T LYMPHOCYTES IN INTRAOCULAR INFLAMMATION

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DECLARATION

The work in chapter two of this thesis was carried out in conjunction with Professor W.R. Lee. The work in chapters three and four was carried out by myself with technical guidance. I have composed the thesis myself and have not submitted it in candidature for any other degree or qualification.

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ABSTRACT

Pathological and immunohistochemical analyses of two forms of human intraocular inflammatory disease, multifocal posterior uveitis and Behcet's disease, were carried out. Studies of post-mortem eyes from a patient with multifocal posterior uveitis revealed a focal lymphocytic infiltrate in the choroid, extending through Bruch's membrane to the retina. Endothelial cells of choroidal vessels at the inflammatory foci had the morphological appearance of "high endothelial venules" known to be involved in the selective trafficking of lymphocytes. Immunohistochemical analysis of the lymphocytic infiltrate showed that 70-80% were T cells. Immunohistochemical analysis of enucleated and post-mortem eyes from Behcet's disease patients showed that the mixed leukocytic infiltrate found in the retina and uveal tract was composed predominantly of T lymphocytes and macrophages. The retinal vessels were hyalinised and narrowed in the post-mortem material and were noted to have IL2 receptor positive CD4 positive T lymphocytes in their walls. Aberrant expression of MHC class II antigens by organ-resident cells in these studies was minimal.

Experimental work on an animal model of intraocular inflammatory disease, experimental autoimmune uveoretinitis, was undertaken to determine the in vivo lymphokine production of T lymphocytes in inflammatory eye disease. The T lymphocyte infiltrate in active EAU was identified immunohistochemically as was interferon-γ protein and IL2 receptor positive cells. cDNA probes specific for rat interferon-γ, IL2, lymphotoxin and IL4 mRNA were amplified, extracted and radiolabelled.

These probes were used to identify autoradiographically mRNA for these lymphokines in the destructive tissue pathology in EAU using the technique of in situ hybridisation. Localised foci of mRNA for all four lymphokines were identified over cells in the areas of T lymphocyte infiltrate. Less than 10% of the infiltrating T cells expressed lymphokine mRNA. The number of positive cells was generally equal for the four lymphokines throughout the disease process. These studies confirm the central role of T lymphocytes in inflammatory eye disease and suggest that production of interferon-γ, IL2, lymphotoxin and IL4 may contribute to the immunopathology.

The findings of these studies are discussed in relation to autoimmunity and inflammatory eye disease. The implications for future work are assessed.

CONTENTS

Abbreviations used in the text

ABC	Avidin-Biotin-Complex
AEC	Amino ethyl carbazole
Ag	Antigen
APC	Antigen presenting cell
APES	3-Aminopropyltriethylsilane
APMPPE	Acute posterior multifocal placoid pigment epitheliopathy
ATP	Adenosine triphosphate
bp	Base pair
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CSF	Cerebro-spinal fluid
CTL	Cytotoxic T lymphocyte
CTP	Cytosine triphosphate
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveoretinitis
EGF	Epidermal growth factor
ELAM	Endothelial leukocyte adhesion molecule
EM	Electron microscopy
G-CSF	Granulocyte colony stimulating factor
GFAP	Glial fibrillary acid protein
GM-CSF	Granulocyte-macrophage colony stimulating factor

GTP	Guanosine triphosphate
HBVP	Hepatitis B virus polymerase
HEV	High endothelial venule
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
ICAM	Intercellular adhesion molecule
IDDM	Insulin dependent diabetes mellitus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRBP	Interphotoreceptor retinoid binding protein
LCA	Leukocyte common antigen
LFA	Lymphocyte function associated antigen
LT	Lymphotoxin
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NK	Natural killer
NOD	Non obese diabetic
OCT	Optimal cutting temperature compound
PAP	Peroxidase anti peroxidase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PMN	Polymorphonuclear leukocyte

RPE	Retinal pigment epithelium
RVE	Retinal vascular endothelium
SLE	Systemic lupus erythematosis
SSC	Standard saline citrate solution
TCR	T cell receptor
TGF	Transforming growth factor
TTP	Thymidine triphosphate
TNF	Tumour necrosis factor
VKH	Vogt Koyanagi Harada syndrome
VLA	Very late activation antigen
VCAM	Vascular cell adhesion molecule

AIMS

The aims of this thesis are:

(a) to describe the processes of immunological tolerance and autoimmunity and their relationship to inflammatory eye disease.

(b) to undertake immunopathological studies carried out on human eyes with intraocular inflammation with the aim of characterising the cell types and mechanisms involved in the disease processes.

(c) to investigate the in vivo lymphokine production by T lymphocytes in ocular inflammation using an animal model of inflammatory eye disease, experimental autoimmune uveoretinitis (EAU), with the aim of identifying the T lymphocyte subsets present and the lymphokine mRNA expressed.

(d) to discuss the significance of the results of this work in relation to current knowledge of immune-mediated inflammatory disease.

CHAPTER 1

INTRODUCTION

1.1 Introduction

This thesis is divided into five chapters. Chapter one is an introduction which covers background information on clinical uveitis, immunology, in particular concepts of immune tolerance and autoimmune disease and clinical and laboratory studies on inflammatory eye disease.

Chapters two and three deal with pathological and immunohistochemical studies on human eyes with multifocal posterior uveitis and Behcet's disease. These are the first such studies to be carried out on these two forms of inflammatory eye disease.

Chapter four covers experimental work using immunohistochemical and molecular biology (in situ hybridisation) techniques on lymphokine production and gene expression in experimental autoimmune uveoretinitis (EAU) an animal model of intraocular inflammation. The work described represents the first application of in situ hybridisation to the investigation of inflammatory eye disease.

Chapter five is a discussion of the results of the studies described in the preceding chapters in the context of immune-mediated inflammatory disease and the potential implications for future work.

1.2 Intraocular inflammation

In clinical ophthalmology the term intraocular inflammatory disease covers a range of conditions which vary in location, severity and chronicity. Since the uveal tract is often centrally involved in these conditions the term "uveitis" is commonly used clinically to refer to intraocular inflammatory disorders. The classification of uveitis as anterior or posterior on the basis of

anatomical location has proven useful since it also divides conditions which are markedly different in terms of both clinical behaviour and immunopathology. This thesis deals with posterior uveitis.

Intraocular inflammation can be caused by a local infection with organisms such as Toxoplasma Gondii (Perkins, 1973); Herpes Zoster, Herpes Simplex and Epstein-Barr viruses which can cause acute retinal necrosis (Lightman, 1991) or cytomegalovirus which can produce a retinitis (Henderley et al.1987). Such infections may in some cases be substantiated by specific laboratory tests whilst in others the diagnosis remains presumptive based solely on clinical findings. In the clinical setting, however, most cases of intraocular inflammatory disease are of unknown aetiology, can have an uncertain prognosis and may be refractory to therapy. These idiopathic forms of intraocular inflammation frequently involve the posterior segment of the eye and the destructive pathology in the choroid, retinal pigment epithelium (RPE) and retina can result in significant visual loss.

Idiopathic, non-infectious forms of intraocular inflammation are widely considered to have an autoimmune basis, a concept which was first introduced by Elschnig in 1911 in relation to the pathogenesis of sympathetic ophthalmitis (Elschnig, 1911). The introductory chapter of this thesis is therefore divided into subsections dealing with the mechanisms of immune tolerance and autoimmune disease and the relationship of these processes to ocular inflammatory disease.

1.3 Immunological tolerance

The ability of an individual to distinguish self from nonself is a fundamental principle of the immune response. The lack of self reactivity is achieved through the processes of immunological tolerance. Since animal populations are outbred, individuals within a population vary substantially in the self ligands expressed on their cells and there is therefore no simple way of producing an immune system which does not react with self antigens. The immune system acquires the knowledge to discriminate self and nonself during its development (Schwartz, 1989). The processes of tolerance induction are therefore closely related to those of lymphocyte ontogeny.

1.3.1. T lymphocyte development

T lymphocytes develop in the thymus from bone marrow derived thymic progenitor cells. Rearrangement of the variable (V), diversity (D) and joining (J) gene segments encoding the α and β chains of the T cell receptor (TCR) allows thymic progenitor cells to express a wide diversity of $\alpha\beta$ T cell receptors (Blackman et al.1990). It is the interaction of the TCR and the T cell accessory molecules CD4 and CD8 with MHC molecules expressed on cells in the thymus which is central to tolerance development.

The $\alpha\beta$ TCR (the $\gamma\delta$ TCR is expressed on a separate T cell lineage making up 5% of peripheral blood T cells which is currently under investigation) is composed of two transmembrane polypeptide chains. These α and β chains have constant (C) and variable (V) regions. The variable regions form combining sites that have contact residues for antigenic peptide and MHC molecule (Marrack et al.1987). In addition the T

cell CD4 and CD8 surface molecules bind determinants on class I and class II MHC respectively to increase the avidity of T cell binding to its target cell (Sprent et al.1990). The antigen fragment is bound by an MHC molecule in the groove between two α helices and a β pleated sheet. Random rearrangement of TCR gene segments at α (Vα,Jα) and β (Vβ,Dβ,Jβ) loci along with N region diversity created by imprecise joining of gene segments or nucleotide insertion could give rise to a T cell repertoire of 10^9 different specificities (Blackman et al.1990). This repertoire is not expressed by any animal because of the processes of negative and positive selection which occur in the thymus.

1.3.2 T lymphocyte selection

Although it had previously been proposed that negative selection of T cell precursors took place in the thymus, the studies of Kappler et al (Kappler et al.1987b; Kappler et al.1987a) provided the first direct evidence that thymocytes are subject to a process of clonal deletion. These experiments showed cells which expressed the Vβ17a gene product in their TCR (recognised by the monoclonal antibody KJ23a) were a major subpopulation (9-14%) in mice not expressing the MHC class II molecule I-E. The Vβ17a T cell subpopulation was virtually absent in F1 crosses and other mice strains which did express I-E. Importantly, unchanged numbers of Vβ17a+ cells were present in the subset of immature thymocytes (CD4+CD8+, "double positive") in the thymuses of I-E positive animals although the numbers were greatly reduced in CD4+CD8- and CD4-CD8+ subsets. This provided evidence that clonal deletion of thymocytes took

place at or shortly after the double positive stage of maturation.

TCR transgenic mice have also been used to study negative selection of thymocytes. Kisielow et al (Kisielow et al.1988a) constructed transgenic mice expressing in a large fraction of their T cells an αβTCR specific for an antigen (H-Y) present on male but not female cells. Very few lymph node cells of male mice proliferated in response to H-Y although one in seven CD4-CD8+ lymph node cells in female mice responded. The peripheral T cells of male mice expressed the transgenic TCR at normal frequency however they expressed very low levels of CD8. The thymuses of male mice had 10 fold fewer cells than females mainly due to loss of double positive cells. These studies provided further evidence that deletion occured at the double positive stage of thymocyte maturation and also suggested that CD4 and CD8 molecules are involved in the deletional process. Sha et al (Sha et al.1988) used a different transgenic model to demonstrate that deletion takes place at the double positive thymocyte stage.

Developing thymocytes undergo a second selective process termed positive selection. It has been established that T cells recognise antigen exclusively in the context of self MHC and that thymocytes "learn" this self MHC recognition during maturation (Blackman et al.1990). Experiments using TCR transgenic mice (Kisielow et al.1988a; Sha et al.1988) have demonstrated that mature thymocytes and peripheral T cells only develop in mice with the same MHC haplotype as the original clone from which the transgenic TCR was isolated. In contrast large percentages of immature thymocytes are found to express the MHC disparate TCR. Most of these thymocytes are found to be CD4+CD8+ demonstrating that positive selection

takes place at this stage of development.

In thymus repopulation experiments (Kisielow et al.1988b) it was demonstrated that thymocytes preferentially differentiate towards a CD4:CD8 ratio determined by the MHC expression in the thymic environment. This observation shows that the CD4/CD8 phenotype of T cells is directed by MHC molecule expression in the thymus and the TCR specificity in immature thymocytes and that the CD4/CD8 accessory molecules are involved in positive selection.

1.3.3. Mechanisms of T lymphocyte selection

These thymic selective events are of fundamental importance to the development of the T cell repertoire and further investigations have been carried out in an attempt to elucidate the mechanisms involved. Experiments have demonstrated that different thymic cell types are involved in negative and positive selection (Blackman et al.1990). Marrack and associates (Marrack et al.1988) used transgenic and bone-marrow chimeric mice to investigate the cell types involved in deletion of Vβ17a+ thymocytes. These experiments showed that bone-marrow derived cells are responsible for tolerance induction due to thymocyte deletion and that expression of peptides by thymic epithelium alone was not sufficient to produce tolerance induction.

Experimental work has in addition indicated that it is the interaction of the thymic epithelial cell population with developing thymocytes that is responsible for positive selection (Blackman et al.1990; von Boehmer et al.1990). It has been argued that this interaction produces positive selection

by preventing programmed cell death (apoptosis) (Sprent et al.1990).

Since the events leading to the positive and negative selection of thymocytes appear to occur at a similar stage of ontogeny the factors which differentiate a positive (selective) response from a negative (deletional) response brought about by the TCR-self MHC/antigen interaction are central to the process of selection of the T cell repertoire. Two theories have been proposed to account for the different thymocyte response in this interaction. The first model is based on receptor affinity and implies that quantitative differences in TCR-MHC interaction and binding determine thymocyte fate (Schwartz, 1989; Blackman et al.1990). This model proposes that thymocytes whose TCRs recognise MHC with high affinity are eliminated by negative selection whereas low affinity TCR-MHC interactions produce positive selection. This theory is supported by recent transgenic experiments carried out by Robey and associates (Robey et al.1992) which demonstrated that a two fold increase in expression of a CD8 transgene in mice expressing a 2C TCR transgene causes a change from positive to negative selection indicating that a quantitative difference in TCR-MHC binding can alter T cell selection.

A second theory on the differentiation of positive and negative selective processes referred to as the "peptide" model argues that qualitative differences in the interaction of thymocyte TCR with MHC on thymic epithelial cells or bone marrow derived cells in the thymus is responsible for the difference in thymocyte response (Marrack et al.1987; Schwartz, 1989; Blackman et al.1990). This model proposes that thymic epithelial cells present a set of peptides on their MHC molecules some of which are unique

and some of which are ubiquitous. Positive selection would occur when a thymocyte recognises a unique peptide on a thymic epithelial cell and recieves a signal for survival. Negative selection would result from thymocyte interaction with an MHC ubiquitous peptide. The data of Marrack and associates (Marrack et al.1988) provide some evidence that the affinity model mechanism was not the only process of selection occuring in transgenic mice where negative selection of transgenic TCR thymocytes was limited yet there was no overselection of these thymocytes as would be predicted by the affinity hypothesis. In addition there is evidence that the MHC molecules on thymic epithelium are not the same as MHC found elsewhere (Marrack et al.1989). An additional possibility is that the stage of thymocyte maturation when it interacts with self MHC may influence its selective response (Blackman et al.1990; Robey et al.1992). Further investigation of the mechanisms mediating the processes of thymocyte selection will be necessary before definitive statements on the molecular events involved can be made.

There is evidence that in addition to clonal deletion there is a mechanism of thymic tolerance induction leading to a functional non-responsiveness, this is termed clonal anergy. Chimeric and transgenic experiments have shown that in animals where specific T cell clones are not deleted there is nevertheless a reduction in responsiveness of these cells (Ramsdell et al.1990). In vitro work by Schwartz and colleagues has characterised the critical change in anergic T cells as a dramatic reduction in the ability of the T cell to produce IL2 on subsequent antigen stimulation (Schwartz, 1990).

It has been proposed that the intrathymic induction of clonal anergy may be the result of the interaction of the TCR on immature thymocytes with self MHC in the absence of a co-stimulatory signal. Presentation of antigen would occur on the thymic epithelium which is unable to provide a "second signal". Alternatively the affinity of the TCR-MHC interaction may determine the type of thymus induced unresponsiveness (Sprent et al.1990). The experimental data to support these theories is at present not available.

1.4 Extrathymic tolerance

1.4.1 Peripheral T lymphocytes

Evidence also exists that mature T lymphocytes can be tolerised. Using a transgenic mouse model which expresses a class II molecule on pancreatic beta cells Lo and co-workers (Lo et al.1988) demonstrated that T lymphocytes were tolerant to this molecule although it was not expressed on thymic or peripheral lymph tissue. In this model there was no immune-mediated attack on the pancreatic beta cells although interestingly the animals did develop diabetes suggesting that an alternative mechanism may act to produce this presumed "autoimmune" phenomenon. Subsequent work has investigated the possible mechanisms of peripherally induced tolerance.

It has been shown that peripheral T cells can be subject to deletion (Rocha et al.1991) and that this deletional process is a consequence of antigen binding to the TCR and occurs as a gradual process in contrast to thymic tolerance where deletion is relatively sudden (Rocha et al.1991). In addition to deletion experimental evidence suggests that peripheral T cells

can be rendered non-responsive or anergic. In the model used by Lo et al (Lo et al.1988) T cells, although not deleted, were rendered unresponsive to class II antigen. In the transgenic experiments of Morahan et al (Morahan et al.1989) peripheral T cells were found to be unresponsive to upregulated MHC class I in vivo although these cells could produce in vitro cytotoxic responses to this antigen suggesting that the unresponsiveness is not due to deletion or permanent clonal anergy. In contrast to this finding Burkly et al (Burkly et al.1989) used a transgenic mouse model with Vβ17a TCR T cells responsive to I-E to demonstrate that clonal anergy could be induced in peripheral T cells (without clonal deletion).

Investigation of the molecular basis for peripheral tolerance has suggested that receptor downregulation on T cells may be of importance. Rocha and von Boehmer (Rocha et al.1991) demonstrated that the progressive peripheral tolerance found in their transgenic mouse model correlated with downregulation of the αβ TCR and CD8 in T cells. Using a different transgenic model Schonrich et al (Schonrich et al.1991) were able to demonstrate a similar result with regard to downregulation of the TCR and CD8. These results are also of importance since they demonstrate that the loss of T cell surface markers is not always correlated with physical deletion of the cell.

1.4.2 B lymphocyte tolerance

It has been demonstrated that B lymphocytes may also be tolerised and that B cell tolerance is not solely due to the absence of autoreactive T cell help (although this may play a significant role) (Goodnow et al.1990).

Studies have shown that tolerance induction occurs in B cells by processes which differ from those inducing T cell tolerance. In vivo experimental work carried out in transgenic mice (Goodnow, 1992) has demonstrated that B cell tolerance may result from both clonal deletion and clonal anergy. It has been proposed that these two tolerising mechanisms may be related to the biphasic nature of B cell development. This consists of an initial phase of immunoglobulin gene recombination in the bone marrow and liver and a later somatic hypermutation producing selective expansion of individual B cell clones and high affinity antibodies. The second phase of B cell maturation necessitates effective peripheral tolerising controls (Goodnow et al.1990). Various factors are thought to play a role in the production of B cell tolerance including the stage of B cell development on encountering antigen, receptor affinty and downregulation and the absence of costimulatory signals although the exact nature of these mechanisms remains to be elucidated.

1.4.3. Tolerance breakdown

The establishment and maintenance of immune tolerance therefore appears to be mediated by several thymic and extrathymic mechanisms based on the processes of clonal deletion and clonal anergy. These mechanisms could act in series to protect an individual from the consequences of an autoaggressive response. Breakdown of self tolerance and subsequent autoimmune disease would therefore require a number of pathological events occuring together or in an ordered sequence. It has been demonstrated that autoreactive T and B lymphocytes are present in

healthy individuals who do not have autoimmune disease (Guilbert et al.1982; Cohen et al.1977). A series of tolerising mechanisms would allow such self reactive lymphocytes to exist without the development of overt autoimmune pathology.

The term autoimmune disease is applied to those situations where the immune system contributes to the pathogenesis of the disease. The events causing tolerance breakdown are central to the pathogenesis of autoimmunity. A critical step in this process is the interaction of the T lymphocyte receptor with antigen in relation to the costimulatory signals applied to the T cell.

1.5 Costimulation of T lymphocytes

1.5.1 Antigen presentation

Class I MHC antigens (encoded by the HLA-A, HLA-B and HLA-C regions) are normally expressed on all nucleated cells. The expression of MHC class II antigens (encoded by the HLA-D locus) is restricted to cells such as macrophages, B lymphocytes and dendritic cells which function as antigen presenting cells (APC).The restricted number of cell types expressing class II antigens serves to limit the situations in which antigen is presented to CD4 positive T cells. Antigen presentation normally involves ingestion of antigen by a professional APC, such as a macrophage, degradation of the antigen by lysosymal enzymes and re-expression on the APC surface (Unanue et al.1984). Alternative, non-lysosomal dependent mechanisms of intracellular proteolysis and antigen presentation have also been documented (Mills, 1986) and these may be sufficient to promote

protein antigen presentation to class II restricted T cells. APCs simultaneously secrete cytokines such as IL1 which activate T lymphocytes.

Cells which do not normally present antigen can be induced to express class II MHC antigens by exposure to interferon γ. However this class II expression does not by itself produce the ability to effectively present antigen to T lymphocytes as shown by the experiments of Geppert and Lipsky (Morahan et al.1989) which additionally demonstrated that class II positive endothelial cells and fibroblasts had different abilities to present antigen to T cells despite comparible levels of class II expression. These and similar findings have led to investigations on the role of costimulatory signals in the process of antigen presentation to T cells.

1.5.2. Costimulatory signals

Experiments carried out on transgenic mice have demonstrated that potentially reactive T lymphocytes may remain unresponsive despite the presence of specfic peripheral tissue antigens (Schonrich et al.1991) and that this tolerance can be overcome by viral infection of the appropriate tissue (Oshashi et al.1991; Oldstone et al.1991). It has been proposed that the tolerance seen in these model systems is due to the presence or absence of costimulatory signals. A discovery of fundamental importance has been that the interaction of antigen with TCR in the absence of a costimulatory signal is not a neutral event and can lead to T cell anergy (Schwartz, 1990). Gaspari and coworkers used an in vitro system to demonstrate that class II positive keratinocytes induced in T cells a state of unresponsiveness to subsequent stimulation with specific antigen (Gaspari

et al.1988). The in vivo relevance of such findings remains uncertain at present, however, it is possible that antigen presentation by non-professional APCs leading to tolerance could be a mechanism of maintaining self-nonself discrimination in the periphery and limiting autoaggressive responses.

The molecular mechanisms by which costimulatory signals determine the outcome of TCR-antigen interaction have not yet been fully elucidated. Schwartz has defined three possible systems (Schwartz, 1990): a) a direct interaction of the costimulatory signal receptor with the antigen specfic TCR and subsequent enhancement of signal transduction; b) an interaction with molecules which increase the avidity of the TCR-antigen binding; and c) the costimulatory signal-receptor interaction initiates its own second messenger cascade which synergises with the antigen-TCR signals distally or at the level of gene activation. The nature of the costimulatory signals themselves may involve cell to cell contact or soluble messengers and is also yet to be defined. The possible candidates for costimulatory function have recently been reviewed by Liu and Linsley (Liu et al.1992): a) The CD28 molecule on T cells has been shown to interact with the B7/BB1 molecule on antigen presenting cells to produce co-stimulation by a signal transduction pathway that is completely independent of early events in antigen specific receptor signaling (Schwartz, 1992). Furthermore, it has been demonstrated that CD28 induced signaling blocks the development of anergy in T cells (Harding et al.1992). The T cell activation molecule CTLA-4 also interacts with B7/BB1 on APCs to produce co-stimulation and experiments using soluble CTLA-4 receptor suggest that blocking this interaction may provide

therapeutic immunosuppression (Schwartz, 1992). b) The interactions of the adhesion molecules LFA3 with CD2, ICAM1/2 with LFA1 and VLA4 with VCAM1 also have the potential to play a costimulatory role. Liu and Linsley also argue that costimulation and T cell activation are linked since differing APCs preferentially stimulate Th1 or Th2 cells.

The further investigation of the nature and role of T cell costimulatory signals is of importance not only to the elucidation of the processes of peripheral T cell tolerance but are also potential sites of immune intervention since the ligands/receptors involved in costimulation are likely to be less polymorphic than MHC or TCR.

1.6 Autoimmune disease

Aspects of human autoimmune disease give valuable clues to the pathogenic mechanisms involved in tolerance breakdown (Sinha et al.1990). Certain clinical conditions thought to be autoimmune in nature have MHC class II associations. These conditions may be either organ specific such as insulin dependent diabetes mellitus (IDDM) and Graves' disease or non organ specific as with systemic lupus erythematosis (SLE). Other presumed autoimmune conditions such as ankylosing spondylitis and psoriasis are associated with specific MHC class I antigens. Although a genetic association may predispose an individual to an autoimmune condition the aetiology is multifactorial since studies of monozygotic twins reveal a substantial discordance (concordance is less than 5% for multiple sclerosis and approximately 30% for IDDM). These findings imply that environmental factors also play a role in disease development and many

autoimmune diseases are known to be associated with preceding infection (Bottazzo et al.1986; Rose et al.1988). Human autoimmune conditions tend to run a chronic course with spontaneous exacerbations and remissions implying a balance between positive and negative regulatory factors.

1.6.1 Autoantibodies

In many organ specific and non-organ specific autoimmune conditions circulating autoantibodies have been demonstrated (Bottazzo et al.1986). Such autoantibodies may interact with cell receptors to produce stimulatory effects, for example thyroid stimulating immunoglobulin in Graves' hyperthyroidism, or to block receptor action such as the anti-acetylcholine receptor antibody in myasthenia gravis. In SLE autoantibodies are generated to several nuclear components and the deposition of immune complexes is thought to play a role in the immunopathology of the condition. However, since B cell responses need T cell help, and there is evidence that T cells are central to the disease processes seen in autoimmunity, it would appear that the humoral immune response may be secondary to cellular mechanisms. Autoantibodies may nevertheless provide useful markers for disease development and progression in human autoimmune conditions (Sinha et al.1990).

1.6.2. Autoreactive T lymphocytes

Studies carried out on organ-specific and non organ-specific autoimmune disease have defined the involvement of T lymphocytes in the disease processes. Autoimmune thyroid disease includes both Graves'

disease and Hashimoto's thyroiditis which has a more destructive course. Surgical specimens of thyroid from Graves' disease patients have an infiltrate of T lymphocytes either scattered throughout the gland or concentrated in small or large foci (Hanafusa et al.1983). These cells were not found to be distributed in relation to the MHC class II positive thyroid follicular cells. In vitro work has shown that MHC class II positive thyroid follicular cells from patients with Graves' disease are capable of presenting influenza virus peptide antigen to cloned human T cells although they could not present whole virus implying that they are incapable of processing complex antigens (Londei et al.1984). This would suggest that aberrant expression of MHC class II antigen by thyroid follicular cells can have a functional role in antigen presentation to infiltrating CD4 positive T lymphocytes.

