR White embedded Human Retina

Electron micrograph showing the absence of staining on an LR White embedded section of an apical region of human ROS, proximal to the RPE, treated with an irrelevant rat IgG MAb and protein A-gold.

Magnification x 31,000

Figure 7.11 Immunocytochemical Localisation of S-Antigen within Lowicryl embedded Sections of Pig Retina by means of MAb S2.4.C5 and a Gold Probe

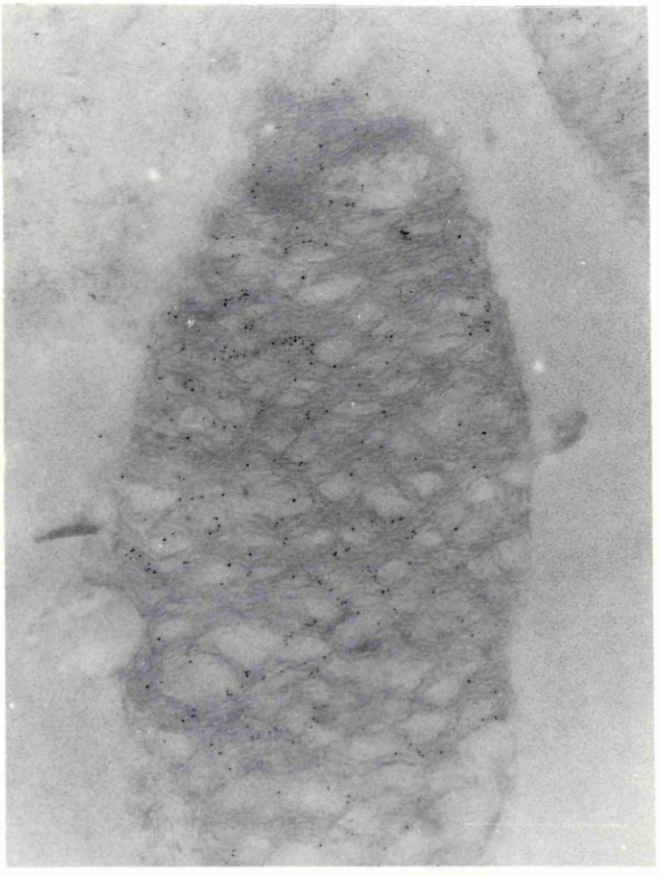
Electron micrograph of a porcine rod outer segment, double immunostained, for S-antigen and opsin. Gold particles, 5nm in diameter indicate the localisation of S-antigen with MAb S2.4.C5, and the 10nm particles indicate the labelling of opsin by means of polyclonal antiserum, raised in rats against bovine ROS membrane protein. Both sizes of gold particles were localised on the disc membranes

Magnification x 76,000

Figure 7.12 Negative Control for Electron immunocytochemistry

Absence of gold label on a section of Lowicryl embedded porcine ROS, in an immunogold cytochemistry procedure involving an irrelevant rat IgG MAb.

Magnification x 43,000



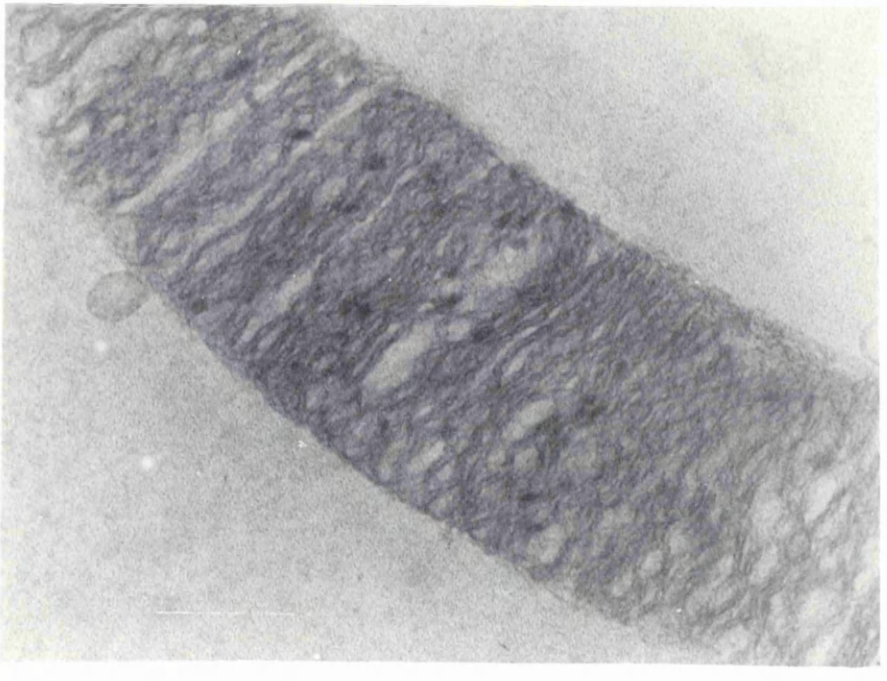


Figure 7.13 Ultrastructural Localisation of S-Antigen within the Perinuclear region of the Rod Cell

a) Electron micrograph of the inner nuclear region of LR White embedded human retina, treated with MAb S2.4.C5 and protein A-gold. The section demonstrates specific labelling of the perinuclear region, where the S-antigen is likely to be synthesised.

Magnification x 53,000

KEY

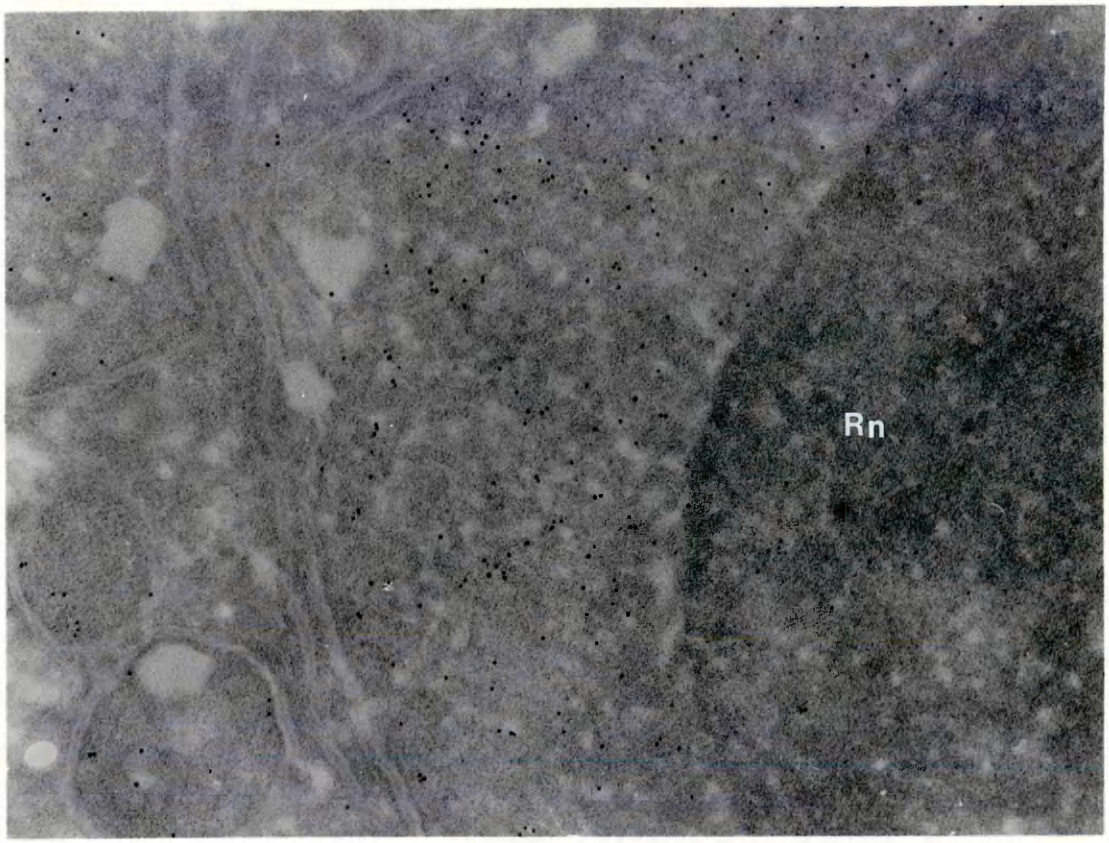
Rn: Photoreceptor rod nucleus

Cn: Photoreceptor cone cell nucleus

b) Electron micrograph of the inner nuclear region of LR White embedded human retina, treated with MAb S2.4.C5 and protein A-gold. The perinuclear region of the rod cell, but not the cone, was labelled. The background reaction apparent on the rod nucleus, might have resulted from the diffusion of S-antigen from the site of synthesis, due to the delay before tissue fixation.

Magnification x 53,000

a



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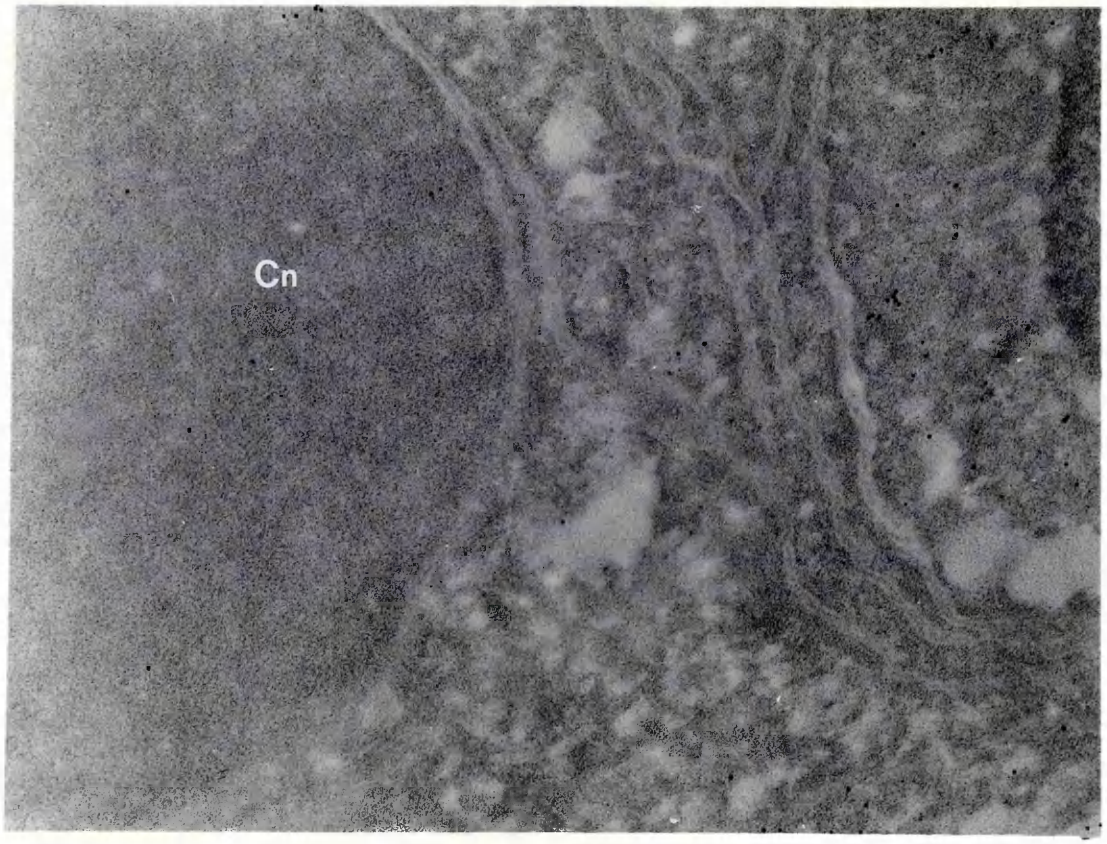


appeared well preserved (Figures 7.7 and 7.8a). The inner segments in both cases were also stained, although less intensely (Figures 7.7 and 7.8b). Gold label was also demonstrated in the connecting cilium (Figure 7.7). In the LR White embedded tissue, there was also intense labelling in the rod perinuclear region (Figures 7.13a,b), suggesting that this is likely to be the site of S-antigen synthesis. This was not clear in the Lowicryl embedded retinae. There appeared to be fewer non-specifically adsorbed gold particles on the cone cell nuclei, than on the rod cell nuclei (Figures 7.13a,b). This presumably resulted from the diffusion of S-antigen, from the site of synthesis, to the rod nuclei, during tissue preparation for electron microscopy, and perhaps during the delay before fixation.

7.6.2 Reaction for the Cone Cells

The cone photoreceptor cells of the human and pig specimens were labelled only at background levels (Figures 7.14a,b, Table 7.1), when incubated with antibody S2.4.C5 or specific antisera to S-antigen. The cone cells were, however, lightly labelled when the grids were treated with certain rat anti-bovine ROS sera since the immunising antigen contained material from the cones in addition to the ROS.

7.6.3 S-Antigen Immunoreactivity within RPE Cells

Figure 7.15 shows a section of an LR White embedded human RPE cell, with the closely associated ROS. Phagosomes containing ROS membranes at various stages of digestion were observed. The ROS and some of the phagocytosed ROS debris were stained with monoclonal antibody S2.4.C5, and protein A-gold (Figure 7.15). Numerous lipofuscin deposits were apparent within the cytoplasm, due to the accumulation

Figure 7.14 Absence of Immunolabelling for S-Antigen within a Human Photoreceptor Cone Cell

Electron micrographs of a Lowicryl embedded cone outer segment (a) and inner segment (b), presenting staining at only background levels after treatment with MAb S2.4.C5 and the immunogold probe.

KEY

COS: Photoreceptor cone outer segment

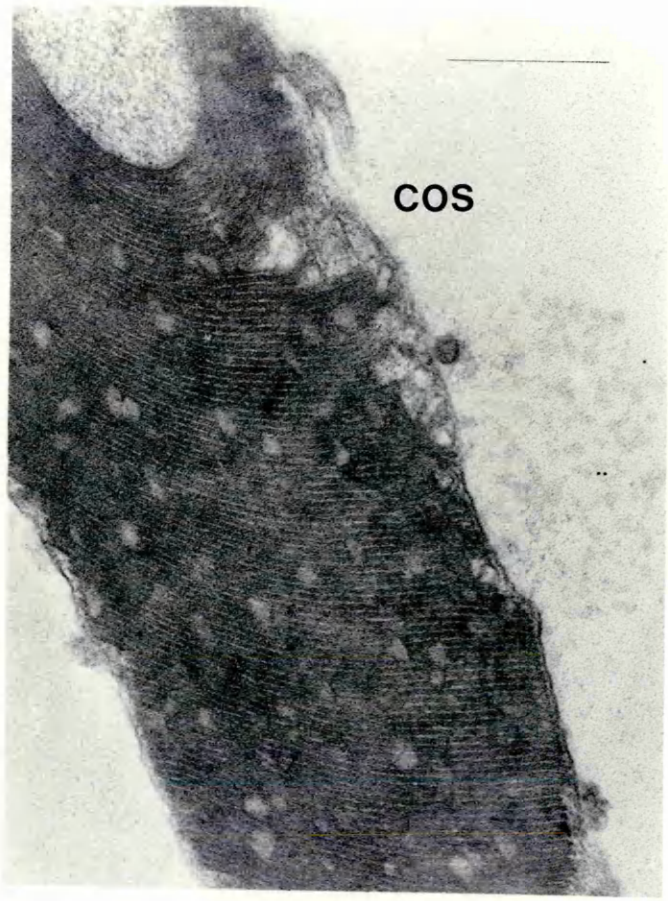
CIS: Photoreceptor cone inner segment

m: Mitochondrion in ellipsoid region of inner segment

a) Magnification x 45,000

b) Magnification x 58,000

a



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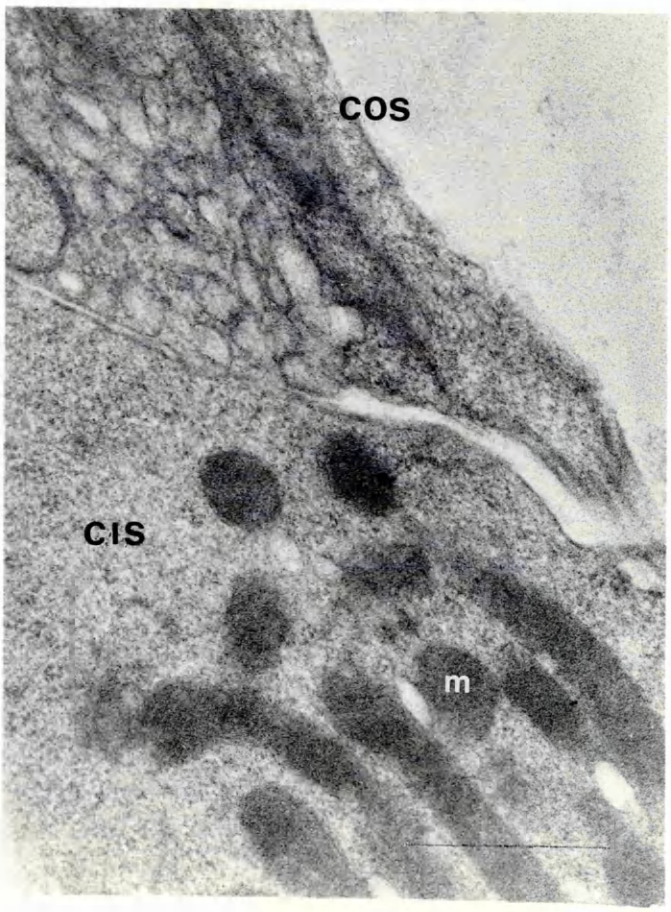


Figure 7.15 Electron Immunocytochemical Localisation of S-Antigen within ROS Membrane Material Phagocytosed by the RPE

Electron micrograph of LR White embedded human retina, showing a portion of pigment epithial cell embedded in LR White. The section shows the tips of the ROS surrounded by sheaths or microvilli, from the RPE. The section was stained with MAb S2.4.C5 and protein A-gold.

KEY

cy: RPE cell cytoplasm

N: RPE nucleus

lf: Lipofuscin deposits

◄ RPE apical microvilli

i) and ii) indicate phagosomes shown at higher magnification in Figures 7.16a,b respectively.

iii) indicates ROS shown in Figure 7.16c

Magnification x 8,200

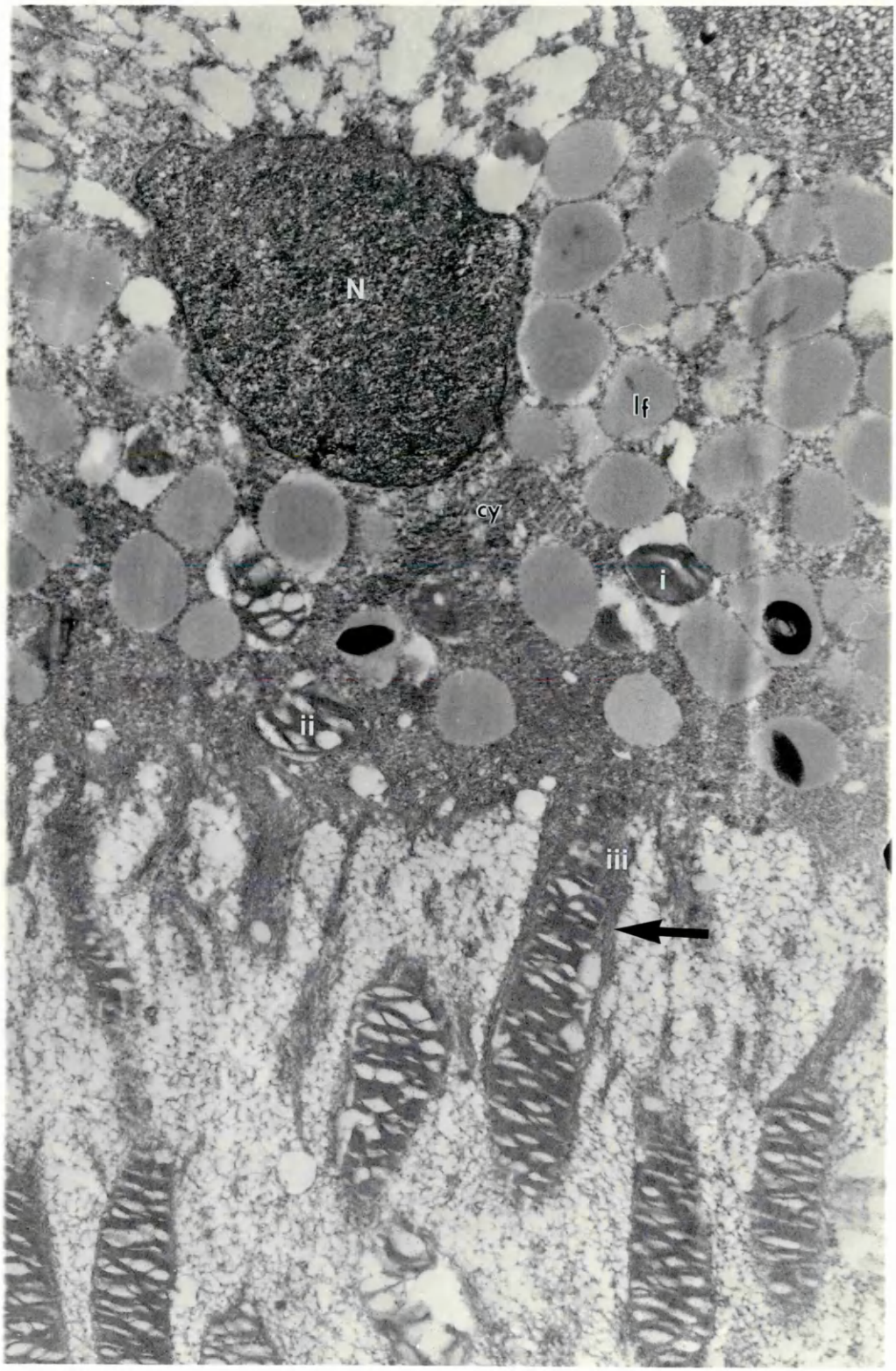

N
lf
cy
i
ii
iii

Figure 7.16 Ultrastructural Localisation of S-Antigen in Phagocytosed ROS at various Stages of Digestion within RPE

a) Electron micrograph of phagosome within an RPE cell. The epitope of S-antigen recognised by MAb S2.4.C5 could no longer be labelled, probably due to enzymatic degradation.

Magnification x 30,000

b) Electron micrograph showing newly phagocytosed ROS membranes labelled for S-antigen with MAb S2.4.C5 and protein A-gold.

Magnification x 16,000

c) Electron micrograph of LR White embedded human retina, showing the tip of an ROS embedded within the RPE cell. The ROS disc membranes were labelled for S-antigen with MAb S2.4.C5 and protein A-gold. There was no indication for specific staining of the RPE sheath surrounding the ROS tip.