Cloned T cells from Graves' disease surgical specimens are predominantly of the CD4 phenotype and have been shown to be capable of specifically recognizing autologous thyroid cells (Londei et al.1985). In contrast cloned T cells from Hashimoto's thyroiditis glands are mostly of the CD8 phenotype and have increased in vitro cytotoxicity in comparison to cloned T cells from Graves' disease thyroids (Feldmann et al.1989) implying a difference in the composition of the T cell infiltrate in these two types of autoimmune thyroid disease. This is in keeping with the more destructive disease process seen in Hashimoto's thyroiditis.

Autoimmune liver disease may take several forms. In primary biliary cirrhosis there is a destructive process involving the intrahepatic bileducts. In liver biopsy specimens from primary biliary cirrhosis patients infiltration of

predominantly CD4 positive T lymphocytes has been demonstrated in the areas of tissue destruction surrounding bileducts which show aberrant expression of MHC class II antigens (Ballardini et al.1984). T cell clones have been generated from patients with another form of autoimmune liver disease, chronic active hepatitis (Wen et al.1990). These T cell clones were found to be predominantly CD4 positive and reacted specifically against liver-membrane antigen implying a role for these cells in the destructive immunopathology.

In insulin dependent diabetes mellitus there is an autoimmune attack on the β cells of the pancreatic islets. These cells aberrantly express MHC class II antigens and in the acute stage of the disease there is a T lymphocyte infiltrate (predominantly CD8 positive) in the area of tissue destruction (Bottazzo et al.1985). The T cells were found to be HLA DR and IL2 receptor positive.

Moore et al (Moore et al.1988) examined the phenotype of lymphocytes present in the active tissue lesions of rheumatoid arthritis and Crohn's disease, two diseases thought to have an autoimmune aetiology. In both diseases they found a predominance of CD4 positive T lymphocytes (with a CD4:CD8 ratio of 2.4 in the samples from the rheumatoid synovium).

The studies outlined above demonstrate that T lymphocytes, in most cases of the CD4 positive subset, are the predominant cell at the sites of autoimmune pathology. T cells may also be involved in tissue damage through lymphokine release. This effect may be direct or indirect. Release of IFNγ, TNF or lymphotoxin could produce direct tissue damage (Tite et al.1985; Tite, 1990b). Activation of other cells such as macrophages, which

are also found at the sites of autoimmune tissue pathology, by T cell lymphokines such as IFNγ, could indirectly contribute to local autoimmune pathology.

1.7 Mechanisms of tolerance breakdown

1.7.1 T cell receptor - antigen - MHC interaction

Tolerance breakdown and subsequent autoimmune disease appears to be the result of several events in predisposed individuals although the exact mechanism involved is not known for any condition (Sinha et al.1990). The interaction of the T cell receptor with antigen and MHC is again central to this process. The target antigens involved are mostly not yet identified in human disease (and animal models) and it is possible that the antigens responsible for initiation of a condition may be different for those involved in perpetuation and effector mechanisms. Where target antigens are sequestered (not subject to the normal immune system surveillance) they must first be exposed to the immune system in a context where they are recognised and can subsequently be immunogenic. This effectively means presentation to a T cell by a suitable antigen presenting cell. It has been demonstrated that there is aberrant expression of MHC class II by tissue resident cells at the sites of autoimmune tissue pathology. For example in autoimmune thyroiditis class II expression has been demonstrated on thyrocytes (Hanafusa et al.1983) and in primary biliary cirrhosis aberrant class II expression has been shown on bile duct epithelium (Ballardini et al.1984). It has been proposed that such aberrant expression of class II could lead to auto-antigen presentation to T cells (Bottazzo et al.1983) and

in vitro it has been shown that thyrocytes can present antigen to T cells but that they cannot process complex antigens (Bottazzo et al.1986). However there is no evidence that such antigen presentation takes place in vivo and it is equally possible that presentation by tissue resident cells (in the absence of costimulation) maintains peripheral tolerance as discussed above (Schwartz, 1990).

Self reactive T cells are a prerequisite for an autoimmune response, but as already discussed these can exist in normal individuals without overt autoimmune disease (Guilbert et al.1982; Cohen et al.1977) and hence are not sufficient by themselves to produce autoimmunity. Recent work has focussed on the TCR involved in autoimmunity. In multiple sclerosis (MS) there appears to be restricted usage of TCRs by the T cells involved in the central nervous system pathology seen in the disease although it is still unclear as to whether this is true also of peripheral T cells (Hafler et al.1988; Cooke, 1991; Wucherpfennig et al.1990). In experimental autoimmune encephalomyelitis (EAE), an experimental model of MS induced by immunisation with myelin basic protein (MBP), encephalitogenic T cell clones specific for residues 1-11 of MBP show extensive TCR homology (both α and β chains) although the T cell clones specific for residues 89-100/101 show extensive Vβ TCR diversity (Sinha et al.1990). It therefore remains uncertain whether there is restriction of TCR gene expression in autoimmune disease since the picture is likely to be complicated by non-specific recruitment of T cells to sites of autoimmune pathology and changes in the profile of target antigens involved which may occur with progression of the disease process. If restricted TCR usage is demonstrated in human

autoimmune disease blocking a specific TCR has the potential to provide a valuable form of treatment.

Studies of human autoimmunity have demonstrated MHC associations. The MHC susceptiblity allelles are considered to be those which are capable of binding and presenting self (or cross-reactive foreign) antigens and selecting anti-self T cells. In addition there appears to be non-MHC genetic associations althought the nature of these at present remains uncertain.

The local presence of lymphokines or other costimulatory signals at the sites of autoimmune pathology is necessary for T cell activation (Schonrich et al.1991; Oshashi et al.1991). It therefore appears that the presence of costimulatory signals is a prerequisite for an autoimmune response since antigen presentation in their absence would lead to tolerance (Schwartz, 1990).

1.7.2. Exogenous stimuli

The initiating event which triggers the processes described above is likely to be some form of environmental insult. Tissue damage following a bacterial or viral infection could allow for the release of sequestered antigens from immunologically priveleged sites and a concomitant non-specific inflammatory response would generate the costimulatory signals necessary for MHC class II expression and effective antigen presentation. There is also evidence that cross-reaction with certain foreign antigens may lead to anti-self responses.

1.7.3. Molecular mimicry

Many exogenous microbial pathogens share common antigenic sites with self components and this phenomenon has been termed molecular mimicry (Oldstone, 1987). Experiments have suggested that molecular mimicry may play a role in the initiation of autoimmune disease. A sequence homology between myelin basic protein and hepatitis B virus polymerase (HBVP) has been documented and it was subsequently found that immunisation with a HBVP peptide could produce humoral and cellular immune responses and tissue pathology similar to immunisation with MBP (Fujinami et al.1985). These findings led to the hypothesis that an immune cross-reaction of a microbial protein with a self component could initiate an autoimmune response. According to this theory the pathogen may no longer be present when the autoimmune pathology becomes manifest as ongoing tissue destruction will generate further autoantigen release and continuing immune-mediated inflammation. In addition the initial insult may be remote from the site of autoimmune attack as seen in rheumatic heart disease where a streptococcal skin or nasopharyngeal infection is though to trigger the autoimmune response.The theory of molecular mimicry has been supported by the finding of potentially cross-reactive self components to epidemiologically associated pathogens in coeliac disease and ankylosing spondylitis (Oldstone, 1987), diseases which are thought to be of autoimmune aetiology.

Autoimmune disease appears therefore to develop in genetically predisposed individuals as a consequence of an environmental agent causing an event which leads to tissue damage and non-specific

inflammation or molecular mimicry or both. The sum of these genetic and environmental factors must be sufficient to override the mechanisms of self tolerance. In relation to inflammatory eye disease studies of the clinical features of these conditions provide background information important to the understanding of the immunopathology involved.

1.8. Intraocular inflammatory disease

As discussed in section 1.2 the term intraocular inflammatory disease covers a range of conditions. The inflammatory processes may be confined to the eye or be part of a systemic inflammatory disorder. Clinical studies of these conditions have attempted to define the immunopathological processes on the basis of the systemic manifestations of the conditions.

1.8.1. Humoral immune responses

Initial clinical studies of immune mechanisms focussed on the humoral immune response. Studies on peripheral blood of Behcet's disease patients with intraocular inflammation showed circulating immune complexes and alterations in serum complement component levels in some patients (Williams et al.1977; Kasp et al.1986). Immune complexes were also found in retinal vasculitis (Dumonde et al.1985; Dumonde et al.1982). High levels of circulating antibodies to Herpes Simplex virus have also been documented in Behcet's disease (Hamzaoui et al.1990a). Humoral immune responses to retinal S antigen have also been investigated in uveitis patients. Although some studies have shown an increase in circulating anti S antigen antibody in clinical uveitis (Gregerson et al.1981), subsequent

longitudinal studies, in which healthy controls were shown to have similar levels of circulating anti S antigen antibody to uveitis patients, have failed to support this (Abrahams et al.1983; Doekes et al.1987; Froebel et al.1989; Forrester et al.1989; Chan et al.1985b).A study of Vogt-Koyanagi-Harada (VKH) syndrome, Behcet's disease and sympathetic ophthalmia (Chan et al.1985b) has suggested that there are circulating antibodies to photoreceptor outer segments and Muller cells in these conditions although only small numbers of patients were investigated. In diabetic patients a significant increase in anti S antigen antibodies was documented after panretinal photocoagulation (Gregerson et al.1982), suggesting that retinal tissue damage may produce these antibodies as an epiphenomenon. Taken together the variability of these studies of peripheral humoral immune responses in uveitis patients and the similarities found in control data have implied that humoral immunity may play a secondary role in the disease processes and have led to investigations on the nature of cellular immune responses in uveitis.

1.8.2. Cellular immune responses

Lymphocyte subpopulations in peripheral blood samples of patients with intraocular inflammation have been analysed in an attempt to elucidate alterations in immune function. Alterations in the CD4:CD8 T lymphocyte ratio have been described in active posterior uveitis (Nussenblatt et al.1983b) although some reports have failed to find this change (Deschenes et al.1986). In Behcet's disease a decrease in CD8 T cell function together with altered CD4:CD8 ratios has been documented in the preactive phase of

the disease (Sakane et al.1982).

Results of studies of T cell activation markers in uveitis patients have been easier to interpret (Deschenes et al.1988). Overall there is an increase in T cells bearing the Interleukin 2 receptor (IL2R) compared to healthy controls, the percentage of IL2R positive T cells (in peripheral blood and intraocular fluids) correlates with disease activity and IL2R expression correlates with HLA DR antigen expression. Exclusion of uveitis associated with systemic disease did not affect these findings. It has also been demonstrated that there is an increased frequency of peripheral blood lymphocytes capable of producing IL2 in response to S antigen in uveitis patients (Opremcak et al.1991). These results suggest that there is a T cell mediated systemic immune response in ocular inflammatory disease without overt systemic involvement.

Cellular immune responsiveness to retinal S antigen has been documented in some peripheral blood studies on uveitis patients (Nussenblatt et al.1980; Nussenblatt et al.1982; Doekes et al.1987) although other investigations have failed to confirm this (Froebel et al.1989) or have given equivocal results (de Smet et al.1990). The recent detailed study of DeSmet and coworkers analysed cellular proliferation in response to S antigen, IRBP and peptide fragments of these proteins in patients with a variety of intraocular inflammatory conditions. In general the proliferative responses were at very low levels and reached statistical significance in only three subgroups ($P<0.05$ in Behcets disease, pars planitis and birdshot chorioretinopathy) each of which contained patients with no proliferative response at all. T cell responses in peripheral blood of uveitis patients are

therefore by no means consistent. Responsiveness to interphotoreceptor retinoid binding protein (IRBP) and uveitogenic fragments of these antigens appears to parallel S antigen (Hirose et al.1988; de Smet et al.1990). An increased lymphocyte proliferative response to S antigen (and its uveitogenic peptide fragments) has been found in diabetic patients following panretinal photocoagulation suggesting that chorioretinal tissue trauma may stimulate a cellular immune response without the development of an ongoing intraocular inflammatory process (Vrabec et al.1990). The significance of the decreased T cell proliferation (Young et al.1988) and increased cytotoxicity (Hamzaoui et al.1990b) to Herpes Simplex virus which have been documented in Behcet's disease remains uncertain.

Results of these studies on cell -mediated immunity in intraocular inflammatory disease imply that the current tests of immune function carried out on peripheral blood lack the sensitivity and specificity necessary to dissect and analyse localised immune-mediated pathology in the eye.

1.8.3. Genetic background of uveitis patients

Investigation of the genetic makeup of patients with ocular inflammatory disease have also been carried out. Studies of patients with birdshot chorioretinopathy have revealed a strong association between this disease and the HLA A29 antigen (Nussenblatt et al.1982; Priem et al.1988). Nussenblatt and coworkers found HLA A29 to be present in 80% of birdshot chorioretinopathy patients compared to 7.4% of controls, Priem and Oosterhuis found a prevalence of 95.8% in patients compared to 7.2% of controls. These results give a relative risk of developing the disease of 49.9

and 224.4 for individuals with the HLA A29 antigen and this association is amongst the highest of any disease entity.

Sympathetic ophthalmia has been found to be associated with the HLA A11 antigen (Reynard et al.1983). Behcets disease is associated with HLA B5 (in particular the HLA BW 51 split antigen which shows a relative disease risk of 6.1) and VKH syndrome is associated with HLA BW54, DWa, DR4 and MT3 (which has a relative disease risk of 74.5) although these associations only apply to Japanese patients with VKH syndrome (Ohno, 1981).

Investigation of the systemic manifestations of immune responses in intraocular inflammatory disease indicates that T cell mediated immune mechanisms play a role in the pathogenesis of these conditions. The favourable clinical response of many cases to the T cell specific immunosuppressive agent cyclosporin (Nussenblatt et al.1983a; Nussenblatt et al.1985; Graham et al.1985; de Vries et al.1990; Masuda et al.1989) supports this view. Present methods of investigating systemic immune mechanisms are not, however, sensitive enough to be of use in diagnosis and treatment of intraocular inflammation or to give us useful information on the pathogenesis of the ocular disease. Studies of the local immunopathology are necessary to define the detailed disease mechanisms.

1.9 Immunopathology of intraocular inflammation

The development of increasingly accurate and specific peripheral blood tests in the future will depend on a greater understanding of the local immunopathology of intraocular inflammatory disease. Investigation of active

clinical disease is at present possible using the technique of chorioretinal biopsy (Peyman et al.1981). This technique is invasive and has potential severe complications (Peyman et al.1986; Peyman et al.1987). Careful patient selection is therefore critical for this procedure (Foulds et al.1985; Chan et al.1991). This is illustrated by the report of two cases of inflammatory eye disease investigated by chorioretinal biopsy where the findings of the immunopathologic studies of the tissue obtained directed the therapeutic approach (Chan et al.1991). An obvious application of the technique is in the differentiation of immune-mediated inflammatory eye disease, neoplastic disease and intraocular infection.

Pathological and immunopathological studies have been carried out on eyes from patients suffering from various forms of intraocular inflammatory disease. These studies must be interpreted with the caveat that in general they represent eyes that have been enucleated following prolonged intraocular inflammation often complicated by conditions such as neovascular glaucoma which will produce further pathological changes. Such eyes have also usually been subject to extensive local and systemic medication. Although the findings of such investigations may represent end-stage pathology which may differ from the pathology of active disease, they nevertheless provide information on the immunopathology in these conditions. Previous studies have characterised the local immunopathology in four forms of intraocular inflammatory disease: sympathetic ophthalmitis, pars planitis, VKH syndrome and sarcoidosis.

1.9.1. Sympathetic ophthalmitis

Studies of eyes enucleated because of sympathetic ophthalmitis (Green, 1986a; Lubin et al.1980) have demonstrated a marked granulomatous inflammation primarily affecting the uveal tract. The choroid is infiltrated by lymphocytes, macrophages, epithelioid cells and giant cells in both the inciting and the sympathizing eyes. Dalen-Fuchs nodules are seen to be at the level of the RPE and are composed mainly of epithelioid cells. Immunohistochemical analysis of sympathetic ophthalmitis eyes (Jakobiec et al.1983; Chan et al.1986c) has shown that the choroidal infiltrate consists mainly of T lymphocytes which are predominantly of the CD4 phenotype although the relative number of CD8 positive T cells tends to increase as the disease progresses. 60-90% of these T cells are IL2 receptor positive (although in one described patient who was on heavy immunosuppression including cyclosporin A only 1% of T cells were IL2R positive). B lymphocytes are generally less than 20% of cells in the choroidal infiltrate. Choroidal granulomas consist predominantly of MHC class II positive cells of monocyte/macrophage lineage. Dalen-Fuchs nodules are composed mainly of MHC class II positive monocyte/ macrophage derived cells along with T lymphocytes, depigmented RPE cells and very few B lymphocytes.

1.9.2. Pars planitis

Pathological studies of pars planitis (Green et al.1981) have shown that the exudative "snowbank" covering the pars plana and anterior retina is composed of condensed vitreous, hyperplastic non-pigmented ciliary body

epithelium and fibrous astrocytes in end-stage enucleation specimens. Retinal neovascularisation has been demonstrated extending into this tissue. Vitreous aspirates obtained during active disease are composed of lymphocytes, plasma cells, macrophages, fibroblasts, epithelioid cells and giant cells (Green et al.1981). Immunohistochemical analysis of an eye with pars planitis (Wetzig et al.1988) showed the inflammatory infiltrate in the snowbank consisted mainly of T lymphocytes in a CD4:CD8 ratio of 10:1 (although the patient had normal peripheral blood T cell populations). T cells were also prominent in retinal vasculitic infiltrates. B lymphocytes were found in the iris and ciliary body infiltrates. Most of the inflammatory cells were HLA DR positive however very few of the T cells were IL2R positive. Within the snowbank most cells were GFAP (glial fibrillary acid protein) positive, stained positively with an anti- Muller cell monoclonal antibody and were HLA DR positive. In vitro it has been shown that Muller cells proliferate in response to lymphocyte derived products (Roberge et al.1985) and can present antigen to T lymphocytes (Roberge et al.1988) when stimulated (although only after non-physiological modification by trypsinisation and glutaraldehyde fixation). Other in vitro studies have shown that Muller cells inhibit T lymphocyte proliferation to antigen presentation by conventional antigen presenting cells and T cell clonal expansion to IL2 as well as reducing IL2 production and IL2R expression (Caspi et al.1987). It is therefore possible that in response to the intraocular inflammation Muller cells play an active role in the ongoing immunopathology in pars planitis although whether this role is in upregulation or downregulation of the immune response is at present uncertain. The degree of glial response seen

in pars planitis is not seen in other forms of intraocular inflammation.

1.9.3. VKH syndrome

In VKH syndrome, histopathological examination of enucleated eyes has shown a panuveitis which may be granulomatous or non-granulomatous (Perry et al.1977). Infiltrating lymphocytes and plasma cells, and proliferating RPE cells were noted in these eyes. Immunohistochemical study of an eye from a patient with long-standing disease (Chan et al.1988b) showed a T lymphocyte infiltrate in the uvea but in contrast to other forms of intraocular inflammation which have been studied, foci of B lymphocytes were also found. Together with the finding of plasma cells within ocular tissue in VKH (Perry et al.1977) this would suggest that the role of humoral immunity may be greater in the intraocular immunopathology in VKH syndrome than in other forms of inflammatory eye disease.

1.9.4. Sarcoidosis

The characteristic tissue pathology in sarcoidosis is a non-caseating granuloma (Daniele et al.1980) which has macrophages, epithelioid cells and giant cells centrally, surrounded by a ring of T and B lymphocytes. Examination of an eye enucleated because of ocular inflammation secondary to sarcoidosis revealed widespread granulomatous tissue consisting of lymphocytes, epithelioid cells and multinucleate giant cells (Chan et al.1987). Immunohistochemistry showed the lymphocytes were mainly CD4 positive T cells (with a CD4:CD8 ratio of 10:1) many of which stained positively for IL2 receptors and interferon γ. Numerous cells positive

for macrophage markers were also present and many cells were found to be positive for MHC class II antigens. This is similar to the findings in other tissue foci in sarcoidosis and it is notable that, in contrast to this, the number of T cells and the proportion of CD4 positive cells declines in peripheral blood in active disease (Daniele et al.1980) suggesting that there is recruitment of these cells to the tissues.

1.9.5. MHC expression

Expression of the MHC class II antigen HLA DR has been noted on various cells in eyes with intraocular inflammation. T and B lymphocytes and cells of monocyte/ macrophage lineage have been shown to be class II positive in many types of intraocular inflammation (Jakobiec et al.1983; Chan et al.1986c; Chan et al.1987; Kim et al.1987; Wetzig et al.1988; Hooks et al.1986). Organ resident cells have also been demonstrated to be class II positive in enucleated eyes. RPE cells (Chan et al.1986a; Chan et al.1987; Kim et al.1987; Hooks et al.1986), ciliary body epithelium (Chan et al.1987), retinal vascular endothelium (RVE) (Chan et al.1987; Kim et al.1987), Muller cells (Kim et al.1987; Wetzig et al.1988), fibroblasts (Kim et al.1987), keratocytes and corneal epithelial cells (Chan et al.1987) have all been shown to express class II in immunohistochemical studies of ocular inflammatory disease. RPE cells have also been shown to express class II antigens in retinitis pigmentosa, a group of genetically determined degenerative retinal conditions (Detrick et al.1986).

The lymphokines interferon-γ and IL2 have been demonstrated immunohistochemically to be present in enucleated eyes with intraocular

inflammation (Hooks et al.1986). These were found to be present on infiltrating cells in the choroid and retina related to HLA DR positive infiltrating cells and RPE. In vitro studies have shown that human RPE cells can express class II antigens in response to stimulation by interferon-γ (Liversidge et al.1988a). This combined evidence would suggest that lymphokines, and interferon-γ in particular, are responsible for the in vivo class II expression seen in various organ resident cells in human intraocular inflammation.

1.9.6. Adhesion molecules

The posterior segment of the eye does not contain lymphatics and therefore lymphocytic interactions with retinal antigens must occur at the blood-retinal barrier. The cells constituting this are the RPE (and the adjacent Bruchs' membrane) and the RVE (retinal vascular endothelium). Adhesion molecules are considered to play an important role in T lymphocyte interactions in immune recognition. The role of the LFA-1/ICAM-1 and LFA-3/CD2 adhesion pathways at the blood-retinal barrier has been investigated in vitro (Liversidge et al.1990). Results indicate that the RPE-T cell interaction is LFA-1/ICAM-1 dependent in the absence of lymphokine pretreatment and that the RVE-T cell interaction has both LFA-1/ICAM-1 and LFA-3/CD2 dependent systems.

The studies of local immunopathology in intraocular inflammation outlined above demonstrate that cell-mediated immunity is central to the pathogenesis of these conditions. CD4 positive T lymphocytes are consistently found in high numbers within the eyes implying that they play a

major role in the tissue pathology. These studies, however, cannot address questions on the aetiology and the precise pathogenic mechanisms of intraocular inflammatory disease and animal models of uveitis have been employed to investigate further these disease processes.

1.10 Animal models of intraocular inflammation

It was originally thought that the immunogenic autoantigen in ocular inflammatory disease was related to uveal pigment, and attempts by Woods to demonstrate immune responses to uveal pigment in patients with sympathetic ophthalmitis (Woods et al.1933) showed a positive cell mediated response to intracutaneous injection of crude bovine uveal tissue. Collins then produced ocular inflammation in an animal model (guinea pig) by systemic injection of heterologous and homologous uveal preparations in adjuvant (Collins, 1949). Autoantigenicity was subsequently discovered in the retina (Wacker et al.1965) and this led to the present experimental models of immune-mediated intraocular inflammatory disease.

1.10.1. Experimental autoimmune uveoretinitis

Experimental autoimmune uveoretinitis (EAU) has been induced in rabbit, rat, mouse and primate models using several ocular antigens. A range of tissue responses is seen in these models which can be altered by varying the method of immunisation and the species of animal.

Retina was first used as an inducing antigen by Wacker and Lipton (Wacker et al.1965), this preparation was then separated by ultracentrifugation into soluble (S) and particulate (P) fractions, with the

subsequent finding that only the S fraction produced uveitis (Wacker et al.1971). Wacker and Donoso extracted and purified "S antigen" from this fraction and found that it was potentially uveitogenic in partially purified and purified forms (Wacker et al.1977). S antigen has been characterised as an intracellular 48 kDa protein which regulates phosphodiesterase activity in light signal transduction (Pfister et al.1985) and has been localised to rod outer segments (Rodrigues et al.1987). S antigen is a phylogenetically conserved molecule which is highly uveitogenic in most species (Faure, 1980; Gery et al.1986), and is also located in the pineal gland.

Analysis of the structure of the S antigen protein has been carried out using a combination of enzymatic cleavage, fragment analysis and amino acid sequencing (Donoso et al.1990). Synthetic peptides have been constructed to investigate the properties of various domains of the protein. From these experiments uveitopathogenic sites, T lymphocyte proliferation sites and adoptive transfer sites have been identified within the protein (Gregerson et al.1989). Comparison of these amino acid sequences with those of other bacterial, viral and fungal proteins from a protein database showed sequence homology with a yeast histone peptide (Singh et al.1989) and three viral proteins (Singh et al.1990) which have subsequently proven to be uveitogenic, and to produce proliferative responses in S antigen specific T lymphocytes. These findings imply that viral or fungal infection could sensitize T cells to cross-react with S antigen because of the molecular mimicry between the proteins and thus initiate an autoimmune response.

Interphotoreceptor retinoid binding protein (IRBP) is a 140 kDa

extracellular glycoprotein which functions in Vitamin A transport between RPE and photoreceptors (Lai et al.1982). IRBP has also been found in the pineal (Rodrigues et al.1986). IRBP has induced EAU in rats (Fox et al.1987), rabbits (Eisenfeld et al.1987), and primates (Hirose et al.1986) but is poorly uveitogenic in guinea pigs (Vistica et al.1987). In mice, which are often refractory to EAU, IRBP has been found to be a more potent uveitogen than S antigen (Caspi et al.1988b). The immunopathology produced by IRBP is similar to that produced by S antigen (Gery et al.1986; Fox et al.1987). The IRBP molecule has been analysed using a similar approach to that used for S antigen. From this analysis uveitogenic peptides have been isolated (Redmond et al.1988).

Other antigens have also been found to be uveitogenic. Although early preparations of rhodopsin may have been contaminated with S antigen, highly purified rhodopsin preparations have subsequently been found to produce EAU (Marak et al.1980). "A" antigen, transducin and cGMP phosphodiesterase are also considered to produce uveitic changes (Gery et al.1986) although the properties of these antigens are less well investigated.

The failure to produce models of ocular inflammation by systemic injection before the 1940s has been attributed to the lack of suitable adjuvants. Complete Freund's adjuvant (CFA) is a mixture of mycobacteria and mineral oils which is commonly used in the induction of EAU. It has been shown that the simultaneous administration of Bordetella pertussis organisms or pertussis toxin intraperitoneally or intravenously further enhances the induction of EAU (De Kozak et al.1981b) and it has been suggested that this effect may be due to the sensitization of vascular

endothelial cells to vasoactive amines (Mochizuki et al.1984). It is notable that no disease is induced in animals injected with antigen without proper adjuvant. In mice, which are resistant to EAU, it is necessary to pre-treat with low dose cyclophosphamide to reliably produce disease (Caspi et al.1988b). This procedure is considered to reduce the numbers of lymphocytes involved in immune suppression mechanisms.

1.11. Immunopathology of EAU

At present the most widely used EAU model is the Lewis rat. 12 to 16 days following immunisation with S antigen in CFA there is a rapid influx of inflammatory cells to the vitreous, iris, ciliary body, anterior and posterior chambers. This is accompanied by an infiltrate of inflammatory cells in the photoreceptor layer along with patches of retinal oedema and photoreceptor destruction. 2 to 4 days after onset the choroid becomes heavily infiltrated with inflammatory cells. There is increasingly heavy inflammatory infiltration and destruction of the retina which eventually forms a glial scar after approximately 2 weeks of active inflammation. Polymorphonuclear leukocytes (PMN) are the predominant cell type in the anterior segment infiltrate whereas mononuclear cells (lymphocytes and macrophages) predominate in the posterior segment (Faure, 1980; De Kozak et al.1981b; Gery et al.1986). Immunohistochemical analysis of the lymphocytic infiltrate in EAU has shown that the vast majority of these cells are T lymphocytes and that the ratio of CD4:CD8 positive T cells (using the W3/25 and OX8 antibodies) is 5:1-5:2 in the early stages of the disease course and 1:1 to 1:2 in the later stages (Chan et al.1985a). These findings have been interpreted

as indicating that CD4 positive T cells are responsible for the acute disease whereas CD8 cells function to downregulate the disease process (Gery et al.1986).

Electron microscopic studies of the Lewis rat model of EAU have recently shown that retinal vascular endothelial cells (but not choroidal vascular endothelium) at the sites of active inflammation take on the appearence of high endothelial venules (HEV) (Dua et al.1991). This morphological change is associated with biochemical changes resulting in lymphocyte homing through receptor interactions with endothelial cells (Duijvestijn et al.1989; Butcher, 1990). These changes may therefore play a role in recruitment and migration of lymphocytes through the blood-retinal barrier.

Other species of rat are also susceptible to EAU. In the black hooded Lister rat immunisation with S antigen produces a mild, chronic form of EAU which is confined to the posterior segment of the eye (Stanford et al.1987). Retinal vasculitis is a prominent feature of this model and it may therefore be a more appropriate model for investigation of intraocular inflammatory conditions such as Behcets disease in which retinal vasculitis is a marked clinical feature.

The guinea pig was used for many of the early studies of EAU (Collins, 1949). In this model the pathology is centered on the choroid because of the lack of a retinal circulation (Forrester et al.1985). The guinea pig model has also been used to demonstrate the variation in EAU produced by different doses of S antigen (Rao et al.1979). An acute endophthalmitis was produced by very high doses, moderate S antigen dose produced a

granulomatous uveitis and smaller dose levels produced a non-granulomatous uveitis composed mainly of mononuclear cells. EAU has been less well investigated in the rabbit although both S antigen and IRBP have been shown to produce disease (Rao et al.1986; Eisenfeld et al.1987).

The primate model of EAU is considered to be closest to human disease. EAU induced by S antigen in the Rhesus monkeys is characterised by a more delayed onset and a more chronic course than EAU in other animals (Nussenblatt et al.1981a). Histopathologically there is a retinal perivasculitis with focal loss of the photoreceptor layer in relation to the inflamed vessels. There is a subretinal lymphocytic infiltration and relatively few inflammatory cells in the choroid. Animals also exhibit cell-mediated responses to S antigen. IRBP induced disease in primates has a greater degree of choroidal involvement (Hirose et al.1986) suggesting that immunisation with a retinal antigen can under some cicumstances produce pathology centered on the choroid. The inflammation in this model was granulomatous in nature with many giant cells similar to the changes found in human sympathetic ophthalmia and VKH syndrome.

In general mice are less susceptible to EAU than other animals. Recently, using S antigen and IRBP with an intensified immunisation protocol it has been possible to induce disease in several strains of mouse (Caspi et al.1988b). In the B10.A strain of mouse EAU is a relatively chronic disease with focal lesions in the retina and choroid, granuloma formation and vasculitis. Lesions resembling Dalen-Fuchs nodules and subretinal neovasculisation have also been found in this model.

Studies of the expression of MHC class II antigens in EAU (Chan et

al.1986b; Chan et al.1988a) have shown that there is class II expression on RPE cells prior to onset of clinical or histological evidence of disease and that this persists after the active phase of the disease is over. There is also widespread expression of class II on infiltrating inflammatory cells.

In all the EAU models outlined above lymphocytes are found in significant numbers within the inflammatory infiltrates at the sites of destructive lesions in the posterior segment. Immunohistochemistry has shown that cells are predominantly T cells and that very few B cells are present. Treatment of rats with anti-CD4 antibody has been shown to completely inhibit the development of EAU following active immunisation (Atalla et al.1990). This is evidence that cell mediated immunity plays a central role in the tissue pathology in EAU.

1.12. Immune mechanisms in EAU

1.12.1. Adoptive transfer

It has been demonstrated that EAU can be adoptively transferred from immunised donor animals to genetically identical naive recipients with T lymphocytes but not with serum (Mochizuki et al.1985). T cells must be of the CD4 subgroup to have the potential to transfer disease. Furthermore, in the Lewis rat model, long term T cell lines have been developed which are capable of transferring EAU (Caspi et al.1986). These T cell lines are CD4 positive, IL2R positive, MHC class II restricted and S antigen specific. The immunopathology of this form of EAU starts 4-5 days after transfer of disease and follows a similar pattern to EAU induced by active inflammation (Chan et al.1988a). Very small numbers of these S antigen specific T cells can be

detected in the eyes at the time of disease onset (Lightman et al.1987) suggesting that they function to recruit other inflammatory cells following antigen recognition. It is notable that in order to mediate EAU uveitogenic T cells must be activated with specific antigen or T cell mitogen prior to transfer (Mochizuki et al.1985). These activated T cells produce lymphokines and it may be that this is an essential component of the mechanism of induction of EAU.

Lymphocytes (both CD4+ and CD8+), macrophages and neutrophils are present in the retinal destructive lesions in EAU, however the identity of the cells causing the tissue damage is unknown. Since EAU can be adoptively transferred without a detectible accumulation of antigen specific T cells in the eye (Lightman et al.1987) the numbers of T cells seen in these lesions must be produced by clonal expansion of transferred antigen specific cells, recruitment of non-antigen specific T cells or a combination of both these processes. Antigen specific T cells must also initiate the mechanisms of chemotaxis by which macrophages and neutrophils accumulate in the destructive lesions. Lymphokine secretion is fundamental to the ability of T lymphocytes to act as cytotoxic effector cells and to exert effects on other cells, for example migration and induction of cytotoxic action (Feldmann et al.1989).

1.12.2 Immune modulation

It has been shown that treatment with the specific T cell inhibitor cyclosporin A from the day of immunisation totally inhibits the development of EAU (Nussenblatt et al.1981b). Higher dosage commenced at day 7 post-

immunisation also inhibited disease. Systemic cell mediated immune responses to S antigen are altered in animals treated with cyclosporin A in line with the inhibition of disease (Nussenblatt et al.1983c). These findings emphasize the central role of cell mediated immunity in the induction and development of EAU.

Anti-S antigen and anti-IRBP antibodies can be detected following immunisation with these antigens but antibody production does not correlate with a uveitogenic response (De Kozak et al.1981b; Caspi et al.1988b). In adoptive transfer models of EAU, disease can be induced without detectable antibody levels (Mochizuki et al.1985). Furthermore, it is not possible to transfer EAU with serum. In cyclosporin A treated animals disease inhibition occurs without a decrease in systemic antibody levels (Nussenblatt et al.1981b). It seems unlikely therefore that humoral immunity plays a major role in EAU pathogenesis. It may however play an auxiliary role in EAU regulation through antiidiotype antibody mechanisms whereby antibodies may form a network following recognition of idiotypes on other antibodies (or lymphocytes). There is experimental evidence to suggest that EAU can be modified through manipulation of idiotypic networks (De Kozak, 1990).

1.12.3. Downregulation of EAU

Several mechanisms may play a role in the downregulation of the immune response and tissue destruction in EAU. The increase in numbers of CD8 positive T lymphocytes seen in the later stages of the inflammation in EAU (Chan et al.1985a) along with the termination of active disease has led to speculation that these cells may act to downregulate the immune

response. Further evidence for a role for cells of the CD8 subtype has come with the development of a CD8 (OX8) positive T cell line which can inhibit the proliferation of uveitogenic T cells in vitro and downgrade EAU in vivo when adoptively transferred to actively immunised animals (Caspi et al.1988a).

Non-lymphoid organ resident cells such as Muller cells may act locally to suppress the immune response. Evidence for this has come from in vitro experiments showing the negative effect of Muller cells on antigen presentation to T cells as well as T cell IL2 production and IL2R expression (Caspi et al.1987). It has also been shown that modified Muller cells can act as antigen presenting cells to T cells (Roberge et al.1988). Iris and choroidal cells have the capacity to produce a soluble inhibitor of lymphocyte proliferation in vitro (Hooper et al.1991). The in vivo role of such local immune regulation by organ resident cells remains uncertain.

1.12.4. Genetic influences in EAU development

The genetic background of animals used in the study of EAU appears to be of importance in disease induction. Studies on the mouse model suggest that the haplotype of a strain may determine its susceptibility to EAU (Caspi et al.1990). Only mouse strains carrying the H-2^k haplotype in the I-A region of their MHC are susceptible to EAU induction. Interestingly the strains studied appeared to respond to either IRBP or S antigen but not to both.

Strains of rat used in EAU studies can be grouped into high or low responders on the basis of their genetic makeup. In rats there is a strong

correlation between the susceptibility to EAU and to EAE. EAE susceptibility in rats has been linked to the RT1 major histocompatibility gene and this relationship appears to hold for EAU (Gery et al.1986). The role of mast cells in the local release of vasoactive amines has been studied in EAU. It has been demonstrated that there is a build up of choroidal mast cells following immunisation and a massive degranulation, which would cause increased blood vessel permeability, immediately prior to disease onset (De Kozak et al.1981a). Studies of choroidal mast cells in different strains of rat show that high numbers of mast cells correlate with disease susceptibility whereas strains with low numbers are less susceptible (Mochizuki et al.1984). The extra-MHC genes controlling mechanisms of vasoactive amine action on blood vessels may therefore be of importance in EAU susceptibility also.

1.13 Relevance of EAU to human intraocular inflammation

1.13.1. Intraocular antigens

Investigation of the immune responses of patients with intraocular inflammatory disease to S antigen and IRBP, the most commonly used antigens for the induction of EAU, has shown that while some patients do have an increase in humoral and cell-mediated responses to these antigens this is by no means a consistent finding (Williams et al.1977; Kasp et al.1986; Dumonde et al.1985; Doekes et al.1987; Froebel et al.1989; Forrester et al.1989). Furthermore, immune responsiveness to these antigens is found in patients following retinal trauma (Gregerson et al.1982) and in healthy individuals (Doekes et al.1987; Froebel et al.1989; Forrester et al.1989). It is therefore uncertain whether these antigens are involved in

the induction of human intraocular inflammation. It is notable, however, that S antigen and IRBP are capable of inducing ocular inflammation in sub-human primates (Nussenblatt et al.1981a; Hirose et al.1986) and that this disease model is closely analogous to human disease suggesting that they at least have the potential to be uveitogenic in humans. It is possible that multiple ocular antigens are involved in any particular form of human disease. Since clinical studies are done on peripheral blood samples and autoreactive T cells specific for a small organ such as the eye may constitute only a very small proportion of the total lymphocyte pool, it is likely that our present clinical tests lack the sensitivity to pick up subtle changes in a T cell population.

1.13.2. Immunopathology

Many of the histological and immunohistological features seen in human intraocular inflammation are reproduced in the animal models of EAU (Forrester et al.1990). The marked T cell involvement in immunohistological studies of human disease is paralleled by the predominance of T cells in EAU. The retinal vasculitic changes seen in the more chronic models of EAU, particularly the primate model (Nussenblatt et al.1981a) is similar to that seen in many forms of clinical disease. Nodules formed at the chorioretinal interface in EAU (in particular the IRBP primate model (Hirose et al.1986)) show similarities to the Dalen-Fuchs nodules seen in sympathetic ophthalmia, VKH and other forms of posterior uveitis. The widespread MHC class II expression on infiltrating and organ-resident cells seen in EAU is analogous to that seen in tissue from many forms of

clinical intraocular inflammation.

1.13.3. Genetic factors

The genetic predisposition to certain clinical types of intraocular inflammation such as birdshot chorioretinopathy (Nussenblatt et al.1982; Priem et al.1988) is also seen in the susceptibility of certain strains of animal to induction of EAU. The response of EAU to immunosuppressive treatment, for example cyclosporin A (Nussenblatt et al.1981b), appears to be similar to the response seen clinically in intraocular inflammation and is of importance in the development of new therapies for human disease.

Although it is uncertain if EAU is exactly analogous to any form of human intraocular inflammation it does provide a range of experimental models which have proved of value in the investigation of human disease.

1.14 The role of autoimmunity in inflammatory eye disease

Idiopathic intraocular inflammatory disease and in particular sympathetic ophthalmitis, have for many years been thought to be an organ-specific autoimmune disease. This theory remains unproven. There are, however, several lines of evidence which suggest that immune system mechanisms are centrally involved in the evolution of these conditions and support the view that they represent autoimmune disease.

Firstly there is a severe inflammatory response, usually localised to intraocular tissues, in the absence of a demonstrable exogenous stimulus such as an infective agent or foreign antigen (Forrester, 1991). This is despite extensive investigations carried out on actively inflammed eyes and

on systemic studies of patients with inflammatory eye disease. This does not preclude the possibility of a transient infection providing an initiating event for ongoing inflammation. Since the subsequent immune-mediated inflammation is inappropriate in the absence of an infective agent and has a deleterious effect on the ocular tissues the response could justifiably be termed autoimmune.

Associations with specific MHC antigens have been demonstrated in several diseases thought to be of autoimmune nature (Sinha et al.1990) and the MHC associations of certain subtypes of inflammatory eye disease (Nussenblatt et al.1982; Priem et al.1988; Reynard et al.1983; Ohno, 1981) is also suggestive of an autoimmune aetiology.

The central role of T lymphocytes in the immunopathology of inflammatory eye disease (sections 1.8 and 1.9) is analogous to the situation found in many forms of autoimmune disease (section 1.6) and implies that similar immune-mediated disease mechanisms may be active in these diverse conditions. Although studies on the immune responsiveness of uveitis patients to retinal antigens have given inconclusive results (section 1.8) the demonstration that certain patients do react to ocular autoantigens is suggestive that in at least a proportion of patients these antigens may be providing an autoimmune stimulus. The inconsistent results found in these studies may be accounted for by the relative insensitivity of currently available systemic tests of immune processes ocurring in the eye.

Overall, although there is no conclusive evidence of an autoimmune aetiology in inflammatory eye disease, aspects of current knowledge gained from clinical observation and experimental work provide support for the

theory that this range of conditions represents manifestations of an organ specific autoimmune disease.

To extend current knowledge on the immune-mediated processes involved in intraocular inflammatory disease this thesis has investigated the cellular inflammatory response in two forms of human disease: multifocal posterior uveitis and Behcet's disease which have not been previously studied. Subsequent experimental work was undertaken on experimental autoimmune uveoretinitis to produce the first in situ hybridisation studies of lymphokine gene expression in intraocular inflammatory disease.

CHAPTER 2

STUDIES OF HUMAN INTRAOCULAR INFLAMMATORY DISEASE

A: MULTIFOCAL POSTERIOR UVEITIS

2.1 Introduction

The previous chapter has outlined studies of the systemic immune response to intraocular inflammatory disease and its local immunopathology. These studies demonstrate that although systemic immune abnormalities can be detected these are often inconsistent and are not sufficient to allow an understanding of the immunopathologic mechanisms of intraocular inflammation (or to monitor its clinical course and response to treatment). Studies of the local human intraocular immunopathology are therefore essential to provide a basis for clinical and experimental research. Information from such studies must always be interpreted with the caveat that it may represent an inactive or end stage of the disease and may be subject to modification by treatment.

This chapter describes light microscopic, electron microscopic and immunohistochemical studies carried out on post-mortem material from a form of human intraocular inflammation: multifocal posterior uveitis. The general aims of these studies are firstly to describe the ocular pathology and characterize the infiltrating inflammatory cells in the condition and secondly to relate these to clinical findings, experimental work and to the pathology found in other immune-mediated conditions.

2.2 Multifocal posterior uveitis

This term covers forms of posterior uveitis which have multiple inflammatory foci scattered across the posterior segment of the eye. Within this group of conditions it may be possible to assign cases to specific diagnostic entities.

In many cases, however, this is not possible and these are often termed idiopathic posterior uveitis. The case outlined below highlights some of the diagnostic difficulties in clinical uveitis.

2.3 Materials and Methods

2.3.1. Case Report

A 50 year old woman who had a one-month history of progressive blurred vision was noted to have mild anterior uveitis, optic disc oedema and bilateral total exudative retinal detachments. She had no systemic illness and was not on any medication. Extensive investigation including viral serology gave negative results. Following treatment with high-dose systemic steroids the exudative detachments regressed and multiple round cream-coloured patches were observed across both optic fundi (Fig. 1). These were thought to be at the level of the retinal pigment epithelium and a diagnosis of acute posterior multifocal placoid pigment epitheliopathy (APMPPE) (Gass, 1968) was made. Fluorescein angiography showed characteristic patchy late hyperfluorescence. There were several recurrences of crops of similar discrete, pale fundal lesions over the subsequent year against a background of low-grade inflammation resulting in patchy depigmentary chorioretinal scarring, with mottled pigmentation of the maculae (Fig. 2). Treatment with systemic steroids was necessary for a total of five years. After steroids were withdrawn there was a mild residual posterior uveitis and good preservation of visual function. The patient died from an acute myocardial infarction nine years after the original episode of ocular inflammation and the eyes were obtained for pathological examination. Six weeks prior to death her vision

was 6/9 right and left, there was a mild bilateral anterior uveitis, and patches of chorioretinal scarring which did not show any clinical evidence of active inflammation.

2.3.2. Light and electron microscopy

The eyes were removed approximately 12 hours following death. The left eye was fixed in 4 % formal saline and the right in 2% cacodylate buffered glutaraldehyde. Both eyes were of normal dimensions and were divided horizontally above and below the macula. Paraffin embedded serial sections 6μm thick, from both eyes were stained with haematoxylin and eosin, periodic-acid-Schiff, and stains for axons (Bodain), myelin (Loyez), and iron (Perls) for light microscopic examination. Individual lesions of varying appearance were selected macroscopically from the glutaraldehyde fixed eye for electron microscopy. Fifteen blocks were embedded in Araldite and 1μm thick sections were stained with toluidene blue. Eight of these blocks were studied by semiserial sectioning (at 50 or 100 μm steps) and, where appropriate, ultrathin sections taken and stained conventionally for examination using a Philips 301 electron microscope.

2.3.3. Immunohistochemistry

Immunohistochemical staining was done on sections from the formalin fixed eye using the avidin-biotin-complex (ABC) method. Sections were dewaxed through toluene and graded alcohols and washed in phosphate buffered saline (PBS) pH 7.3 for 5 minutes. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide in 50% methanol for

20 minutes. Slides were then washed in PBS for 5 minutes and normal (horse) serum applied for 20 minutes. The serum was tipped off and the primary monoclonal antibody applied for 30 minutes followed by washing in PBS for 5 minutes. Biotinylated secondary antibody was applied for 30 minutes and after a further wash in PBS, avidin-biotin-complex applied for 45 minutes. Slides were then washed in PBS, developed in amino ethyl carbazole (AEC) to give a red final reaction product and counterstained in haematoxylin. Coverslips were mounted using an aqueous based mountant (Dako glycergel). Table 1 (page 99) lists the primary antibodies used and their specificities. Estimates of numbers of T (UCHL1 antibody) and B lymphocytes (L26 antibody) were made by counting positive cells (demonstrating red membrane staining) in ten high power fields.

Control studies for the immunohistochemical staining were carried out by (a) omitting the primary antibody to provide a negative control and (b) on known positive tissues (inflammatory eyelid or orbital lesions) to verify the efficacy of the monoclonal antibodies and immunohistochemical method. Control studies were also performed on sections of normal eyes using each primary monoclonal antibody.

2.4 Results

2.4.1. Macroscopic examination

Both globes had similar macroscopic appearances. The anterior segments were normal and the vitreous was clear. There were meridional folds at the pars plana but no evidence of "snowball" or "snowbank" accumulations of leucocytes. Focal lesions, varying in size between 0.5 and

1.5 mm diameter, were scattered throughout the fundus, predominantly in the mid-periphery (Figs. 3 & 4). The lesions varied in the degree of their pigmentation. Smaller lesions in general had less hyperpigmentation while larger lesions were often heavily pigmented with variable focal areas of depigmentation. The maculae, peripapillary retina and optic discs appeared normal for post-mortem material.

2.4.2. Light and electron microscopic findings

2.4.2.1. General pathological findings

The appearances of the conjunctiva, cornea, outflow system and lens were normal with no evidence of an ongoing inflammatory process. Histologically there were scattered lymphocytes, occasionally forming aggregates, in the iris and ciliary body but no evidence of inflammatory cells in the vitreous. There was non-specific hyperplasia in the epithelial layers of the pars plana.

In general the retina was well preserved with the nerve fibre layer and optic nerve containing axons in normal density. Occasional drusen and degenarative areas of the RPE were noted in the far periphery. Clusters of lymphocytes were noted in the posterior choroid and in the adventitia of some of the posterior ciliary vessels and nerves.

Focal destructive lesions involving the retina, RPE and choroid were found throughout the posterior segment and corresponded to the foci seen clinically and macroscopically. These lesions were grouped into (a) non-pigmented - figure 3, lesion b ; figure 4, lesions a, d and e. (b) partially pigmented - figure 3, lesions a,c,d and f ; figure 4, lesion c, and (c) heavily

pigmented - figure 3, lesion e ; figure 4, lesion b, on the basis of their macroscopic appearance.

2.4.2.2. Small non-pigmented lesions

The small, non-pigmented lesions were of variable histological appearance. Three of the blocks examined individually (Fig 4 lesions a,d & e) had focal collections of lymphocytes in the choroid (Fig. 5) with infiltration of lymphocytes and macrophages in the hypopigmented retinal pigment epithelium. The overlying retina had a minimal degree of photoreceptor atrophy. Ultrastructural analysis showed the cells within the RPE were macrophages (Fig.6). Glial scar tissue replaced the RPE and in some foci penetrated Bruch's membrane causing destruction of the choriocapillaris.

2.4.2.3. Partially pigmented lesions

Partially pigmented lesions also varied in their histologic appearance (Fig. 3 lesions a,c,d & f; Fig. 4 lesion c). There was a consistent finding of lymphocytes in the choroid below these foci. The choriocapillaris was either normal, infiltrated by inflammatory cells or obliterated by collagenous tissue. In some foci Bruch's membrane had short breaks (Fig. 3 lesions c,d & f; Figs 7 & 8) which in some instances contained inflammatory cells (Fig. 9). The RPE was multilayered and infiltrated by lymphocytes and macrophages in some foci (Figs 8,9 & 10) and was destroyed in others. The outer retina was usually atrophic in these foci and in some cases, where the inflammatory process was more destructive, Bruch's membrane and the outer retina were completely lost. In the more advanced lesions there was invasion of the retina by lymphocytes, macrophages and RPE cells and the extension of choroidal blood vessels into the retina (Fig. 11).

2.4.2.4. Heavily pigmented lesions

Lesions which appeared heavily pigmented macroscopically (Fig. 3 lesion e; Fig. 4 lesion b) consisted of atrophic retina over multilayered and hyperplastic RPE cells infiltrated by macrophages (Fig. 12). There was a dense choroidal lymphocytic infiltrate in these lesions. At the periphery of even advanced lesions the photoreceptor architecture was preserved, and there was evidence of photoreceptor phagocytosis by exogenous macrophages (Fig. 13)

2.4.2.5. Common pathological features in inflammatory foci

In none of the sections examined at light or ultrastructural level was it possible to identify structures with protazoal, viral or bacterial characteristics.

Certain features were consistently found in all the inflammatory foci studied. The lesions were restricted, the adjacent retina being spared and the choroidal lymphocytic infiltrate being focal (Figs. 5 & 10). Lesions were often related to a myelinated nerve in the choroid. Retinal glial cells penetrated Bruch's membrane to extend to the choroid and collections of RPE cells were often found in the choroid at the site of inflammatory foci (Figs. 5 & 14) irrespective of degree of destruction. There was evidence of extension of choroidal vascularisation towards the retinal side of the destructive focus in some lesions (Fig. 11). Very few plasma cells were found in any of the tissues examined. There was no hyalinisation of the intima or media or luminal narrowing in either choroidal or retinal vessels which would have suggested previous vascular occlusive disease.

The endothelial cells of choroidal capillaries and venules had marked swelling and cytoplasmic rarefaction irrespective of the degree of

inflammatory destruction (Figs. 6,7,11,14-18). This change was restricted to the foci of inflammation and was not seen in the adjacent uninvolved choroid. Lymphocytes were often noted in relation to the swollen endothelial cells (Fig. 7). At the ultrastuctural level the junctions between these endothelial cell were noted to be intact. In some vascular lumina there were platelet clusters (Fig 8), neither fibrin nor thrombus deposits were identified.

2.4.3. Immunohistochemical findings

Within the choroidal infiltrates an estimated 70 - 80 % of the lymphocytes were identified as T cells (Fig. 19). B cells constituted 5 - 10 % of lymphocytes in the choroid. The majority of UCHL1 positive T cells were also OPD4 positive and these cells were noted to be prominent within the choriocapillaris (Fig. 20) and in relation to areas of RPE disruption . The lymphocytes in the iris and ciliary body were also noted to be UCHL1 and OPD4 positive T cells. Cells which stained positively with the monocyte/macrophage monoclonal antibody were found in small numbers scattered throughout the choroid and also in the destructive retinal foci. The antibody specific for the MHC class II antigen HLA DR α chain was positive on drusen-like deposits at the level of the RPE and also on occasional cells in the choroid and ciliary body. No vascular endothelial cells were HLA DR positive. There were no significant deposits of IgG or IgM.

Studies of normal eyes revealed very occasional OPD4 positive and UCHL1 positive T cells in the choroid. There was also a very small number of HLA DR positive cells in the choroid.