Magnification x 30,000

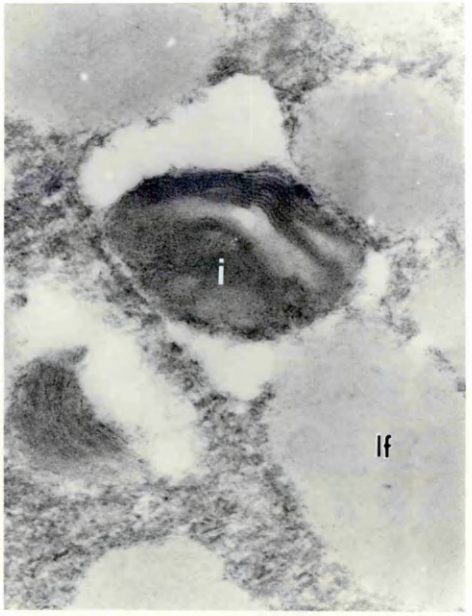
KEY

cy: RPE cell cytoplasm

lf: Lipofuscin deposits

i, ii and iii are also shown in Figure 7.15

a



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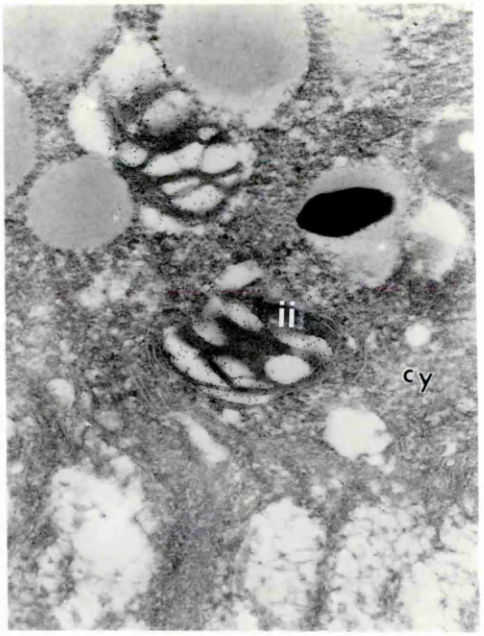
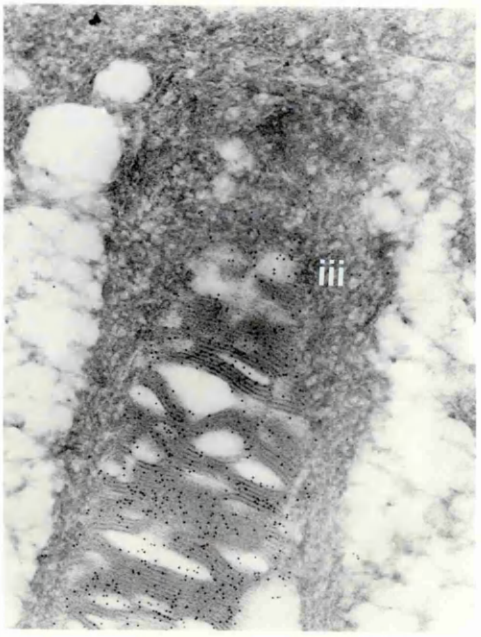


c



accumulation and condensation of undigestable ROS material and old melanin granules, within the aging RPE cells (Figures 7.15 and 7.16a,b).

Figure 7.16 presents enlargements of three regions of the area of RPE shown in Figure 7.15. Phagosomes containing newly phagocytosed ROS membranes were labelled with the S2.4.C5 monoclonal antibody (Figure 7.16b). Stained and unstained regions within a phagosome could be identified. Unstained phagocytosed material, presumably at more advanced stages of digestion were also observed (Figure 7.16a). No specific staining was identified within the RPE cytoplasm, Bruch's membrane or the choroid.

7.7 ULTRASTRUCTURAL LOCALISATION OF A 39K RETINAL PROTEIN

7.7.1 Localisation within Rod Photoreceptors

The pattern of staining for the R1.2.D2 IgM rat monoclonal antibody was similar at both light (Figure 7.4) and electron microscopical levels. The pattern of reactivity contrasted to that for S-antigen (Section 7.2.1 and 7.6). The antibody reacted intensely along the length of the ROS disc membranes (Figures 7.17a,b and 7.18a). Negligible staining was present within the rod inner segments and very little label was observed in the region of the connecting cilium (Figure 7.17b and 7.18a). There was perhaps an increase in staining around the nuclei of the rods where the antigen is likely to be synthesised.

The staining with antibody R1.2.D2, was apparent as far as the rims of the discs, and the gold label could also be detected on the rod plasma membrane. The thin cytoplasmic extensions of the inner

Figure 7.17 Ultrastructural Localisation of the 39K Antigen within Lowicryl embedded Human Rod Photoreceptors

a) Electron micrograph showing the localisation of a 39K antigen recognised by the rat IgM MAb R1.2.D2. The immunogold label was specifically associated with the ROS disc membranes.

Magnification x 42,000

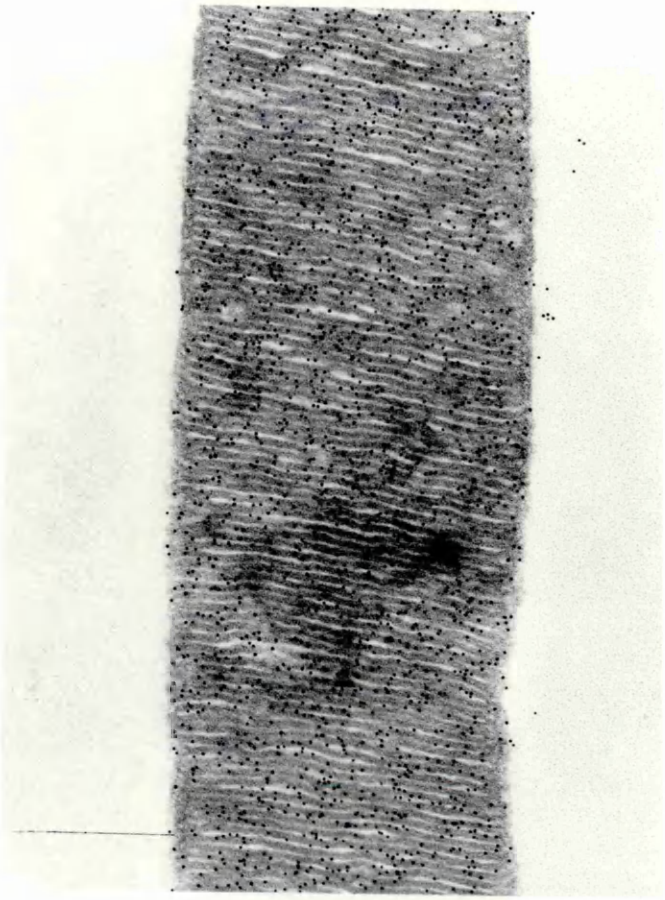
KEY

cc: Connecting cilium

b) Electron micrograph showing the region of the connecting cilium of a human rod cell. Although the ROS disc membranes were intensely labelled, there were very few gold particles within the connecting cilium or the inner segment.

Magnification x 42,000

a



b

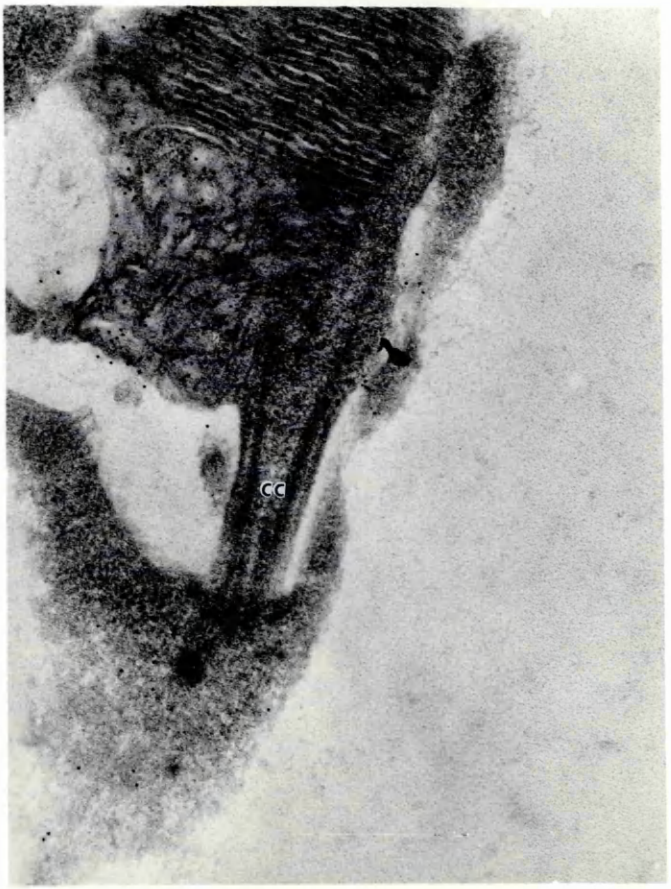


Figure 7.18 Electron Immunocytochemical Localisation of the 39K Antigen within Lowicryl embedded Human Rod Photoreceptors

a) Electron micrograph showing the localisation of a 39K antigen recognised by the rat IgM MAb R1.2.D2. The immunogold label was specifically associated with the ROS disc membranes. There was only a slight staining in the region of the connecting cilium, and even less in the inner segments of the rod cells.

Magnification x 44,000

KEY

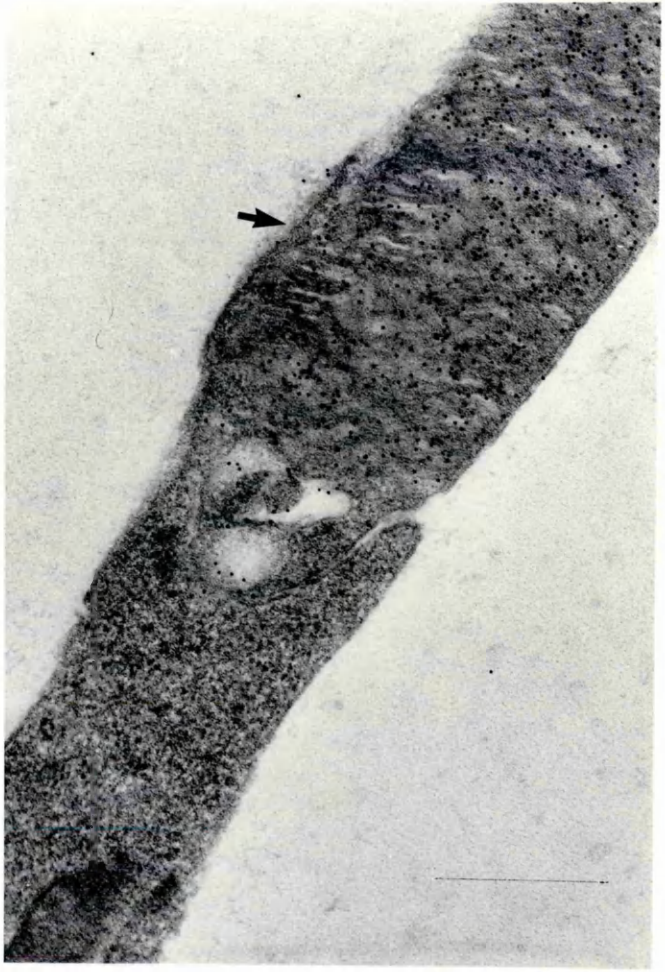
m: Mitochondrion

◄ Cytoplasmic extension

b) Electron micrograph showing the faint staining of a rod inner segment, with MAb R1.2.D2 and immunogold.

Magnification x 46,000

a



b

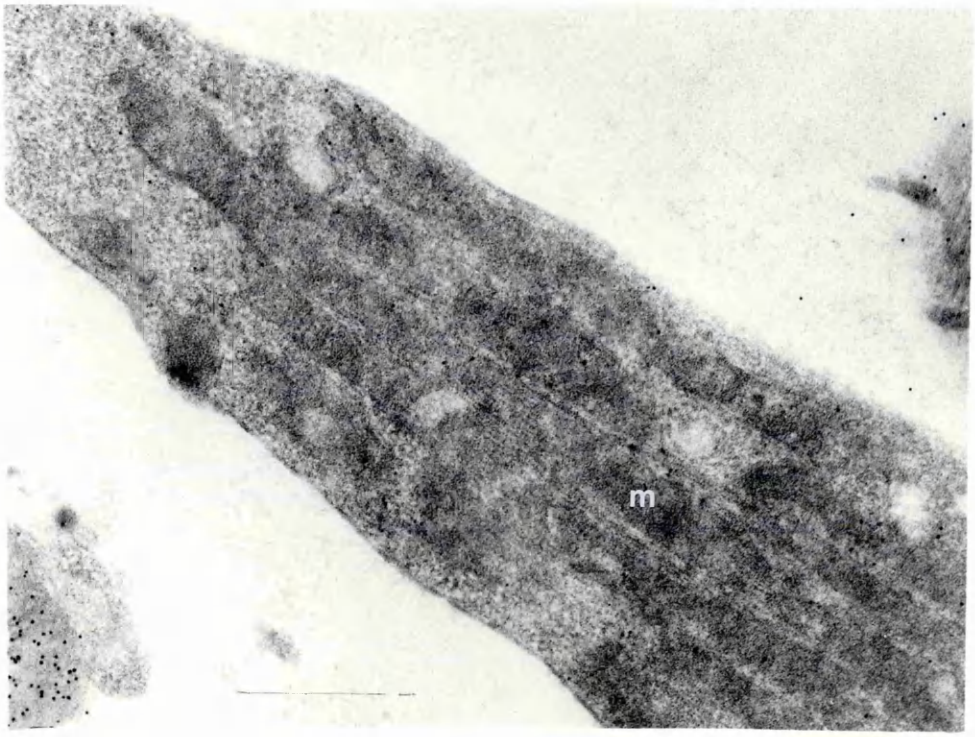


Figure 7.19 Localisation of the 39K Antigen in the region of the Rod Cell Plasma Membrane

a) Electron micrograph of a Lowicryl embedded human ROS, labelled with MAb R1.2.D2 and immunogold. In this rod cell the rims of the discs and the plasma membrane appeared to be preferentially labelled.

Magnification x 90,000

KEY

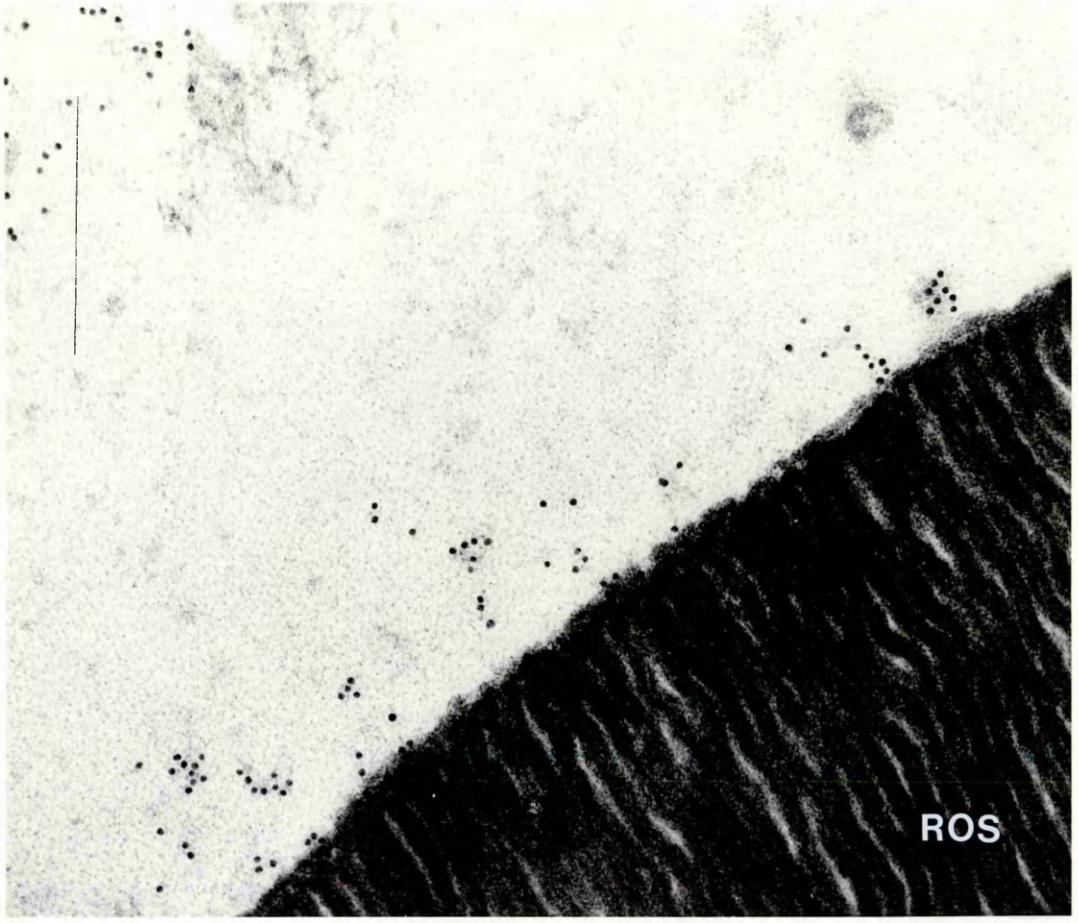
ROS: Rod outer segment

RIS: Rod inner segment

b) Electron micrograph showing labelling of a rod inner segment plasma membrane with MAb R1.2.D2 and immunogold. Few gold particles were identified within the cytoplasm of the inner segment.

Magnification x 90,000

a



b

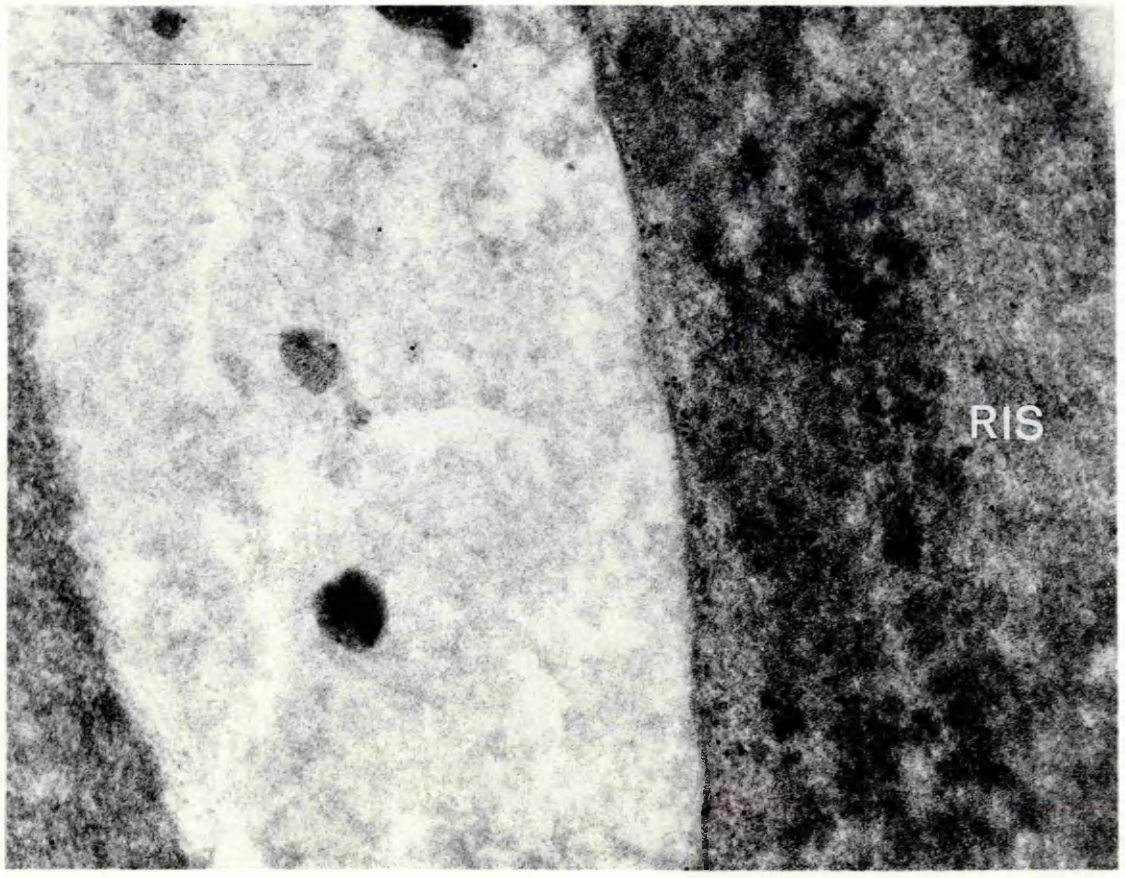


Figure 7.20 Absence of Immunogold labelling for the 39K Antigen in Lowicryl embedded Human Cone Cells

a) Electron micrograph showing a low power view of a human cone outer and inner segment. There was no indication for any specific staining with MAb R1.2.D2 and the immunogold probe.

Magnification x 24,000

KEY

COS: Cone outer segment

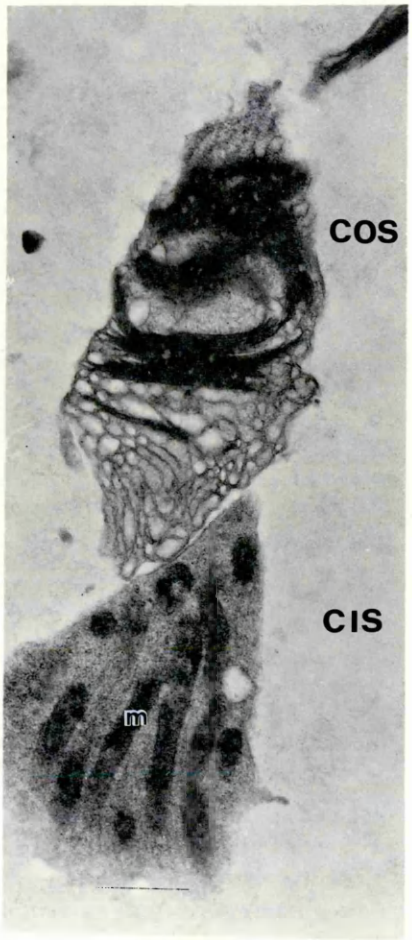
CIS: Cone inner segment

m: Mitochondrion

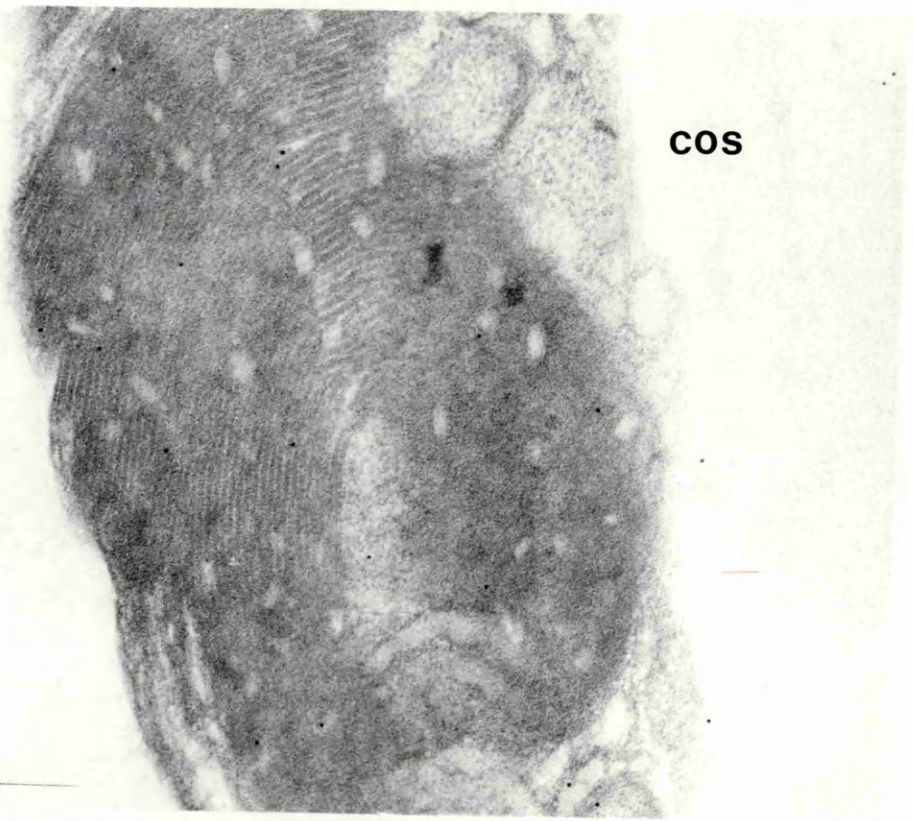
b) Electron micrograph of a human cone outer segment, treated with MAb R1.2.D2 and immunogold. The gold particles were present at only background levels.