2.5 Discussion

2.5.1 Clinical features

Exudative retinal detachments are not commonly associated with APMPPE but have been reported (Bird et al.1972). The initial clinical report of APMPPE described a self-limiting condition (Gass, 1968) but further studies have stressed that it may be recurrent or chronic (Gass, 1983; Damato et al.1983; Williams et al.1989). It has also been suggested that APMPPE may form a continuum of clinical disease with Harada's disease (Wright et al.1978). Although the initial clinical impression was that this was a case of APMPPE the presentation with exudative retinal detachments and subsequent clinical course are more typical of Harada's disease (Ohno et al.1977; Snyder et al.1980). The condition probably best fits an intermediate group as defined by Wright, Bird and Hamilton (Wright et al.1978). An infective aetiology is unlikely in this case as the presentation and subsequent clinical course was not characteristic of the defined infective causes of posterior uveitis. Other diagnoses which were considered were (a) birdshot chorioretinopathy (Ryan et al.1980; Nussenblatt et al.1982; Priem et al.1988) however the initial total exudative retinal detachments and subsequent hyperpigmentation of many of the fundal lesions (Figs. 3 & 4) are not described in this condition; (b) geographic chorioretinopathy (Hamilton et al.1974) although the focal, non-progressive nature of the fundal lesions and the good visual preservation are not seen in this condition; (c) punctate inner choroiditis (Nozik et al.1991; Dreyer et al.1984; Watzke et al.1984; Morgan et al.1986) which usually presents with multiple small depigmented chorioretinal inflammatory lesions unlike the varied,

often hyperpigmented, lesions seen in this case (Figs 3 & 4).

This case illustrates some of the diagnostic difficulties common to many forms of intraocular inflammation. Because of the diagnostic difficulties outlined, the pathology described above is best considered in the broader clinical context of multifocal posterior uveitis.

2.5.2. Pathology and immunopathology

The pathological changes reported in this case represent the ongoing features of the processes involved (nine years after the initial onset) and the study of the tissue is therefore aimed towards the ongoing pathology rather than the initial aetiology. Unlike much of the human pathological material available in inflammatory eye disease the disease has not been complicated by other pathology and was not subject to medication when it was obtained. Identifying an aetiological agent is a problem common to many studies of the immunopathology of human intraocular inflammation since the initiating insult may be transient and no longer detectable by the time the disease comes to clinical attention. Some of the observed negative findings are relevant in this respect. It has been suggested that both pigment epithelial disease and multifocal choroiditis may be initiated by choroidal ischaemia (Wright et al.1978; Hayreh, 1975; Deutman et al.1977; Young et al.1980; Gaudric et al.1982) but no evidence of concomitant vascular occlusive disease was found.

The study attempted to correlate the clinical and macroscopic appearance of the inflammatory foci with their histological appearance. However, no overall pattern was found in that lesions of similar macroscopic appearance varied widely in the nature of their pathological change. It was

notable that in general the lesions were pathologically active yet were considered to be clinically quiescent.

The restricted, focal nature of the lesions was notable and suggests that localised stimuli are producing the ongoing inflammation. Finding inflammatory cells within small breaks in Bruch's membrane suggests that these cells may gain access to the inflammatory foci by this route.The demonstration of photoreceptor phagocytosis by exogenous macrophages is evidence that these cells could form part of the effector cell mechanisms in this type of intraocular inflammation.

Glial cell migration from the retina to the choroid has been described in syphilis (Blodi et al.1968) and other chorioretinal inflammatory conditions (Green, 1985). The occurence of this abnormality in multifocal posterior uveitis is further evidence of the non-specific nature of the glial cell activity. Similarly migration of RPE cells into the choroid is regarded as non-specific (Green, 1985) as is neovasculisation in a glial scar (Green, 1986b).

In VKH syndrome the pathological changes have been described as a mild, diffuse uveitis consisting of lymphocytes and plasma cells and RPE atrophy (Perry et al.1977). This is in contrast to the inflammatory infiltrate described above which although non-granulomatous was severe in discrete foci and contained very few plasma cells. Immunohistological study of VKH syndrome (Chan et al.1988b) has revealed that the majority of infiltrating cells are T lymphocytes with a high proportion of CD4 positive cells and an increase of GFAP positive (glial) cells in the retina (although this study was carried out on end-stage material). The immunohistochemical findings described above are broadly similar to the results of this study. The

predominance of T lymphocytes correlates with the findings from studies of other forms of intraocular inflammation such as sympathetic ophthalmitis (Jakobiec et al.1983; Chan et al.1986c), sarcoidosis (Chan et al.1987) and pars planitis (Wetzig et al.1988) and is further evidence of the central role of cell-mediated immune responses in intraocular inflammatory disease. The interpretation of the minimal MHC class II antigen expression found in these eyes must be made with caution since the HLA DR α chain monoclonal antibody used on the formalin fixed tissue in this study may be less sensitive than the anti-class II antibodies used in studies of frozen tissue from other forms of human intraocular inflammation (Dako: unpublished communication).

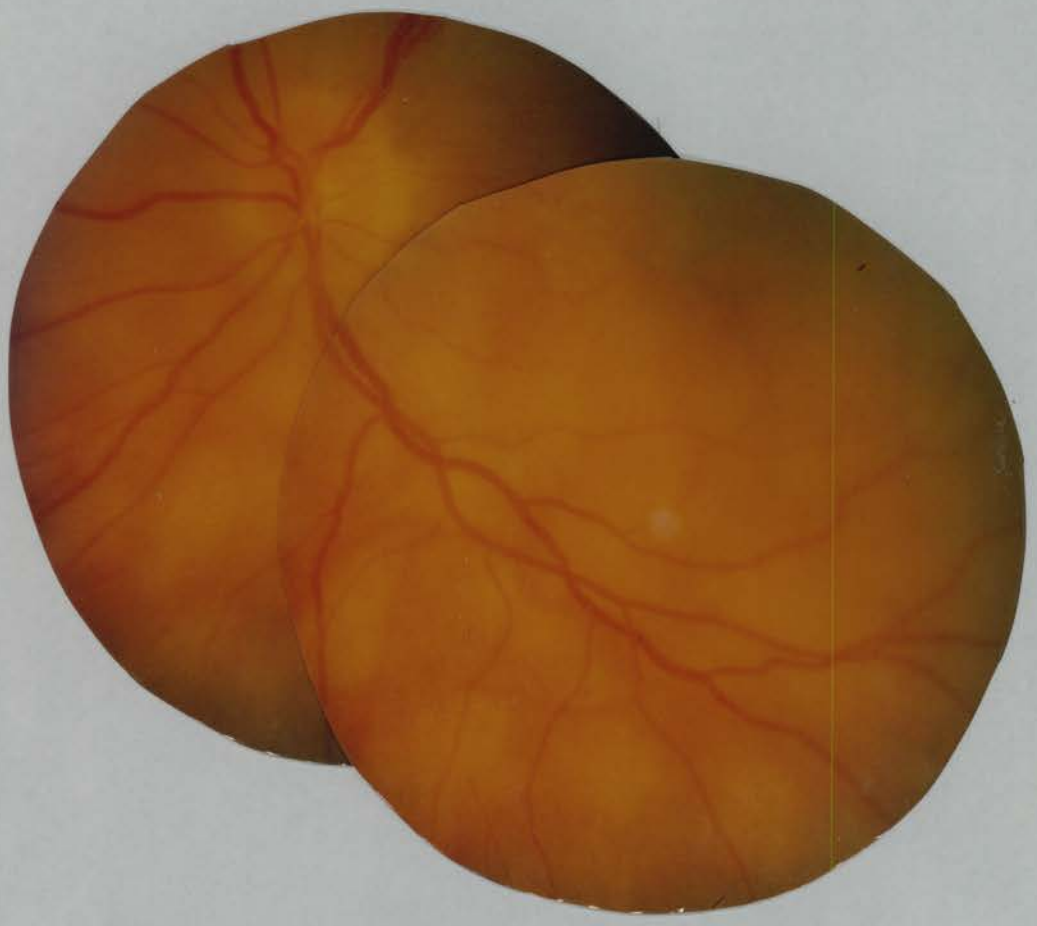


Figure 1

Left fundal appearance at the time of presentation in 1978. Multiple cream coloured patches are seen across the central retina at the level of the RPE.

Figure 2

Appearance of the left optic fundus one year after initial onset of posterior uveitis. There is patchy chorioretinal scarring with mottled hyperpigmentation at the fovea.

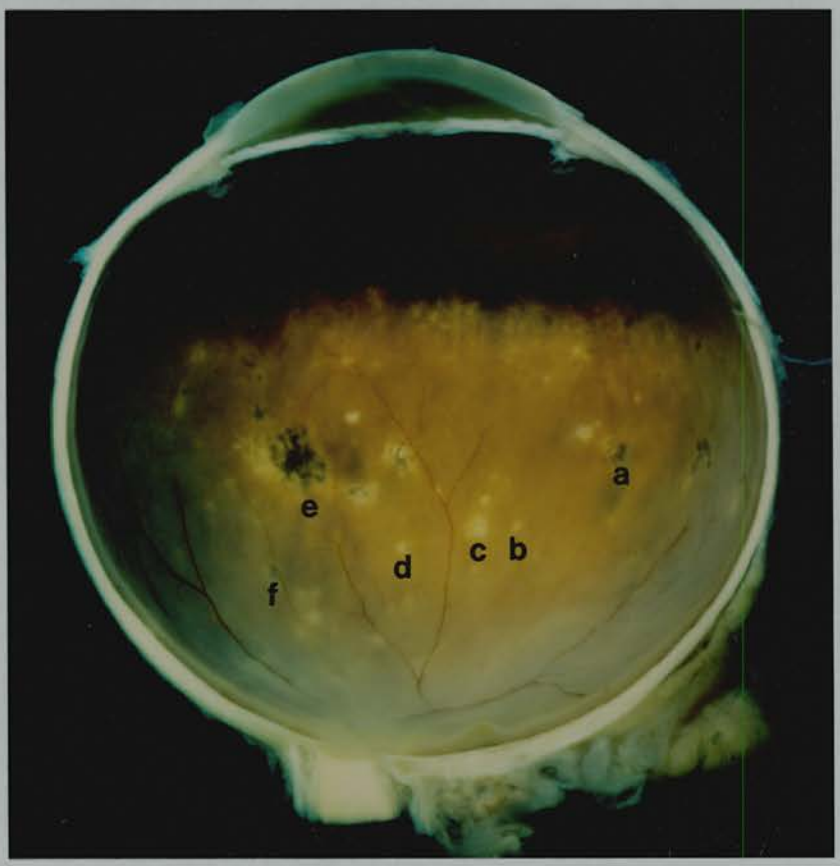


Figure 3

Inferior calotte of the right globe demonstrating the macroscopic appearance of the lesions. Plastic embedded blocks of the lesions designated a-f were examined individually. (Non-pigmented - b; partially pigmented - a,c,d, and f ; heavily pigmented - e)

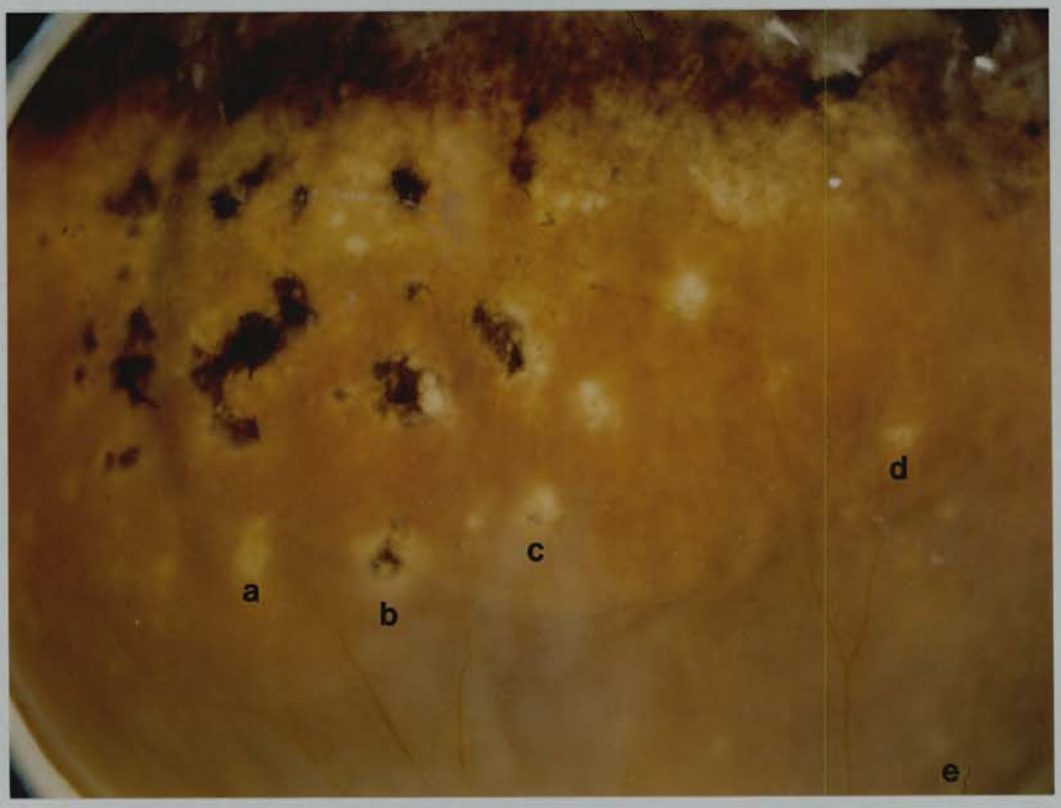


Figure 4

Superior calotte of right eye showing the variable appearance of the fundal lesions. Blocks of the lesions designated a-e were examined individually. (Non-pigmented - a,d and e ; partially pigmented- c ; heavily pigmented - d)

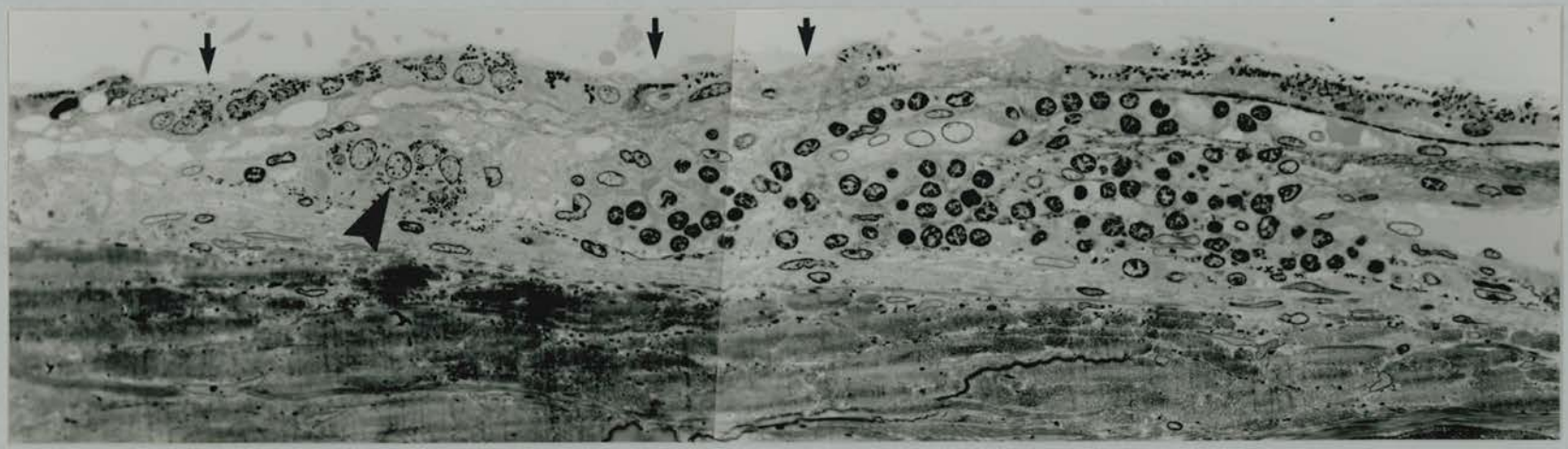


Figure 5

Focal collection of lymphocytes in choroid and choriocapillaris.

The RPE is disorganised (arrows) and there is a cluster of RPE

cells in the choroid (arrowhead). x300

Figure 6

Electron micrograph demonstrating macrophages (arrows) infiltrating the RPE. Where the choriocapillaris is intact the endothelial cells are swollen (arrowhead) x1700

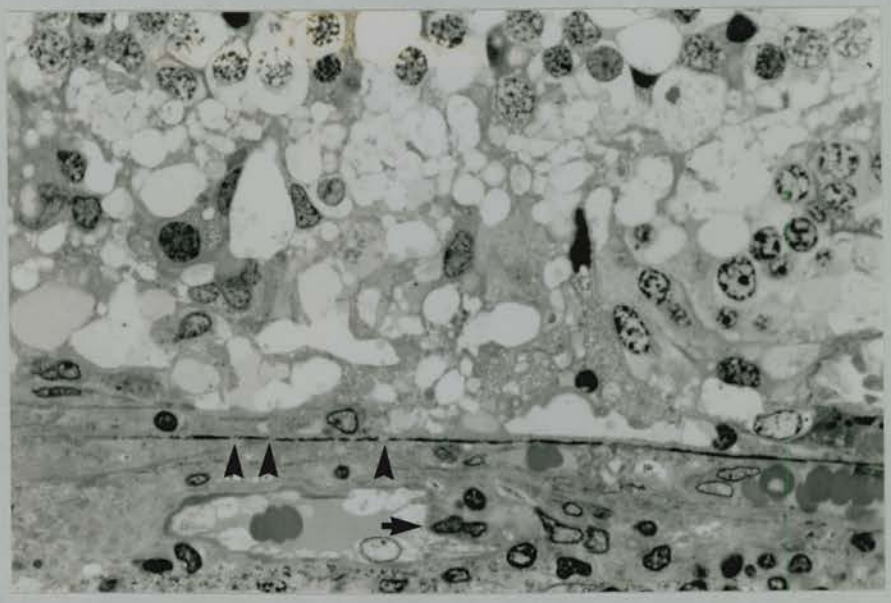


Figure 7

Inflammatory focus in which gliotic retina abuts on to Bruchs' membrane which has small breaks (arrowheads to examples). Inflammatory cells (arrow) are closely related to HEV in choroidal vessel. x375

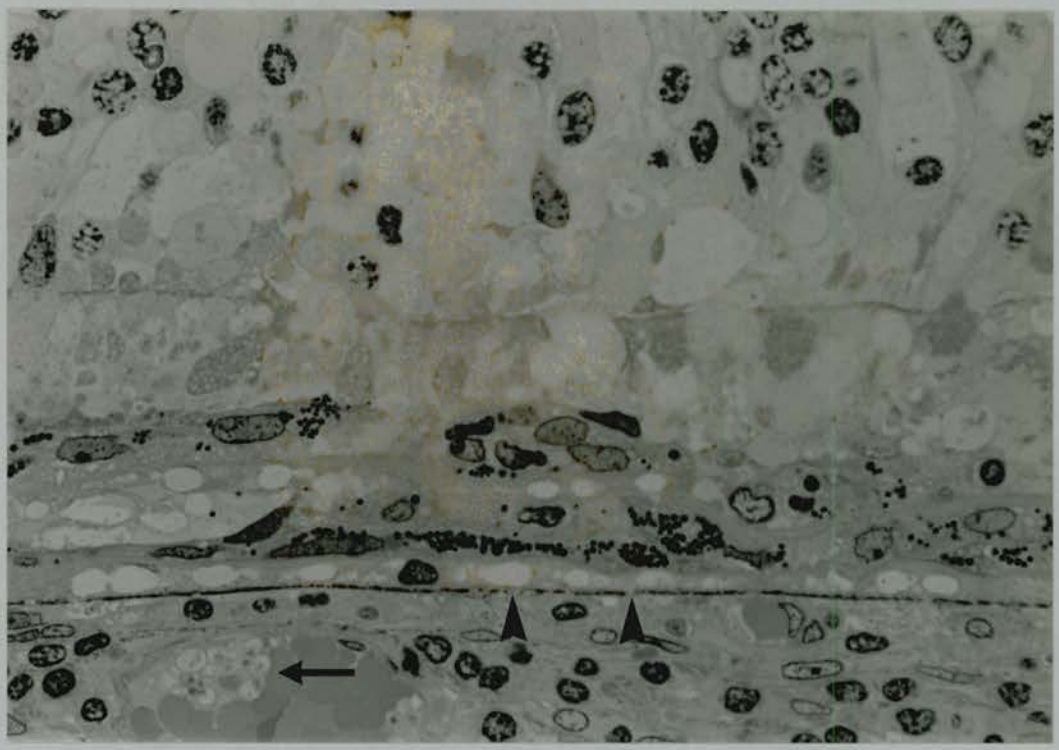


Figure 8

Partially pigmented lesion with multilayered RPE, short breaks in Bruchs' membrane (arrowheads) and a platelet aggregate in a choroidal vessel (arrow). x650

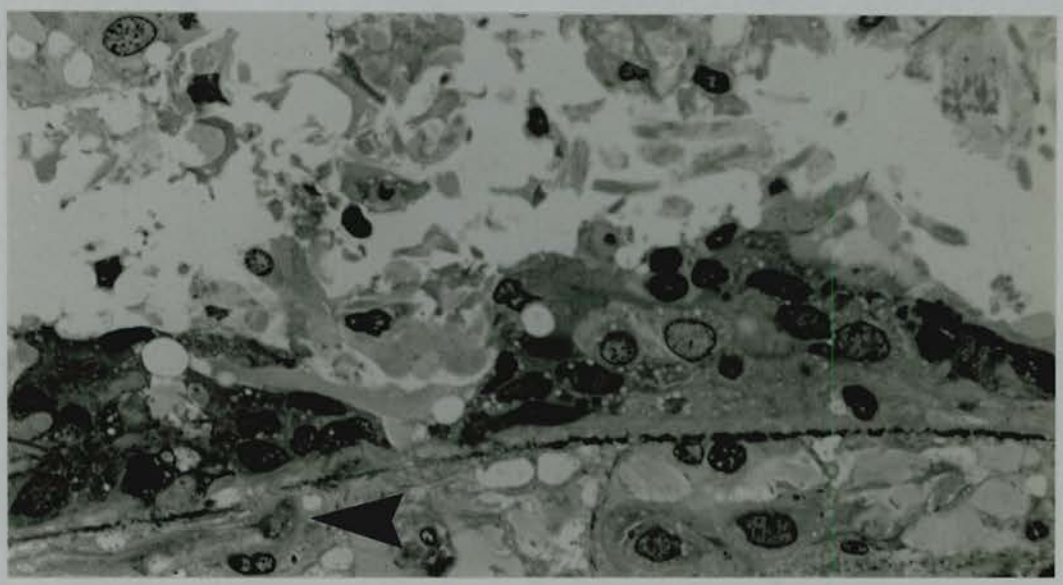


Figure 9

Fragmentation of photoreceptors over clusters of RPE cells, macrophages and lymphocytes. A macrophage (arrowhead) is seen in a break in Bruchs' membrane. x 650

Figure 10

Multilayered RPE (arrow) in a focal area of outer retinal destruction. Bruchs' membrane shows several breaks (arrowheads) and there is an accumulation of lymphocytes in the choroid. x325

Figure 11

Choroidal venule (arrow) and an intraretinal new vessel (arrowhead) showing endothelial cells of HEV morphology. Bruchs' membrane (white arrow) is broken and a macrophage (white arrowhead) is in the process of penetrating through the membrane into the retina. x 1500

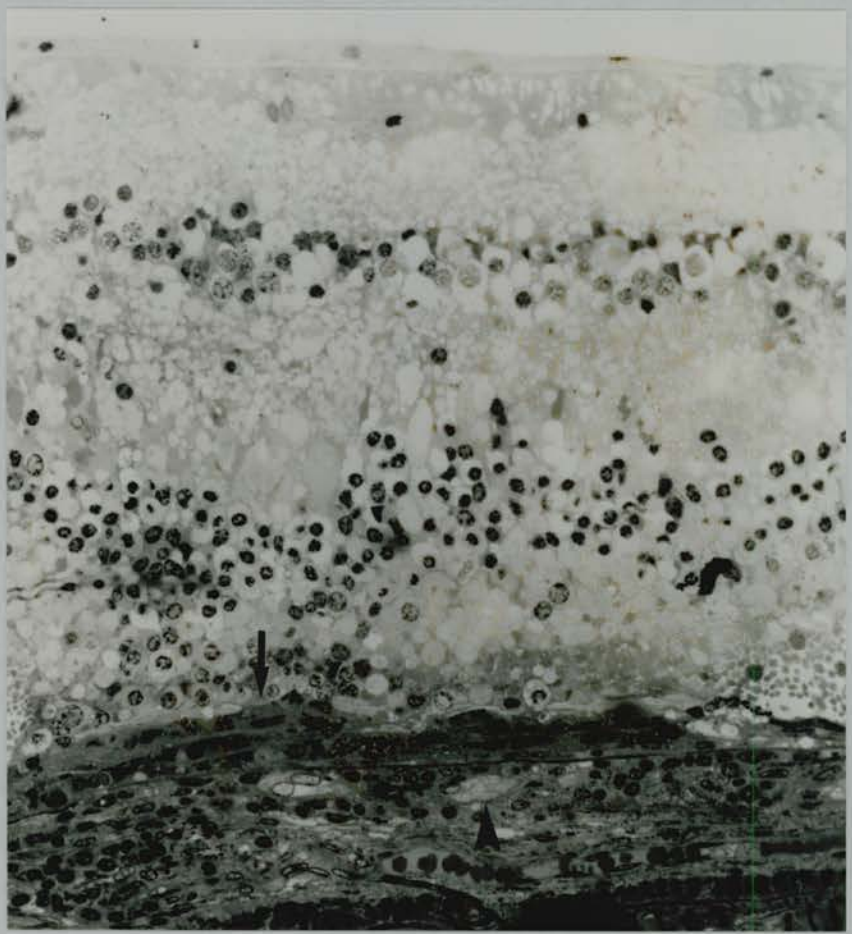


Figure 12

Heavily pigmented lesion (lesion b in figure 4) demonstrating multilayering of the RPE (arrow) with infiltrating macrophages. Note that choroidal vessel endothelial cells are swollen (arrowhead). x350