Magnification x 70,000

a



b



segments which surround the lower regions of the ROS were labelled (Figure 7.18a), indicating further that the antigen may be present on the ROS plasma membrane. Specific decoration of the membrane surrounding the ROS was difficult to discern, however, due to the poor preservation of the plasma membrane. It is also possible that the antigen was subject to leaching off the plasma membrane during the tissue processing. In some rods, the plasma membrane of the outer and inner segments appeared labelled, although the cytoplasm of the inner segment was unstained (Figure 7.19a,b). A few rods were found in one section, which were labelled preferentially at the periphery of the ROS, apparently staining the rims of the discs and/or the ROS plasma membrane (Figure 7.19a). The phenonemon may have been a staining artifact, and was not seen in the sections labelled with the S2.4.C5 monoclonal antibody to S-antigen.

7.7.2 Reaction with Cone Photoreceptors

Antibody R1.2.D2, was unreactive with the cones of human (Figure 7.20a,b) and pig retina.

7.8 SPECIFICITY CONTROLS FOR IMMUNOCYTOCHEMICAL LOCALISATION

7.8.1 Specificity of Antibodies

The specificity of labelling was indicated, in part, by the absence of label on irrelevant structures in the section, such as the photoreceptor nuclei. The specificities of the monoclonal antibodies, and of the antisera were also established in immunoblotting experiments (Sections 6.3.2 and 3.8.1, respectively). Antibody S2.4.C5 was apparently totally specific for S-antigen (Figure 6.7), indicating that the localisation was unambiguous. Similarly, the specificity of monoclonal antibody R1.2.D2, was shown in immunoblotting experiments (Figure 6.8b).

7.8.2 Comparison With conventional Polyclonal Antisera

Rat antiserum against S-antigen served as a useful comparison for the reaction with monoclonal antibody S2.4.C5, and also provided a positive staining control. The antisera gave rise to a similar staining pattern to that of the monoclonal antibody, primarily labelling the ROS disc membranes. In this study, anti-opsin sera was also used. Rhodopsin (and opsin) are known to be localised in the disc membranes, and thus antisera to this protein provided a useful disc staining control, and labelled the ROS disc membranes virtually exclusively (see double immunolabelling for opsin and S-antigen in Figure 7.11).

7.8.3 Negative Controls

The omission of the primary antibody provided one negative control, ensuring the specificity of the gold probes. The use of a negative antibody control, however, is very important. In this study, preimmune rat serum provided a negative control for the test antisera, and was used at the same or lower dilutions. This gave an indication of the possible nonspecific reactions with polyclonal antisera (Table 7.1). An irrelevant monoclonal rat IgG, to Vibrio cholerae, was employed as a negative control for the monoclonal antibodies (Figures 7.10 and 7.12). The control monoclonal antibody was used as the cell free culture supernatant or as a 10 fold concentrate in PBS (Table 7.1).

7.9 QUANTIFICATION OF STAINING

The intensity of labelling with the immunogold probe was assessed by counting the number of gold particles per square micron, with the aid of a template laid over the electron micrographs. The results for the quantitation of labelling of S-antigen are shown in Table

Table 7.1 Estimation of specific labelling of disc membranes by monoclonal antibody S2.4.C5

	Density of Gold Label*		
	Rod Outer Segments	Rod Inner Segments	Rod Nuclei
Human Retina			
S2.4.C5 x 1/2	40 +/-0.6 n=6	15.5 +/-2.96 n=4	0.45 +/-0.50 n=11
Anti-S x 1/200	44.3 +/-8.8 n=6	17.3 +/-6.9 n=9	4.0 +/-1.76 n=9
Anti-Opsin x 1/200	20.3 +/-6.1 n=4	6.5 +/-3.0 n=4	2.0 +/-1.58 n=4
MAb to V.cholerae x10	1.0 +/- 1.2 n=6	0.8 +/-0.75 n=5	n/d
Preimmune x 1/100	0.75 +/-0.8 n=4	1.25 +/-0.97 n=8	3.33 +/-0.47 n=3
Preadsorption **	1.09 +/-1.2 n=11	2.0 +/-0.89 n=5	0.8 +/-0.75 n=5
Ab blank	n/d	0.5 +/-0.5 n=4	0.5 +/-0.87 n=4
Pig Retina			
S2.4.C5 x 1/2	62 +/-21.4 n=11	27.7 +/-4.5 n=6	3.83 +/-1.34 n=6
Anti-S x 1/200	36.6 +/-9.0 n=12	23.5 +/-1.5 n=2	5.0 +/-1.22 n=4
Anti-Opsin x 1/200	76.4 +/-12.5 n=7	n/d	n/d
MAb to V.cholerae x10	0.86 +/-0.64 n=7	0.67 +/-0.5 n=3	n/d
Preimmune x 1/200	3.86 +/-1.8 n=4	n/d	n/d
Ab blank	3.83 +/-1.3 n=6	n/d	2.7 +/-1.09 n=4
	Pig Cone Outer segment	Pig Cone Inner Segment	
S2.4.C5	2.33 +/-0.47 n=3	3.25 +/-1.71 n=8	

* Expressed as mean particles per square micron +/- standard deviation.
** 100µg/ml S-antigen 1:1 S2.4.C5

7.1. Only the results obtained with the immunogold detection method were quantified.

Due to the hydrophilic nature of the Lowicryl and LR White resins, gold particles tended not to adhere non-specifically to it, and only rarely was gold observed on the resin. Thus, the resin could not provide a true indication of background reaction. Background staining was indicated by the deposition of gold particles on irrelevant structures such as the photoreceptor nuclei. The number of gold particles on the photoreceptor rod cell nuclei was always larger than the number present on other retinal nuclei, for example, those of the inner nuclear layer. This phenomenon may result from the diffusion of S-antigen during tissue preparation for electron microscopy, from the perinuclear area, where the protein is probably synthesised (Section 7.6.1).

Nonspecific reactions with irrelevant structures, and general background staining tended to be greater with the control positive and negative sera than with the monoclonal antibody S2.4.C5. Similarly, background was virtually absent when an irrelevant rat IgG monoclonal antibody was used as a negative control, rather than pre-immune rat serum (Table 7.1). Omission of the primary antibody also resulted in negligible staining.

7.10 Discussion

This study has revealed further information on the nature and physiology of photoreceptor antigens.

The electron immunocytochemistry results involving the specific monoclonal antibody S2.4.C5 to S-antigen, indicated that the protein

was located within the rod, but not the cone photoreceptors (Figures 7.7 and 7.14, respectively). The pattern of labelling within the rods suggested that this protein was synthesised in the perinuclear region of the rods (Figure 7.13), where the staining was more intense than in the inner segment (Figure 7.8b). It is in this region of the photoreceptor cell, that the rough endoplasmic reticulm and Golgi apparatus are located, although these membranous structures were not easily identified in the Lowicryl and LR White embedded retinae. S-antigen is probably transported from the perinuclear area to the apical region of the inner segment for its incorporation into the ROS. S-antigen was localised specifically on the disc membranes, and the gold label could also be identified within the region of the connecting cilium (Figures 7.7). This was in agreement with a previous study using polyclonal antiserum and similar techniques (McKechnie et al., 1985). Specific labelling of the ROS plasma membrane was less readily determined, due to the poorer preservation during tissue processing for electron microscopy.

At the level of the light microscope, S-antigen could be detected throughout the entire photoreceptor cell, including the synapses (Figure 7.2). It is not yet known whether S-antigen has a physiological role in this region of the cell. There is evidence that the protein may be involved in the control of the visual cycle (Section 1.2.3), however, S-antigen does seen to distributed in the rod cell, in a different pattern to that of opsin. A preliminary study at the light microscopical level, has suggested that S-antigen may be distributed in a different pattern under conditions of dark adaptation (Figures 7.3a,b; Section 7.2.1.2). If this is shown to be reproducible, similar experiments at the level of the electron

microscope would allow the pattern of staining to be more precisely determined, and also enable quantitation of labelling.

At the electron microscopical level S-antigen could also be detected in the newly phagocytosed ROS, but was apparently absent in some phagosomes which were presumably at more advanced stages of digestion (Figures 7.15 and 7.16). S-antigen was not localised within the RPE cytoplasm, the basement membrane or the choriod.

The 39K protein was localised on the rod disc membranes using the monoclonal antibody R1.2.D2 (Figure 7.17 and 7.18). Very light labelling was obtained for the epitope recognised by R1.2.D2, in the rod inner segments and connecting cilium (Figures 7.17b and 7.18a), which contrasted to that observed for S-antigen (Figure 7.7). This pattern of staining was also observed at the light microscopical level (Figure 7.4). The plasma membrane around the inner segment appeared labelled in some sections. The identity of the 39K antigen is yet to be determined, and it may be a subunit of one of the larger ROS proteins, eg. transducin (Section 8.6).

The epitope of bovine S-antigen recognised by the monoclonal antibody S2.4.C5, was shared by the pig, human and rat. Similarly, antibody R1.2.D2 was raised against bovine antigen, and is capable of recognising the human, rat and pig form of the antigen. This provides further evidence for the conserved nature of photoreceptor specific antigens.

CHAPTER EIGHT

GENERAL DISCUSSION

Chapter 8

GENERAL DISCUSSION

8.1 OVERVIEW

This study has investigated the question of whether humoral autoimmunity to retinal antigens is expressed in human retinitis pigmentosa and uveitis, and the retinal dystrophic RCS rat model. Specific serum antibody was sought to purified S-antigen, crude retinal extract and ROS membranes (Chapter 4). Human and rat B lymphocytes were modified, to allow long term culture enabling the potential anti-retinal immune response to be further studied (Chapter 5). In this study, the frequency of positive serum responses to retinal antigens, and the frequency of clones secreting antibody to S-antigen or ROS, was greater in the retinal dystrophic rats than in the control rats, and greater in the uveitis patients than in the RP patients or in the healthy controls.

The second aspect of this thesis involved the characterisation of rat monoclonal antibodies to purified S-antigen and a rod outer segment membrane protein. An IgG monoclonal antibody was raised against bovine S-antigen, and an IgM monoclonal antibody was produced to a rod disc membrane protein with an apparent molecular weight of 39K (Chapter 6). Both of these antibodies, derived from hyperimmunised DA rats were used to localise the respective antigens at the electron microscopical level on human and pig photoreceptor rod disc membranes (Chapter 7). The affect of one monoclonal antibody, on S-antigen induced EAU was also investigated.

8.2 PROPERTIES OF RETINAL S-ANTIGEN

8.2.1 Presence of Carbohydrate on S-Antigen

S-antigen has been tested for the presence of a carbohydrate group by means of the periodic acid Schiff's (PAS) reaction. A faint positive PAS reaction was obtained for the S-antigen prepared for this study, suggesting the presence of a relatively small carbohydrate moiety, which is perhaps easily lost during the purification procedures. Both positive and negative reactions have been reported (Borthwick and Forrester, 1983; Wacker et al., 1977, respectively). Blotting of S-antigen transferred to nitrocellulose paper from SDS polyacrylamide gels, with radio-labelled lectins could yield useful information on the nature of this carbohydrate moiety. This technique offers a greater sensitivity than does the PAS reaction.

8.2.2 Uveitopathogenic capacity of S-Antigen and the effect of Monoclonal Antibody S2.4.C5 in EAU

Duncan-Hartley guinea pigs, injected in the hind foot pad with a single dose of 50µg of S-antigen mixed 1:1 with CFA, developed severe clinical uveitis within 17 days after the injection. Histopathological examination revealed that the morphology of the EAU was similar to that described by Forrester et al. (1985). The intraperitoneal injection of 1mg of the rat IgG1 monoclonal antibody S2.4.C5, with specificity to S-antigen (Section 6.3), simultaneously with the foot pad injection of S-antigen in CFA, resulted in an exacerbation of the clinical and histopathological features of EAU. This is in contrast to the situation in similar experiments with Lewis rats, were monoclonal antibody S2.4.C5 prevented the development of the disease (Section 6.6).

The results with the Lewis rats are similar to the findings of de Kozak et al. (1985) and Merryman et al. (1986), who found that their mouse monoclonal antibodies to S-antigen prevented the induction of EAU in rats. In such experiments, it is unlikely that anti-idiotypic antibodies to the monoclonal antibodies are involved, either in the suppression of EAU by inducing T suppressive anti-idiotypes, as suggested by de Kozak et al. (1985), or in the exacerbation of the disease by acting as surrogate antigen. Experience has shown that the production of anti-idiotypes is difficult to achieve, and requires injection with large quantities of the monoclonal antibody (Ghosh and Campbell, unpublished observations). It is unlikely that many anti-idiotypic antibodies, which truely resemble the original antigen, could be generated in the short period of time, (within two weeks) after injection. In experiments with rats, the monoclonal antibodies may facilitate the removal of S-antigen, reducing its exposure to the immune system. In the guinea pigs, the foreign rat monoclonal antibody might have resulted in the boosting of the immune response in general, perhaps involving immune complexes with the rat monoclonal antibody, and guinea pig anti-rat antibodies. The results are however, difficult to fully explain until the experiments are repeated, with additional tests. Further work is required in order to monitor of the animals' responses to the injected antibody, eg. assay for anti-idiotypic antibody to S2.4.C5, and the determination of the effect of the monoclonal antibody acting alone, in the induction of EAU. Important controls for such experiments include the injection of irrelevent antigen with the monoclonal antibody, and also the injection of an irrelevant monoclonal antibody with S-antigen.

De Kozak et al. (1985) reported that the disease was only inhibited if the antibody was administered at the time of injection of S-antigen, and not after 7 or 14 days. Since the level of circulating monoclonal antibody, measured by ELISA, did not persist at high levels after 7 days (de Kozak et al., 1985), it may have complexed with autologous antigen released from the retina, or with anti-idiotypic antibodies. It seems more likely, however, that the mouse antibody was removed by the rat immune system, as foreign protein, since the damage to the retina was unlikely to occur during the first week after immunisation.

In contrast to the present study and that of de Kozak et al. (1985), Merryman et al. (1986) injected their experimental rats with preformed immune complexes of S-antigen with two mouse monoclonal antibodies to S-antigen. One of the monoclonals did not have any effect on the EAU, and the second antibody was capable of preventing EAU in 85% of the animals, when 200µg of complexes were injected. The remaining 15% had a delayed onset of the disease. At higher doses, 300µg of complexes, all animals developed clinical disease although it was delayed in onset. Merryman et al. (1986) suggested that their second monoclonal antibody modified the S-antigen in a way in which the first antibody did not, and that immune complexes may play a role in the regulation of this T cell mediated experimental disease. It seems likely, however, that the immunising complexes would be broken up and processed fairly rapidly within the rats, both components of the complexes being foreign protein. In this respect, the complexes may have been rapidly cleared, thus reducing the chances of developing specific cell mediated immune mechanisms. Perhaps the ineffective antibody

of Merryman et al. (1986) was of a lower affinity, and did not readily remain complexed in vivo, and thus the S-antigen could persist longer and evoke the cell mediated responses which are responsible for the development of EAU. Furthermore, it is not possible to obtain true complexes of monoclonal antibody and antigen, since by nature monoclonal antibodies usually only recognise one epitope. Thus the ratio of antibody to antigen is only 1:1, or possibly 1:2, with an IgG monoclonal monoclonal antibody. Antigen complexes with IgM monoclonal antibodies can be larger, since they are polyvalent, but these will be very much smaller than than those which can be obtained with polyclonal antisera.

The activation or suppression of S-antigen induced EAU, by monoclonal antibodies, is likely to depend on many factors, including the relative doses of antigen and antibody, routes and time of injection, the affinity and isotype of the antibody, the nature of the appropriate epitope on S-antigen, and the species or strain of experimental animal. This is a new area of research into EAU, and may provide further information into the mechanisms of the induction of the disease.

Although the DA rats employed for the production of monoclonal antibodies were immunised with 2 to 3 injections of doses up to twice that required for the induction of EAU in guinea pigs, none of the animals examined demonstrated any significant ocular pathological changes. DA rats were utilised because of their ability to produce good immune responses to injected antigens. After a single injection, the DA rats were capable of developing titres of several thousand. These rats received subcutaneous

injections in the thigh and neck, in contrast to the animals involved in most EAU studies, where the animals were injected in the foot pad. It is thought that injection into the foot pad is more efficient in the induction of a cell mediated immune response, through the recruitment of lymphocytes to the draining lymph nodes, and EAU is thought to be largely cell mediated (Section 1.3.5). It is possible that the induction of specific suppressor T cells reduced the T cell mediated damage. The pathological changes which occur in EAU tend to be focal in distribution (Forrester et al., 1985). Perhaps, the sections of S-antigen immunised DA rat eyes which were examined represented the healthy regions of retina and choroid.

8.2.3 Fragments of S-Antigen Retain Immunological Activity

It has recently been suggested that a 40K antigen, isolated from ROS, contains most, if not all of the antigenic determinants of S-antigen (Gregerson and Putterman, 1984). This 40K antigen might be a precursor or breakdown product of S-antigen. However, this information was based on an immunoprecipitation experiment involving polyclonal antiserum to S-antigen. In the present study there was no strong evidence to support this hypothesis, since in immunoblots against crude retinal antigens, where a 40K band could be identified, both rat antisera and monoclonal antibody S2.4.C5, reacted only with S-antigen.

Partial proteolytic digestion of S-antigen in Cleveland gels, followed by immunoblotting with monoclonal antibody S2.4.C5, indicated that all fragments produced, contained the S2.4.C5 antigenic determinant. The smallest fragment obtained under these conditions was approximately 9.0 to 9.4K (Section 6.4.2), which

would contain in the region of 50 amino acids. Although smaller fragments could not be generated using this technique, cleavage with cyanogen bromide (CNBr) can produce smaller fragments (Gregerson and Putterman, 1984). It would be interesting to identify and purify the smallest fragment of S-antigen, bearing the S2.4.C5 epitope, and investigate its role in EAU. Perhaps the CNBr digestion technique of Gregerson and Putterman (1984) could be used in combination with immunoblotting. Similarly, it would be useful to identify and characterise the epitopes recognised by naturally occurring anti- S-antigen antibodies, from rats and humans (Chapter 4).

Gregerson and Putterman (1984) produced 7 major fragments with molecular weights ranging from 18 to 199 amino acids in length. Of these 7 fragments, 6 were purified and their amino acid sequences determined. In addition, a minor fragment was similarly characterised. All of these fragments were reactive in the ELISA with polyclonal antiserum, but their uveitopathogenic capacity has not yet been reported. In a short communication Stein (1984), presented evidence that a mixture of S-antigen fragments, mainly of 10 to 30K, was capable of inducing EAU in about 50% of Lewis rats. However, the successful induction of EAU with a mixture of fragments is not surprising, since the development of EAU is thought to involve T cells (Section 1.3.5), sensitised by small fragments of antigen processed and presented on the surfaces of antigen presenting cells, such as macrophages, in association with Class II histocompatability antigens. More information is to be gained from experiments involving the induction of EAU with purified fragments of S-antigen. Kamada et al. (1985) produced 3 peptides of S-antigen by digestion with α-chymotrypsin. Only the

24K peptide and not those of 16 and 12K, reacted with the rabbit polyclonal antisera in the ELISA. However, the reactivity of the antisera with S-antigen in the ELISA is dependent on the collective affinities of the polyclonal antibodies, and on the dilution used. This was also seen in the Cleveland blot experiments involving 3 different antisera shown in Figure 6.15. The smallest fragment detected in this experiment was 9.7K. These sera were used at 1:400 dilution, and it is possible that at a 1:200 dilution, the 9.2 and 9.4K bands would also be detected. The 24K fragment identified by Kamada et al. (1985) was uveitopathogenic, but this is a relatively large fragment (50% of the original protein). It is quite probable that there are several regions of the molecule which are capable of inducing the disease, and together they have a synergistic effect, since they will activate separate clones of T cells with different specificities.

Together, polyclonal and monoclonal antibodies will aid the identification of the major antigenic determinants of S-antigen. It is quite possible that different epitopes of S-antigen might be antigenic, or uveitopathogenic in some species, but not others. Therefore antibodies from different species, in addition to different individuals, should be investigated. Recently it has been suggested that the majority of antigenic determinants are conformational, rather than linear (Tainer et al., 1985; Barlow et al., 1986). However, the epitope recognised by S2.4.C5 is more likely to be linear in nature since it has survived treatments likely to be detrimental to many conformational determinants, ie. enzymatic digestion followed by SDS-PAGE and binding to nitrocellulose paper, and fixation in 4% gluteraldehyde (Figure 7.2), processing for electron microscopy.

8.3 EXTENT OF AUTOIMMUNITY IN UVEITIS AND RP PATIENTS

In recent years, there have been numerous studies investigating the immune mechanisms of RP and uveitis patients, as well as other occular patients (Section 1.4). However, the data supporting the view of associated autoimmunity, with many of these diseases, is largely unconvincing, and often contradictory. The contradictions between groups probably arise, in part, from the differences in the sensitivities of the techniques employed, the low numbers of patients and controls investigated and also the interpretation of the data. Often the results for the various immunological tests suggests that the majority of the patients responses lie within the normal range. However, two or three individual patients generating high readings, raise the mean response of the whole group slightly, but statistically significantly, above the mean of the controls (eg. Nussenblatt et al., 1980; Nussenblatt et al., 1983; and Gregerson et al., 1981).

8.3.1 Estimation of Serum Anti-retinal Immune Responses of RP and Uveitis Patients

The preliminary study described in Section 5.3, sought to investigate the relative frequencies of circulating B lymphocytes in RP and uveitis patients, which were capable of secreting antibody against S-antigen or ROS after activation by EBV. The patients' serum titres to these antigens, were also recorded (Section 4.2.4) so that comparisons could be made. In this limited study, only one of the 11 control sera, gave an indication of a positive titre to S-antigen and ROS in the ELISA (Figures 4.12a,b), and was obtained from a user of the antigens, who probably became passively sensitised through contact (Section 4.2.5). Only 5 RP,

and 6 uveitis patients were tested in serum study. It is interesting that although none of the RP patients generated positive serum titres to either antigen (Figures 4.10a,b), five of the six uveitis patients tested either responded to S-antigen or to ROS, but not both (Figures 4.11a,b) (Sections 4.2.5). It is also interesting that all of the positive human sera identified S-antigen and the 40K antigen in immunoblots, regardless of the ELISA specificities (Figure 4.14). These two components were also recognised by the positive RCS and Wistar rat sera (Figures 4.7 and 4.8) (Sections 4.1.8-9). The results with the human sera suggest that there were determinants on S-antigen which were exposed in the immunoblotting technique, but were not readily detected in the ELISA. Conversely, human (and rat) autoantibody to the ROS insoluble proteins, such as opsin, were not readily detected by this method, probably due to the denaturation of the relevant epitopes. It is also possible that the relative low affinity and/or avidity of the antibodies resulted in the failure to demonstrate autoantibody to opsin in the immunoblots.

The 40K antigen, present in the crude retinal extract (predominantly soluble antigens), was not seen in the S-antigen and ROS preparations in significant quantities, and therefore was probably not readily detected in the ELISA assay systems employed. The 40K antigen was thought not to be a breakdown product of S-antigen, since rat monoclonal antibody S2.4.C5, specific for S-antigen, did not recognise it (Figure 6.7). The retention of the S2.4.C5 epitope in fragments of S-antigen is further exemplified by the Cleveland blotting experiments (Figure 6.13).

It is interesting that S-antigen and the 40K antigen appeared to

dominate the autoimmune response in both the positive rat and human sera. Perhaps this is a reflection of the high antigenicity of these ROS components, compared with the other retinal antigens. The high solubility of S-antigen, and perhaps also of the 40K antigen, might facilitate their leakage from the photoreceptors, during degeneration or inflammation, and the stimulation of immunocompetent cells.

However, until a larger scale study is completed, it is not possible to draw any conclusions on the significance of the patients' responses, shown in the ELISA assays and immunoblots. Others have found that there is little difference in the serum antibody response to S-antigen in the ELISA of uveitis patients and controls (Forrester, personal communication, 1986). Further studies are required in order to determine the immunological importance of the 40K antigen.

8.3.2 Presence of Anti-Retinal B Lymphocytes in Uveitis and RP

In the transformation study (Sections 5.3.6-11), only one of the 5 control samples generated a possible positive clone (Section 5.3.6). It is, of course possible, that in a larger study, a greater proportion of positive serum responses, or positive clones would be detected. The transformation results did not fully correspond to the patients titres. One uveitis patient with a relatively high titre to ROS, did not generate any positives upon transformation (Sections 5.3.8 and 5.3.11). Another patient, with RP, who did not demonstrate a significant serum response to either antigen in the ELISA, generated several clones positive to both antigens upon transformation of the peripheral B lymphocytes (Section 5.3.7 and 5.3.11). These findings might be an indication

of the nature of the B cells which were transformed. It is not yet known whether EBV invades primary small B lymphocytes, memory cells or more mature B lymphocytes. The virus may infect B lymphocytes at more than one stage of maturation, and yet be selective as to the nature of the surface receptors on the B cells, perhaps including the antigen specificity, and this could vary between individuals. There is evidence that EBV may selectively transform B cells which are just beginning to secrete antibody, but not mature plasma cells (Steel et al., 1986).

Three samples obtained from one uveitis patient, on separate occasions, generated almost identical serum titres to S-antigen (Figure 4.11a), yet the transformation of the B cells yielded differing frequencies of clones positive to S-antigen and ROS (Figures 5.17) (Sections 5.3.8.1 and 5.3.11). This might be a reflection of the different phases in the regulation of the immune system. There are, however, many other factors which might have an effect on the number of clones obtained, including the physiological state of the patient and recent infections. Even the time of day at which blood samples are taken can affect the number of lymphocytes obtained, if the circulating cells follow a circadian rhythm. Little is known of the physiological variations which occur in the immune system, perhaps on a daily basis. Others have reported variations in the production of IL-2 and α-interferon by lymphocytes isolated from the same individuals on different days (BenEzra et al., 1984; Hendricks and Fishman, 1986) (Section 1.4.3.6). Clearly, a detailed longitudinal investigation of the circulating immunoglobulins and cells of the immune system, in healthy individuals, would provide a valuable reference for all studies involving tests of immune function.

The involvement of the immune system is even less certain in RP than in uveitis, and no obvious inflammation has been found in RP, although the presence of cells in the vitreous is a constant feature of RP (Marmor et al., 1983). Undoubtedly the immune system is involved in uveitis, since it is the cells of the immune system which infiltrate into the uveal tissues and vitreous (Section 1.4.4.1). However, the specificity of this inflammatory response is yet to be accurately determined. It is possible that increased vascular permeability or changes in the pigment epithelium occurring in some patients, gives the potentially autoreactive antibodies or T cells, the opportunity to localise within the vitreous or uveal tissues. It is also possible that in uveitis disease there is a malfunction in the regulation of the immune system, such that the normally present low affinity autoantibodies or antibodies, which crossreact with self antigens, eg. retinal proteins, could take on a pathogenic role. Similar antibodies in healthy subjects, are relatively harmless. It has been suggested that anti-idiotypic antibodies could have a protective role, since retinal vasculitis patients with high levels of circulating immune complexes, tended to have less severe disease (Drumonde et al., 1982).

Much of the published work cannot be verified until a large, perhaps double masked, study is undertaken, preferably funded by a relevant body and monitored by a scientific committee, as recommended by BenEzra et al. (1984). The RP and uveitis patients should be clearly classified, as far as possible, and numerous age matched controls included, since age is a relevant factor in the consideration of autoimmunity.

Should a full evaluation of the responses of human transformed B lymphocytes reveal no significant difference between the patients and controls, the value of the transformation technique remains as a step towards the development of human monoclonal antibodies. A human monoclonal antibody, derived from an individual not artificially immunised, may identify an interesting epitope of an antigen, not readily detected in hyperimmune animal serum. It may be that patients with uveitis disease produce specific antibody, or antibody which crossreacts with S-antigen, recognising a certain group of antigenic determinants, which are not recognised by the sera of healthy controls, although the control sera may identify other, perhaps less pathogenic epitopes.

8.4 HUMORAL AUTOIMMUNITY IN RCS RATS

8.4.1 Immunocytochemistry with RCS Rat Sera

Previous reports regarding humoral autoimmunity in the retinal dystrophic RCS rat utilised the relatively insensitive immunofluorescence technique and unspecified numbers of animals (Brinkman and Broekhuyse, 1981; Chant and Meyers-Elliott, 1982). These studies employed undiluted sera, or very low serum dilution. Under such conditions, the chances of nonspecific binding of antibody due to charge attractions (ionic and Van der Waals forces) as well as physical interactions with the tissue section are maximised. In addition, many cells have fc receptors to which serum immunoglobulin will bind in immunocytochemisty. Furthermore, low serum dilutions enhance the interaction of low concentrations of autoantibodies to non-tissue specific antigens such as contractile proteins, nuclear and mitochondrial antigens (Spalton et al., 1978b; Murray, 1986). In this study, the immunofluorescence

technique was found to be unreliable when neat serum was used on the frozen sections of rat eye, since the results were poorly reproducible, and that similar frequencies of apparent (or false) positives were detected in RCS dystrophic rat sera and PVG control sera (Table 4.31). Clearly section thickness and a differential washing between sections could also affect the intensity of an apparent specific reaction. The choroid usually demonstrated intense fluorescence, presumably due to the endogenous rat immunoglobulin in this highly vascular tissue. Where fluorescence was observed within the retina, the reaction tended to be located in the photoreceptor cell bodies rather than the ROS, similar to the observation of Chant and Meyers-Elliot (1982). Ideally, a wide range of serum dilutions should be tested, and a large number of rats tested, including different strains, the immune response genes may lead to considerable differences between strains (Ruscetti et al., 1975; see also Roitt et al., 1985).

The present study also utilised the more sensitive indirect peroxidase immunocytochemistry procedure, the exquisitely sensitive ELISA and a sensitive immunoblotting technique (Section 4.1.2.2). In the immunoperoxidase cytochemistry assay, only one Campbell rat serum sample, at a dilution of 1:80, was found to give a faint positive reaction with the photoreceptor outer segments. At dilutions lower than 1:80, there was a higher proportion of weak, perhaps normally present autoantibodies reacting with the non-tissue specific antigens, however the general background reaction present at lower dilutions was too high. In a further study, it would be useful to investigate the reaction of RCS rat sera in tissue sections using the peroxidase anti-peroxidase (PAP) technique, which offers sensitivity at least 100 fold greater than

that of the indirect peroxidase procedure (see Polak and Van Norden, 1984).

Immunocytochemical assays are often used to test for autoantibody, since they offer a wide variety of tissue antigens. In addition, the antigens are often considered to be close to their native form, especially in unfixed frozen sections. However, the antigens of either frozen or dewaxed paraffin sections of tissue, such as retina, will be differentially exposed and also exist at different concentrations. Under the conditions of assay, soluble antigens like S-antigen, are easily washed away. Therefore light fixation is necessary. Cross-linking fixatives such as formaldehyde (usually as formalin) and gluteraldehyde were not used to fix tissue to be used for the assay of potentially autoimmune sera, since the relevant epitopes are likely to be altered, and also at the low dilutions necessary to detect the autoantibodies, the background reaction would be very intense. Thus 95% ethanol or acetone was utilised to insolubilise the normally soluble proteins. Therefore, in immunocytochemical assays the antigens are not in the native form.

8.4.2 Identification of Autoimmune Rat Sera in the ELISA

Sera from 3 of the 10 Campbell RCS rats at the age of 6 weeks, demonstrated responses to S-antigen in the ELISA, significantly above those of the apparently unresponsive littermates (Figure 4.3b) (Sections 4.1.5 and 4.1.9). Similar responses were not detected at the age of 3 weeks (Figure 4.3a). Only one of the three rats retained a detectable positive response at the age of 9 weeks, at which time a fourth Campbell rat became seropositive (Figure 4.3c). Two of the RCS rat sera, reactive with S-antigen, also recognised ROS antigen(s), albeit at lower titres (Figure 4.3b).

The lower magnitude of responses to the ROS antigen, was not thought to be a reflection of a lower sensitivity of the assay compared to the S-antigen ELISA system. It is possible that the relevant epitopes of opsin, which accounts for most of the ROS membrane protein, are denatured upon binding to the plastic microtitre plates (Berkowitz and Webert, 1981). In order to partially compensate for this, the ELISA plates were coated with ROS at a greater protein density (5µg/ml) compared with S-antigen (1µg/ml). The amount of antigen adsorption probably varies with the nature of the antigen, and it was not possible to determine the relative binding of the two antigens in the ELISA. This would be possible in a radioimmunoassay system, where binding could be more readily assessed by radiolabelling the antigen. However, the efficiency of labelling of the different antigens would provide another variable.

The reason for the drop in titres at the age of 9 weeks, of the two of the Campbell rats seropositive at 6 weeks, for S-antigen, might reflect a minor reduction in the secretion of autoantibody to S-antigen, such that the antibodies could no longer be detected in the ELISA. It is possible that in these animals a suppressor T cell response modified the specific anti- S-antigen response. The development of self tolerance usually occurs in neonatal life, but may continue for a few weeks. The animals used in this study were relatively young, from 3 to 9 weeks in age, and might still have been susceptible to the induction of tolerance. The production of anti-idiotypic antibodies during the control of the immune response might be responsible for sequestering the autoreactive antibodies, or perhaps more likely, minute quantities of antigens released from the degenerating ROS, was enough to sequester the small

concentration of autoantibody present. Further work, however, is required to verify this postulation, perhaps involving assays for soluble immune complexes.

No similar positive titres to S-antigen were detected within the 10 Hunter RCS rats tested (Section 4.1.6). It is interesting that 1 of the 5 Wistar control rats, but none of the 15 pigmented controls, was identified as positive to S-antigen (at 6 weeks only) (Section 4.1.4). Thus, the positive serum responses were detected predominantly in the pink-eyed strain of dystrophic rat, and also in one albino Wistar rat. This raises the possibility that the apparent sensitisation of the animals to retinal antigens, S-antigen in particular, arises from light induced damage to the retina, rather than from the genetic degenerative process. Indeed the presence of pigment within the RPE and choroid has been found to slow the degenerative processes by about a week (Yates et al., 1974; La Vail and Battelle, 1975). The phenomenon of light induced retinal damage has been well documentd since its first report in albino rats by Noell et al., in 1966. High light intensities and long exposures result in the total disruption of photoreceptor ultrastructure, beginning with the ROS, and resulting in the eventual loss of visual cells (La Vail, 1976c; Duncan and O'Steen, 1985). Perhaps the degeneration of the RCS rat retina increases the susceptibility of the Campbell strain to light induced damage, which results in the exposure of the immune system, in some animals, to previously hidden retinal antigens. Previous reports on the reaction of dystrophic rat sera with retinal antigens in sections (Brinkman and Broekhuyse, 1981; Chant and Meyers-Elliott, 1982), only examined the pink-eyed strain. Where positive reactions were detected, the possibility of a light induced exposure of the

immune system to retinal antigens was not considered (Chant and Meyers-Elliott, 1982).

It would be interesting to identify the class of autoantibodies in the RCS rats using specific probes. The weak positive responses could either be due to the presence of low affinity antibody, probably IgM, or a very small amount of higher affinity IgG.

Thus, the humoral autoimmune response in the RCS rats does not appear to be general, since the majority of animals did not demonstrate a detectable response. In this study, the sera of 10 Campbell RCS rats were tested at three different time intervals. If fewer animals were tested, and if only one time interval was chosen, it would have been possible to miss the positive sera. Previous reports studying the potential anti-retinal autoimmune response of RCS rats did not state the number of rats involved in the study. Further work is required to acsertain the frequency of positive titres among the RCS rats to the 40K antigen.

8.4.3 Certain RCS Rat Sera identify S-Antigen and a 40K Antigen

The specificity of the anti- S-antigen immune response observed in the four Campbell and one Wistar positive sera was assessed in immunoblots against crude retinal extract, solubilised ROS membranes and purified S-antigen (Sections 4.1.8 and 4.1.9). The Wistar and two of the four Campbell sera, positive to S-antigen in the ELISA, also demonstrated a reaction with the protein in immunoblots (Figures 4.7 and 4.8). Some of the RCS rat autoantibodies may have been directed against epitopes on the native S-antigen, which became denatured after SDS-PAGE and binding to nitrocellulose paper, since the sera of two Campbell rats,

positive in ELISA, were unreactive in immunoblots. Alternatively, the relative affinities of some of the autoantibodies may have been too low to detect under the conditions employed in immunoblots. Evidently some autoantibodies produced by the rats were of high enough affinity and present in sufficient quantity for detection, and directed to epitopes which survived the conditions of immmunoblotting. The two RCS and the one Wistar rat sera also recognised a 40K antigen in the crude retinal extract, and one of the RCS rats also weakly reacted with a 35K antigen. The 40K antigen is probably the same band seen in the blots with the human patients' sera, and the 35K antigen may be a breakdown product of S-antigen. Again, similar to the situation with the human sera, autoantibodies were not detected to opsin in this technique (Section 8.3.1).

It should be remembered that both the ELISA and especially the immunoblotting techniques involve several washing steps. During the washing procedure, antibodies of lower affinity and specificity may be leached from the plate or nitrocellulose paper. These techniques tend to be selective for high affinity antibodies, which may be used at low concentrations. However, autoimmune serum, especially when the degree of specific sensitisation is not great, contains antibodies of relatively low affinity and avidity. Such antibodies might take longer to bind to the relevant antigens in the immunoassays (Mason and Willams, 1980) and if the antibodies are of low avidity, they may easily be washed off. Also if the association and dissociation of the antibody with the antigen is rapid, the antibodies would easily leach from the immobilised antigen.

8.4.4 Identification of RCS Splenic B Cell Clones with Specificity to S-Antigen and ROS

The hybridoma study was somewhat different from in the serum study, since it was not possible to control the number of tests (clones) per animal. Although numerous clones secreting antibody reactive with S-antigen and ROS were detected in the ELISA, the majority of these were obtained from one RCS rat fusion (Table 5.1). This fusion was derived from a Hunter rat, yet in the serum study, responses as high as those seen in Campbell rats, were not found. In the hybridoma study, only one fusion with a Campbell rat generated viable cells (Table 5.5). Similarly, relatively few primary clones were obtained in fusions with PVG lymphocytes. The reason for the poor fusion frequency obtained in many amimals in this study may be due in part, to the differential response of the newly fused cells to minor variations in tissue culture conditions. However, the reason probably lies with the splenic B lymphocytes. Generally, hybridoma technology and the generation of monoclonal antibodies, involves the intraperitoneal boosting of the immune system with small doses of the immunogen, 3 to 4 days prior to fusion (see Campbell, 1984). This stimulates blastogenesis of the antigen specific clones. Cells in the blastoid stage have been found to be the optimal fusion partners for the myeloma cell lines, not because fusion frequency is greater, by because the nuclei of the two cell types are likely to enter mitotic division at the same time (Løvborg, 1982).

However, in spite of the above difficulties, the fact remains that one rat, at least, generated a fusion frequency and a frequency of positive clones, comparable to a fusion derived form an immunised

rat. This indicates that several B cell clones were undergoing division at the time of sacrifice for fusion. Since several clones were positive to retinal antigens, the individual may have become autosensitised to the degenerating retina. However, cloning of three of these primary growths, and further characterisation of the monoclonal autoantibodies, indicated that they were of relatively low affinity compared to the monoclonal antibodies obtained from hyperimmunised animals (Section 6.3.1.1). The relatively low affinity of the RCS rat monoclonal autoantibodies, might be a reflection of their class, which was IgM. Thus, the rats were probably in the early stage of an immune reponse to the retinal antigens, which may have matured to give higher affinity antibodies should the rats have been spared.

Although the Campbell strain generated a higher frequency of positives in the serum study, the individuals utilised in the hybridoma study, yielded few or no clones (Table 5.5). This have been an indication that few B cells in these individuals were undergoing blastogenesis in response to retinal or other antigens. Similarly, the PVG control fusions did not yield high fusion frequencies (Tables 5.6-8).

Until more successful fusions are obtained with the control rats, it is not possible to conclude that retinal autoreactive B cells can only be derived from retinal dystrophic rats. However, it seems unlikely that such a high frequency of positive clones to retinal antigens would be obtained from a healthy rat, as that found with Hunter rat 1 (Table 5.1).

8.4.5 Possible Dual Specificity of Monoclonal Autoantibody H10.1.D2

The three monoclonal autoantibodies derived from two Hunter RCS rats, H9.1.C2, H9.5.A4 and H10.1.D3, respectively recognised S-antigen, opsin and crossreacted with S-antigen and opsin (Sections 6.3.1.2 and 6.3.2.2). The interesting crossreactivity of antibody H10.1.D3, is perhaps more likely to reflect dual specificity of the antibody, than to suggest shared carbohydrate residues or amino acid sequences. In this situation the antibody binding region has the capacity to react with more than one epitope, which may be unrelated and located on different antigens (Ghosh and Campbell, 1986). Such phenonema are more common in IgM than IgG antibodies (reviewed by Ghosh and Campbell, 1986). However, many of IgM antibodies, derived from hyperimmunised animals, do exibit good specificity and affinity.

The monoclonal autoantibodies produced in this study, might recognise epitopes which are not 'seen' by hyperimmune serum, which tends to recognise the dominant determinants. Further studies, involving suitably modified Cleveland blots, with concentrated antibody, could generate some interesting data.

8.4.6 Significance of the Anti- S-Antigen Response in Albino RCS and Wistar Rats

The significance of the low positive titres to S-antigen, found in 40% of the Campbell strain, and the high frequency of positive clones from a Hunter rat fusion, is difficult to evaluate. Since the genetic defect in the rat retinal dystrophy is known to be expressed in the RPE cells (Section 1.5.4) an autoimmune response to the retina is unlikely to be responsible for the initiation of

the disease. A sensitisation to the retinal antigens, secondary to the primary defect in the RPE, may occur in some animals, particularly in the pink-eyed Campbell strain, where the light induced retinal damage can exacerbate the condition.

There is evidence that in the RCS rat, there is a breakdown, and decrease in number, of the cell junctional complexes which normally form a belt-like arrangement around the RPE cells, thus forming the barrier between the choriocapillaris and the retina (Caldwell and McLaughlin, 1984; Caldwell et al., 1984). In the albino RCS rat, these junctions become abnormally permeable to tracer substances after 3 weeks. These changes are not apparent in the pigmented RCS rats until the age of 6 weeks (Caldwell and McLagchlin, 1983). Furthermore, there are several reports of degenerative changes in albino rat RPE cells, prior to or concomitant with light induced photoreceptor cell degeneration (see Semple-Rowland and Dawson (1986). There is also evidence that the albino RPE is also functionally altered after exposure to extremely low light intensities (Semple-Rowland and Dawson, 1986). Although the rats employed in the present study were not exposed to extremes of light intensity, it is possible that in some individuals, changes did occur in the RPE, resulting in a breach of the blood-retinal barrier. Thus the circulating components of the immune system could be exposed to previously hidden antigens. Minute amounts of the highly immunogenic photoreceptor antigens may diffuse through the RPE layer to the choriocapillaris, or perhaps the incoming macrophages which phagocytose the ROS membrane debris, present the antigenic debris to the T cells.

In view of the very small size of the retina relative to the whole

body, and the microscopic regions where there may be a breach in the blood-retinal barrier, it may be unreasonable to expect a strong stimulation of the immune system. Immunised animals, producing high titres are usually injected with 30 to 100µg of S-antigen, or ten fold greater concentrations for other retinal antigens (Section 1.3), mixed 1:1 with CFA which nonspecifically activates the immune system, thus facilitating a strong response to the immunogen. Under these conditions the antigens are slowly released, providing exposure of the immune system to the antigen over a period of time, thus aiding the maturation of the immune response.

The fact that hyperimmunised DA rats utilised for monoclonal antibody generation, did not develop EAU, would suggest that a low titre, such as those demonstrated by the 4 Campbell and one Wistar rat, is not likely to be immunopathogenic. However, the RCS rats may develop a breached blood-retinal barrier (Caldwell and McLaughlin, 1984), which would initally result in the autosensitisation, whereas the experimentally induced immune response in the DA rats would have to cause a breach in this barrier in order to initiate EAU.

If a humoral immune response against the retina was an important secondary development in the RCS rat, it might be expected that the response would be more readily detectable, and the antibodies to be of higher affinity and present in all individuals of every strain of the dystrophic rat.

Finally, it should be noted that heterologous (bovine) antigens were used to screen the rat and human sera, and cell culture

supernatants, in the ELISA assays. Although strong evidence exists for the conservation of S-antigen and opsin (Section 1.2.2) (O'Tousa et al., 1985), and probably other photoreceptor specific antigens, the possiblity remains that some antibodies recognised epitopes specific to the homologous antigen (ie. rat or human). This would result in a lower serum titre and also a decrease in the detected frequency of positive primary immortalised B cell clones.

8.5 IMMUNOCYTOCHEMICAL LOCALISATION WITH MONOCLONAL ANTIBODIES

Lampson and Fisher (1985) recommend that in situations where a monoclonal antibody is to be used to locate an antigen in a tissue sample, the homogenised tissue be first immunoblotted to ensure that no crossreactive material is present. The immunoblot of the monoclonal antibody S2.4.C5 indicates high specificity for S-antigen so that the immunocytochemical localisation is unambiguous. In addition to crude retinal extract and ROS, the antibody was tested against soluble extracts of several rat tissues and found negative in all cases (Section 6.3.2.1). The apparent high affinity of monoclonal antibody S2.4.C5, as suggested by its rapid reaction in the ELISA (Figure 6.1), further emphasised the suitability of this antibody for immunocytochemistry, at both light and electron microscopical levels (Polak and Van Noorden, 1984).

In addition to specificity, monoclonal antibodies offer a reduction of the background signal inevitably present in polyclonal serum so that there is no non-specific reaction outwith the main antigenic sites. This study confirms such observations, since the localisation is very much better defined with the monoclonal antibody in comparison to conventional antisera (Figure 7.1a,b; 7.4

and 7.5).

Monoclonal antibodies are generally assumed to be superior reagents for immunocytochemical localisation of antigens because of their fine specificity. However, even with these probes, unexpected immunocytochemical crossreactions are occasionally observed. A high affinity IgG antibody generally displays high specificity and gives good localisation, whereas IgM monoclonal antibodies sometimes generate ambivalent data (reviewed by Ghosh and Campbell, 1986). Consequently, the IgG class of the S2.4.C5 antibody employed for the localisation of S-antigen was a relevant contributory factor to the favourable signal to noise ratio. Such advantages were probably also shared by the mouse IgG monoclonal antibodies of Faure et al., 1984; Mirshahi et al., 1984; Mirshahi et al., 1985 (Section 1.2.2). However, most IgM monoclonal antibodies probably do not crossreact, and many highly specific IgM antibodies have been generated. Antibody R1.2.D2 was an IgM antibody, which demonstrated specific cellular localisation (Figures 7.4 and 7.17).