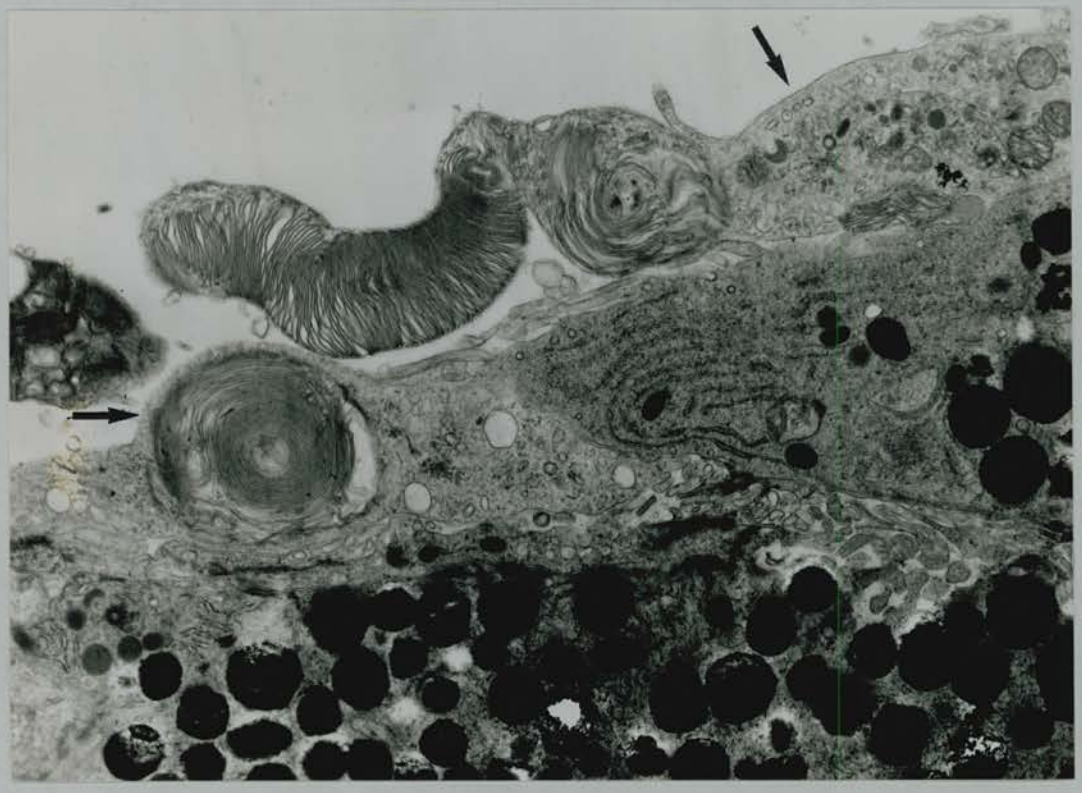


Figure 13

Macrophages (arrows) phagocytosing photoreceptor outer segments within an inflammatory focus. x 6000

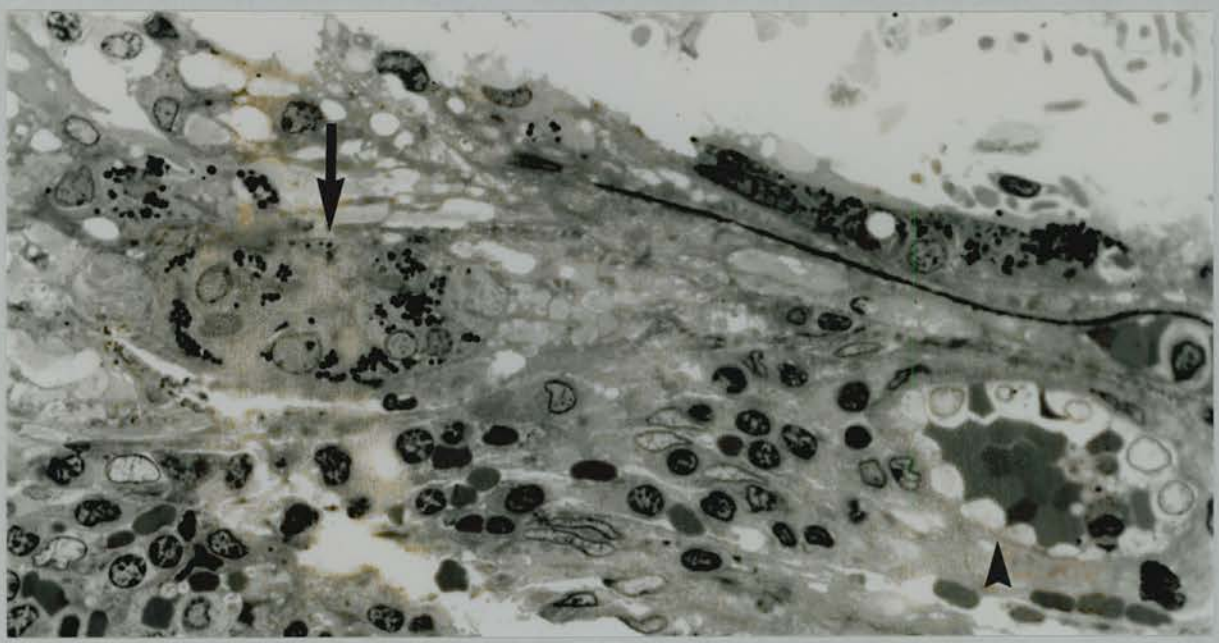


Figure 14

Lesion c in figure 4 showing a collection of RPE cells in the choroid (arrow) adjacent to an area of destruction of the RPE and Bruchs' membrane. The endothelial cells of a choroidal venule have an HEV like appearance (arrowhead). x 650

Figure 15

Swollen endothelial cells (arrow) in the choriocapillaris below intact Bruchs' membrane and RPE cells in an inflammatory focus.

x 2500

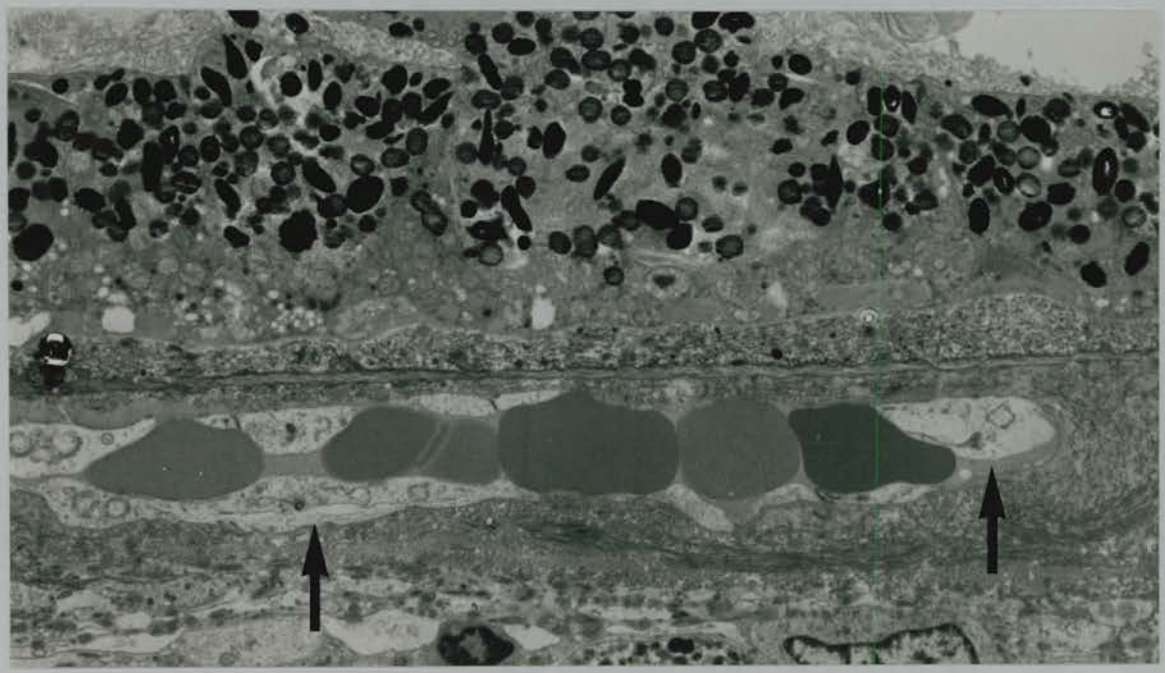


Figure 16

HEV like endothelial cells (arrows) in the choriocapillaris below intact Bruchs' membrane and RPE cells in an inflammatory focus.

x 3000

Figure 17

Early endothelial cell swelling in a choroidal venule (arrowhead) in an inflammatory focus below an intact Bruchs' membrane. x 1500

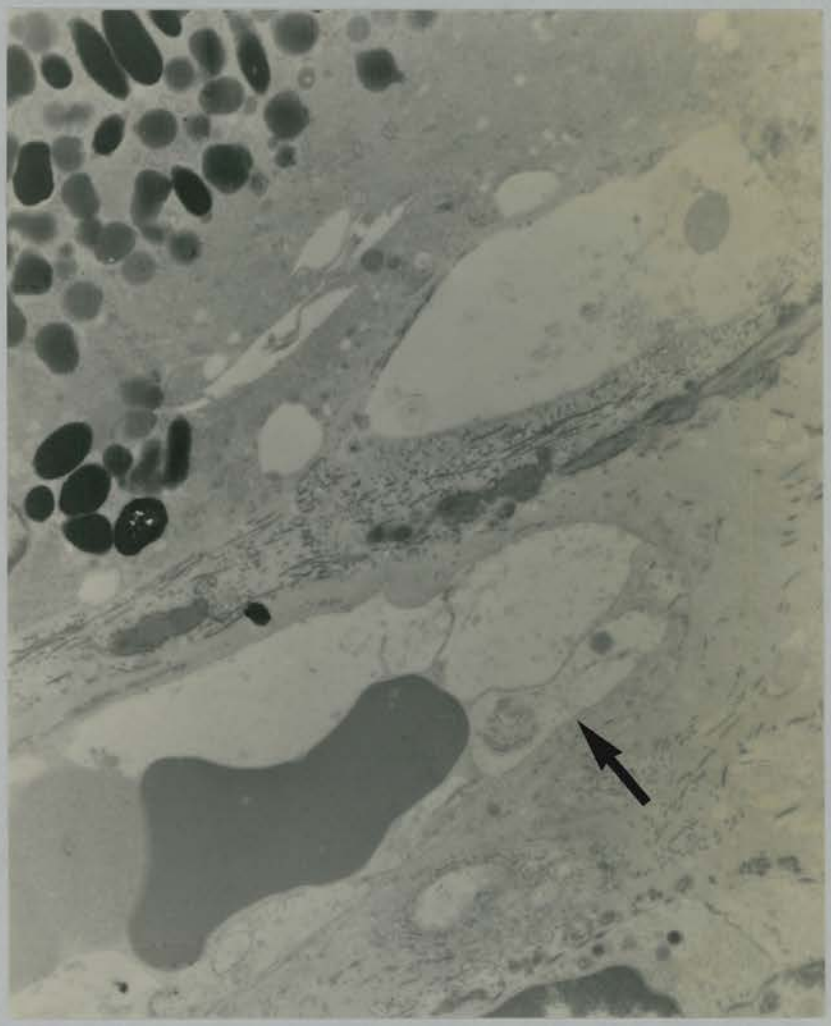


Figure 18

High power electron micrograph demonstrating swollen endothelial cells (arrow) in an inflammatory focus. x 4500

Figure 19

UCHL1 positive T lymphocytes (arrows to examples) within choroidal infiltrate at the site of an inflammatory focus. x 450

Figure 20

OPD4 positive T lymphocytes (arrows) in the choriocapillaris below intact RPE in an area of inflammatory infiltrate. x 450

TABLE 1 MULTIFOCAL POSTERIOR UVEITIS : PRIMARY MONOCLONAL ANTIBODIES

ANTIBODY	SOURCE	DILUTION	SPECIFICITY
UCHL1	Dako	1:100	T lymphocytes *
OPD4	Dako	1:10	T lymphocytes **
L26	Dako	1:50	B lymphocytes
MAC 387	Dako	1:100	Monocytes/macrophages
HLA DR alpha	Dako	1:50	Alpha chain of HLA DR Ag
IgG	Serotec	1:1000	IgG heavy chain
IgM	Serotec	1:1000	IgM heavy chain

* Norton et al. 1986, Terry et al. 1988

** Yoshino et al. 1989, Poppema etal. 1991

CHAPTER 3

STUDIES OF HUMAN INTRAOCULAR INFLAMMATORY DISEASE

B. BEHCET'S DISEASE

3.1 Behcet's disease

3.1.1. Clinical features of Behcet's disease

Behcet's disease is a multisystem inflammatory disorder. Ocular manifestations, which occur in 70-85% of patients, are a recurrent iridocyclitis (classically with a transient hypopyon), choroiditis and retinal vasculitis. These may be severe and be complicated by macular oedema and optic atrophy leading to visual loss (Dinning, 1986; Chajek et al.1975; BenEzra et al.1986). The underlying disease mechanism for the widespread manifestations of this condition is an occlusive vasculitis (Michelson et al.1982), however the aetiology is unknown.

3.1.2. Systemic immunopathology

Studies of aqueous humour (Fenton et al.1964) and peripheral blood (Williams et al.1977; Shimada et al.1974) showing alterations in levels of complement components, led to the proposition that immune complex deposition resulted in the immunopathology of Behcet's disease. Herpes simplex virus genome has been found in mononuclear cells (Eglin et al.1982) and in blood samples (Bonass et al.1991) from Behcet's disease patients. Likewise, increased levels of anti-HSV antibodies (Hamzaoui et al.1990a), reduced proliferative responses of CD4 and CD8 positive T lymphocytes to HSV (Young et al.1988) and elevated cytotoxic responses by CD4 and CD8 positive T cells to cells infected with HSV (Hamzaoui et al.1990b) have also been found in Behcet's disease in studies carried out on peripheral blood. Taken together these findings implicate HSV in the pathogenesis of Behcet's disease.

Streptococcal antigens have been found to induce systemic symptoms

(The Behcet's disease research committee of Japan., 1989) and to activate T cells (Niwa et al.1990) in Behcet's patients leading to speculation that streptococcal infection may play a role in the pathogenesis of the condition. These theories, however, remain unproven and the aetiology of this disease is still uncertain.

3.1.3. Tissue immunopathology

Immunohistological studies of localised tissue pathology in Behcet's disease such as erythema nodosum like lesions (Yamana et al.1986a; Kaneko et al.1985), oral ulcers (Kaneko et al.1985; Poulter et al.1986), and terminal ileum (Yamana et al.1986b) have revealed infiltrates of predominantly T lymphocytes suggesting that these cells produce the tissue damage seen in Behcet's disease. Alterations in peripheral blood T cell populations have been found (Lim et al.1983) and a decrease in suppressor T cell activity has been noted in the pre-active stage of Behcet's disease patients (Sakane et al.1982). These studies are evidence that cell-mediated immune responses are centrally involved in the tissue pathology seen in Behcet's disease.

The studies described below were undertaken with the aim of analysing the local immunopathology of ocular Behcet's disease which is often the most severe manifestation of the condition. These are the first published immunopathological investigations of the localised ocular inflammation found in Behcet's disease and the results of the studies are considered together.

3.2 Materials and methods

Initial immunohistochemical analysis was carried out on five enucleated eyes which were formalin fixed and was directed towards the characterisation of the infiltrating inflammatory cell types in the chronic stages of Behcet's disease. Post-mortem eyes then became available which represented an earlier stage of the condition, one of these eyes was fixed in glutaraldehyde and the other was frozen allowing a more detailed immunohistochemical analysis. On the frozen tissue it was possible to investigate lymphocyte activation using the interleukin 2 receptor monoclonal antibody and to study MHC class II expression using more sensitive monoclonal antibodies.

3.2.1. Patient details

3.2.1.1. Fixed tissue

Five eyes, enucleated for the complications of prolonged ocular inflammation characteristic of Behcet's disease, were obtained from the archives of the Institute of Ophthalmology. In all cases there was a clear history of Behcet's disease confirmed by contacting the clinician involved. Details of the clinical histories are outlined in table 2 (page 131).

3.2.1.2. Frozen tissue

The eyes from a thirty-eight year old male patient who died from Behcet's disease were obtained post mortem. The patient was twenty-nine years old when he diagnosed as having Behcet's disease in 1981 on the basis of a three year history of recurrent retinal vasculitis, anterior uveitis and oropharyngeal aphthous ulcers. These manifestations were controlled initially by high dose oral prednisolone. In 1984 he had two episodes of

deep venous thrombosis and was treated with oral anticoagulants. Between 1985 and 1990 his systemic condition deteriorated with the development of erythema nodosum, multiple joint symptoms and meningoencephalitis which resulted in epilepsy. Treatment with chlorambucil, cyclosporin, cyclophosphamide and azathioprine failed to halt the progression of the disease. The patient died in October 1990 when central nervous system involvement resulted in cerebral vascular leakage causing severe cerebral oedema and raised intracranial pressure which failed to respond to treatment.

During the period 1981-90 he had recurrent episodes of anterior uveitis (with hypopyon) and retinal vasculitis in both eyes. This resulted in retinal ischaemia and visual loss despite systemic immunosuppression and orbital steroid injections. A dense cataract developed in the left eye early in the disease process, but cataract extraction was not considered justified because of the advanced retinal ischaemia. On his last ophthalmic review three months prior to death he had no light perception in either eye. The right optic disc was swollen and surrounded by multiple retinal haemorrhages and retinal oedema (Fig. 21). The retinal vessels in the right eye were sheathed. There was no view of the left fundus due to the lens opacity. Anterior uveitis was minimal in both eyes. Intraocular pressures were normal throughout the course of the disease. Immunosuppressive treatment at the time of death was high dose systemic prednisolone, azathioprine and cyclophosphamide.

3.2.2. Tissue fixation

The enucleated eyes were formalin fixed and paraffin embedded.

Horizontal sections of the whole eye were cut at 6-8 μm thickness. The post-mortem material was processed as follows ; after removal the left eye was frozen in OCT and the right eye was fixed in 4% glutaraldehyde. Horizontal sections, through the pupil and optic nerve, of both globes were cut at 6 μm thickness. Initial staining was carried out using conventional haematoxylin and eosin.

3.2.3. Immunohistochemistry

Immunohistochemical staining on the fixed tissue was then carried out by the peroxidase-anti-peroxidase (PAP) method using a panel of monoclonal and polyclonal antibodies (Table 3, page 132). Sections were dewaxed through toluene and graded alcohols then washed in phosphate buffered saline (PBS) pH 7.3. Endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide in 50% methanol. The slides were washed in PBS and background staining blocked with normal serum appropriate to the secondary antibody at a concentration of 10% in PBS with 2% ovalbumin for 30 minutes. The primary antibody was applied at optimum concentration (ranging from 1:10 to 1:1500) for 45 minutes. Slides were washed and secondary antibody applied at a dilution of 1:50 for 30 minutes. After further washing peroxidase-anti-peroxidase complex was applied for 45 minutes at a dilution of 1:75 in PBS. Slides were washed again and amino ethyl carbazole (AEC) was used as the reaction substrate to give a red final reaction product. Sections were counterstained with haematoxylin and coverslips applied using aqueous based mountant (Dako glycergel).

Negative control sections were processed in identical fashion omitting primary antibody. Control studies were also carried out on eyes with (a)

active sympathetic ophthalmitis - to compare the findings in Behcet's disease with another form of chronic immune-mediated uveitis, and (b) neovascular glaucoma - to compare the findings to minimally inflamed eyes with the complications of the type seen in Behcet's disease. Positive control studies were also carried out on sections known to be positive for each antibody used.

On the post-mortem material sections of the frozen (left) eye were stained immunohistochemically using a panel of monoclonal antibodies (Table 4, page 133) by the avidin-biotin-complex method as described in section 2.3.3.

Sections from each level examined were stained immunohistochemically as above with the omission of the primary monoclonal antibody to provide negative controls. Frozen sections from a normal eye were stained with each monoclonal antibody to demonstrate the distribution of each cell type in uninflamed ocular tissue and known positive tissues (inflammatory eyelid or orbital lesions) were stained with each antibody used.

3.2.4. Cell counting

To quantify the numbers of cells staining with the monoclonal antibodies to leukocyte common antigen (LCA), UCHL1 (T lymphocytes), CD43 (T lymphocytes), OPD4 (T lymphocytes) and L26 (B lymphocytes) cell counts were made from twenty randomly selected high power fields in (a) the choroid and in (b) the retina (and periretinal scar tissue). Cells were considered positive if they had mononuclear morphology and exhibited a ring of red stain around their cell membrane.

3.3 Results

3.3.1. Pathological findings

The five fixed eyes varied in the severity of the intraocular inflammation. The pathological findings in each eye are summarised in table 2 (page 131). Total retinal detachment and periretinal fibrovascular tissue (of varying degrees) were consistent findings in these eyes. Two of the eyes had evidence of secondary neovascular glaucoma complicating the inflammation. The retinae were markedly thinned and degenerate in all the eyes. Inflammatory cell infiltrates were noted around the episcleral vessels, within the optic nerve, within the iris, ciliary body and choroid, around and within the walls of the retinal vessels (Fig. 22) and within the fibrovascular scar tissue. These infiltrates were composed almost entirely of mononuclear cells, neutrophils being a very infrequent finding. In the sections studied there was no evidence of thrombus in any of the retinal vessels. In three of the five eyes there was moderate hyaline thickening of the blood vessel walls in the iris, ciliary body, retina and periretinal scar tissue associated with minimal luminal narrowing.

The pathological findings were similar in both the frozen and glutaraldehyde fixed eyes and represent a less advanced stage of the inflammatory process than the pathological changes seen in the enucleated eyes. There was a mild lymphocytic infiltrate of the iris and ciliary body, this extended to the choroid where there were scattered lymphocytes in all sections examined. The retinal vessels were patent but showed marked hyaline thickening of their walls and luminal narrowing (Fig. 23). There were

infiltrating lymphocytes in this hyaline tissue. There were limited foci of retinal detachment with underlying patches of subretinal fluid. In the right eye the optic nerve head was oedematous and there was a lymphocytic perivasculitis of the central retinal vessels extending into the nerve. The left optic nerve head was not oedematous but had a perivasculitis and a degree of loss of neural tissue.

3.3.2. Immunohistochemical findings

3.3.2.1. Fixed tissue

Throughout the fixed eyes the majority of infiltrating cells were positive for the leucocyte common antigen (LCA) marker. T lymphocytes, identified by the UCHL1, CD43 and OPD4 markers were a consistent finding in the infiltrates (Fig. 24) and were prominent in the perivascular infiltrates in the retina, fibrovascular tissue (Fig. 25) and uveal tract and in the choriocapillaris below disrupted retinal pigment epithelial cells (Fig. 26).

Markers for IgG and IgM immunoglobulins generally gave a faint stain of serous fluid in the subretinal space and within vessels. This staining was observed to be no greater than that in the non-inflamed neovascular glaucoma control eye. In a total of three areas in two eyes there were small collections of cells which showed cell membrane staining for immunoglobulins: two for IgG and one for IgM (Figs. 27 & 28). These cell aggregates corresponded to the B cell clusters seen with the L26 marker. There was no evidence of immunoglobulin deposits within blood vessels or elsewhere within the inflamed tissue. Both the C3c and C1q markers faintly stained the serous fluid within blood vessels and the subretinal fluid. There was no evidence of any solid deposition of complement components.

In the control eye with sympathetic ophthalmitis numerous T and B lymphocytes were found in the choriodal and retinal cellular infiltrates consistent with previous reports (Jakobiec et al.1983; Chan et al.1986c).The lymphocytic infiltrate in the iris of the eye with neovascular glaucoma consisted of minimal numbers of T and B lymphocytes but no inflammation was seen in the retina and choroid demonstrating that the findings in the retina and choroid in the Behcet's disease eyes did not result from the secondary neovascular glaucoma which had developed in two of the eyes.

3.3.2.2. Frozen tissue

Positive immunohistochemical staining for T lymphocytes was seen in cells in the hyalinised retinal vessel walls (Fig. 29) and surrounding the optic nerve head vessels (Fig. 30). These cells also stained positively with the CD4 monoclonal antibody however the small number of cells present precluded cell counts of T cell subset ratios. The perivascular T cells also stained positively with the IL2 receptor monoclonal antibody. Lymphocytes in the iris, ciliary body and choroid were also positive for the T lymphocyte monoclonal antibody although they were generally IL2 receptor negative. No CD8 positive T lymphocytes or B lymphocytes were found. A few cells in the hyalinised vessel walls were positive for the macrophage primary monoclonal antibody. No neutrophils were identified in the vasculitic lesions. A small number of cells in the optic nerve head and retinal vascular endothelium were HLA DR positive. There were also numerous HLA DR positive cells in the retinal pigment epithelium.

In the normal control eye there were very occasional cells in the choroid which were positive for the T lymphocyte and CD4 monoclonal

antibodies. There were also small numbers of cells in the choroid which were positive for the HLA DR monoclonal antibody.

3.3.2.3. Cell counts

The results of the cell counts are presented in tables 5-8 (pages 134-137). The cell counts from the choroid, retina and periretinal scar tissue were analysed together to give an overall picture of the infiltrating cells. T lymphocytes (CD43 positive) made up a mean of 38% of the LCA positive cell population in the choroid, retina and periretinal fibrovascular scar tissue. The OPD4 monoclonal antibody stained a similar number of T cells to the CD43 antibody.

B lymphocytes were an infrequent and variable finding. Overall in the LCA positive population less than 1% of cells in the choroid, retina and epiretinal scar tissue were positive for the B cell marker. In two of the eyes no B cells were seen in the choroid and in three eyes no B cells were found in the retina and fibrovascular scar tissue. B cells did occur in small clusters within the choroidal and perivascular infiltrates (Fig. 31). NK cells, identified by the Leu7 marker, were absent in three of the globes and were a very infrequent finding within the leukocyte infiltrates in the other two.

Macrophages were a consistent finding within the T lymphocyte containing infiltrates being prominent in the fibrovascular scar tissue (Fig. 32) where they constituted an estimated 20-40% of the leucocyte population in some areas. Numerous lymphocytes and macrophages within the leucocyte infiltrates showed positive staining for HLA DR α (Fig. 33). In four of the five eyes small numbers of RPE cells were weakly positive for HLA DR α however very few vascular endothelial cells in the choroid, retina and

periretinal scar tissue were HLA DR α positive.

3.4 Discussion

3.4.1. T lymphocytes in Behcet's disease

These investigations have demonstrated that T lymphocytes are the principle infiltrating cell type in inflammatory foci in Behcet's disease. B lymphocytes, neutrophils and NK cells were not prominent findings nor were there deposits of immunoglobulin or complement components suggestive of immune complex deposition contributing to the pathology. The high proportion of T cells implies a central role for these cells in the immunopathogenic mechanisms responsible for the ocular inflammation in Behcet's disease and helps to explain the favourable response of ocular Behcet's to cyclosporin A (Nussenblatt et al.1983a; Graham et al.1985; BenEzra et al.1986). The dominance of T cells found in the end stage, fixed eyes was subsequently supported by the same finding of CD4 positive T cells in the non-end stage frozen tissue even though the overall cell numbers were much less in this tissue. This finding is in accordance with the results of the immunohistochemical study of multifocal posterior uveitis reported in chapter two and with the results reported by other authors in various forms of intraocular inflammatory disease (Jakobiec et al.1983; Chan et al.1986c; Chan et al.1987; Wetzig et al.1988) it is possible therefore that while the the clinical manifestations of the various forms of intraocular inflammation may differ they may share common immunopathologic mechanisms.

T lymphocytes are also the predominant cell type in other affected

tissue foci in Behcet's disease (Yamana et al.1986a; Kaneko et al.1985; Poulter et al.1986; Yamana et al.1986b) and together with the data in this study this is strongly suggestive that cell mediated immunity is responsible for the tissue damage in Behcet's disease. It is notable that the findings of many of the peripheral blood studies in Behcet's disease (Fenton et al.1964; Williams et al.1977; Shimada et al.1974) do not reflect the tissue immunopathology. This is a similar situation to the findings in sarcoidosis (Daniele et al.1980; Chan et al.1987) where normal or low levels of circulating CD4 positive T cells are found in peripheral blood in contrast to the large numbers of T cells in the affected tissues.