In all immunohistochemical studies, stringent controls are essential, including a negative antibody control. The most appropriate control for a monoclonal antibody consists of an irrelevant monoclonal antibody, raised in the same species, of the same class and used at the same concentration. Supernatants (concentrated or otherwise) or ascites from the hybridoma parent cell line (eg. NS1 for the mouse system or Y3-Ag.1.2.3 for the rat system) are inappropriate, since such cells are either non-secretors (NS1) or secrete only the immunoglobulin light chain (Y3-Ag.1.2.3). Thus, such samples do not provide the same amount of antibody as do the experimental monoclonal antibodies.

It is often stated that immunohistochemistry requires a compromise between preservation of antigenicity and ultrastructure (Polak and Van Noorden, 1984). Frozen sections and techniques may result in a greater proportion of native antigens. However, this is not without considerable sacrifice in terms of ultrastructural preservation of membranes and organelles. Light fixation and low temperature embedding techniques for electron immunocytochemistry, such as those employed by McKechnie et al., (1986) and in the present study, gave rise to satisfactory preservation of ultrastructure. The antigenicity of S-antigen was also adequately maintained, as indicated by successful staining with a monoclonal antibody as well as with antiserum.

8.5.1 Distribution of S-Antigen at the Light Microscopical level

S-antigen, extractable from mammalian photoreceptor rod outer segments, has been localised throughout the photoreceptor rod cells by light microscopy in an immunofluorescence procedure, using guinea pig antiserum to purified S-antigen (Wacker et al., 1977). The recent development of monoclonal antibodies to S-antigen has made more definative immunocytochemical localisation of the protein possible. In this study, S-antigen was localised throughout the photoreceptor cell layer, using monoclonal antibody S2.4.C5 (Figure 7.1) in a pattern similar to that observed by Wacker (1981). In addition to the retina, S-antigen has also been identified in the mammalian pineal gland, using similar techniques (Kalsow and Wacker, 1977; Korf et al., 1985).

Monoclonal antibody technology has enabled the phylogenic distribution of distinct epitopes to be studied (Faure et al.,

1984; Mirshahi et al., 1984; Mirshahi et al., 1985). These authors have detected epitopes of S-antigen in the photoreceptors and pinealocytes of species from every class of vertebrates, and in the photoreceptors of certain invertebrates, using mouse IgG monoclonal antibodies and rat polyclonal antisera to S-antigen, in immunofluorescence techniques (Section 1.2.2). The rat monoclonal antibody S2.4.C5, utilised in the present study, was raised and detected against bovine S-antigen, although the antibody appeared to recognise human, pig and rat S-antigens equally well. It would be interesting to further study the phylogenic distribution of the S2.4.C5 epitope.

It would be valuable to compare immunoblots of extracts of photoreceptor cells, and pineal organs, with the monoclonal antibodies of Mirshahi et al. (1985). This would give information about the molecular weights of the related S-antigens, as well as ascertaining the specificity of the monoclonals for S-antigen. Cleveland mapping of the different S-antigens, together with Cleveland blotting, would yield very interesting information with respect to the extent of conservation of the S-antigen epitopes. It is possible that the proteins recognised by these mouse monoclonal antibodies were recognising epitopes on one or more related proteins, which have a similar function to that of S-antigen.

8.5.2 Effect of Fixation Method

The present study, described in Section 7.1, has shown that different patterns of staining can be obtained for S-antigen, with monoclonal antibody S2.4.C5, depending on the fixative employed. When the tissue was fixed in 70% alcohol, the protein was localised throughout the entire photoreceptor cell, the peroxidase reaction

appearing most intense within the ROS and the rod synapses (Figure 7.1a,b). This is similar to the distribution observed by Wacker et al. (1977) and Wacker (1981) on unfixed frozen sections, with antiserum in an immunofluorescence technique. However, in gluteraldehyde fixed tissue, S-antigen appeared to be largely confined to the ROS layer, with only a faint reaction in the inner segments and synapses (Figure 7.2). The area around the photoreceptor cell nuclei also appeared stained, although less intensely than the ROS. Such a fixative effect, apparent at the electron microscopical level has been reported by others. Broekhuyse et al., (1985b) suggested that S-antigen easily moves from its situation in the ROS, to the inner segment plasma membrane, during fixation by immersion. If the antigen is capable of this shift prior to immobilisation by the fixative, then it might be capable of such a shift, during dark adaptation in vivo (Section 8.5.3).

8.6.3 Localisation of S-Antigen under conditions of Light and Dark Adaptation

At the light microscopical level, S-antigen in light adapted, alcohol fixed eyes was localised throughout the photoreceptor cell, using both monoclonal and polyclonal antibodies (Section 7.2.1.2). The reaction in the ROS was more intense than in the inner segments in all specimens examined. However, in a 16 hour dark adapted specimen (Section 7.2.1.2), the immunoperoxidase reaction obtained in the inner segment appeared as intense as that in the ROS (Figure 7.3b). These observations are very similar to those of Broekhuyse et al. (1985b), who used polyclonal antisera and immunofluorescence to localise S-antigen in light and dark adapted rat retinae. If in the dissected eye, S-antigen is capable of diffusing from the ROS

through the connecting cilium to the inner segment in the relatively short time prior to fixation (Broekhuyse, et al., 1985b), the protein might conceivably undergo such a migration under conditions of long term dark adaptation (overnight). This is in keeping with the theory that S-antigen becomes closely associated with the disc membranes upon illumination, and that the protein is more easily extractable from the ROS membranes in the absence of light (Pfister et al., 1984; Pfister et al., 1985).

In the context of localisation in light versus dark adapted specimens of retinae, it is interesting that Balkema and Drager (1985) report a mouse monoclonal antibody which reacts with ROS in dark adapted, but not in light adapted specimens. The antigen had not yet been identified, but it was thought not to be rhodopsin. It is also interesting that two mouse monoclonal antibodies reactive against a highly phosphorylated neurofilament subunit, reacted with light, but not dark adapted ROS (Balkema and Drager, 1985). These antibodies reacted with the amacrine, horizontal and ganglion cells of the neural retina, irrespective of illumination. At least one of the antibodies was found to crossreact with rhodopsin, presumably at the phosphorylation sites (Balkema and Drager, 1985).

8.5.4 Ultrastructural Localisation of Retinal S-Antigen within the Rod Photoreceptor Cell

Information concerning the distribution and precise localisation of S-antigen within the photoreceptor cell will aid the elucidation of its physiological role. Section 7.6 describes the electron immunocytochemical localisation of S-antigen on the ROS disc membranes of human and pig photoreceptors by means of the highly specific rat IgG monoclonal antibody, raised to bovine S-antigen.

Electron microscopically, it was possible to discern that the cone photoreceptors laked S-antigen (Figures 7.14a,b). This was in agreement with the findings of McKechnie et al. (1986). At the ultrastructural level, monoclonal antibody S2.4.C5, revealed that S-antigen was located throughout the photoreceptor cell, from the outer segment to the rod spherule, with the majority of the gold label concentrated on the rod disc membranes (Figures 7.7 and 7.8a). Staining of the inner segments was less intense (Figure 7.8b). The perinuclear region was also fairly intensely stained (Figure 7.13). The pattern of staining was consistent with the intensity of reaction of the monoclonal antibody in immunoperoxidase experiments at the light microscopical level. This distribution is not, perhaps, unexpected since the protein is likely to be synthesised in the perinuclear area, where the Golgi apparatus and rough endoplasmic reticulum are located, and transported through the inner segment, before its incorporation into the outer segment. There is biochemical evidence that the outer segment proteins are synthesised in the inner segment and transported to the base of the ROS for their incorporation (Hall et al., 1969; Young, 1971, 1974).

The protein has been localised at the electron microscopical level, on rat ROS disc membranes of fixed, frozen tissue (Broekhuyse et al., 1985a) and on the ROS disc membranes of fixed human retina (McKechnie et al., 1986) using rabbit polyclonal antisera with protein A-gold immunocytochemical techniques. Yajima et al. (1983) and Uusitalo et al.(1985) obtained staining of fixed rodent rod outer segment plasma and disc membranes, using conventional antisera and direct immunoperoxidase and peroxidase anti-peroxidase

staining procedures respectively. In these studies, the labelling of the inner segments was less intense, in comparison with the ROS. The findings were similar to those with the monoclonal antibody S2.4.C5. The peroxidase techniques do not offer the advantage of relative quantitation which is possible with the immunogold and protein A-gold techniques. In addition, the peroxidase reaction product tends to obsure the underlying ultrastructure. Yajima et al. (1983) and Uusitalo et al. (1985) did not present any evidence that the protein which they localised was, in fact, the S-antigen originally identified by Wacker (1973).

The method of pre-embedding immunoperoxidase staining of tissue blocks, employed by Uusitalo et al. (1985), has the disadvantage of only a limited penetration of the reagents, and the pattern of staining was very variable. However, these authors were able to identify an intense reaction on the disc membranes. The choroid and RPE apparently remained unstained. The findings were generally in agreement with the observations in this study, with the exception of the absence of a reaction in the cytoplasm of the outer nuclear layer, although the plasma membrane in this region was stained. This discrepancy might have resulted from the penetration problem experienced with the pre-embedding staining technique.

In the present study, there was no evidence of specific labelling of the photoreceptor plasma membrane, in agreement with the observations of Broekhuyse et al., (1985a) and McKechnie et al., (1986). The membrane around the photoreceptor ROS, however, was not as clearly preserved as the disc membranes. The absence of plasma membrane decoration could be artifactual, resulting from the deleterious effects of tissue preparation, whether frozen or fixed.

The frozen section of a rod cell, stained with rabbit antiserum to S-antigen and protein A-gold, presented by Broekhuyse et al. (1985a) as showing light plasma membrane staining in addition to predominant disc labelling, demonstrated poorer membrane preservation compared with the Lowicryl embedded tissue of McKechnie et al. (1986), also used in the present study. The interpretation of a reaction in this area is probably subjective. Possibly soluble membrane associated proteins like S-antigen, were more easily leached from the plasma membrane prior to complete fixation.

The reason for the presence of S-antigen throughout the photoreceptor cell, remains to be discovered. Some of the S-antigen localised within the inner segment might be the result of diffusion from the outer segment before fixation, as suggested by Broekhuyse et al. (1985b). Alternatively, it may reflect a different mechanism of transport of this soluble protein, compared to the insoluble proteins, eg. rhodopsin, of which little is detected within the inner segment using monoclonal antibodies (Hicks and Molday, 1986). It is also possible that the observed pattern of staining is related to the rate of synthesis of S-antigen. Perhaps the answer lies with more than one of these explanations. The presence of S-antigen within the photoreceptor synapses is more difficult to explain. It may have some, as yet unidentified function in this area, or indeed within the inner segment also. Perhaps S-antigen is also involved in neurological signalling in photoreceptor rod cells. Further research into the physiological role or roles of this protein are required to answer these questions.

All previous studies on the localisation of S-antigen at the

ultrastructural level have employed polyclonal antisera. The present work employed a monospecific monoclonal antibody, which allowed the identification of S-antigen within the photoreceptor cell and the RPE with greater certainty.

8.6.5 Pattern of staining within the ROS

The epitopes of S-antigen recognised by the monoclonal antibody S2.4.C5 and the polyclonal antisera, were apparently uniformly exposed along the length of the ROS in human and pig specimens, as quantitative studies gave little indication for a graded labelling (Section 7.6). McKechnie et al., (1986) observed differential labelling of the proximal and apical regions of the ROS. Such a manifestation could also be an artifact of fixation or serum dilution. S-antigen could leach from the ROS prior to fixation, resulting in a lower concentration of the protein, perhaps especially in the apical regions since in this tissue the RPE was not retained. This differential concentration, whether as a result of leaching of the soluble antigen, or a true property of the ROS, might only be detected at certain dilutions of the antiserum or monoclonal antibody. Perhaps in this study, the antibody was used at saturating conditions, and a lower concentration of the antibody might have given similar results as observed by McKechnie et al. (1986).

Differential expression of the epitopes of S-antigen have been implicated at the light microscopical level, using mouse monoclonal antibodies with immunofluorescence (Mirshahi et al., 1985) and radioimmunocytochemistry (Das et al., 1984). Both these immunocytochemical techniques give rise to difficulties with resolution, making it difficult to distinguish labelling of the

plasma membrane from that of the cell interior, in a narrow cell like the photoreceptor. Differential longitudinal expression is, however, easier to discern.

Of the two monoclonal antibodies of Das et al. (1984), RSA1/83 apparently recognised an epitope of S-antigen which is distributed throughout the photoreceptor cells. The other monoclonal antibody, RSA2/83, preferentially labelled the apical region of the ROS. The monoclonal antibodies were applied as ascites fluid, which generally contain high concentrations of monoclonal antibody (Campbell, 1984). The control consisted of non-secreting NS1 myeloma ascites. The radioimmunocytochemical technique appeared to give a very poor resolution and a very high background with the NS1 ascites control, which was virtually indistinguishable from the reaction given by antibody RSA1/83. Apart from the difficulty in the determination of the specificity of the reactions, a further problem with these experiments was the use of bovine retina, with ascites fluid which could contain mouse antibody to the foetal calf serum antigens, and thus could cause unwanted reactions.

In agreement with the observations presented in Section 7.6.1, for monoclonal antibody S2.4.C5, Mirshahi et al., (1985) found that in an indirect fluorescence technique, four of their monoclonal antibodies to S-antigen outlined the entire photoreceptor cell, excluding the nucleus. A fifth monoclonal antibody predominantly labelled the perinuclear area. The authors suggested that this antibody was recognising an epitope of the native protein which became masked upon further processing. Alternatively, the antibody may have recognised a precursor molecule. Irrelevant monoclonal antibodies were employed as negative controls in their study.

It is also possible, however, that epitopes of S-antigen are exposed differentially in different regions of the photoreceptor cell, eg. at the site of manufacture, or transport. The antigenic determinants of S-antigen may be differentially exposed along the ROS, perhaps as the discs age, or in preparation for phagocytosis. McKechnie et al. (1986), suggest that longitudinal differences in antigenic expression may account for some of the morphological differences in EAU reported by different groups. It is also possible that a binding protein is involved in the transport of the protein from its site of synthesis to the ROS base.

8.5.6 Fate of S-antigen within the RPE

In this study, S-antigen was also localised in the ROS phagosomes of the human pigment epithelium, but was absent in the other retinal cells and in the choroid. The presence of gold staining with monoclonal antibody S2.4.C5, in the phagosomes of the pigment epithelium, indicates that the epitope is conserved after initial phagocytosis (Figures 7. and 7.a,b,c). The survival of the epitope recognised by antibody S2.4.C5 is not surprising, in view of the Cleveland blotting results described in Section 6.4, where reactivity was detected after considerable digestion.

A recent study using rabbit polyclonal antisera with an immunoperoxidase staining method, has suggested that S-antigen may be taken up by the RPE microvilli during phagocytosis, and transported through the cytoplasm and the basal infoldings of the RPE, across Bruch's membrane, to the endothelium of the choriocapillaris after a period of 30 hours exposure to light (Yajima et al., 1985). The retinal tissues used in the present

study would have been light adapted due to the illuminated environment of dissection. S-antigen was not be detected in the region of Bruch's membrane or the choriocapillaris, and the immunogold stain was confined to the phagocytosed membrane material, where it was apparently fully degraded. The retinae studied by Yajima et al. (1985) were derived from albino rabbits. A period of 30 hours light adaptation is perhaps a little severe, especially to the albino eye. It probably would be more relevant to follow the fate of S-antigen within pigmented retinae, with an immunogold rather than a peroxidase technique, to avoid a nonspecific deposition of the peroxidase substrate on the melanin, and to allow relative quantitation of staining to be made within each experiment.

8.6 LOCALISATION OF THE R1.2.D2 EPITOPE

The rat IgM antibody, derived from an animal immunised with bovine ROS membranes, reacted with a protein with an apparent molecular weight of 37 to 39K in immunoblots, after SDS-PAGE (Figure 6.8). It is possible that the antigen recognised by R1.2.D2 is the alpha or beta subunit of transducin, the photoreceptor G-protein. These subunits have molecular weights of approximately 39K and 36K, respectively (see Shichi et al., 1984).

At the light and electron microscopical levels, antibody R1.2.D2 reacted with a similar pattern to that given by the rat antisera raised against ROS membranes. At the level of the light microscope, the photoreceptor ROS were intensely stained, while the inner segment was virtually devoid of the peroxidase reaction product

(Figure 7.4). In contrast to the localisation of S-antigen (Section 8.6.3), the pattern of staining appeared independent of illumination. Likewise, the localisation of opsin, using polyclonal rat anti-ROS sera did not appear related to illumination, in agreement with Broekhuyse et al. (1985b). Fekete and Barnstable (1983) describe a mouse monoclonal antibody (Ret P2) which reacts preferentially with the ROS in tissue sections, and with a 38K antigen in immunoblots, and was unreactive with opsin. Thus, the epitopes recognised by antibodies R1.2.D2 and Ret P2 might be presented on the same antigen.

Electron microscopically, the R1.2.D2 epitope was similarly distributed, with intense specific decoration of the disc membranes and very little label in the connecting cilium or inner segment (Figures 7.17b). This pattern of staining is very similar to that reported for rhodopsin, using a panel of mouse monoclonal antibodies (Hicks and Molday, 1986). However, no specific reaction was obtained with antibody R1.2.D2 in immunoblots against rhodopsin. The rod outer and inner segment plasma membrane might also have been labelled (Figures 7.19a,b). The pattern of staining contrasted with that obtained for S-antigen, where the entire rod photoreceptor was stained (Figure 7.7). There was no reaction with the R1.2.D2 antibody and the cone photoreceptors (Figures 7.20a,b).

At the electron microscopical level, some rods stained with R1.2.D2 and immunogold, appeared specifically labelled at the edges of the rods, perhaps reacting with the disc rims and/or the plasma membrane (Figures 7.19a,b). Further studies are required to determine the importance of this reaction, which may depend on the dilution of the antibody. The result suggested, however, that there

might be a relationship between the R1.2.D2 antigen and the 35K subunit of peripherin, a recently described disc rim protein (Molday et al., 1986). However, monoclonal antibodies to peripherin tend not to react with the lamellar regions of the discs (Molday et al., 1986), and antibody R1.2.D2 clearly reacted in this region in most cases (Figures 7.17a,b and 7.18a).

8.7 FUTURE WORK

The role of retinal antigens such as S-antigen in the development of human uveitis, RP or other ocular conditions of unknown aetiology, remains to be established. A large well defined, double blind, longitudinal study is required. This should include numerous cases of well classified uveitis, RP and possibly other conditions, and at least an equal number of controls. It would also be useful to include patients with established autoimmune disease, such as rheumatoid arthritis. A variety of assays for humoral and cell mediated assays should be used, with standardised and controlled conditions. It would be important to take blood samples from the subjects on different days and perhaps also at different times of the day, since the population of circulating cells seem to vary (BenEzra et al., 1984; Hendricks and Fishman, 1986). The results should be confirmed in separate laboratories. BenEzra et al. (1984) recommended that such a study be sponsored by a relevant organisation, for example the RP Foundation, and monitored by a scientific body.

Although the investigation suggested above is the more important, it may be useful to further study the role of the immune system in animal models of retinal dystrophy, especially where naturally

occurring, since if certain groups of ocular conditions, like RP, are found to involve abnormalities in the immune system, animals demonstrating similar immune responses, might provide suitable models.

Should specific retinal or choroidal autoimmune mechanisms be found to be important in certain subgroups of RP or uveitis, then the human monoclonal antibody system might provide a useful tool for investigating the role of humoral antibody, and in the identification of the relevant antigens and their important epitopes. However, such a study might best be postponed until the human monoclonal antibody technology has further advanced. It may be possible to generate monoclonal autoantibodies from other strains of retinal dystrophic rat, and mice, should they be found to demonstrate significant retinal autoimmunity. The RCS rat monoclonal antibodies generated in this study might recognise epitopes relevant in the human response. Further characterisation of these monoclonal autoantibodies, in this context, may prove to be of value.

It seems likely that S-antigen is in fact the '48K' protein of the retina, and binds to photoactivated disc membranes (Pfister et al., 1984; Pfister et al., 1985). It is possible that the protein, S-antigen, is no longer associated with the disc membranes in the absence of light (Pfister et al., 1984; Pfister et al., 1985). Future investigations using monoclonal antibody S2.4.C5 and immunogold, comparing the distribution of S-antigen under various degrees of light and dark adapted retinae, of laboratory animals, might verify this hypothesis at the electron microscopical level. If S-antigen interacts with photoactivated rhodopsin, it would be

interesting to determine whether or not certain anti-rhodopsin, or anti-transducin antibodies might demonstrate a light dependent reaction due either to a shared binding site with S-antigen, or to steric hindrance caused by that protein.

It would be interesting to investigate the phylogenic distribution of the epitopes of monoclonal antibodies S2.4.C5 and R1.2.D2, in the various photoreceptor types and pineal structures. Studies, involving the localisation of S-antigen and the 39K protein in the developing photoreceptors, might also prove valuable. Such studies may be useful in further determining the physiological role of S-antigen and the 39K antigen, and may give some clues as to its localisation of S-antigen within the rod synapse.

Studies are presently in progress, further investigating the effects of monoclonal antibody S2.4.C5, in S-antigen induced EAU in rats and guinea pigs. The antibody has been found to exacerbate the disease in guinea pigs, but in rats, the development of EAU was was inhibited. The effect of antibody dose in EAU will be determined, in addition to the effect of the time of its administration. It will also be important to determine the effects of the monoclonal antibody alone, since it may be capable of inducing EAU. Control experiments with irrelevant monoclonal antibodies should also be undertaken. It may be useful to identify and purify the smallest fragment of S-antigen, bearing the S2.4.C5 epitope, and investigate its role in EAU, probably after coupling to a carrier protein.

Further characterisation of the R1.2.D2 antibody, and the identification of the 39K antigen is required. It might be possible to purify the R1.2.D2 antigen using an affinity column with the

monoclonal antibody. The purified antigen could then be further characterised. Similar EAU studies will be possible with monoclonal antibody R1.2.D2, and the crude, or purified antigen.

EPILOGUE

To me, we have been as children,

Playing on the sea-shore,

And diverting ourselves in now and then

Finding a smoother pebble or prettier shell than ordinary,

Whilst the great ocean of truth lay undiscovered before us.