Examination of the earlier stage pathology seen in the frozen tissue showed a perivascular and intramural lymphocytic infiltrate in the retinal and optic nerve vessels together with marked hyaline thickening of the vessel walls and luminal narrowing. This feature has been reported in pathological studies of tissue from the eye (Fenton et al.1964; Shikano, 1966) and other affected foci (Michelson et al.1982) in Behcet's disease. The lymphocytes were identified in the study of the frozen tissue to be CD4 positive IL2 receptor positive T cells. The perivascular lymphocytes in ocular sarcoidosis (Chan et al.1987) and pars planitis (Wetzig et al.1988) have also been identified as T cells.

3.4.2. Cell counts

Neither UCHL1 nor CD43 are specific for CD3 positive T lymphocytes. The UCHL1 antibody recognises a peptide of the CD45 family (Terry et al.1988) which is present on 72% of CD4 positive and 35% of CD8 positive T cells (Norton et al.1986) although it may stain small numbers of

macrophages and myeloid cells. The CD43 antibody (Dako DFT1) stains a high percentage of T cells but also some B cells, neutrophils and macrophages (Stross et al.1989). Since few B cells were found in the infiltrates (see results) and neutrophils and macrophages could be distinguished morphologically the cell counts with the CD43 antibody were used to represent the T cell population in the assessment of ratios of cell types. The OPD4 monoclonal antibody reacts against the CD45RO determinant and selects a similar population of T lymphocytes to the UCHL1 antibody (Poppema et al.1991) although it does not react against monocytes or granulocytes, the initial report of this antibody suggested that it was reactive against a helper/inducer T cell subpopulation (Yoshino et al.1989).

3.4.3. Localisation of pathological changes in Behcet's disease

In Behcet's disease the early stage pathology seen in the post-mortem frozen tissue is primarily focussed on the retinal and optic nerve vasculature with associated destruction of related retina and optic nerve tissue. The choroiditis appears to be a secondary event. In multifocal posterior uveitis, by comparison, the primary pathological damage is at the level of the RPE and outer retina and there is a marked, focal choroidal infiltrate. Additionally the advanced vascular wall thickening and luminal narrowing seen in the retina and optic nerve in Behcet's disease was not seen in multifocal posterior uveitis where the vascular changes were found focally in the choroid. Therefore, although there may be shared immunopathological processes, the anatomical site of the tissue pathology (and also, by implication, the inciting antigens) may differ between forms of intraocular inflammation.

In experimental autoimmune uveoretinitis there is often marked pathological change in both the retina and choroid (where there are no limiting anatomical factors) although "high endothelial venule" changes have been reported to occur only in the retinal vasculature (Dua et al.1991) in the Lewis rat model, implying that these are the sites of lymphocyte trafficking, and that this model is closest to human intraocular inflammation where retinal vasculitis is the dominant feature. It would be of interest to carry out electron microscopic examination on the vasculature of human eyes with active retinal vasculitis to investigate the morphology of the vascular endothelial cells.

3.5.3. Antigen presentation in Behcet's disease

The cellular expression of the MHC class II antigen HLA DR was found to be similar in both the unfixed frozen tissue and the formalin fixed tissue. Expression of class II by tissue resident cells such as the RPE and vascular endothelium (which constitute the the blood-retinal barrier where antigen presentation could potentially happen) was generally at a lower level than described in other forms of intraocular inflammation (Chan et al.1986a; Chan et al.1987; Chan et al.1988b; Wetzig et al.1988). Aberrant expression of MHC class II antigens has been demonstrated on endogenous tissue cells in conditions thought to be of autoimmune aetiology (Hanafusa et al.1983; Ballardini et al.1984) and it has been suggested that these cells may play a role in antigen presentation to infiltrating CD4 positive T lymphocytes (Bottazzo et al.1983) such as those demonstrated in these studies. However as discussed in chapter 1 the expression of MHC class II antigen does not by itself confer the ability to present antigen (Morahan et

al.1989) and may indeed lead to T cell anergy in the absence of an appropriate co-stimulatory signal (Schwartz, 1990; Gaspari et al.1988). Given the low level of aberrant class II expression by tissue resident cells found in the above studies and the numerous class II positive macrophages present in the later stages of the disease process it appears likely that macrophages are the major antigen presenting cells at this stage. The role of MHC class II positive tissue resident cells, such as RPE and vascular endothelial cells, in vivo remains uncertain and may be of central importance to disease progression since these cells have the potential to induce anergy (and also possibly to activate T cells if the necessary co-stimulatory signals are present). The balance of the in vivo result of antigen presentation by macrophages and by tissue resident cells with autoreactive T cells could therefore determine the outcome of the interaction of such T cells with self antigen following an initial ocular insult. Macrophage presentation of antigen to T cells producing upregulation of the anti-self response and subsequent autoimmune disease whereas antigen presentation by tissue resident cells leading to down regulation and acting to limit the autoaggressive response.

3.4.5. Treatment of Behcet's disease

It is notable that despite heavy immunosuppressive therapy the perivascular T cells in the frozen tissue continued to express IL2 receptors. This demonstrates the difficulty in adequately treating the severe vasculitis seen in Behcet's disease. The success of future immunotherapy for Behcet's disease depends on targetting specific cellular components involved in the pathogenesis of the tissue destruction. The demonstration of IL2 receptor

positive, CD4 positive T lymphocytes in the vasculitic lesions in ocular Behcet's disease suggests that downregulating the effects of these cells would be beneficial in controlling the severe ocular inflammation.

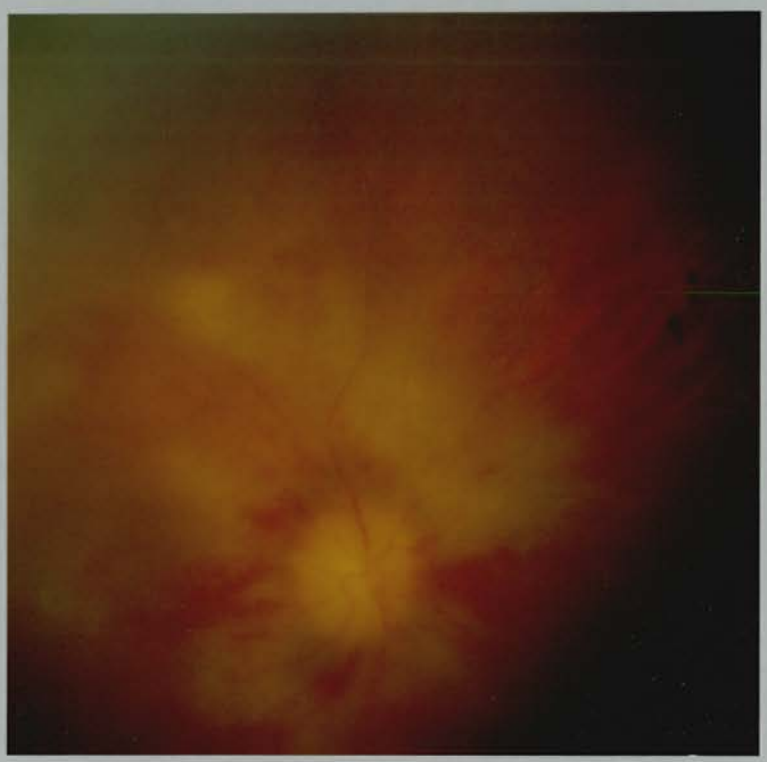


Figure 21

Right optic fundus demonstrating swollen optic disc with surrounding retinal oedema and haemorrhage.

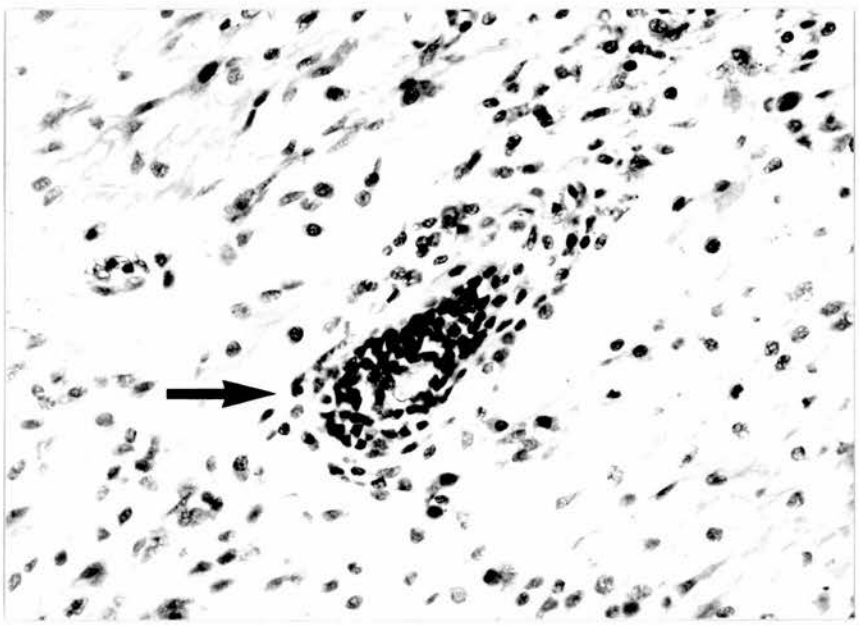


Figure 22

Behcet's disease: perivasculitis with lymphocytic infiltration in fibrotic retina.

x 360 H&E

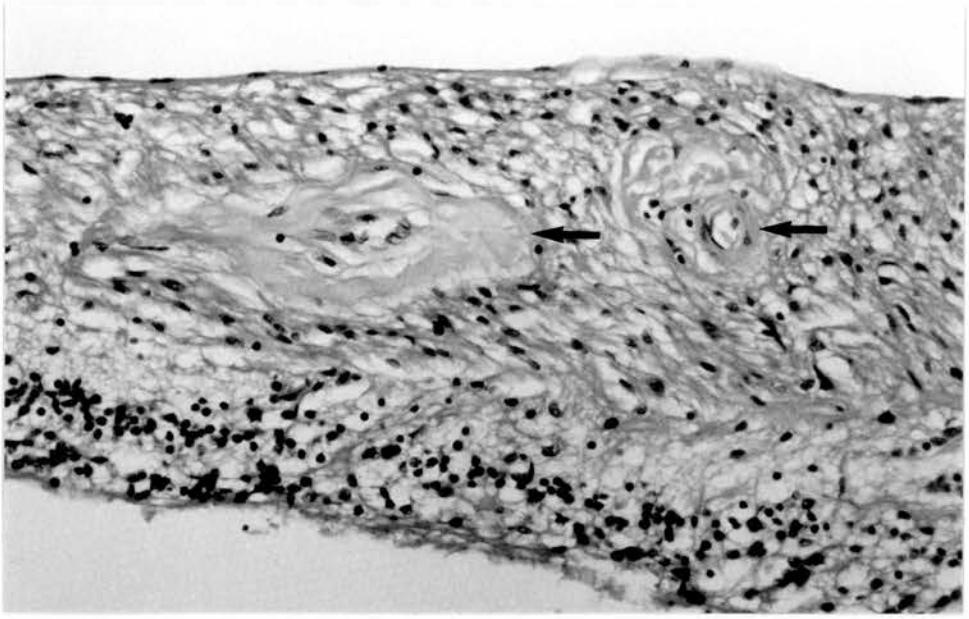


Figure 23

Hyaline thickening and luminal narrowing (arrows) of vessels in disorganised retina. x 280 H&E

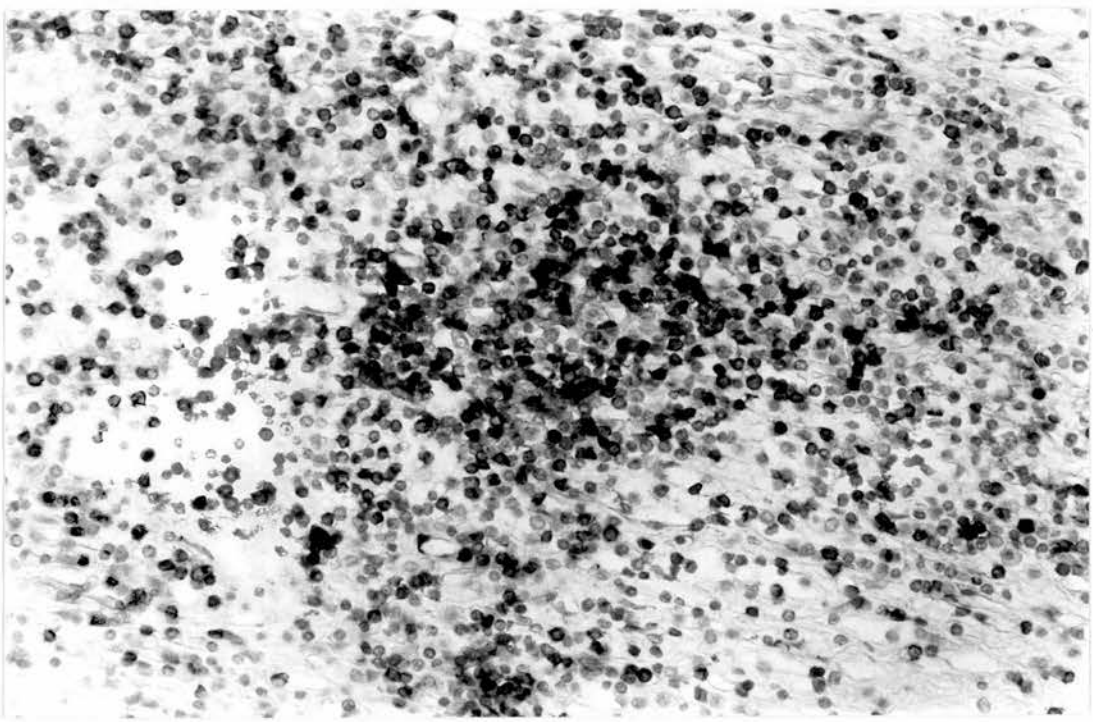


Figure 24

OPD4 positive T lymphocytes (identified by a ring of cell membrane staining) in fibrovascular scar tissue in ocular Behcet's disease. x350 haematoxylin counterstain.

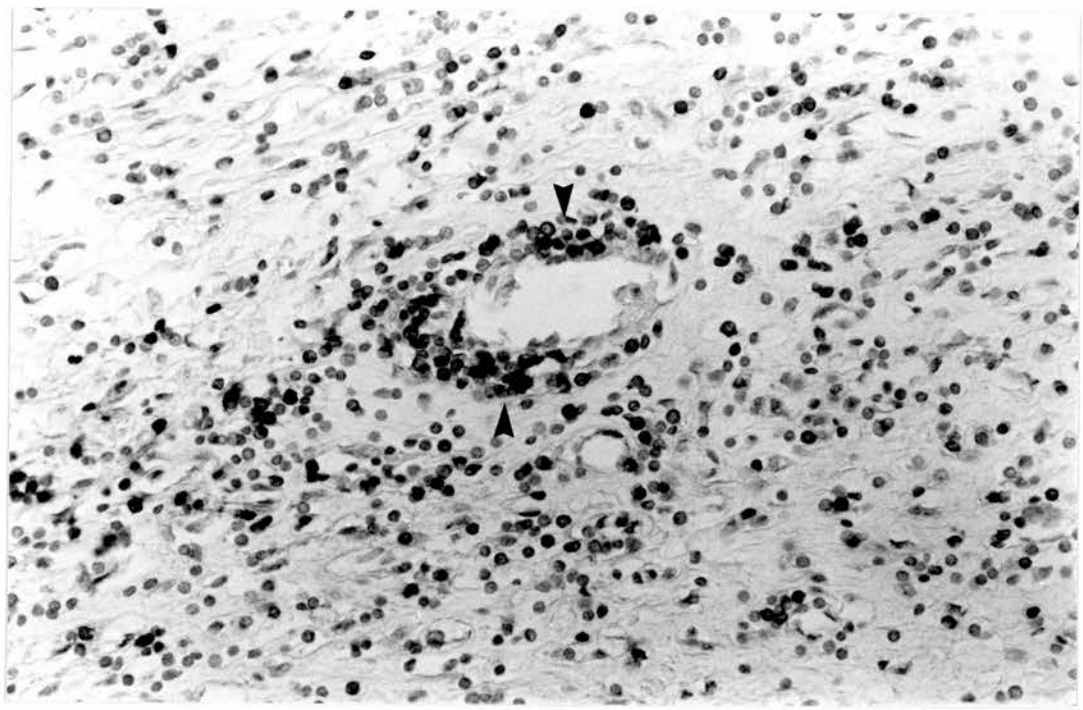


Figure 25

Perivascular distribution of OPD4 positive T lymphocytes (arrowheads, identified by a circle of cell membrane staining) in fibrovascular tissue in advanced Behcet's disease. x 250, haematoxylin counterstain.

Figure 26

UCHL1 positive T lymphocytes (arrows) in choroid below disrupted RPE (arrowheads). x 450

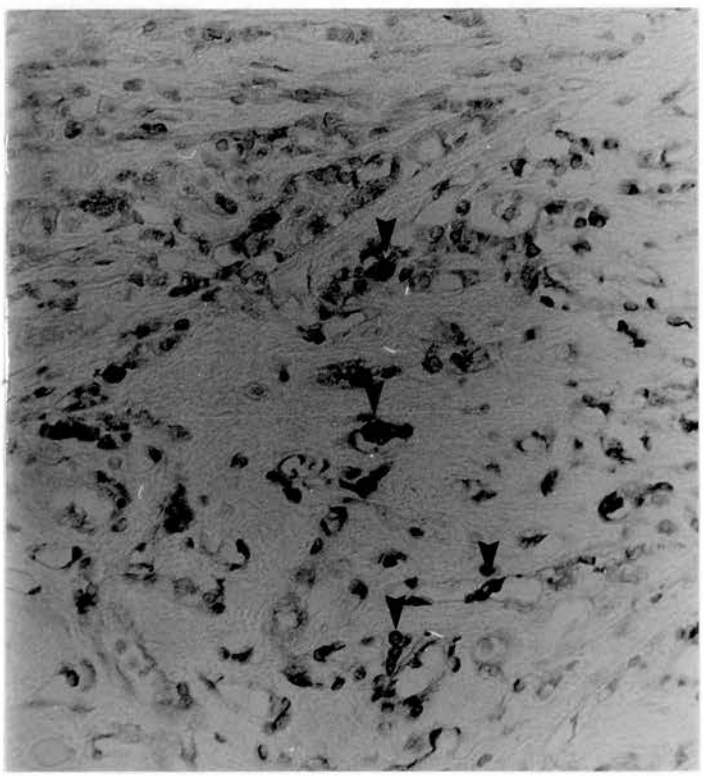


Figure 27

Positive staining for IgM immunoglobulin (arrowheads) on cells within an inflammatory infiltrate. x 250, haematoxylin counterstain.

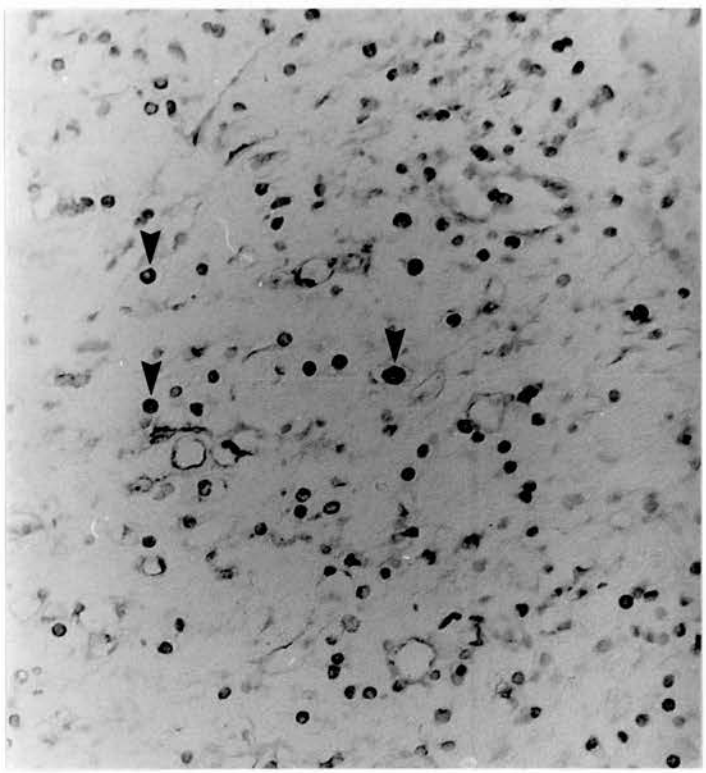


Figure 28

Scattered cells positive for IgG immunoglobulin (identified by a circle of cell membrane staining, arrowheads to examples) in an inflammatory infiltrate. x 250, haematoxylin counterstain.

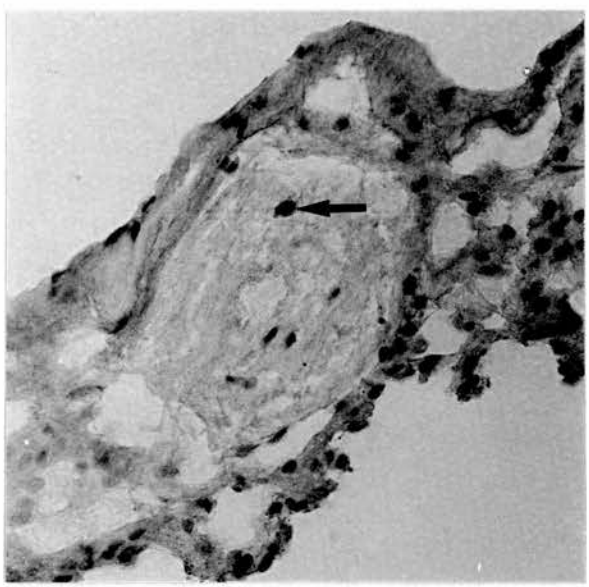


Figure 29

T lymphocyte (arrow) in the wall of a hyalinised retinal vessel (T3 monoclonal antibody). x360, haematoxylin counterstain.

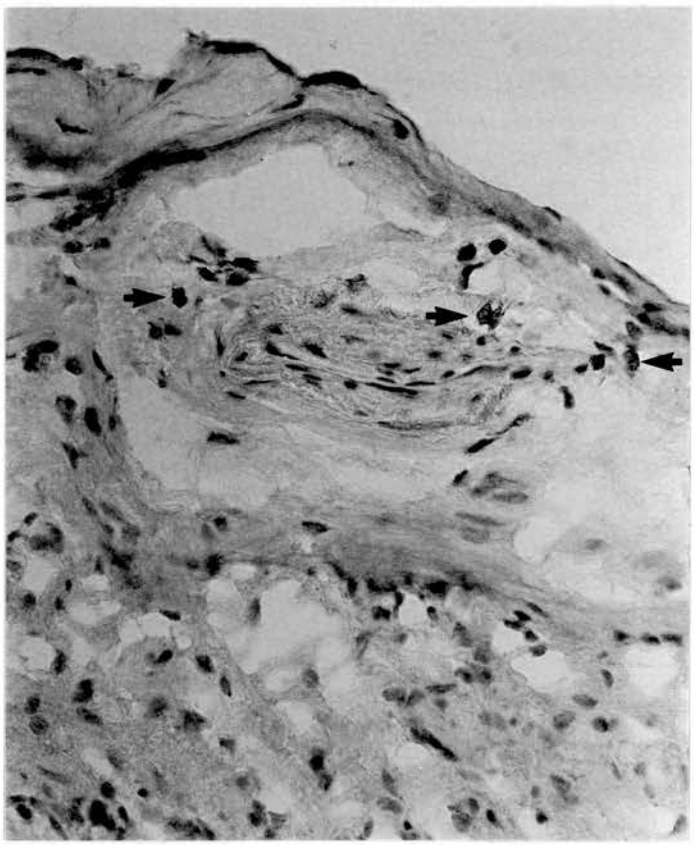


Figure 30

T lymphocytes (arrows) around vessels in optic nerve head. x 360, T3 monoclonal antibody, haematoxylin counterstain.

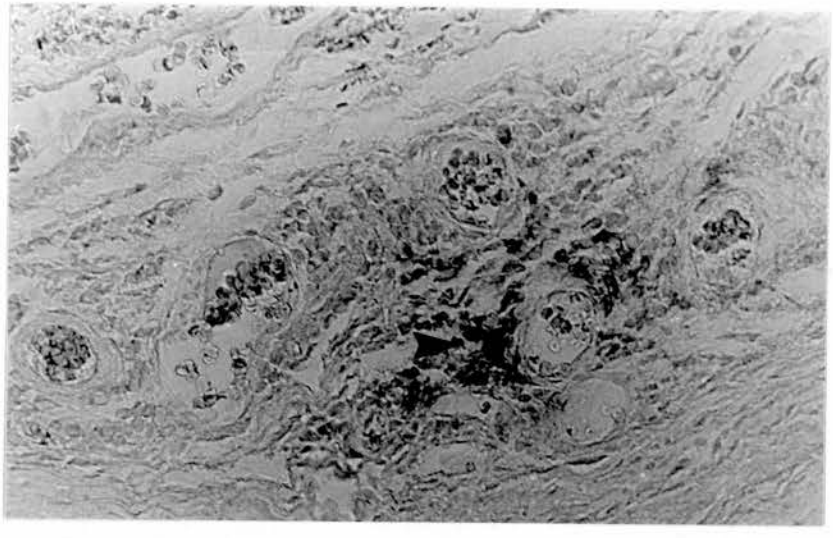


Figure 31

A small cluster of B lymphocytes (arrowhead) related to a vessel in fibrovascular scar tissue. x 200, L26 monoclonal antibody, haematoxylin counterstain.

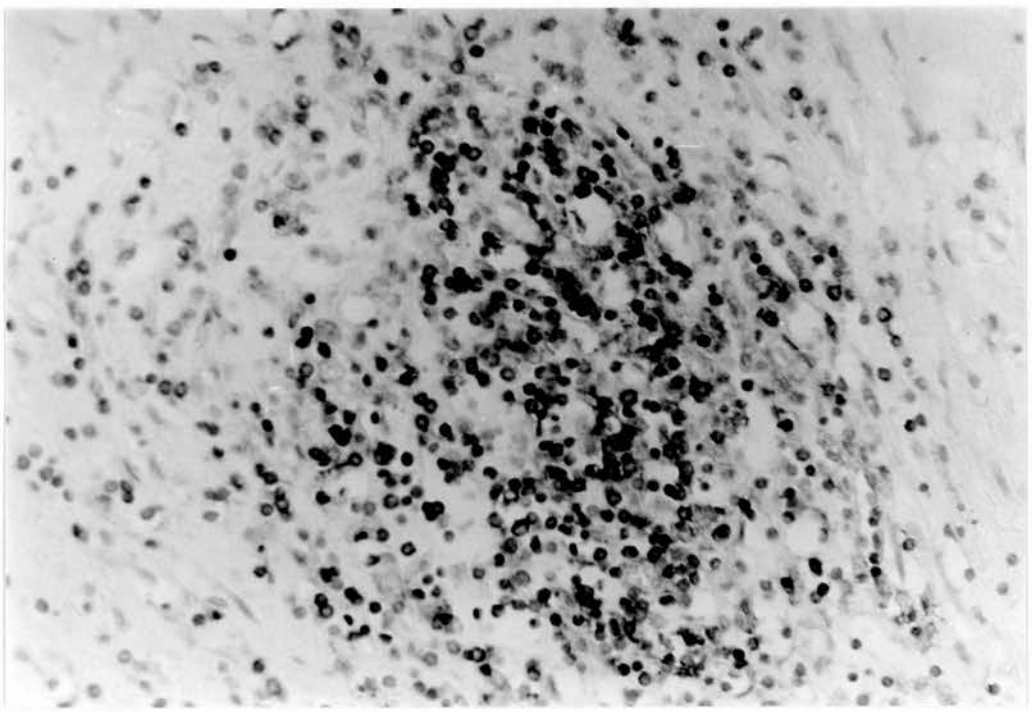


Figure 32

Numerous macrophages (MAC 387 monoclonal antibody, identified by a circle of cell membrane staining) within fibrovascular tissue. x 250, haematoxylin counterstain.

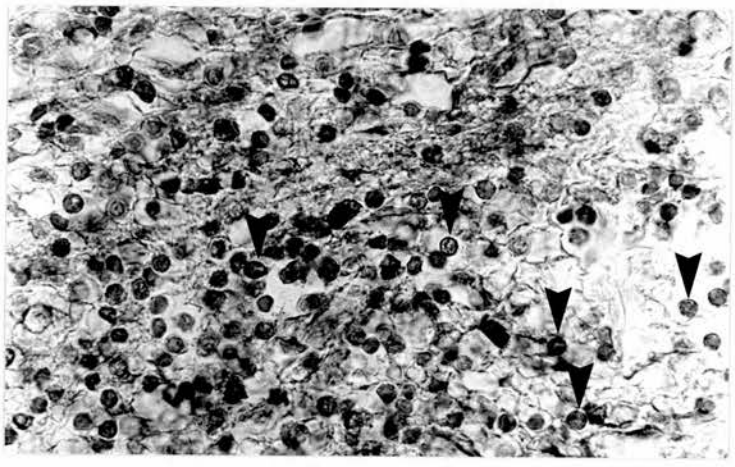


Figure 33

HLA DRα positive inflammatory cells (arrowheads to examples) within fibrovascular scar tissue. x 350, haematoxylin counterstain.

TABLE 2

BEHCET'S DISEASE :

Clinical and pathological details of formilin fixed specimens

Patient No	Age/ Sex	Disease duration	Clinical features/ treatment	Systemic features	Pathological findings
1 1090/89	16 M	3 years	Panuveitis Recurrent hypopyons Rubeosis Blind Painful Systemic steroids	Oral ulceration	Panuveitis Granulation tissue Microabscesses Rubeosis
2 2100/81	37 M	NR	Panuveitis	NR	Panuveitis Vascular scarring
3 1974/80	27 M	3 years	Panuveitis Macular oedema Unilateral Blind Painful Systemic steroids	Orogenital ulceration	Nongranulomatous uveitis Retinal detachment Intraocular haemorrhage
4 1301/89	36 M	NR	Panuveitis Rubeosis Glaucoma Blind Painful	NR	Iritis Mild focal choroiditis Retinal detachment Rubeosis Intraocular haemorrhage
5 1392/89	19 F	2 years	Panuveitis Painful Blind Systemic steroids Azathioprine	NR	Low grade uveitis Retinal vasculitis Fibrovascular epiretinal membrane Microabscesses

NR = not recorded

TABLE 3

BEHCET'S DISEASE :

PRIMARY ANTIBODIES USED ON FIXED TISSUE

ANTIBODY	Source	Dilution	Specificity
Leucocyte Common Antigen (LCA)	Dako	1:50	Leucocytes (CD45)
UCHL1	Dako	1:100	T lymphocytes *
DFT 1	Dako	1:75	T lymphocytes (CD43) **
OPD4	Dako	1:10	T lymphocytes
L26	Dako	1:50	B lymphocytes
Leu 7	Becton Dickinson	1:100	NK cells
MAC 387	Dako	1:100	Monocytes/macrophages
HLA DR alpha	Dako	1:50	Alpha chain of HLA DR Ag.
IgG	Serotec	1:1000	IgG heavy chain
IgM	Serotec	1:1000	IgM heavy chain
C1q	Serotec	1:1500	Complement component C1q
C3c	Serotec	1:1500	Complement component C3c

* Norton et al. 1986, Terry et al. 1988

** Stross et al 1989

All mouse monoclonal antibodies except C1q and C3c : sheep polyclonal

TABLE 4

BEHCET'S DISEASE : PRIMARY MONOCLONAL ANTIBODIES USED ON FROZEN TISSUE

ANTIBODY	SOURCE	DILUTION	SPECIFICITY
T3	Dako	1:50	Pan T lymphocytes
T4	Dako	1:10	CD4+ T lymphocytes
T8	Dako	1:100	CD8+ T lymphocytes
L 26	Dako	1:50	B lymphocytes
IL2 R	Dako	1:100	Interleukin 2 receptor
MAC 3	Dako	1:200	Monocytes/macrophages
HLA DR	Dako	1:100	HLA DR antigen expression

TABLE 5

BEHCET'S DISEASE: (formalin fixed specimens)

Cell counts - Mean positive cell numbers per high power field

(40 fields counted for each antibody per specimen)

	Antibody				
Specimen	LCA	UCHL1	CD43	OPD4	L26
1 (1090/89)	55.2	16.7	19.1	26.6	0.5
2 (2100/81)	53.3	14.6	15.2	16.7	0
3 (1974/80)	23.6	3.3	18.5	9.2	0.5
4 (1301/89)	18.0	3.8	3.8	11.0	0.1
5 (1392/89)	25.6	1.5	10.0	3.3	0.9

TABLE 6

BEHCET'S DISEASE:

Overall mean cell counts per high power field of formalin fixed eyes (40 fields counted in each of 5 specimens)

Antibody	Mean cells per HPF	Standard deviation
LCA	35.1	17.7
UCHL1	8.0	7.1
CD43	13.3*	6.4
OPD4	13.4	8.8
L26	0.4**	0.4

* CD43 = 38% of LCA positive cells

** L26 = 1% of LCA positive cells

T cell : B cell ratio (CD43:L26) = 33:1

Standard error of difference between CD43 and L26 counts = 2.8

(See notes on statistical methods appendix, page 222)

Significance of difference between CD43 and L26 counts $P<0.001$

TABLE 7

BEHCET'S DISEASE:

RETINA AND PERIRETINAL SCAR TISSUE
Cell counts - Mean positive cell numbers per high power field

(20 fields counted for each antibody per specimen)

	Antibody				
Specimen	LCA	UCHL1	CD43	OPD4	L26
1 (1090/89)	29.5	7.8	10.4	26.6	0.1
2 (2100/81)	104.6	28.8	29.0	31.0	0
3 (1974/80)	22.8	3.0	13.8	1.4	0
4 (1301/89)	14.7	1.0	1.2	11.0	0.1
5 (1392/89)	12.4	0.8	2.2	1.0	0

TABLE 8

BEHCET'S DISEASE:

CHOROID

Cell counts - Mean positive cell numbers per high power field
(20 fields counted for each antibody per specimen)

	Antibody				
Specimen	LCA	UCHL1	CD43	OPD4	L26
1 (1090/89)	80.8	25.6	27.8	26.5	0.8
2 (2100/81)	2.0	0.5	1.4	1.4	0
3 (1974/80)	24.4	3.7	23.3	17.2	1.0
4 (1301/89)	21.2	6.6	5.5	11.1	0
5 (1392/89)	38.8	2.2	17.8	5.6	1.8

CHAPTER 4

IN VIVO LYMPHOKINE PRODUCTION IN EXPERIMENTAL AUTOIMMUNE UVEORETINITIS

4.1 Introduction

Experimental autoimmune uveoretinitis is a well characterised animal model of intraocular inflammatory disease and is reviewed in chapter 1. Investigation of the posterior segment inflammatory cellular infiltrate in the Lewis rat model of EAU has shown that there is a predominance of mononuclear cells (lymphocytes and macrophages) and that these cells are mostly T lymphocytes (Faure, 1980; Gery et al.1986; Chan et al.1985a). In this respect the model corresponds to the T cell infiltrate found in the studies of multifocal posterior uveitis and Behcet's disease described in chapters 2 and 3, and to the findings in other forms of human intraocular inflammatory disease (Jakobiec et al.1983; Chan et al.1986c; Chan et al.1987; Wetzig et al.1988). Furthermore the T cell infiltrate seen in the early stages of EAU in this model is predominantly of the CD4+ subset (Chan et al.1985a) a similar finding to most studies of human disease. The Lewis rat model of EAU therefore provides a basis for the experimental study of posterior segment ocular immunopathology which can be considered analogous to the immunopathology seen in human intraocular inflammatory disease.

4.2 Investigation of effector cells in EAU

Immunohistochemical analysis has demonstrated the presence of lymphocytes and macrophages in the destructive pathology in EAU and has also classified the lymphocyte subsets. This information does not, however, tell us the role that these cells play in the immunopathological processes responsible for the tissue destruction and in particular which cells are the effector cells. An understanding of these processes will help us to design

more specific therapeutic modalities for the treatment of human intraocular inflammation.

4.2.1. Cytokine production by effector cells

Cytokines are of major importance in the tissue destruction produced in immune-mediated disease. Cellular interactions mediated by cytokines can produce MHC antigen upregulation, cellular chemotaxis and recruitment, and the activation and proliferation of effector cells, processes central to the development of immune-mediated tissue pathology. In addition cytokines may directly produce tissue destruction. The lymphocyte-derived cytokine lymphotoxin, for example, has a cytotoxic action (Tite et al.1985; Paul et al.1988; Feldmann et al.1989) and could potentially cause local tissue damage.

The function of T lymphocytes has been correlated with their lymphokine production patterns (Mosmann et al.1986; Powrie et al.1988; Powrie et al.1989; McKnight et al.1991; Paliard et al.1988; Salmon et al.1989; Mason et al.1990). Analysis of the in vivo lymphokine production by T lymphocytes would therefore help define the functional nature of these cells. Blocking the action of the effector cells in intraocular inflammation has the potential to provide a means of controlling the damage produced by chronic intraocular inflammation.

4.2.2. Analysis of cytokines

Several approaches are available in the analysis of the cytokine action in vivo. Samples of tissue fluid can be analysed for the presence of bioactive

cytokine using specific bioassays, this approach is of limited value in the investigation of ocular disease since samples of such fluid are rarely available for analysis and are not necessarily representative of the local tissue effects of cytokines which may act only in the microenvironment of the secreting cell. Monoclonal antibodies reactive against cytokine protein are now available for some cytokines which allow the immunohistochemical analysis of tissue sections for cytokine protein. This approach, however, does not define the cell of origin of the secreted protein or the number of cells actively producing a cytokine.

Analysis of cytokine mRNA can be carried out using molecular biology techniques. Using specific DNA or RNA probes cytokine mRNA can be localised on tissue sections using in situ hybridisation. The principle of in situ hybridisation is the use of a labelled, specific DNA or RNA probe to hybridize with mRNA within a cell (Penschow et al.1987; Penschow et al.1986). The resulting hybrid is then detected using autoradiography if the probe is radiolabelled or an enzymatic detection system such as avidin-biotin or digoxigenin. The tissue section or cell smear can then be analysed for the presence of signal. This approach has the advantage of detecting cells which are actively producing cytokine mRNA within tissues. The mRNA may also be extracted and analysed using the "northern" blotting technique or can be amplified using the polymerase chain reaction (PCR) (Bell, 1989) where the mRNA is present in small copy numbers (as is the case for most cytokines).

4.2.3. Aims of the investigation of cytokines in EAU

The aim of the experimental work described in this chapter was to analyse the pattern of expression of mRNA for the lymphokines interferon-γ, IL2, lymphotoxin, and IL4 in vivo in EAU at various time points during the development of the disease and to relate this to the cellular infiltrate. Interferon-γ, IL2, lymphotoxin and IL4 are well characterised cytokines with multiple actions (Trinchieri et al.1985; Haworth et al.1989; Paul et al.1988; Paul et al.1987). All are produced by T lymphocytes which have been shown to be the predominant cell type in the destructive tissue pathology of EAU. These lymphokines were selected for study to produce an overall picture of the destructive process in EAU. Interferon-γ, IL2 and IL4 are important in cytotoxic T lymphocyte (CTL) differentiation (Chen et al.1986; Haworth et al.1989) and also in B lymphocyte activation (Haworth et al.1989). Interferon-γ and lymphotoxin appear to be important mediators of CD4+ T cell cytotoxicity (Tite et al.1985).

The actions of an individual cytokine in a complex cytokine network in the microenvironment of destructive pathology is difficult to determine. The cellular secretion pattern of these four cytokines does, however, define the functional cellular subtype of the infiltrating cells. With regard to murine CD4+ T cells interferon-γ, IL2 and lymphotoxin are produced by the Th_1 subset thought to function as effector cells and IL4 is produced by the Th_2 subset thought to act as helper cells (Mosmann et al.1986). Although the subdivision of CD4+ T cells appears to be more complex in rats and humans (Powrie et al.1988; Powrie et al.1989; McKnight et al.1991; Paliard et al.1988; Salmon et al.1989; Mason et al.1990) in the rat the essential

subdivision appears to be between CD4+ T cells producing IL2 and interferon-γ and those producing IL4 (Powrie et al.1988; Powrie et al.1989; McKnight et al.1991).

The pattern of cellular secretion of these four lymphokines will therefore help identify the functional nature of the T cells involved in the destructive pathology in EAU. Analysis of lymphokine secretion was carried out using a combination of (a) immunohistochemistry using primary antibodies to lymphocytes and, where available, to cytokines and cytokine receptors, and (b) in-situ-hybridisation using molecular probes to cytokine mRNA. No previous studies have analysed the localisation of cytokine protein or mRNA in EAU.

4.3 Materials and methods

4.3.1. Animal model

Female Lewis rats (bred under SPF conditions, St Thomas' Hospital Medical School), 100-150 grams weight, 6-8 weeks old were used in all experiments. Rats were immunised subcutaneously in a hind footpad and base of tail with a total of 50 μg of purified bovine S-antigen (prepared at the Institute of Ophthalmology, London) (Dorey et al.1982) (Fig. 34) in an emulsion in complete Freund's adjuvant supplemented with Mycobacterium tuberculosis organisms to a final concentration of 2.5 mg/ml. Animals were simultaneously given heat inactivated Bordetella pertussis organisms in 150 μl PBS intraperitoneally. Animals were killed by cervical dislocation following ether anaesthesia at days 10,11,12,13,14, 17 and 21 post-immunisation, the eyes rapidly enucleated, embedded in optimal cutting

temperature compound (OCT) and snap-frozen in acetone and dry-ice. Specimens were stored at -70^0 C until used.

4.3.1.1. Preparation of tissue sections

Horizontal sections of the whole eye were cut on a cryostat, these were cut as serial sections where technically feasible. For in-situ-hybridisation sections were cut at 12 μm thickness and mounted on slides prepared as follows: washed slides were dipped in a solution of 0.25% gelatin (300 bloom swine) and 0.025% chromium potassium sulphate, dried and dipped again. For immunohistochemistry 6 μm sections were mounted on slides coated in 3-Aminopropyltriethylsilane (APES) (2% in methylatred spirit). Slides were stored at -70^0 C until used to preserve mRNA.

4.3.2. Probe preparation

Four cDNA probes were used for the in-situ-hybridisation. The rat interferon γ probe consisted of a 528-bp comprising two identical repeats of a 264-bp sequence corresponding to the last 21 residues of exon 2, all of exon 3 and the first 60 residues of exon 4 as deduced from the rat IFN γ gene structure (Dijkema et al.1991). The rat IL2 probe was a full-length 740-bp fragment corresponding to rat IL2 mRNA (McKnight et al.1989). The rat IL4 probe consisted of the first 406-bp of the specific rat IL4 gene structure (McKnight et al.1991). The lymphotoxin probe was a 1.42 kbp coding sequence derived from the murine lymphotoxin gene (Li et al.1987). Lymphotoxin is a highly evolutionary conserved protein (the human protein (Nedwin et al.1985) has 74% homology to the murine protein (Li et al.1987; Paul et al.1988)) and the murine probe reacts with rat lymphotoxin

mRNA.

4.3.2.1. Probe amplification

Probe containing plasmids (with ampicillin resistance gene) were amplified in E. Coli bacteria (HB 101, ampicillin sensitive) using the following method. A single colony of E. Coli was grown overnight in 50 ml of LB medium (1% bactrotryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) at 37^0C. These were spun twice at 4500 RPM and resuspended in Tfx solution (30mM NaCl, 5mM $MgCl_2$, 5mM Tris-Cl pH 7.4), spun again and resuspended in 5 ml Tfx. 200 μl of cells were then aliquoted into eppendorf tubes and plasmid added at concentrations of 50ng/10ml and 1ng/10ml in duplicate. These were incubated on ice for 1 hour and heat shocked at 42^0C for two minutes, 0.6 ml LB medium added and incubated for 30 minutes at 37^0C. 40μl from each sample was then plated onto LB agar/ampicillin plates. The eppendorfs were then centrifuged in a microcentrifuge, the supernatant discarded and the final 40μl including the residual pellet plated as above. These plates were incubated at 37^0C overnight and single colonies were selected and again grown overnight.

4.3.2.2. Analysis of plasmid DNA

To verify the above bacterial transformation a "mini-prep" analysis of the bacteria was carried out as follows: a single colony was grown in 5ml LB medium (supplemented with 50μg/ml ampicillin), 1.5ml was then transferred to a eppendorf tube and centrifuged for 20 seconds to pellet the bacteria. The supernatant was removed and the pellet resuspended in 100μl 50mM dextrose, 25mM Tris-HCl pH 8.0, 10mM Na_4EDTA pH8.0 and left at room temperature for 7 minutes. To this tube 200μl of 1% SDS, 0.2N NaOH was

added and this was incubated on ice for 7 minutes. 150μl of ice cold 0.1M K acetate solution was added and this was left on ice for 7 minutes and centrifuged for 15 minutes. Supernatant was transferred to a fresh eppendorf tube 0.25ml saturated phenol, 0.25ml $CHCl_3$-isoamylalcohol (24:1) added and microcentrifuged for 15 seconds at room temperature for phase separation. The top (aqueous) phase containing plasmid DNA was removed, 1ml 95% ethanol added, mixed and left for 15 minutes at room temperature. The tube was then centrifuged for 5 minutes to pellet the DNA and the alcohol supernatant poured off. The pellet was then rinsed in 70% ethanol, centrifuged again, the alcohol poured off and the pellet air dried. This pellet was then resuspended in 50μl TE buffer (10 mM Tris pH 8.0, 0.1M EDTA pH 8.0). 5μl of this solution was then used for enzyme digestion.

Plasmid DNA digestion was carried out using restriction endonuclease enzymes specified for the individual probe/plasmid combination (EcoRI and BamHI for the interferon gamma, IL2 and lymphotoxin probes, Hind III and BAM H1 for the IL4 probe) together with the appropriate buffer solution. Digestions were carried out at 37^0C for 2 hours. DNA was then denatured by heating to 65^0C for 2 minutes. Gel loading buffer (glycerol 50%, 1xTAE buffer with 1% bromophenol blue, 1% xylene cyanol) was added to the digest sample and to controls at 1/5th of the final volume. Control DNA samples used were (a) Hind III cut λ phage and (b) 123 bp ladder. Samples and controls were run together on gel electrophoresis apparatus (at approx. 60V) using 0.9% agarose (typeII, medium EEO) gel with 12 μl ethidium bromide (10mg/ml). Photographs of the gels were taken using UV transillumination. By comparison of digest DNA with control DNA it was possible to verify the

presence of the appropriate bp sequence of the probe within the plasmid extracted from the transformed bacteria.

4.3.2.3. Probe extraction (large-scale)

Single colonies of plasmid containing E. Coli were grown at 37^{0}C in 100 ml of ampicillin supplemented LB broth overnight. The medium was then spun at 5000 rpm for 10 minutes, the pellet resuspended in 4 ml ice-cold TEG buffer (50mM glucose, 25 mM Tris.HCl, 10mM EDTA, pH=8.0) 8 ml 0.2M NaOH, 1% SDS added and left on ice for 5 minutes. 6ml of 3M NaAcetate pH5.2 was then added, mixed and left on ice 5 minutes and spun at 15000 rpm for 20 minutes. The supernatant was taken and 18 ml of isopropanol added, mixed, left at room temperature for 5 minutes and spun at 7000 rpm for 10 minutes. The supernatant was poured off, the pellet washed in 70% ethanol and spun again at 7000 rpm for 10 minutes. The pellet was then redissolved in 0.5 ml TE buffer, 5μl of 10mg/ml RNAaseA (in 10mM Tris.HCl pH 7.5) and incubated at 37^{0}C for 30 minutes. DNA was extracted by mixing with 0.25 ml Phenol/Tris, 0.5 ml Phenol/$CHCl_3$ (three times), 0.5 ml $CHCl_3$ and 0.5 ml ether (twice) and taking the aqueous layer each time after seperation. 50μl of 3M NaAcetate pH5.2 was then added together with an equal volume of isopropanol, left at room temperature for 10 minutes and spun on a microcentrifuge for 10 minutes. The pellet was then washed in 70% ethanol, spun and resuspended in 200μl of TE buffer.

The concentration of DNA in solution was measured reading the optical density using a spectrophotometer. 5μl of sample with 995μl of water was read at a wavelength of 260 nm: the DNA concentration (μg/μl) is equal to 10

times the optical density. The purity of the sample was assessed by taking a second reading at 280 nm. A ratio of the optical densities at 260 nm: 280 nm equal to 2.0 is due to pure nucleic acids, if the ratio is less than 1.6 then the sample has significant protein contamination. Where protein contamination was present in a sample a further extraction with Phenol/$CHCl_3$ and ethanol precipitation was performed.

4.3.2.4. Probe labelling

Probes were radiolabelled using the random-primer technique (Boehringer mannheim). 25-100 ng of probe was used for each labelling. Probe was initially denatured by heating to 95^0C for 10 minutes then cooled on ice. To the denatured probe 5μl of ^{35}S (=25μCi, specific activity >1000Cl/mmol) dCTP (CTP was used for initial experiments), 1μl of dATP, dGTP and dTTP, 2μl of reaction mixture (hexanucleotide in 10x concentrated reaction buffer), 1μl of Klenow enzyme and 8μl of distilled water were added. This was incubated at 37^0C for 30 minutes and 2μl of EDTA (0.2mol/l, pH 8.0) was then added to stop the reaction. This was adjusted to 100μl with G50 column buffer (0.1M NaCl, 0.05M Tris-HCl pH8.0, 0.001M EDTA, 0.02% SDS). A G50 sepharose column was then prepared by running through 3ml TE buffer, the probe was then run through the column and collected in a second aliquot of 400 μl TE buffer. Radioactivity was measured on a scintillation counter by measuring 1μl with 99μl scintillation fluid. Probe activity was then adjusted to $2x10_5$ CPM/ml in hybridisation buffer (see below) prior to hybridisation.

4.3.3. In situ hybridisation

All glassware and solutions were treated with 1% diethylpyrocarbonate (DEPC) followed by autoclaving to ensure that there was no exogenous RNAase activity during hybridisation. Slides were warmed to room temperature and fixed for five minutes in 5% glutaraldehyde in 0.1M phosphate buffer pH7.2 (0.1M Na_2HPO_4, 0.1M KH_2PO_4) with 20% ethylene glycol then rinsed twice in hybridisation buffer (600mM NaCl, 50mM $NaPO_4$ pH7.0, 5mM EDTA, 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.1% DNA (salmon testis) and 50% deionised formamide). The slides were then soaked in hybridisation buffer for one hour (found to be the optimal time to prevent background signal) rinsed in ethanol and air dried. The radiolabelled probe in hybridisation buffer was heated to 90^0C for 10 minutes, cooled and 100μl applied under a parafilm coverslip. Sections were left to hybridize at room temperature for 72 hours in a humidified chamber. Post hybridisation slides were immersed in 2xSSC (2xSSC=0.3M NaCl, 0.03M Na citrate) until the coverslips dislodged, rinsed in 2xSSC and washed at 40^0C for 30 minutes in1xSSC. Slides were then rinsed briefly in distilled water, in 70% ethanol for five minutes, in 95% ethanol for five minutes and allowed to dry.

4.3.4. Autoradiography

Slides were taped to blotting paper and overlaid with an X-ray film in a film cassette and exposed for 3-4 days. This film was then developed to evaluate the strength of the hybridisation signal. Slides were then dipped in K5 (Ilford) photographic emulsion diluted 1:1 in 0.5% glycerol and left to

expose over silica gel at 4^0C for 21-24 days depending on the hybridisation signal strength on the X-ray plate. The slides were then developed for 3.5 minutes in D19 developer (Kodak) fixed in Unifix (Kodak), washed in distilled water, counterstained with haematoxylin and coverslips mounted.

4.3.5. Controls

The following control studies were carried out simultaneously with the in-situ-hybridisation described above.

4.3.5.1. Non-immunised animals

Eyes from non-immunised Lewis rats were processed in the same manner as above to define the lymphokine gene expression in normal eyes.

4.3.5.2. RNAase

To confirm that positive signal was produced by probe binding to RNA on the section, slides from each eye studied were treated with RNAase to abolish specific binding to tissue RNA. Sections were fixed for five minutes in glutaraldehyde buffer, washed once in 2xSSC with 5% Tween and three times in 2xSSC. 100μl of RNAase A 1mg/ml in 2xSSC was applied to each slide under a parafilm coverslip and incubated at 37^0C for 1 hour, slides were then washed in 2xSSC and hybridisation carried out as above.

4.3.5.3. Irrelevant probe

To determine the specificity of the lymphokine probe hybridisation, sections from each eye were also hybridised with a ^{35}S labelled probe to the P53 oncogene which was considered to be irrelevant to the EAU disease process. This hybridisation was carried out using an identical protocol to the procedure detailed above.

4.3.6. Immunohistochemistry

Immunohistochemistry was carried out using the avidin-biotin-complex method. Slides were warmed to room temperature then fixed for 7 minutes in acetone and endogenous peroxidase activity blocked by immersion in 3% hydrogen peroxidase in 50% methanol. Normal serum was then applied for 20 minutes. This was tipped off and primary monoclonal antibodies (Table 9, page 186) applied in appropriate dilution in PBS for 30 minutes. Slides were then washed in PBS and rat-adsorbed biotinylated secondary antibody applied for 30 minutes . Following further washing in PBS, ABC complex was applied for 45 minutes. The slides were again washed, developed in AEC to give a red final reaction product and counterstained with haematoxylin. Coverslips were mounted using aqueous-based mountant.