Sir Isaac Newton

1642-1727

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APPENDIX

TABLES

4.1 - 4.30

4.33 - 4.36

Table 4.1 ELISA Responses for Individual Campbell Rats to S-Antigen, at the Age of 3 Weeks

	Serum Dilution 20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.112	0.035	0.013	0.001	0.021	0.014	0.008	0.001	0.010
Mean	0.473	0.140	0.113	0.103	0.020	0.035	0.049	0.029	0.066
Rat 2 SD	0.018	0.009	0.013	0.001	0.008	0.001	0.001	0.008	0.000
Mean	0.095	0.061	0.039	0.034	0.068	0.050	0.065	0.071	0.097
Rat 3 SD	0.005	0.010	0.001	0.056	0.012	0.002	0.004	0.008	0.005
Mean	0.291	0.114	0.087	0.066	0.025	0.001	0.011	0.034	0.033
Rat 4 SD	0.014	0.019	0.006	0.003	0.019	0.033	0.007	0.005	0.007
Mean	0.254	0.154	0.100	0.100	0.100	0.115	0.045	0.050	0.086
Rat 5 SD	0.020	0.004	0.006	0.029	0.021	0.000	0.025	0.020	0.011
Mean	0.289	0.182	0.058	0.068	0.059	0.031	0.068	0.083	0.076
Rat 6 SD	0.020	0.010	0.001	0.001	0.028	0.011	0.013	0.011	0.006
Mean	0.296	0.114	0.074	0.053	0.031	0.018	0.009	0.013	0.016
Rat 7 SD	0.024	0.008	0.003	0.011	0.030	0.011	0.013	0.022	0.025
Mean	0.505	0.217	0.122	0.057	0.044	0.051	0.056	0.039	0.045
Rat 8 SD	0.002	0.024	0.009	0.013	0.007	0.006	0.014	0.001	0.008
Mean	0.121	0.041	0.015	0.009	0.019	0.018	0.014	0.021	0.026
Rat 9 SD	0.012	0.011	0.024	0.009	0.000	0.009	0.044	0.009	0.004
Mean	0.145	0.049	0.018	0.031	0.027	0.034	0.086	0.048	0.043
Rat 10 SD	0.026	0.016	0.012	0.007	0.000	0.013	0.008	0.010	0.011
Mean	0.399	0.152	0.052	0.024	0.000	0.009	0.008	0.007	0.022
Mean	0.287	0.122	0.068	0.054	0.039	0.036	0.041	0.040	0.051
SD	0.141	0.058	0.038	0.031	0.029	0.032	0.029	0.024	0.029
Range	0.095	0.041	0.015	0.009	0.000	0.001	0.008	0.007	0.016
	0.505	0.217	0.122	0.103	0.100	0.115	0.086	0.083	0.097

Results for apparently unresponsive Rats 2,3,6,7,9 & 10

Mean	0.289	0.118	0.065	0.044	0.033	0.027	0.039	0.035	0.043
SD	0.153	0.062	0.037	0.017	0.023	0.021	0.034	0.023	0.029
Range	0.095	0.049	0.018	0.024	0.000	0.001	0.008	0.007	0.016
	0.505	0.217	0.122	0.066	0.068	0.051	0.086	0.071	0.097

Table 4.2 ELISA Responses for Individual Campbell Rats to S-antigen, at the Age of 6 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.123	0.057	0.011	0.018	0.007	0.035	0.026	0.033	0.030
Mean	1.594	0.570	0.255	0.158	0.103	0.103	0.109	0.114	0.114
Rat 2 SD	0.083	0.005	0.006	0.006	0.017	0.006	0.002	0.013	0.009
Mean	0.220	0.072	0.049	0.040	0.043	0.035	0.028	0.033	0.069
Rat 3 SD	0.003	0.010	0.020	0.016	0.030	0.010	0.027	0.018	0.013
Mean	0.376	0.133	0.105	0.092	0.082	0.080	0.083	0.133	0.061
Rat 4 SD	0.013	0.018	0.040	0.006	0.076	0.033	0.018	0.023	0.016
Mean	1.444	0.894	0.551	0.261	0.122	0.071	0.065	0.056	0.047
Rat 5 SD	0.016	0.008	0.019	0.005	0.012	0.012	0.008	0.001	0.003
Mean	0.250	0.163	0.144	0.120	0.128	0.123	0.135	0.043	0.084
Rat 6 SD	0.072	0.004	0.012	0.002	0.025	0.016	0.008	0.011	0.016
Mean	0.518	0.232	0.129	0.042	0.072	0.031	0.018	0.028	0.020
Rat 7 SD	0.015	0.017	0.054	0.010	0.001	0.028	0.030	0.003	0.016
Mean	0.450	0.165	0.076	0.007	0.008	0.039	0.026	0.020	0.016
Rat 8 SD	0.021	0.189	0.048	0.008	0.016	0.004	0.013	0.018	0.016
Mean	1.553	0.769	0.311	0.109	0.033	0.029	0.009	0.023	0.011
Rat 9 SD	0.088	0.062	0.053	0.033	0.029	0.045	0.042	0.058	0.064
Mean	0.532	0.155	0.052	0.024	0.020	0.032	0.030	0.042	0.057
Rat 10 SD	0.054	0.031	0.019	0.016	0.018	0.000	0.000	0.002	0.006
Mean	0.204	0.097	0.031	0.012	0.013	0.000	0.000	0.001	0.004
Mean	0.714	0.325	0.170	0.086	0.063	0.054	0.050	0.049	0.048
SD	0.576	0.302	0.162	0.080	0.045	0.038	0.046	0.042	0.036
Range	0.204	0.072	0.031	0.007	0.008	0.000	0.000	0.001	0.004
	1.594	0.894	0.551	0.261	0.128	0.123	0.135	0.133	0.114

Results for apparently unresponsive Rats 2,3,6,7,9 & 10

Mean	0.383	0.142	0.074	0.036	0.040	0.036	0.031	0.043	0.038
SD	0.144	0.056	0.037	0.031	0.031	0.026	0.028	0.046	0.028
Range	0.204	0.072	0.031	0.007	0.008	0.000	0.000	0.001	0.004
	0.532	0.232	0.129	0.092	0.082	0.080	0.083	0.133	0.069

Table 4.3 ELISA Responses for Individual Campbell Rats to S-Antigen, at the Age of 9 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.007	0.002	0.006	0.003	0.024	0.016	0.007	0.012	0.027
Mean	0.323	0.108	0.076	0.059	0.063	0.082	0.104	0.020	0.035
Rat 2 SD	0.008	0.082	0.049	0.029	0.004	0.004	0.012	0.008	0.000
Mean	0.338	0.169	0.096	0.053	0.076	0.069	0.025	0.063	0.081
Rat 3 SD	0.031	0.037	0.006	0.033	0.022	0.031	0.037	0.015	0.008
Mean	0.285	0.163	0.118	0.099	0.083	0.095	0.112	0.028	0.053
Rat 4 SD	0.037	0.024	0.024	0.010	0.013	0.001	0.008	0.005	0.037
Mean	0.262	0.144	0.075	0.068	0.019	0.013	0.025	0.031	0.063
Rat 5 SD	0.025	0.027	0.042	0.019	0.004	0.011	0.013	0.004	0.015
Mean	0.753	0.457	0.208	0.079	0.042	0.029	0.028	0.039	0.053
Rat 6 SD	0.043	0.062	0.052	0.043	0.022	0.015	0.008	0.011	0.010
Mean	0.304	0.146	0.082	0.042	0.033	0.015	0.033	0.058	0.036
Rat 7 SD	0.023	0.010	0.013	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.218	0.047	0.010	0.000	0.000	0.000	0.000	0.000	0.000
Rat 8 SD	0.008	0.026	0.025	0.006	0.017	0.012	0.017	0.018	0.016
Mean	0.844	0.382	0.163	0.070	0.023	0.008	0.012	0.023	0.011
Rat 9 SD	0.016	0.013	0.011	0.005	0.000	0.006	0.008	0.005	0.001
Mean	0.417	0.139	0.050	0.036	0.020	0.013	0.055	0.031	0.050
Rat 10 SD	0.040	0.008	0.016	0.011	0.006	0.003	0.000	0.004	0.014
Mean	0.361	0.108	0.035	0.008	0.007	0.004	0.000	0.005	0.010
Mean	0.411	0.186	0.091	0.051	0.037	0.033	0.039	0.030	0.039
SD	0.213	0.129	0.059	0.031	0.029	0.035	0.040	0.020	0.026
Range	0.218	0.047	0.010	0.000	0.000	0.000	0.000	0.000	0.000
	0.844	0.457	0.208	0.099	0.083	0.095	0.112	0.063	0.081

Results for apparently unresponsive Rats 2,3,6,7,9 & 10

Mean	0.321	0.129	0.065	0.040	0.037	0.033	0.038	0.031	0.038
SD	0.068	0.045	0.041	0.035	0.035	0.039	0.042	0.026	0.030
Range	0.218	0.047	0.010	0.000	0.000	0.000	0.000	0.000	0.000
	0.417	0.169	0.118	0.099	0.083	0.095	0.112	0.063	0.081

Table 4.4 ELISA Responses for Individual Campbell Rats to ROS, at the Age of 3 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.065	0.001	0.029	0.004	0.032	0.002	0.015	0.018	0.000
Mean	0.206	0.171	0.182	0.244	0.148	0.094	0.099	0.141	0.058
Rat 2 SD	0.020	0.047	0.016	0.020	0.004	0.005	0.001	0.062	0.005
Mean	0.186	0.202	0.180	0.077	0.074	0.082	0.078	0.048	0.058
Rat 3 SD	0.004	0.001	0.008	0.002	0.025	0.006	0.006	0.004	0.001
Mean	0.297	0.280	0.163	0.138	0.119	0.074	0.065	0.079	0.064
Rat 4 SD	0.037	0.032	0.020	0.008	0.006	0.003	0.049	0.027	0.002
Mean	0.290	0.227	0.153	0.129	0.084	0.037	0.061	0.077	0.077
Rat 5 SD	0.020	0.040	0.016	0.000	0.001	0.016	0.006	0.052	0.007
Mean	0.266	0.194	0.133	0.121	0.094	0.087	0.087	0.059	0.014
Rat 6 SD	0.006	0.005	0.025	0.043	0.010	0.007	0.017	0.016	0.016
Mean	0.295	0.127	0.086	0.068	0.049	0.051	0.079	0.054	0.073
Rat 7 SD	0.001	0.002	0.027	0.002	0.007	0.011	0.000	0.030	0.010
Mean	0.226	0.145	0.127	0.103	0.068	0.045	0.098	0.061	0.051
Rat 8 SD	0.014	0.006	0.001	0.002	0.019	0.017	0.011	0.008	0.003
Mean	0.232	0.243	0.190	0.195	0.117	0.053	0.058	0.031	0.030
Rat 9 SD	0.027	0.035	0.027	0.003	0.001	0.028	0.044	0.006	0.013
Mean	0.235	0.224	0.133	0.117	0.082	0.066	0.055	0.037	0.015
Rat 10 SD	0.001	0.017	0.005	0.013	0.010	0.004	0.000	0.007	0.004
Mean	0.450	0.279	0.167	0.088	0.057	0.021	0.001	0.005	0.015
Mean	0.268	0.209	0.151	0.128	0.089	0.061	0.068	0.059	0.045
SD	0.074	0.052	0.032	0.054	0.031	0.024	0.028	0.036	0.025
Range	0.186	0.127	0.086	0.068	0.049	0.021	0.001	0.005	0.014
	0.450	0.280	0.190	0.244	0.148	0.094	0.099	0.141	0.077

Results for apparently unresponsive Rats 2,3,6,7,9 & 10

Mean	0.282	0.210	0.143	0.099	0.075	0.057	0.063	0.047	0.046
SD	0.093	0.065	0.034	0.026	0.025	0.022	0.034	0.025	0.025
Range	0.186	0.127	0.086	0.068	0.049	0.021	0.001	0.005	0.015
	0.450	0.280	0.180	0.138	0.119	0.082	0.098	0.079	0.073

Table 4.5 ELISA Responses for Individual Campbell Rats to ROS, at the Age of 6 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.033	0.009	0.018	0.055	0.004	0.018	0.030	0.043	0.003
Mean	0.753	0.480	0.274	0.260	0.233	0.163	0.146	0.065	0.078
Rat 2 SD	0.006	0.001	0.006	0.003	0.003	0.021	0.006	0.001	0.018
Mean	0.314	0.157	0.106	0.066	0.045	0.082	0.041	0.068	0.030
Rat 3 SD	0.045	0.046	0.056	0.015	0.001	0.031	0.049	0.001	0.001
Mean	0.454	0.287	0.256	0.170	0.082	0.054	0.113	0.083	0.094
Rat 4 SD	0.049	0.010	0.001	0.016	0.019	0.010	0.002	0.001	0.020
Mean	0.840	0.615	0.422	0.488	0.263	0.134	0.148	0.068	0.050
Rat 5 SD	0.032	0.035	0.016	0.005	0.004	0.006	0.004	0.002	0.002
Mean	0.369	0.154	0.149	0.087	0.084	0.057	0.149	0.062	0.076
Rat 6 SD	0.029	0.008	0.058	0.032	0.007	0.013	0.003	0.001	0.016
Mean	0.227	0.137	0.167	0.149	0.059	0.080	0.044	0.041	0.052
Rat 7 SD	0.013	0.028	0.011	0.010	0.002	0.001	0.006	0.008	0.008
Mean	0.328	0.161	0.138	0.126	0.111	0.104	0.087	0.089	0.082
Rat 8 SD	0.005	0.016	0.133	0.004	0.004	0.045	0.017	0.033	0.001
Mean	0.353	0.277	0.256	0.165	0.104	0.045	0.058	0.076	0.097
Rat 9 SD	0.011	0.031	0.030	0.015	0.011	0.004	0.005	0.047	0.001
Mean	0.455	0.269	0.210	0.134	0.043	0.054	0.028	0.065	0.077
Rat 10 SD	0.003	0.048	0.042	0.043	0.057	0.000	0.000	0.000	0.010
Mean	0.258	0.118	0.058	0.033	0.040	0.000	0.000	0.000	0.007
Mean	0.435	0.266	0.204	0.168	0.106	0.077	0.081	0.062	0.064
SD	0.205	0.164	0.104	0.129	0.079	0.047	0.055	0.025	0.029
Range	0.227	0.118	0.058	0.033	0.400	0.000	0.000	0.000	0.007
	0.840	0.615	0.422	0.488	0.263	0.163	0.149	0.089	0.097

Results for apparently unresponsive Rats 2,3,6,7,9 & 10

Mean	0.339	0.188	0.156	0.113	0.063	0.062	0.052	0.058	0.057
SD	0.096	0.071	0.071	0.052	0.028	0.036	0.041	0.033	0.034
Range	0.227	0.118	0.058	0.033	0.040	0.000	0.000	0.000	0.007
	0.455	0.287	0.256	0.170	0.111	0.104	0.113	0.089	0.094

Table 4.6 ELISA Responses for Individual Campbell Rats to ROS, at the Age of 9 Weeks

Serum Dilution

	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.028	0.005	0.060	0.020	0.037	0.024	0.005	0.019	0.002
Mean	0.274	0.294	0.253	0.178	0.155	0.074	0.065	0.061	0.048
Rat 2 SD	0.018	0.065	0.010	0.011	0.004	0.010	0.023	0.007	0.006
Mean	0.205	0.153	0.090	0.084	0.084	0.080	0.055	0.052	0.046
Rat 3 SD	0.019	0.022	0.016	0.016	0.009	0.011	0.004	0.002	0.018
Mean	0.272	0.220	0.170	0.113	0.056	0.065	0.033	0.037	0.045
Rat 4 SD	0.005	0.011	0.001	0.006	0.025	0.001	0.011	0.015	0.009
Mean	0.267	0.122	0.092	0.082	0.070	0.071	0.045	0.045	0.042
Rat 5 SD	0.017	0.052	0.024	0.012	0.004	0.013	0.011	0.015	0.011
Mean	0.291	0.237	0.134	0.119	0.056	0.084	0.079	0.045	0.059
Rat 6 SD	0.013	0.018	0.008	0.011	0.012	0.002	0.005	0.002	0.036
Mean	0.226	0.170	0.086	0.090	0.079	0.087	0.082	0.086	0.061
Rat 7 SD	0.019	0.044	0.006	0.000	0.003	0.003	0.008	0.007	0.035
Mean	0.316	0.246	0.105	0.082	0.043	0.040	0.042	0.045	0.071
Rat 8 SD	0.035	0.004	0.004	0.001	0.012	0.001	0.026	0.028	0.000
Mean	0.244	0.185	0.066	0.088	0.068	0.085	0.062	0.056	0.044
Rat 9 SD	0.183	0.039	0.003	0.016	0.028	0.001	0.004	0.002	0.003
Mean	0.348	0.133	0.055	0.073	0.052	0.052	0.079	0.089	0.032
Rat 10 SD	0.001	0.001	0.004	0.011	0.006	0.004	0.004	0.000	0.008
Mean	0.477	0.200	0.124	0.079	0.043	0.022	0.013	0.000	0.005
Mean	0.292	0.196	0.118	0.099	0.071	0.066	0.056	0.052	0.045
SD	0.077	0.054	0.058	0.031	0.033	0.022	0.022	0.025	0.018
Range	0.205	0.122	0.055	0.073	0.043	0.022	0.013	0.000	0.005
	0.477	0.294	0.253	0.178	0.155	0.087	0.082	0.089	0.071

Results for apparently unresponsive Rats 2,3,6,7,9 & 10

Mean	0.307	0.187	0.105	0.087	0.060	0.058	0.051	0.052	0.043
SD	0.099	0.043	0.039	0.014	0.018	0.025	0.027	0.033	0.023
Range	0.205	0.133	0.055	0.073	0.043	0.022	0.013	0.000	0.005
	0.477	0.246	0.170	0.113	0.084	0.087	0.082	0.089	0.071

Table 4.7 ELISA Responses for Individual Hunter Rats to S-Antigen, at the Age of 3 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.018	0.004	0.014	0.001	0.006	0.008	0.026	0.019	0.005
Mean	0.511	0.205	0.163	0.092	0.038	0.017	0.061	0.079	0.083
Rat 2 SD	0.010	0.007	0.010	0.012	0.030	0.010	0.001	0.006	0.017
Mean	0.197	0.170	0.184	0.063	0.049	0.061	0.069	0.069	0.075
Rat 3 SD	0.007	0.009	0.018	0.018	0.001	0.010	0.004	0.011	0.006
Mean	0.139	0.046	0.018	0.013	0.014	0.014	0.009	0.014	0.009
Rat 4 SD	0.023	0.034	0.030	0.007	0.011	0.006	0.023	0.002	0.008
Mean	0.402	0.210	0.109	0.061	0.046	0.050	0.047	0.080	0.035
Rat 5 SD	0.011	0.006	0.018	0.000	0.000	0.000	0.000	0.000	0.001
Mean	0.161	0.104	0.043	0.000	0.000	0.000	0.000	0.000	0.006
Rat 6 SD	0.132	0.037	0.052	0.055	0.031	0.030	0.006	0.022	0.041
Mean	0.619	0.328	0.275	0.184	0.152	0.127	0.122	0.092	0.044
Rat 7 SD	0.004	0.007	0.008	0.006	0.008	0.006	0.000	0.001	0.003
Mean	0.177	0.124	0.082	0.013	0.006	0.004	0.000	0.001	0.008
Rat 8 SD	0.004	0.014	0.009	0.018	0.005	0.011	0.014	0.000	0.004
Mean	0.199	0.120	0.126	0.115	0.094	0.013	0.032	0.094	0.024
Rat 9 SD	0.028	0.013	0.018	0.040	0.001	0.002	0.008	0.007	0.000
Mean	0.453	0.215	0.121	0.068	0.048	0.042	0.011	0.005	0.000
Rat 10 SD	0.004	0.014	0.013	0.000	0.010	0.009	0.002	0.004	0.011
Mean	0.168	0.086	0.064	0.037	0.057	0.083	0.056	0.063	0.059
Mean	0.303	0.161	0.119	0.065	0.050	0.041	0.041	0.050	0.034
SD	0.176	0.082	0.075	0.056	0.045	0.041	0.039	0.040	0.030
Range	0.139	0.046	0.018	0.000	0.000	0.000	0.000	0.000	0.000
	0.619	0.328	0.275	0.184	0.152	0.127	0.122	0.094	0.083

Table 4.8 ELISA Responses for Individual Hunter Rats to S-Antigen, at the Age of 6 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.016	0.018	0.011	0.048	0.004	0.029	0.025	0.019	0.052
Mean	0.284	0.177	0.153	0.095	0.056	0.053	0.018	0.070	0.059
Rat 2 SD	0.001	0.003	0.023	0.022	0.035	0.008	0.058	0.020	0.032
Mean	0.219	0.167	0.106	0.095	0.027	0.045	0.041	0.035	0.022
Rat 3 SD	0.069	0.025	0.022	0.007	0.002	0.009	0.023	0.011	0.013
Mean	0.496	0.151	0.093	0.034	0.013	0.031	0.033	0.040	0.039
Rat 4 SD	0.018	0.033	0.000	0.007	0.018	0.021	0.006	0.033	0.004
Mean	0.136	0.052	0.042	0.015	0.033	0.024	0.044	0.046	0.054
Rat 5 SD	0.029	0.049	0.045	0.019	0.013	0.000	0.000	0.000	0.000
Mean	0.144	0.087	0.034	0.014	0.009	0.000	0.000	0.000	0.000
Rat 6 SD	0.014	0.006	0.040	0.039	0.044	0.035	0.005	0.000	0.008
Mean	0.332	0.225	0.176	0.141	0.148	0.151	0.094	0.084	0.071
Rat 7 SD	0.015	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.148	0.037	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Rat 8 SD	0.056	0.004	0.001	0.012	0.016	0.011	0.001	0.013	0.023
Mean	0.924	0.278	0.173	0.176	0.042	0.049	0.045	0.050	0.065
Rat 9 SD	0.042	0.016	0.026	0.021	0.008	0.023	0.007	0.000	0.000
Mean	0.307	0.180	0.093	0.073	0.052	0.016	0.021	0.000	0.000
Rat 10 SD	0.008	0.000	0.001	0.053	0.016	0.021	0.008	0.036	0.012
Mean	0.079	0.065	0.013	0.041	0.049	0.059	0.053	0.037	0.045
Mean	0.307	0.142	0.088	0.068	0.043	0.043	0.035	0.036	0.036
SD	0.249	0.079	0.065	0.058	0.042	0.043	0.028	0.029	0.028
Range	0.079	0.037	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	0.924	0.278	0.176	0.176	0.148	0.151	0.094	0.084	0.071

Table 4.9 ELISA Responses for Individual Hunter Rats to S-Antigen, at the Age of 9 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.023	0.028	0.010	0.014	0.004	0.013	0.023	0.015	0.004
Mean	0.395	0.167	0.128	0.176	0.090	0.065	0.090	0.056	0.039
Rat 2 SD	0.004	0.022	0.002	0.017	0.022	0.003	0.018	0.002	0.007
Mean	0.106	0.065	0.081	0.056	0.103	0.122	0.071	0.087	0.077
Rat 3 SD	0.004	0.030	0.037	0.020	0.015	0.009	0.023	0.011	0.007
Mean	0.596	0.202	0.113	0.068	0.048	0.031	0.033	0.040	0.035
Rat 4 SD	0.054	0.011	0.013	0.010	0.038	0.067	0.041	0.007	0.023
Mean	0.395	0.168	0.080	0.061	0.093	0.071	0.086	0.062	0.073
Rat 5 SD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.165	0.121	0.066	0.027	0.018	0.000	0.000	0.000	0.006
Rat 6 SD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.124	0.052	0.003	0.000	0.000	0.000	0.000	0.000	0.000
Rat 7 SD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.439	0.255	0.171	0.168	0.171	0.149	0.039	0.122	0.083
Rat 8 SD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.475	0.364	0.209	0.202	0.166	0.149	0.086	0.051	0.064
Rat 9 SD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.050	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000
Rat 10 SD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.044	0.000	0.000	0.000	0.001	0.000	0.000	0.002	0.002
Mean	0.279	0.139	0.085	0.076	0.069	0.059	0.041	0.042	0.038
SD	0.202	0.116	0.072	0.078	0.066	0.062	0.040	0.042	0.034
Range	0.044	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	0.056	0.364	0.209	0.202	0.171	0.149	0.090	0.122	0.083

Table 4.10 ELISA Responses for Individual Hunter Rats to ROS, at the Age of 3 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.024	0.005	0.006	0.012	0.005	0.002	0.011	0.001	0.028
Mean	0.383	0.247	0.253	0.122	0.128	0.103	0.080	0.071	0.037
Rat 2 SD	0.053	0.003	0.029	0.019	0.002	0.028	0.001	0.028	0.009
Mean	0.409	0.301	0.250	0.170	0.168	0.042	0.055	0.050	0.088
Rat 3 SD	0.021	0.001	0.013	0.001	0.011	0.002	0.020	0.018	0.004
Mean	0.261	0.173	0.133	0.108	0.076	0.087	0.048	0.063	0.053
Rat 4 SD	0.059	0.027	0.004	0.042	0.035	0.010	0.018	0.001	0.028
Mean	0.439	0.324	0.269	0.136	0.125	0.086	0.034	0.071	0.068
Rat 5 SD	0.016	0.001	0.013	0.004	0.000	0.006	0.000	0.000	0.000
Mean	0.108	0.050	0.015	0.006	0.000	0.004	0.000	0.000	0.000
Rat 6 SD	0.011	0.021	0.000	0.013	0.006	0.006	0.014	0.011	0.016
Mean	0.557	0.491	0.346	0.271	0.189	0.153	0.145	0.016	0.024
Rat 7 SD	0.007	0.011	0.009	0.000	0.012	0.000	0.000	0.000	0.000
Mean	0.355	0.214	0.108	0.054	0.013	0.000	0.000	0.000	0.000
Rat 8 SD	0.008	0.014	0.022	0.018	0.027	0.008	0.007	0.003	0.007
Mean	0.089	0.089	0.106	0.094	0.113	0.136	0.068	0.022	0.061
Rat 9 SD	0.028	0.034	0.031	0.007	0.028	0.016	0.001	0.004	0.018
Mean	0.415	0.389	0.249	0.168	0.150	0.095	0.084	0.015	0.066
Rat 10 SD	0.051	0.029	0.177	0.040	0.001	0.016	0.033	0.035	0.031
Mean	0.576	0.404	0.381	0.150	0.065	0.060	0.065	0.061	0.065
Mean	0.359	0.268	0.211	0.128	0.103	0.077	0.058	0.037	0.046
SD	0.165	0.141	0.116	0.072	0.063	0.051	0.043	0.029	0.030
Range	0.089	0.050	0.015	0.006	0.000	0.000	0.000	0.000	0.000
	0.576	0.491	0.381	0.271	0.189	0.153	0.145	0.071	0.088

Table 4.11 ELISA Responses for Individual Hunter Rats to ROS, at the Age of 6 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.036	0.029	0.018	0.006	0.002	0.008	0.016	0.008	0.006
Mean	0.253	0.153	0.187	0.094	0.064	0.067	0.088	0.060	0.026
Rat 2 SD	0.005	0.013	0.040	0.006	0.001	0.028	0.006	0.011	0.016
Mean	0.275	0.175	0.167	0.095	0.088	0.059	0.070	0.051	0.059
Rat 3 SD	0.031	0.023	0.015	0.016	0.033	0.017	0.007	0.004	0.019
Mean	0.236	0.134	0.095	0.076	0.093	0.062	0.093	0.071	0.081
Rat 4 SD	0.013	0.013	0.008	0.003	0.004	0.008	0.011	0.001	0.004
Mean	0.302	0.217	0.183	0.165	0.079	0.071	0.053	0.058	0.059
Rat 5 SD	0.028	0.008	0.004	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.033	0.006	0.002	0.000	0.000	0.000	0.000	0.000	0.000
Rat 6 SD	0.011	0.014	0.049	0.018	0.018	0.011	0.002	0.005	0.004
Mean	0.230	0.178	0.160	0.076	0.066	0.061	0.048	0.048	0.018
Rat 7 SD	0.008	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.035	0.019	0.003	0.000	0.000	0.000	0.000	0.000	0.000
Rat 8 SD	0.015	0.007	0.018	0.000	0.016	0.017	0.004	0.011	0.019
Mean	0.273	0.214	0.164	0.117	0.045	0.037	0.031	0.034	0.026
Rat 9 SD	0.033	0.005	0.008	0.034	0.033	0.006	0.001	0.011	0.004
Mean	0.279	0.151	0.184	0.066	0.055	0.061	0.095	0.076	0.022
Rat 10 SD	0.016	0.020	0.024	0.028	0.002	0.023	0.004	0.027	0.016
Mean	0.317	0.267	0.154	0.140	0.083	0.046	0.063	0.065	0.047
Mean	0.223	0.151	0.130	0.083	0.057	0.047	0.054	0.046	0.034
SD	0.103	0.083	0.072	0.053	0.034	0.026	0.035	0.027	0.027
Range	0.033	0.006	0.002	0.000	0.000	0.000	0.000	0.000	0.000
	0.317	0.267	0.187	0.165	0.093	0.071	0.095	0.076	0.081

Table 4.12 ELISA Responses for Individual Hunter Rats to ROS, at the Age of 9 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.018	0.010	0.005	0.008	0.015	0.006	0.006	0.006	0.001
Mean	0.280	0.170	0.194	0.077	0.066	0.065	0.028	0.024	0.019
Rat 2 SD	0.039	0.006	0.011	0.023	0.003	0.003	0.008	0.011	0.023
Mean	0.277	0.215	0.114	0.065	0.039	0.052	0.090	0.018	0.049
Rat 3 SD	0.018	0.003	0.000	0.032	0.006	0.013	0.001	0.008	0.018
Mean	0.255	0.157	0.146	0.119	0.099	0.083	0.065	0.077	0.052
Rat 4 SD	0.014	0.005	0.025	0.013	0.006	0.016	0.013	0.017	0.025
Mean	0.383	0.277	0.182	0.153	0.091	0.075	0.075	0.084	0.054
Rat 5 SD	0.016	0.001	0.013	0.004	0.000	0.006	0.000	0.000	0.000
Mean	0.108	0.050	0.015	0.006	0.000	0.004	0.000	0.000	0.000
Rat 6 SD	0.011	0.049	0.004	0.013	0.006	0.006	0.014	0.011	0.002
Mean	0.358	0.241	0.143	0.071	0.089	0.053	0.045	0.016	0.034
Rat 7 SD	0.013	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.095	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Rat 8 SD	0.001	0.003	0.027	0.013	0.006	0.012	0.001	0.002	0.009
Mean	0.301	0.195	0.125	0.064	0.040	0.021	0.014	0.037	0.022
Rat 9 SD	0.008	0.009	0.027	0.007	0.022	0.009	0.004	0.025	0.017
Mean	0.266	0.215	0.148	0.104	0.077	0.093	0.093	0.071	0.079
Rat 10 SD	0.004	0.030	0.013	0.011	0.000	0.006	0.011	0.006	0.002
Mean	0.310	0.237	0.176	0.086	0.080	0.054	0.081	0.071	0.058
Mean	0.263	0.179	0.124	0.075	0.058	0.050	0.049	0.040	0.037
SD	0.094	0.081	0.066	0.047	0.037	0.032	0.036	0.033	0.026
Range	0.095	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	0.383	0.277	0.194	0.153	0.099	0.093	0.093	0.084	0.079

Table 4.13 ELISA Responses for Individual PVG Rats to S-Antigen, at the Age of 3 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.004	0.009	0.009	0.011	0.013	0.021	0.010	0.041	0.002
Mean	0.100	0.037	0.015	0.035	0.031	0.039	0.051	0.054	0.071
Rat 2 SD	0.006	0.028	0.002	0.009	0.001	0.001	0.000	0.000	0.000
Mean	0.129	0.078	0.024	0.006	0.001	0.001	0.000	0.000	0.000
Rat 3 SD	0.006	0.015	0.088	0.006	0.005	0.001	0.034	0.012	0.003
Mean	0.357	0.197	0.099	0.124	0.071	0.065	0.072	0.050	0.066
Rat 4 SD	0.004	0.037	0.004	0.004	0.005	0.017	0.008	0.006	0.002
Mean	0.198	0.126	0.065	0.065	0.061	0.063	0.018	0.058	0.041
Rat 5 SD	0.024	0.004	0.030	0.006	0.006	0.006	0.002	0.003	0.004
Mean	0.258	0.149	0.121	0.054	0.004	0.004	0.001	0.002	0.003
Rat 6 SD	0.017	0.001	0.004	0.042	0.001	0.000	0.000	0.000	0.001
Mean	0.183	0.111	0.074	0.040	0.001	0.000	0.000	0.000	0.001
Rat 7 SD	0.008	0.001	0.007	0.011	0.006	0.001	0.001	0.003	0.018
Mean	0.284	0.137	0.094	0.037	0.049	0.024	0.092	0.057	0.042
Rat 8 SD	0.019	0.015	0.001	0.018	0.016	0.004	0.008	0.001	0.006
Mean	0.267	0.138	0.053	0.034	0.033	0.008	0.006	0.006	0.015
Rat 9 SD	0.017	0.001	0.009	0.011	0.018	0.010	0.004	0.016	0.009
Mean	0.156	0.071	0.037	0.027	0.016	0.014	0.014	0.016	0.010
Rat 10 SD	0.006	0.027	0.029	0.004	0.009	0.002	0.040	0.003	0.020
Mean	0.282	0.156	0.163	0.124	0.024	0.013	0.028	0.020	0.014
Mean	0.222	0.120	0.075	0.055	0.029	0.023	0.028	0.026	0.026
SD	0.081	0.047	0.046	0.040	0.025	0.024	0.033	0.025	0.027
Range	0.100	0.037	0.015	0.006	0.001	0.000	0.000	0.000	0.000
	0.357	0.197	0.163	0.124	0.071	0.065	0.092	0.058	0.071

Table 4.14 ELISA Responses for Individual PVG Rats to S-Antigen, at the Age of 6 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.004	0.017	0.002	0.009	0.000	0.001	0.012	0.004	0.001
Mean	0.111	0.068	0.039	0.034	0.044	0.068	0.071	0.023	0.022
Rat 2 SD	0.013	0.002	0.030	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.082	0.045	0.031	0.000	0.000	0.000	0.000	0.000	0.000
Rat 3 SD	0.028	0.008	0.030	0.047	0.019	0.008	0.009	0.002	0.002
Mean	0.171	0.058	0.057	0.061	0.022	0.047	0.075	0.060	0.045
Rat 4 SD	0.002	0.004	0.003	0.006	0.004	0.004	0.026	0.006	0.029
Mean	0.154	0.057	0.038	0.033	0.028	0.011	0.046	0.058	0.068
Rat 5 SD	0.001	0.000	0.000	0.000	0.000	0.005	0.001	0.000	0.000
Mean	0.023	0.000	0.000	0.000	0.000	0.003	0.001	0.000	0.000
Rat 6 SD	0.013	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Rat 7 SD	0.022	0.009	0.015	0.007	0.006	0.003	0.004	0.007	0.001
Mean	0.299	0.180	0.058	0.066	0.094	0.043	0.045	0.031	0.005
Rat 8 SD	0.004	0.016	0.004	0.008	0.000	0.000	0.004	0.000	0.000
Mean	0.090	0.049	0.020	0.005	0.000	0.000	0.003	0.000	0.000
Rat 9 SD	0.002	0.001	0.001	0.002	0.003	0.001	0.004	0.004	0.007
Mean	0.109	0.052	0.034	0.016	0.002	0.001	0.007	0.004	0.005
Rat 10 SD	0.002	0.065	0.044	0.047	0.035	0.035	0.000	0.030	0.007
Mean	0.165	0.069	0.053	0.048	0.063	0.025	0.000	0.022	0.074
Mean	0.122	0.058	0.033	0.026	0.025	0.020	0.025	0.020	0.022
SD	0.082	0.050	0.021	0.026	0.033	0.025	0.031	0.024	0.029
Range	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	0.299	0.180	0.058	0.066	0.094	0.068	0.075	0.060	0.074

Table 4.15 ELISA Responses for Individual PVG Rats to S-Antigen, at the Age of 9 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.054	0.044	0.039	0.030	0.047	0.037	0.015	0.022	0.001
Mean	0.166	0.109	0.062	0.051	0.065	0.068	0.082	0.084	0.071
Rat 2 SD	0.029	0.013	0.011	0.011	0.022	0.000	0.000	0.000	0.000
Mean	0.158	0.065	0.042	0.008	0.016	0.000	0.000	0.000	0.000
Rat 3 SD	0.016	0.026	0.008	0.018	0.004	0.007	0.008	0.045	0.007
Mean	0.282	0.257	0.161	0.075	0.112	0.087	0.095	0.063	0.067
Rat 4 SD	0.007	0.013	0.013	0.000	0.001	0.008	0.008	0.001	0.008
Mean	0.178	0.082	0.072	0.038	0.055	0.075	0.089	0.084	0.087
Rat 5 SD	0.010	0.008	0.000	0.025	0.021	0.006	0.000	0.000	0.000
Mean	0.051	0.059	0.000	0.018	0.015	0.004	0.000	0.000	0.000
Rat 6 SD	0.020	0.071	0.040	0.018	0.019	0.021	0.006	0.007	0.014
Mean	0.014	0.050	0.029	0.013	0.014	0.015	0.007	0.006	0.010
Rat 7 SD	0.008	0.007	0.000	0.001	0.001	0.012	0.007	0.016	0.009
Mean	0.326	0.190	0.115	0.081	0.082	0.076	0.079	0.058	0.076
Rat 8 SD	0.028	0.028	0.003	0.048	0.028	0.023	0.013	0.028	0.035
Mean	0.203	0.118	0.098	0.034	0.020	0.016	0.009	0.020	0.025
Rat 9 SD	0.019	0.021	0.016	0.020	0.019	0.006	0.016	0.005	0.003
Mean	0.144	0.075	0.058	0.027	0.014	0.017	0.028	0.020	0.004
Rat 10 SD	0.028	0.033	0.042	0.054	0.006	0.006	0.000	0.000	0.016
Mean	0.583	0.299	0.116	0.038	0.058	0.074	0.000	0.000	0.011
Mean	0.211	0.130	0.075	0.038	0.045	0.043	0.039	0.033	0.035
SD	0.161	0.088	0.048	0.025	0.035	0.035	0.042	0.035	0.036
Range	0.014	0.050	0.000	0.008	0.014	0.000	0.000	0.000	0.000
	0.583	0.299	0.161	0.081	0.112	0.087	0.095	0.084	0.087

Table 4.16 ELISA Responses for Individual PVG Rats to ROS, at the Age of 3 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.013	0.024	0.002	0.011	0.001	0.001	0.006	0.045	0.008
Mean	0.205	0.200	0.193	0.127	0.096	0.090	0.061	0.066	0.032
Rat 2 SD	0.016	0.021	0.001	0.010	0.001	0.001	0.023	0.018	0.044
Mean	0.294	0.160	0.110	0.092	0.094	0.071	0.073	0.076	0.057
Rat 3 SD	0.041	0.036	0.015	0.004	0.013	0.022	0.000	0.000	0.004
Mean	0.228	0.069	0.050	0.085	0.071	0.065	0.088	0.098	0.041
Rat 4 SD	0.030	0.006	0.004	0.010	0.030	0.039	0.004	0.002	0.013
Mean	0.224	0.193	0.061	0.059	0.071	0.056	0.024	0.082	0.039
Rat 5 SD	0.013	0.013	0.003	0.001	0.001	0.002	0.024	0.004	0.011
Mean	0.215	0.109	0.073	0.093	0.087	0.092	0.055	0.042	0.067
Rat 6 SD	0.023	0.021	0.004	0.006	0.017	0.001	0.015	0.016	0.023
Mean	0.224	0.163	0.072	0.047	0.079	0.093	0.062	0.026	0.046
Rat 7 SD	0.006	0.017	0.011	0.012	0.028	0.011	0.004	0.028	0.039
Mean	0.215	0.124	0.102	0.087	0.052	0.055	0.035	0.061	0.056
Rat 8 SD	0.016	0.025	0.004	0.008	0.004	0.002	0.035	0.004	0.002
Mean	0.259	0.160	0.108	0.063	0.041	0.062	0.056	0.039	0.054
Rat 9 SD	0.022	0.016	0.007	0.028	0.011	0.036	0.025	0.021	0.028
Mean	0.190	0.151	0.110	0.070	0.070	0.062	0.069	0.053	0.066
Rat 10 SD	0.012	0.012	0.020	0.000	0.008	0.007	0.002	0.001	0.003
Mean	0.182	0.160	0.128	0.107	0.074	0.078	0.087	0.064	0.037
Mean	0.224	0.149	0.101	0.083	0.074	0.072	0.061	0.061	0.050
SD	0.033	0.039	0.041	0.024	0.017	0.015	0.020	0.022	0.012
Range	0.182	0.069	0.05	0.047	0.041	0.055	0.024	0.026	0.032
	0.294	0.200	0.193	0.127	0.096	0.093	0.088	0.098	0.067

Table 4.17 ELISA Responses for Individual PVG Rats to ROS, at the Age of 6 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.029	0.036	0.000	0.003	0.004	0.004	0.011	0.021	0.004
Mean	0.285	0.144	0.098	0.091	0.086	0.086	0.061	0.050	0.036
Rat 2 SD	0.008	0.019	0.004	0.003	0.021	0.018	0.023	0.030	0.016
Mean	0.208	0.134	0.090	0.082	0.063	0.080	0.070	0.037	0.072
Rat 3 SD	0.019	0.008	0.000	0.003	0.004	0.008	0.003	0.023	0.001
Mean	0.161	0.148	0.106	0.093	0.079	0.072	0.086	0.037	0.036
Rat 4 SD	0.018	0.006	0.004	0.004	0.006	0.005	0.007	0.003	0.005
Mean	0.155	0.104	0.093	0.071	0.077	0.070	0.070	0.069	0.065
Rat 5 SD	0.014	0.002	0.004	0.006	0.032	0.009	0.001	0.006	0.001
Mean	0.184	0.069	0.060	0.063	0.071	0.082	0.005	0.007	0.050
Rat 6 SD	0.008	0.023	0.018	0.006	0.008	0.012	0.032	0.018	0.007
Mean	0.209	0.143	0.082	0.095	0.049	0.056	0.068	0.056	0.033
Rat 7 SD	0.010	0.007	0.008	0.006	0.019	0.021	0.018	0.011	0.006
Mean	0.250	0.163	0.107	0.088	0.071	0.068	0.068	0.024	0.024
Rat 8 SD	0.008	0.015	0.004	0.010	0.001	0.016	0.019	0.018	0.033
Mean	0.173	0.151	0.109	0.090	0.083	0.056	0.057	0.048	0.055
Rat 9 SD	0.007	0.000	0.008	0.003	0.006	0.018	0.003	0.008	0.002
Mean	0.238	0.195	0.084	0.090	0.085	0.084	0.042	0.072	0.048
Rat 10 SD	0.037	0.016	0.001	0.005	0.014	0.001	0.012	0.004	0.032
Mean	0.168	0.138	0.103	0.092	0.088	0.039	0.073	0.074	0.061
Mean	0.203	0.139	0.093	0.086	0.075	0.069	0.060	0.047	0.048
SD	0.043	0.034	0.015	0.010	0.012	0.015	0.022	0.022	0.016
Range	0.155	0.069	0.060	0.063	0.049	0.039	0.005	0.007	0.024
	0.285	0.195	0.109	0.095	0.088	0.086	0.086	0.074	0.072

Table 4.18 ELISA Responses for Individual PVG Rats to ROS, at the Age of 9 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.010	0.001	0.002	0.006	0.013	0.010	0.002	0.022	0.006
Mean	0.249	0.156	0.104	0.086	0.085	0.074	0.022	0.081	0.021
Rat 2 SD	0.018	0.035	0.000	0.027	0.008	0.001	0.019	0.006	0.001
Mean	0.185	0.159	0.052	0.065	0.074	0.075	0.057	0.087	0.005
Rat 3 SD	0.000	0.005	0.004	0.011	0.004	0.009	0.018	0.028	0.008
Mean	0.184	0.108	0.092	0.078	0.084	0.082	0.045	0.079	0.047
Rat 4 SD	0.062	0.007	0.001	0.006	0.004	0.010	0.007	0.019	0.020
Mean	0.183	0.163	0.104	0.087	0.082	0.051	0.068	0.050	0.039
Rat 5 SD	0.010	0.001	0.004	0.004	0.023	0.012	0.006	0.004	0.012
Mean	0.185	0.171	0.169	0.106	0.078	0.068	0.079	0.094	0.084
Rat 6 SD	0.023	0.002	0.013	0.001	0.006	0.002	0.003	0.009	0.008
Mean	0.257	0.168	0.070	0.097	0.061	0.058	0.060	0.061	0.052
Rat 7 SD	0.033	0.006	0.006	0.019	0.001	0.009	0.004	0.020	0.006
Mean	0.266	0.116	0.080	0.068	0.083	0.087	0.068	0.059	0.076
Rat 8 SD	0.011	0.021	0.030	0.006	0.019	0.013	0.031	0.003	0.015
Mean	0.242	0.128	0.073	0.088	0.081	0.049	0.046	0.064	0.042
Rat 9 SD	0.021	0.013	0.001	0.016	0.008	0.013	0.003	0.042	0.042
Mean	0.217	0.112	0.099	0.082	0.077	0.062	0.065	0.065	0.066
Rat 10 SD	0.045	0.001	0.021	0.000	0.009	0.008	0.001	0.004	0.013
Mean	0.247	0.193	0.082	0.065	0.058	0.074	0.049	0.046	0.050
Mean	0.222	0.147	0.092	0.082	0.076	0.068	0.056	0.069	0.048
SD	0.034	0.029	0.032	0.014	0.009	0.013	0.016	0.016	0.024
Range	0.183	0.108	0.052	0.065	0.058	0.049	0.022	0.046	0.005
	0.266	0.193	0.169	0.106	0.085	0.087	0.079	0.094	0.084

Table 4.19 ELISA Responses for Individual DA Rats to S-Antigen, at the Age of 3 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.013	0.011	0.019	0.025	0.011	0.008	0.001	0.006	0.004
Mean	0.045	0.008	0.014	0.018	0.020	0.020	0.008	0.031	0.039
Rat 2 SD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.105	0.049	0.031	0.023	0.019	0.014	0.018	0.011	0.011
Rat 3 SD	0.033	0.003	0.009	0.022	0.016	0.001	0.021	0.018	0.002
Mean	0.160	0.070	0.083	0.072	0.076	0.098	0.059	0.060	0.053
Rat 4 SD	0.069	0.004	0.021	0.005	0.025	0.033	0.011	0.004	0.008
Mean	0.056	0.050	0.050	0.040	0.018	0.028	0.029	0.037	0.044
Rat 5 SD	0.018	0.009	0.007	0.001	0.016	0.001	0.003	0.010	0.008
Mean	0.148	0.092	0.049	0.048	0.043	0.053	0.051	0.034	0.054
Mean	0.103	0.054	0.045	0.040	0.035	0.043	0.033	0.035	0.040
SD	0.052	0.031	0.026	0.022	0.025	0.035	0.022	0.017	0.017
Range	0.045	0.008	0.014	0.018	0.018	0.014	0.008	0.011	0.011
	0.160	0.092	0.083	0.072	0.076	0.098	0.059	0.060	0.054

Table 4.20 ELISA Responses for Individual DA Rats to S-Antigen, at the Age of 6 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.018	0.021	0.006	0.004	0.008	0.001	0.015	0.016	0.016
Mean	0.136	0.072	0.029	0.020	0.025	0.022	0.028	0.038	0.040
Rat 2 SD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.160	0.089	0.041	0.011	0.012	0.000	0.000	0.000	0.000
Rat 3 SD	0.047	0.004	0.006	0.032	0.005	0.026	0.000	0.025	0.016
Mean	0.270	0.124	0.126	0.053	0.007	0.018	0.000	0.018	0.030
Rat 4 SD	0.031	0.005	0.025	0.010	0.026	0.011	0.002	0.018	0.038
Mean	0.244	0.134	0.081	0.090	0.063	0.064	0.083	0.077	0.070
Rat 5 SD	0.006	0.002	0.001	0.008	0.021	0.005	0.001	0.005	0.001
Mean	0.173	0.103	0.093	0.082	0.050	0.081	0.081	0.047	0.031
Mean	0.197	0.104	0.074	0.051	0.031	0.037	0.038	0.036	0.034
SD	0.057	0.025	0.039	0.036	0.024	0.034	0.041	0.029	0.025
Range	0.136	0.072	0.029	0.011	0.007	0.000	0.000	0.000	0.000
	0.270	0.134	0.126	0.090	0.063	0.081	0.083	0.077	0.070

Table 4.21 ELISA Responses for Individual DA Rats to S-Antigen, at the Age of 9 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.171	0.110	0.107	0.082	0.116	0.114	0.027	0.033	0.029
Rat 2 SD	0.029	0.004	0.035	0.001	0.021	0.010	0.006	0.009	0.021
Mean	0.121	0.061	0.039	0.028	0.015	0.029	0.021	0.050	0.026
Rat 3 SD	0.059	0.000	0.017	0.013	0.003	0.025	0.007	0.003	0.013
Mean	0.183	0.100	0.072	0.054	0.055	0.066	0.053	0.055	0.053
Rat 4 SD	0.027	0.040	0.006	0.014	0.006	0.006	0.013	0.014	0.028
Mean	0.247	0.170	0.086	0.048	0.039	0.086	0.049	0.065	0.050
Mean	0.181	0.110	0.076	0.053	0.056	0.074	0.038	0.051	0.040
SD	0.052	0.045	0.029	0.022	0.043	0.036	0.016	0.013	0.014
Range	0.121	0.061	0.039	0.028	0.015	0.029	0.021	0.033	0.026
	0.247	0.170	0.107	0.082	0.116	0.114	0.053	0.065	0.053

Table 4.22 ELISA Responses for Individual DA Rats to ROS, at the Age of 3 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.010	0.008	0.001	0.006	0.011	0.006	0.009	0.006	0.018
Mean	0.090	0.095	0.090	0.081	0.071	0.067	0.043	0.055	0.053
Rat 2 SD	0.005	0.025	0.013	0.012	0.037	0.010	0.011	0.000	0.000
Mean	0.108	0.104	0.081	0.068	0.066	0.053	0.062	0.068	0.055
Rat 3 SD	0.003	0.007	0.001	0.033	0.023	0.001	0.004	0.015	0.028
Mean	0.119	0.123	0.105	0.067	0.050	0.047	0.048	0.056	0.060
Rat 4 SD	0.004	0.004	0.013	0.011	0.018	0.000	0.028	0.028	0.006
Mean	0.138	0.115	0.064	0.065	0.048	0.055	0.079	0.063	0.072
Rat 5 SD	0.011	0.018	0.011	0.013	0.023	0.008	0.011	0.033	0.008
Mean	0.141	0.053	0.051	0.081	0.079	0.053	0.062	0.063	0.056
Mean	0.119	0.098	0.078	0.073	0.063	0.055	0.059	0.061	0.059
SD	0.022	0.028	0.021	0.008	0.013	0.008	0.014	0.005	0.008
Range	0.090	0.053	0.051	0.065	0.048	0.047	0.043	0.055	0.053
	0.141	0.123	0.105	0.081	0.079	0.067	0.079	0.068	0.072

Table 4.23 ELISA Responses for Individual DA Rats to ROS, at the Age of 6 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.004	0.008	0.013	0.006	0.006	0.010	0.003	0.007	0.006
Mean	0.118	0.109	0.094	0.089	0.090	0.077	0.071	0.064	0.068
Rat 2 SD	0.001	0.015	0.014	0.016	0.014	0.015	0.006	0.001	0.025
Mean	0.187	0.088	0.078	0.071	0.070	0.061	0.046	0.075	0.051
Rat 3 SD	0.021	0.001	0.014	0.025	0.019	0.018	0.019	0.021	0.006
Mean	0.151	0.090	0.065	0.069	0.063	0.056	0.053	0.080	0.028
Rat 4 SD	0.017	0.010	0.011	0.005	0.028	0.023	0.014	0.006	0.015
Mean	0.092	0.098	0.086	0.061	0.064	0.052	0.048	0.070	0.045
Rat 5 SD	0.002	0.004	0.016	0.010	0.013	0.030	0.019	0.027	0.021
Mean	0.126	0.103	0.088	0.075	0.076	0.066	0.063	0.050	0.054
Mean	0.135	0.098	0.822	0.073	0.073	0.062	0.056	0.068	0.050
SD	0.036	0.009	0.011	0.010	0.011	0.010	0.011	0.012	0.015
Range	0.092	0.088	0.065	0.061	0.063	0.052	0.046	0.050	0.028
	0.187	0.109	0.094	0.089	0.090	0.077	0.071	0.080	0.068

Table 4.24 ELISA Responses for Individual DA Rats to ROS, at the Age of 9 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.000	0.000	0.004	0.012	0.010	0.008	0.005	0.021	0.004
Mean	0.133	0.108	0.098	0.082	0.069	0.054	0.076	0.073	0.068
Rat 2 SD	0.025	0.001	0.007	0.008	0.005	0.019	0.019	0.007	0.020
Mean	0.144	0.198	0.083	0.076	0.077	0.085	0.056	0.071	0.047
Rat 3 SD	0.004	0.008	0.007	0.007	0.008	0.018	0.013	0.008	0.002
Mean	0.164	0.103	0.089	0.060	0.089	0.061	0.065	0.059	0.065
Rat 4 SD	0.009	0.001	0.004	0.020	0.008	0.029	0.019	0.023	0.021
Mean	0.119	0.103	0.095	0.068	0.060	0.065	0.063	0.052	0.054
Mean	0.140	0.128	0.091	0.071	0.074	0.067	0.065	0.064	0.058
SD	0.019	0.047	0.006	0.009	0.012	0.013	0.009	0.010	0.009
Range	0.119	0.103	0.083	0.060	0.060	0.054	0.056	0.052	0.047
	0.164	0.198	0.098	0.082	0.089	0.085	0.076	0.073	0.068

Table 4.25 ELISA Responses for Individual Wistar Rats to S-Antigen, at the Age of 3 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.048	0.020	0.004	0.000	0.020	0.014	0.013	0.008	0.005
Mean	0.060	0.030	0.046	0.036	0.041	0.051	0.050	0.057	0.071
Rat 2 SD	0.045	0.003	0.013	0.016	0.029	0.033	0.028	0.010	0.021
Mean	0.287	0.144	0.087	0.044	0.031	0.037	0.024	0.047	0.034
Rat 3 SD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.316	0.086	0.044	0.017	0.032	0.017	0.020	0.036	0.003
Rat 4 SD	0.062	0.004	0.014	0.017	0.004	0.011	0.004	0.011	0.009
Mean	0.318	0.156	0.100	0.072	0.062	0.075	0.074	0.072	0.078
Mean	0.245	0.104	0.070	0.042	0.042	0.045	0.042	0.053	0.046
SD	0.124	0.058	0.028	0.023	0.015	0.024	0.025	0.015	0.035
Range	0.060	0.030	0.044	0.017	0.031	0.017	0.020	0.036	0.003
	0.318	0.156	0.100	0.072	0.062	0.075	0.074	0.072	0.078

Table 4.26 ELISA Responses for Individual Wistar Rats to S-Antigen, at the Age of 6 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.023	0.044	0.045	0.008	0.013	0.011	0.007	0.009	0.007
Mean	0.133	0.066	0.045	0.019	0.034	0.037	0.063	0.076	0.072
Rat 2 SD	0.018	0.009	0.054	0.048	0.013	0.001	0.012	0.022	0.012
Mean	0.556	0.304	0.178	0.137	0.107	0.084	0.057	0.066	0.082
Rat 3 SD	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.273	0.164	0.055	0.045	0.040	0.020	0.029	0.039	0.020
Rat 4 SD	0.034	0.006	0.006	0.003	0.015	0.023	0.004	0.002	0.029
Mean	0.594	0.274	0.117	0.066	0.035	0.026	0.037	0.031	0.048
Rat 5 SD	0.043	0.047	0.021	0.004	0.051	0.011	0.035	0.004	0.026
Mean	0.470	0.158	0.107	0.078	0.068	0.051	0.063	0.071	0.062
Mean	0.405	0.193	0.100	0.069	0.057	0.044	0.050	0.057	0.057
SD	0.196	0.096	0.054	0.044	0.031	0.026	0.016	0.020	0.024
Range	0.133	0.066	0.045	0.019	0.034	0.020	0.029	0.031	0.020
	0.594	0.304	0.178	0.137	0.107	0.084	0.063	0.076	0.082

Table 4.27 ELISA Responses for Individual Wistar Rats to S-Antigen, at the Age of 9 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.038	0.004	0.010	0.000	0.018	0.006	0.030	0.006	0.017
Mean	0.176	0.066	0.087	0.049	0.056	0.062	0.068	0.066	0.083
Rat 2 SD	0.000	0.000	0.001	0.000	0.025	0.033	0.001	0.022	0.016
Mean	0.046	0.089	0.100	0.105	0.082	0.060	0.039	0.066	0.062
Rat 3 SD	0.016	0.001	0.002	0.003	0.009	0.006	0.009	0.001	0.001
Mean	0.274	0.134	0.087	0.050	0.061	0.043	0.064	0.081	0.049
Rat 4 SD	0.044	0.038	0.006	0.031	0.020	0.007	0.001	0.025	0.009
Mean	0.696	0.213	0.125	0.064	0.038	0.048	0.056	0.038	0.056
Mean	0.298	0.126	0.100	0.067	0.059	0.053	0.057	0.063	0.063
SD	0.281	0.065	0.018	0.026	0.018	0.009	0.013	0.018	0.015
Range	0.046	0.066	0.087	0.049	0.038	0.043	0.039	0.038	0.049
	0.696	0.213	0.125	0.105	0.082	0.062	0.068	0.081	0.083

Table 4.28 ELISA Responses for Individual Wistar Rats to ROS, at the Age of 3 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.011	0.004	0.007	0.004	0.015	0.004	0.013	0.000	0.008
Mean	0.197	0.095	0.087	0.086	0.073	0.086	0.056	0.086	0.091
Rat 2 SD	0.008	0.023	0.004	0.021	0.001	0.001	0.010	0.013	0.004
Mean	0.267	0.192	0.104	0.084	0.082	0.087	0.065	0.075	0.078
Rat 3 SD	0.006	0.028	0.012	0.001	0.008	0.018	0.008	0.020	0.003
Mean	0.237	0.204	0.160	0.104	0.089	0.087	0.087	0.080	0.077
Rat 4 SD	0.003	0.040	0.014	0.006	0.040	0.016	0.006	0.008	0.004
Mean	0.277	0.260	0.213	0.177	0.080	0.097	0.076	0.071	0.063
Rat 5 SD	0.012	0.013	0.001	0.004	0.010	0.001	0.004	0.000	0.000
Mean	0.262	0.163	0.108	0.061	0.043	0.043	0.043	0.085	0.074
Mean	0.248	0.182	0.134	0.103	0.073	0.080	0.066	0.079	0.077
SD	0.032	0.060	0.052	0.045	0.018	0.021	0.017	0.007	0.010
Range	0.197	0.095	0.087	0.061	0.043	0.043	0.043	0.071	0.063
	0.277	0.260	0.213	0.177	0.089	0.097	0.087	0.086	0.091

Table 4.29 ELISA Responses for Individual Wistar Rats to ROS, at the Age of 6 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.007	0.021	0.022	0.001	0.006	0.014	0.021	0.011	0.005
Mean	0.282	0.237	0.178	0.126	0.110	0.122	0.079	0.081	0.072
Rat 2 SD	0.037	0.010	0.025	0.018	0.016	0.008	0.033	0.008	0.004
Mean	0.284	0.201	0.126	0.085	0.075	0.086	0.058	0.060	0.053
Rat 3 SD	0.018	0.023	0.001	0.012	0.017	0.021	0.039	0.010	0.021
Mean	0.271	0.183	0.155	0.122	0.044	0.058	0.065	0.081	0.084
Rat 4 SD	0.021	0.002	0.005	0.005	0.017	0.030	0.013	0.011	0.014
Mean	0.356	0.250	0.128	0.092	0.066	0.065	0.068	0.040	0.012
Rat 5 SD	0.033	0.007	0.037	0.000	0.003	0.002	0.028	0.002	0.005
Mean	0.245	0.226	0.148	0.123	0.092	0.078	0.073	0.045	0.036
Mean	0.288	0.219	0.147	0.110	0.077	0.082	0.069	0.061	0.051
SD	0.041	0.027	0.021	0.019	0.025	0.025	0.008	0.019	0.029
Range	0.245	0.183	0.126	0.085	0.044	0.058	0.058	0.040	0.012
	0.356	0.250	0.178	0.126	0.110	0.122	0.079	0.081	0.084

Table 4.30 ELISA Responses for Individual Wistars Rats to ROS, at the Age of 9 Weeks

	Serum Dilution								
	20	40	80	160	320	640	1280	2560	5120
Rat 1 SD	0.025	0.013	0.021	0.015	0.010	0.014	0.008	0.026	0.023
Mean	0.280	0.176	0.153	0.063	0.078	0.077	0.041	0.043	0.061
Rat 2 SD	0.004	0.001	0.027	0.011	0.004	0.010	0.008	0.039	0.018
Mean	0.274	0.221	0.143	0.086	0.089	0.085	0.070	0.060	0.076
Rat 3 SD	0.041	0.002	0.004	0.006	0.013	0.010	0.014	0.018	0.027
Mean	0.244	0.183	0.145	0.097	0.077	0.074	0.063	0.055	0.078
Rat 4 SD	0.011	0.035	0.002	0.013	0.026	0.029	0.014	0.012	0.022
Mean	0.230	0.173	0.108	0.071	0.062	0.064	0.041	0.068	0.035
Rat 5 SD	0.017	0.000	0.000	0.013	0.009	0.007	0.037	0.018	0.007
Mean	0.170	0.137	0.102	0.087	0.080	0.071	0.066	0.045	0.054
Mean	0.240	0.178	0.130	0.081	0.077	0.074	0.056	0.054	0.061
SD	0.044	0.030	0.023	0.014	0.010	0.008	0.014	0.010	0.018
Range	0.170	0.137	0.102	0.063	0.062	0.064	0.041	0.043	0.035
	0.280	0.221	0.153	0.097	0.089	0.085	0.070	0.068	0.078

Table 4.36 ELISA Responses of RP and Uveitis Patients to ROS

Subject		Serum Dilution 10	20	40	80	160	320	640	1280
AA*	X	0.389	0.255	0.150	0.111	0.093	0.043	0.045	0.058
	SD	0.042	0.004	0.008	0.021	0.009	0.006	0.001	0.004
CM+	X	0.321	0.204	0.122	0.073	0.057	0.036	0.068	0.079
	SD	0.010	0.011	0.007	0.001	0.007	0.006	0.020	0.002
EN+	X	0.946	0.876	0.679	0.509	0.351	0.218	0.139	0.083
	SD	0.015	0.062	0.003	0.007	0.023	0.023	0.010	0.004
GA*	X	0.766	0.426	0.298	0.210	0.174	0.136	0.070	0.048
	SD	0.027	0.021	0.004	0.018	0.008	0.062	0.008	0.004
GG*	X	0.273	0.179	0.108	0.047	0.064	0.051	0.054	0.051
	SD	0.030	0.013	0.009	0.010	0.078	0.002	0.008	0.004
HM*	X	0.317	0.164	0.120	0.074	0.062	0.052	0.045	0.038
	SD	0.040	0.001	0.031	0.001	0.005	0.006	0.003	0.005
HS1+	X	0.400	0.246	0.211	0.150	0.136	0.094	0.091	0.085
	SD	0.076	0.008	0.002	0.010	0.017	0.015	0.011	0.007
HS2+	X	0.424	0.310	0.238	0.191	0.167	0.104	-	-
	SD	0.023	0.022	0.004	0.006	0.010	0.002	-	-
IM*	X	0.450	0.331	0.186	0.121	0.093	0.058	0.046	0.050
	SD	0.008	0.009	0.034	0.011	0.004	0.005	0.042	0.001
KM*	X	0.216	0.185	0.082	0.042	0.060	0.022	0.024	0.042
	SD	0.035	0.009	0.017	0.007	0.021	0.015	0.003	0.013
MK+	X	0.679	0.458	0.284	0.163	0.159	0.108	0.076	0.061
	SD	0.046	0.011	0.017	0.010	0.005	0.015	0.004	0.023
UV+	X	0.035	0.272	0.189	0.168	0.140	0.078	0.065	0.054
	SD	0.023	0.005	0.018	0.011	0.004	0.003	0.012	0.001

X = Mean of 2 assays

SD = Standard deviation (n-1)

* = Patient has RP

\+ = Patient has uveitis

Table 4.33 ELISA Responses of Control Human Subjects to S-Antigen

Subject		Serum Dilution 10	20	40	80	160	320	640	1280
AC	X	0.417	0.394	0.253	0.140	0.065	0.051	0.046	0.037
	SD	0.004	0.016	0.019	0.035	0.029	0.003	0.004	0.016
BD	x	0.409	0.331	0.213	0.187	0.157	0.121	0.105	0.102
	SD	0.047	0.007	0.007	0.023	0.019	0.011	0.006	0.013
BM	X	0.425	0.367	0.208	0.153	0.106	0.095	0.067	0.041
	SD	0.013	0.002	0.006	0.019	0.057	0.011	0.036	0.0
CD	X	0.345	0.225	0.138	0.089	0.069	0.068	0.061	0.048
	SD	0.134	0.006	0.013	0.015	0.013	0.005	0.006	0.006
HD	X	0.211	0.172	0.119	0.072	0.072	0.065	0.063	0.057
	SD	0.002	0.002	0.012	0.006	0.011	0.001	0.003	0.003
JF*	X	0.274	0.195	0.129	0.097	0.093	0.061	0.048	0.0
	SD	0.013	0.007	0.011	0.013	0.002	0.0	0.014	0.0
KM*	X	0.257	0.223	0.116	0.065	0.057	0.044	0.042	0.041
	SD	0.256	0.040	0.006	0.004	0.002	0.008	0.003	0.006
LR1*	X	0.925	0.668	0.423	0.253	0.135	0.089	0.078	0.050
	SD	0.010	0.035	0.012	0.013	0.006	0.021	0.020	0.010
LR2*	X	0.891	0.681	0.533	0.293	0.183	0.138	0.070	0.064
	SD	0.055	0.012	0.046	0.006	0.023	0.020	0.014	0.007
MM	X	0.424	0.282	0.191	0.119	0.093	0.078	0.067	0.060
	SD	0.018	0.003	0.014	0.023	0.013	0.007	0.003	0.006
OM	X	0.381	0.293	0.222	0.180	0.152	0.110	0.082	0.090
	SD	0.032	0.008	0.017	0.007	0.023	0.014	0.012	0.016
SG	X	0.452	0.360	0.227	0.143	0.093	0.072	0.050	0.045
	SD	0.011	0.004	0.021	0.001	0.001	0.006	0.0	0.008

X = Mean of 2 assays

SD = Standard deviation (n-1)

* = Users of retinal antigens

Table 4.34 ELISA Responses of RP and Uveitis Patients to S-Antigen

Subject		Serum Dilution 10	20	40	80	160	320	640	1280
AA*	X	0.356	0.282	0.181	0.179	0.117	0.098	0.103	0.062
	SD	0.013	0.010	0.006	0.023	0.009	0.001	0.009	0.015
CM+	X	0.335	0.229	0.128	0.063	0.054	0.042	0.039	0.049
	SD	0.005	0.004	0.014	0.009	0.013	0.006	0.013	0.006
EN+#	X	0.411	0.349	0.275	0.221	0.181	0.110	0.085	0.032
	SD	-	-	-	-	-	-	-	-
GA*	X	0.414	0.233	0.186	0.167	0.137	0.099	0.104	0.053
	SD	0.047	0.075	0.022	0.007	0.011	0.010	0.002	0.0
GG*	X	0.413	0.251	0.150	0.096	0.086	0.079	0.047	0.076
	SD	0.008	0.011	0.004	0.006	0.018	0.099	0.009	0.0
HM*	X	0.334	0.253	0.162	0.100	0.063	0.049	0.049	0.036
	SD	0.016	0.017	0.010	0.007	0.001	0.013	0.008	0.0
HS1+	X	0.811	0.544	0.340	0.188	0.136	0.082	0.063	0.0
	SD	0.035	0.024	0.011	0.008	0.018	0.021	0.015	0.0
HS2+	X	0.808	0.733	0.491	0.284	0.157	0.078	0.076	0.017
	SD	0.054	0.028	0.024	0.007	0.014	0.007	0.013	0.006
IM*	X	0.405	0.294	0.186	0.146	0.097	0.088	0.094	0.093
	SD	0.002	0.004	0.010	0.005	0.006	0.007	0.009	0.007
KM*	X	0.314	0.214	0.130	0.097	0.085	0.061	0.059	0.059
	SD	0.010	0.011	0.017	0.008	0.010	0.002	0.020	0.009
MK+	X	0.390	0.234	0.165	0.103	0.098	0.045	0.034	0.055
	SD	0.018	0.006	0.020	0.021	0.020	0.004	0.008	0.006
UV+	X	0.694	0.549	0.346	0.265	0.163	0.120	0.104	0.064
	SD	0.014	0.020	0.014	0.018	0.009	0.001	0.001	0.008

X = Mean of 2 assays

SD = Standard deviation (n-1)

\# = Results of single assay

* = Patient has RP

\+ = Patient has uveitis

Table 4.35 ELISA Responses of Control Human Subjects to ROS

Subject		Serum Dilution 10	20	40	80	160	320	640	1280
AC	X	0.502	0.398	0.268	0.161	0.123	0.095	0.084	0.081
	SD	0.009	0.009	0.018	0.001	0.006	0.018	0.007	0.007
BD	X	0.478	0.396	0.241	0.166	0.146	0.101	0.073	0.035
	SD	0.040	0.005	0.0	0.113	0.156	0.002	0.001	0.008
BM	X	0.354	0.323	0.207	0.125	0.082	0.079	0.065	0.064
	SD	0.002	0.019	0.006	0.013	0.001	0.003	0.002	0.0
CD	X	0.302	0.178	0.135	0.088	0.087	0.082	0.072	0.048
	SD	0.008	0.004	0.025	0.001	0.002	0.004	0.019	0.004
HD	X	0.323	0.235	0.150	0.076	0.079	0.060	0.080	0.060
	SD	0.005	0.0	0.006	0.004	0.016	0.003	0.038	0.006
JF*	X	0.339	0.210	0.193	0.153	0.138	0.108	0.071	0.045
	SD	0.054	0.025	0.046	0.033	0.042	0.026	-	-
KM*	X	0.218	0.119	0.092	0.051	0.069	0.046	0.044	0.033
	SD	0.019	0.016	0.006	0.026	0.011	0.004	0.007	0.001
LR1*	X	0.907	0.678	0.463	0.254	0.215	0.135	0.091	0.056
	SD	0.043	0.011	0.004	0.004	0.006	0.003	0.004	0.005
LR2*	X	1.029	0.806	0.570	0.386	0.277	0.180	0.114	0.080
	SD	0.023	0.022	0.037	0.304	0.023	0.005	0.002	0.0
MM	X	0.337	0.332	0.238	0.150	0.080	0.071	0.050	0.051
	SD	0.051	0.032	0.016	0.006	0.026	0.006	0.011	0.0
OM	X	0.258	0.207	0.128	0.098	0.064	0.053	0.042	0.046
	SD	0.067	0.008	0.005	0.006	0.003	0.011	0.005	0.006
SG	X	0.553	0.397	0.254	0.139	0.112	0.065	0.068	0.084
	SD	0.098	0.006	0.020	0.013	0.018	0.001	0.001	0.005

X = Mean of 2 assays

SD = Standard deviation (n-1)

* = Users of retinal antigens