4.4 Results

There was consistent induction of bilateral intraocular inflammation in the experimental animals using the immunisation schedule described above.

4.4.1. EAU Pathology

The description below represents the results of observations made on the eyes of 35 immunised animals. These findings are summarised in table 10 (page 187).

4.4.1.1. Disease Onset

Clinical observation of the immunised rats revealed conjunctival hyperaemia in all animals with onset at days 12-13 post-immunisation. In a

minority of animals onset of hyperaemia varied between the eyes by one day.

Eyes from animals killed at days 9-11 post-immunisation did not show any evidence of inflammatory disease. All animals at day 12 showed evidence of early intraocular inflammatory changes. These changes consisted of small numbers of mononuclear cells infiltrating the retina (Fig. 35a) and a mixed infiltrate of neutrophils and mononuclear cells in the iris, ciliary body and anterior chamber (Fig. 35b).

4.4.1.2. Early disease (Day 13)

Histopathologically there was a consistent mixed leucocytic infiltrate of the structures of the anterior segment of all eyes examined. Leucocytes were numerous in the anterior chamber by day 13 post-immunisation (Fig. 36a) with only minimal variation in this pattern. The retinas of all eyes examined contained an infiltrate of predominantly mononuclear leucocytes. In a majority of eyes examined at day 13 early foci of photoreceptor necrosis and oedema were observed (Fig. 36b)

4.4.1.3. Intermediate disease (Days 14-17)

Clinically animals had hyperaemic eyes with consistently hazy media. Most animals also had variable degrees of hypopyon formation in the anterior chamber. Histopathologically all animals had a mixed leucocytic infiltrate in the structures of the anterior segment of similar intensity to that observed at day 13 post-immunisation. All animals had inflammatory changes observed in the posterior segments although the intensity of the inflammation varied. Localised foci of outer retinal oedema and necrosis showed variable degrees of development (Fig. 37a-c) along with localised

areas of retinal detachment with underlying subretinal serous fluid. A mixed infiltrate of inflammatory cells was also found in the vitreous. By day 17 the majority of photoreceptors in most of the animals examined were degenerate. All eyes had a mononuclear cell infiltrate in the choroid, the cells being more numerous in eyes where there was more advanced retinal destruction.

4.4.1.4. Late stage disease (Days 18-21)

Clinically the eyes were less hyperaemic although most still had hypopyons. Histopathologically the anterior segment inflammation was in general less intense. A degree of retinal destruction was seen in all eyes - this varied fron total loss of retinal architecture with extensive retinal detachment (Fig. 38) to more focal retinal destruction and oedema with areas of intact retinal architecture still identifiable. There was a marked accumulation of mononuclear cells in the choroid at this stage of the disease process (Fig. 38).

4.4.2. Cellular Immunohistochemistry

T lymphocytes, identified by the OX 19 monoclonal antibody, were found in inflammatory infiltrates throughout the extraocular tissues, anterior chamber, ciliary body, choroid and retina at all stages of the disease process. Comparison of CD4+ to CD8+ T cells (identified by the W3/25 and OX8 antibodies respectively) found a predominance of W3/25 positive cells in the early and intermediate phases of the disease and increasing numbers of OX8 positive cells as the disease progressed, as has been reported previously (Chan et al.1985a). T lymphocytes in the inflammatory infiltrates

in both the anterior and posterior segments also showed positive staining for IL2 receptor expression (Fig.39). MHC class II expression (OX6 antibody) was found on cells from day 10 post-induction onwards, this increased markedly as the disease progressed and was seen on both organ-resident and infiltrating cells.

4.4.3. Lymphokine Immunohistochemistry

The interferon-γ monoclonal antibody gave positive staining of cells from day 12 post-induction onwards. Mononuclear cells in the extraocular tissues, anterior chamber, ciliary body, choroid, retina, vitreal cavity and subretinal fluid all showed positive staining (Fig. 40). The staining intensity increased as the inflammatory process progressed.

4.4.4. In Situ Hybridisation

Eyes from 24 immunised and 4 control (non-immunised) rats were studied by in-situ-hybridisation. The time points examined were as follows: days 10 and 11, two animals from each day, days 12,13,14,17 and 21, four animals from each day. A total of 398 individual hybridisations were carried out to determine the pattern and reproducibility of the results. The results of all hybridisations were integrated to produce an overall analysis of the findings.

Levels of background signal (the amount of scattered dark grains in the photographic emulsion) varied on the numerous hybridisation procedures carried out but in all experiments, for all four probes, discrete collections of dark grains were identifiable over the background signal. The collections of

autoradiographic signal were seen in sections from eyes at day 12 post-immunisation onwards and occured over mononuclear cells in the areas of the T lymphocyte infiltrate. These foci were interpreted as positive localisation of expression of lymphokine mRNA by lymphocytes.

The characteristics of the pattern of hybridisation for each probe is described in the sections below. To provide an estimate of the relative numbers of cells expressing mRNA for an individual lymphokine at a given time-point in the disease, the number of positive foci in the retina and choroid were counted on each eye section and the mean value taken at each time point. Standard deviations and standard errors of the means of these counts were calculated. These figures were grouped together as early disease (days 12 and 13 post-immunisation), established disease (days 14 and17) and advanced disease (day 21) in the tables and graphs used to illustrate the results for each probe.

4.4.4.1. IFNγ Probe

No positive localisation of hybridisation signal was seen in sections of eyes studied at days 10 and 11 post-hybridisation. Positive hybridisation foci were observed from day 12 post-immunisation onwards. In the early stages of the disease process positive cells were seen in the outer retina and choroid (Fig. 41). These cells were often related to the localised patches of retinal oedema and destruction which were seen in the model at this stage (Fig. 42). There were also numbers of positive cells in the anterior extraocular tissues at the early stage of the disease. As the retinal destructive process progressed increasing numbers of positive cells were seen in the choroid (Fig. 43) as well as in accumulations of inflammatory

cells in the ciliary body and the anterior extraocular region. At the later time points when there was marked retinal destruction positive foci were a variable finding in the necrotic retinal tissue and consistently high numbers of positive foci were found in the choroid .

The relative numbers of positive foci throughout the disease process are documented in table 11(page 188) and illustrated in figure 44. This data demonstrates that there is a significant increase in the numbers of positive foci in late disease and that this is a reflection of the increase in foci counted in the choroid. The numbers of positive foci in the retina declined in established and late disease due to the loss of intact retinal tissue. The standard deviations and standard errors for the counts of positive foci are relatively high especially in the early and established phases of the disease process, this is due to the variation between counts from individual eyes.

It was notable that fewer cells were positive for interferon-γ mRNA than for immunohistochemical localisation of interferon-γ protein as demonstrated using the interferon-γ specific monoclonal antibody. Although there was a heavy infiltrate of mixed inflammatory cells in the anterior chamber no positive signal was seen over any cells in this area.

4.4.4.2. IL2 Probe

There were no localising foci found in the eyes studied at days 10 and 11 post-immunisation. Positive signal for IL2 mRNA was found from day 12 post-immunisation onwards. In the early phase of the disease there was an accumulation of positive cells around the areas of retinal oedema and destruction (Fig. 45). These positive cells were localised to the inner retina despite the focal necrosis of the photoreceptors. At the early disease stage

there were also minimal numbers of positive cells in the choroid and vitreous. In the eyes with established and advanced disease there were numerous positive cells in the retina and vitreous, and markedly increased numbers of positive cells scattered throughout the ciliary body and choroid.

The relative numbers of positive cells present within the eyes of immunised animals are documented in table 12 (page 189) and figure 46. These results show that the number of positive foci increased in the choroid as the disease progressed and the difference from early disease reached statistical significance in the choroid and also in the overall figures. Numbers of positive foci in the retina showed no significant change. The relatively high standard deviations and standard errors reflect the variation between individual sections examinedNo positive signal was seen on cells in the anterior chamber. Numerous positive cells were seen in the extraocular inflammatory infiltrates in both anterior and posterior extraocular regions at all stages of the disease process.

4.4.4.3. Lymphotoxin Probe

Positive hybridisation signal from the lymphotoxin probe was generally less strong than the other probes utilised. No positive cells were seen at days 10 and 11 post-immunisation. In the early phase of the disease positive cells were most numerous in the extraocular inflammatory cell infiltrates but a few scattered positive cells were also found in the choroid and very occasional positive cells in the retina at this stage. Positive foci of hybridisation signal were prominent in the choroidal infiltrate in the later phase of the disease. In general very few lymphotoxin mRNA positive cells were found in the retina. No positive cells were found in the anterior

chamber.

Numbers of positive cells increased as the disease progressed (Fig. 47), these results are documented in table 13 (page 190) and illustrated in figure 48. The data presented in this table and figure demonstrates that there is a significant increase in the number of positive cells in the late phase of the disease and that this is due to the increased numbers present in the choroid at this stage. The relatively high standard deviations and standard errors again reflects the variation between individual sections.

4.4.4.4. IL4 Probe

Hybridisations with the IL4 probe revealed positive cells from day 12 post-immunisation onwards, there were no positive foci at days 10 and 11 post-immunisation. Positive foci of hybridisation were seen in the inflamed retina (mainly the inner retina, related to patches of retinal necrosis and destruction), choroid and extraocular infiltrates in the early phase of the disease. In the established and late stages of the disease (Fig. 49) there was a marked increase in the numbers of positive cells found in the ciliary body and choroid. Small numbers of cells in the retina and subretinal fluid were positive in the later stages of the disease process. Again, no positive signal was seen on any of the numerous inflammatory cells in the anterior chamber.

The numbers of positive cells throughout the disease process are documented in table 14 (page 191) and figure 50. The significant increase in positive cells in late disease seen with the IL4 probe was a consistent finding in the uveal tract. In the retina numbers of positive foci decreased in late disease due to destruction and loss of retinal tissue.

4.4.4.5. Overall Results of In-situ-hybridisation

The numbers of positive cells for each probe was generally a small percentage (less than 10%) of the total T lymphocyte number as defined by the OX19 antibody. On the frozen sections examined immunohistochemically it was not possible to make accurate cell counts of the T cell numbers because of the difficulty in defining individual cells with positive immunohistochemical stain in areas where there was a marked accumulation of infiltrating mononuclear cells. Hence it was not feasible to calculate the percentage of cells giving positive hybridisation signal within the T cell population. It was, however, possible to compare the mean overall number of positive cells for each lymphokine probe within the eyes (in all areas) throughout the disease process, this is illustrated in figure 51. This figure shows that the increase in positive cell numbers in late disease was consistent to all four probes. The increase in cell numbers is also seen in the established phase of the disease (days 14-17) for the IL4 probe and to a lesser degree also the IL2 probe. Because the protocol used was not standardised between probes valid statistical comparison of the probes is not possible.

4.4.5. Controls

4.4.5.1. Non-immunised Animals

No autoradiographic signal suggestive of localisation of lymphokine mRNA was found using any of the probes in any of the four non-immunised animals studied. Immunohistochemical analysis showed no expression of MHC class II antigens within the eyes and only very occasional T

lymphocytes in the anterior extraocular region which were not IL2 receptor positive.

4.4.5.2. RNAase Pretreatment

Analysis of sections pretreated with RNAase at a concentration of 0.1mg/ml for 30 minutes revealed faint residual traces of concentrations of autoradiographic signal at the sites of positive foci found on the non-pretreated sections. The concentration of RNAase was therefore increased to 1mg/ml and the incubation with RNAase increased to one hour, it was found that this regime abolished all localising signal on the sections of all the positive eyes for each probe (Figs. 45,47 &49).

4.4.5.3. P53 Probe

Sections hybridised with the P53 oncogene probe revealed a generalised background signal over areas of inflamed tissue without any focal cellular localisation.

4.5 Technical aspects of EAU studies

Immunohistochemical staining was carried out on unfixed tissue since the monoclonal antibodies used do not recognize epitopes on fixed tissue, this results in morphology which is in general less well preserved. The initial immunohistochemical staining on the EAU eyes was performed with non-adsorbed secondary antibody and gave high levels of background stain, subsequent staining was therfore carried out using a rat-adsorbed secondary monoclonal antibody which abolished this problem.

Modification of various parts of the techniques used in these studies would be of benefit in the further development of the investigations. Cutting

whole eye sections of frozen rat eyes on a cryostat is technically difficult due to the delicate nature of the tissues and the lack of support provided by the vitreous cavity to the unfixed retina. Brief fixation of the eyes (for example with paraformaldehyde) could potentially produce an improvement in morphology without compromising immunohistochemical staining or mRNA hybridisation. Sectioning unfixed eyes to allow penetration of fixative was performed in an earlier study however this resulted in retinal detachment in all eyes and disruption of intraocular morphology.

Matching lymphokine mRNA expression to individual cells stained immunohistochemically was not possible in this study although it was feasible to localize such mRNA expression to areas of T lymphocyte infiltrate. Serial sectioning of eyes would be desirable to match cells and tissues on any given slide to an adjacent slide processed using a different technique. Cutting serial sections of frozen, unfixed ocular tissues is extremely difficult both because of the delicacy of the tissues themselves and the variation in section thickness caused by minor fluctuations in cryostat temperature. A potential solution to this problem would be to use a double staining immunohistochemical technique (although this would not have been possible for rat cytokines since IFN-γ is the only cytokine to which monoclonal antibodies are available) or to combine immunohistochemical staining and in-situ-hybridisation on a single slide, a method which would involve considerable technical difficulties due to the different requirements of the two methods.

Beta irradiation from the ^{35}S labelled probe tends to spread within the photographic emulsion and the resulting signal is therefore not precisely

localised over a cell. In this investigation the results were not compromised by this effect however a more accurately localised signal could be obtained by using a probe radiolabelled with an alternative radioisotope. An alternative to radiolabelling of the probe is to use an enzymatic detection system such as digoxigenin which has proven useful in the detection of viral genomes (Furuta et al.1990). Enzymatic methods provide rapid results by avoiding prolonged autoradiographic exposures and avoid the constraints and risks of the use of radioactivity in laboratories, however it is uncertain whether enzymatic systems are at present sensitive enough to detect low-copy genes such as those for cytokines. In the investigations it was notable that radiolabelling the DNA probe with CTP rather than dCTP gave similar overall results, although its probe incorporation was much reduced this was compensated by using a more concentrated hybridisation mixture. Occasional slides were found to be unexpectedly negative when hybridised, having been positive on previous hybridisations, this can be explained by a contamination with RNAase during the hybridisation procedure.

Alternative methods could be employed to further investigate the production of lymphokines by infiltrating cells in EAU. Cells could be extracted from the eyes and analysed phenotypically on smears (or using flow cytometry) lymphokine mRNA could then be analysed in a semi-quantitative manner using northern blot analysis or the polymerase chain reaction (Bell, 1989) if only small quantities of mRNA message were found to be present. Extracted cells could also be cultured as cell lines and the lymphokine secretion measured by these molecular methods and supported by assays of biological activity. Experiments involving the in vitro stimulation

of rat T lymphocytes failed to detect IL4 mRNA by in-situ-hybridisation which was found only using the polymerase chain reaction technique (McKnight et al.1991). The demonstration of IL4 mRNA by in-situ-hybridisation in vivo in EAU suggests that the in vivo stimulus is more potent and produces a greater expression of IL4 mRNA.

Photomicrography of autoradiographic slides presents difficulties in focussing on both the tissue section and the grains in the photographic emulsion which are on different focal planes. This problem is greatest at higher powers of magnification where the depth of field of the objective lens may not be sufficient to compensate. Although in general the best results are obtained by focussing on the photographic grains it may be necessary to compromise by focussing midway between focal planes to provide a degree of morphological definition of the tissue.

Further development of the in-situ-hybridisation technique employed in these studies could provide a method of quantification of lymphokine mRNA present. This would involve a standardisation of the probe activity along with incubation and exposure times used in the experimental protocol and subsequently counting grains within the photographic emulsion. The liquid emulsion used does not produce a coating which is even or of a standard thickness and would therefore make results unreliable. This problem could be overcome by the use of transparent photographic film of a standard thickness which could be laid over a slide and would give reproducible results. As a development of this method the EAU model could be modified by the use of various therapies and the effects on the production of individual cytokines assessed.

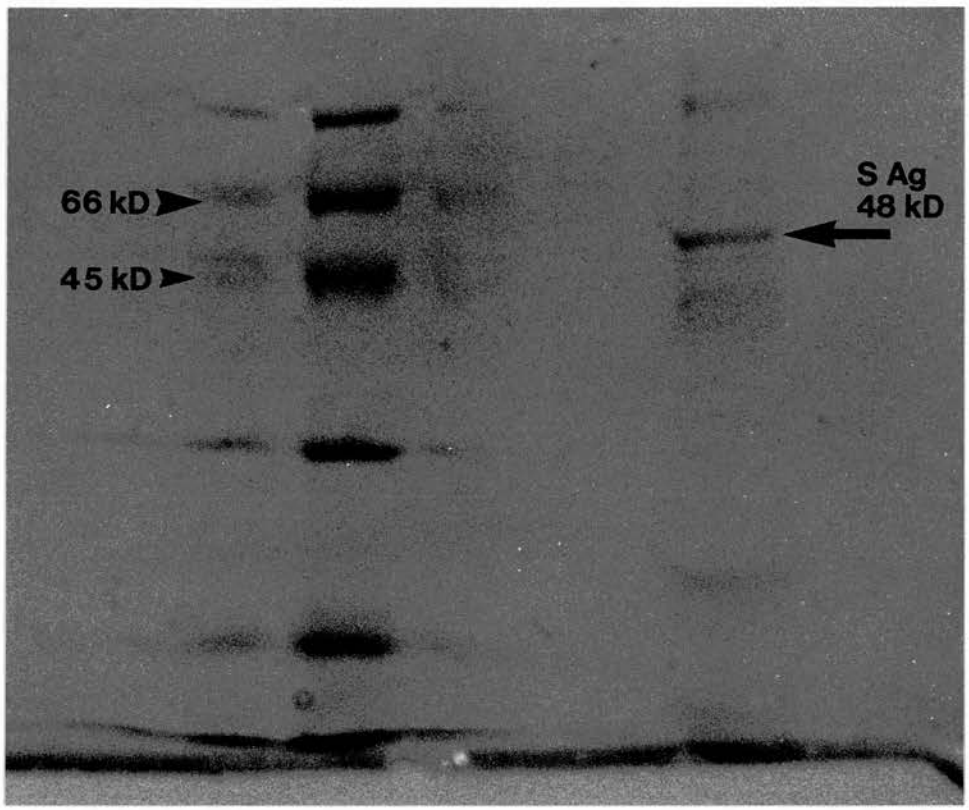
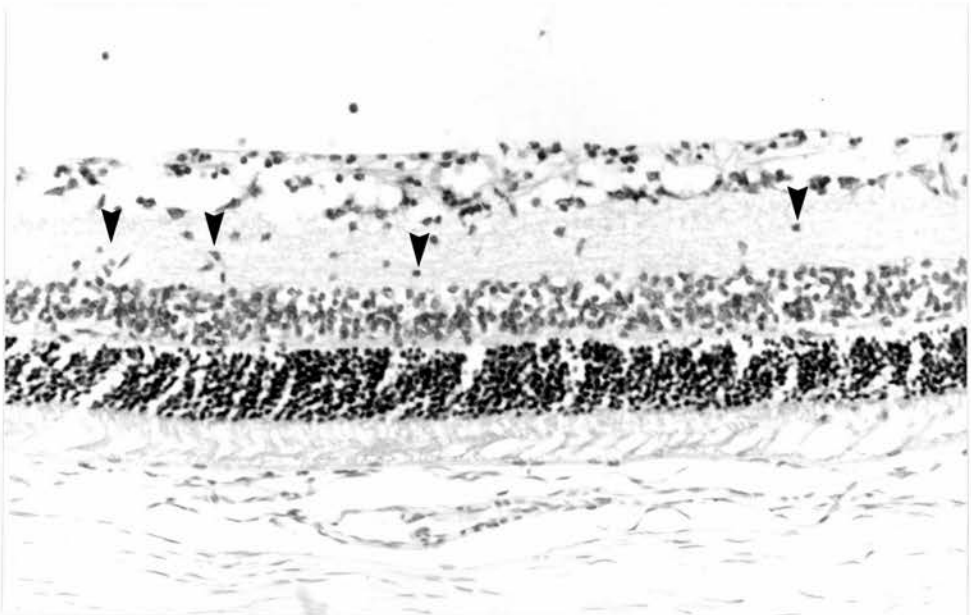


Figure 34

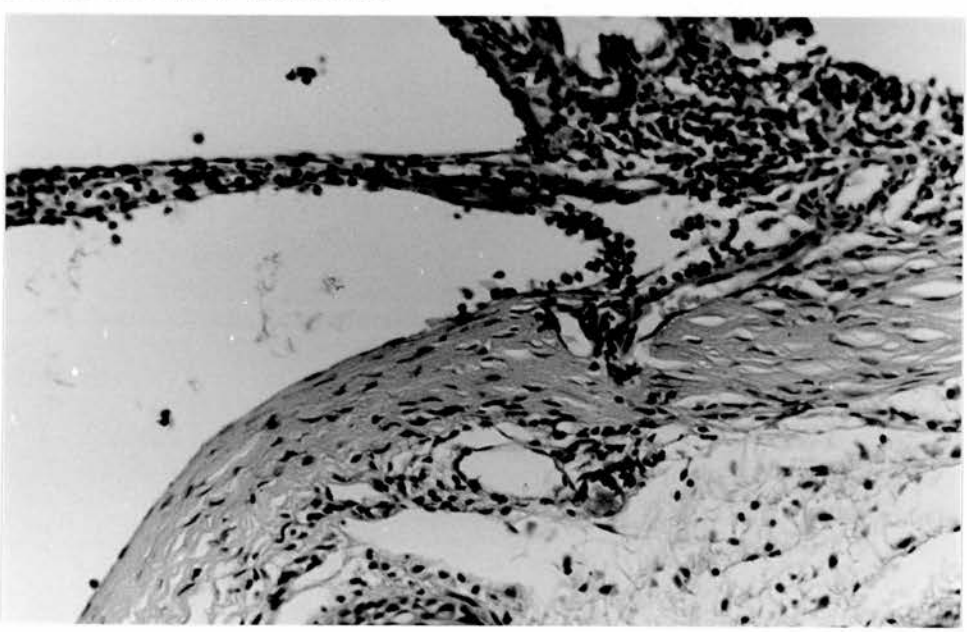
Electrophoretic gel (12.5% agar) demonstrating purified S antigen (arrow, 48kD) and molecular weight ladder (large arrowhead = 66kD, small arrowhead = 45 kD)

Figure 35

EAU - disease onset



a) Small numbers of leukocytes (arrowheads) infiltrating the retina which retains its normal architecture.



b) Mixed leukocytic infiltrate within iris, ciliary body and drainage angle x 200, H&E.

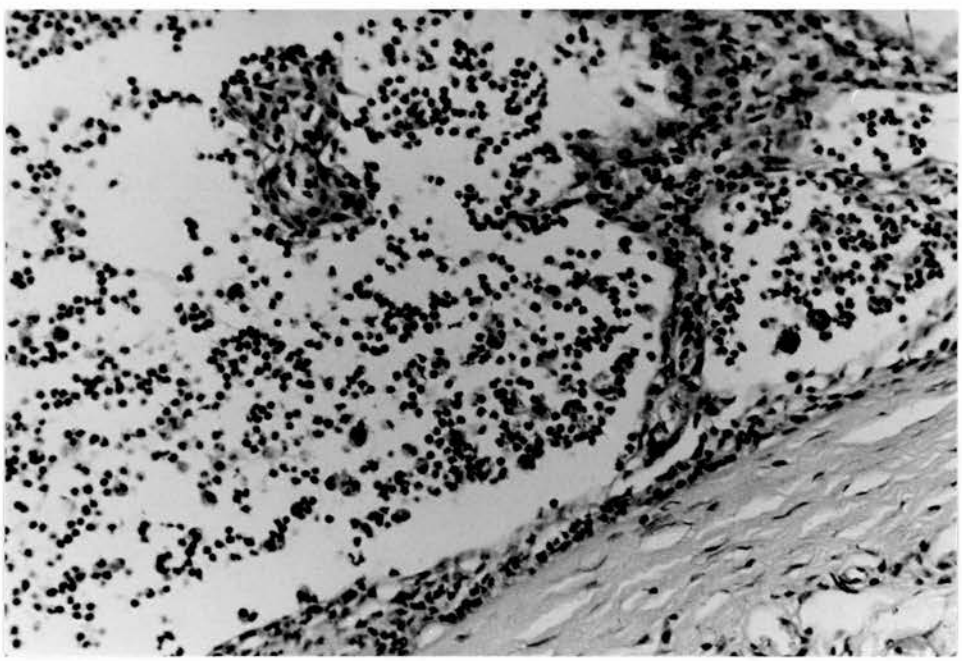


Figure 36 (a) EAU - early disease. Numerous mixed leukocytes in the anterior chamber and infiltrating iris and ciliary body.
x 200, H&E

Figure 36 (b) Retina in early EAU with a leukocytic infiltrate to the inner retina (arrowheads) and an early focus of photoreceptor necrosis and outer retinal oedema (arrow). Inflammatory cells are also seen in the vitreous cavity.

x 200, H&E.

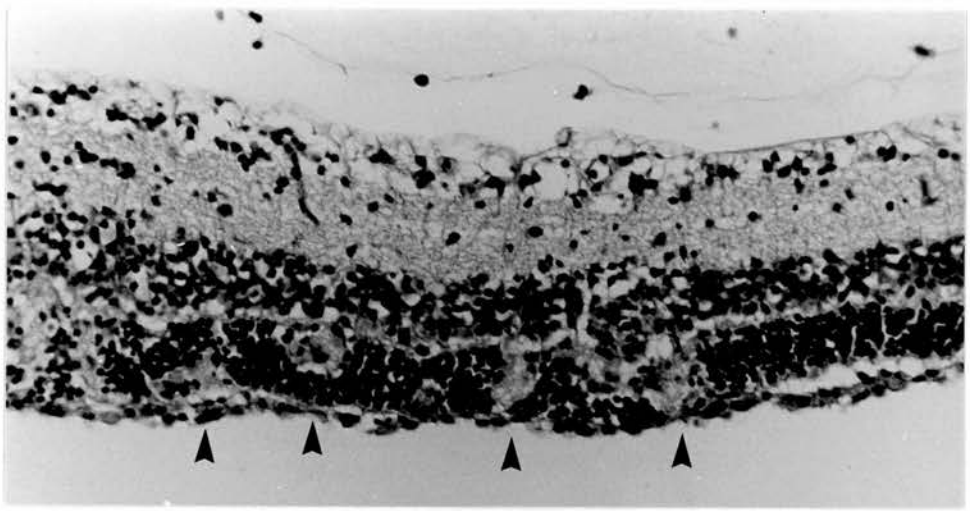


Figure 37 (a)

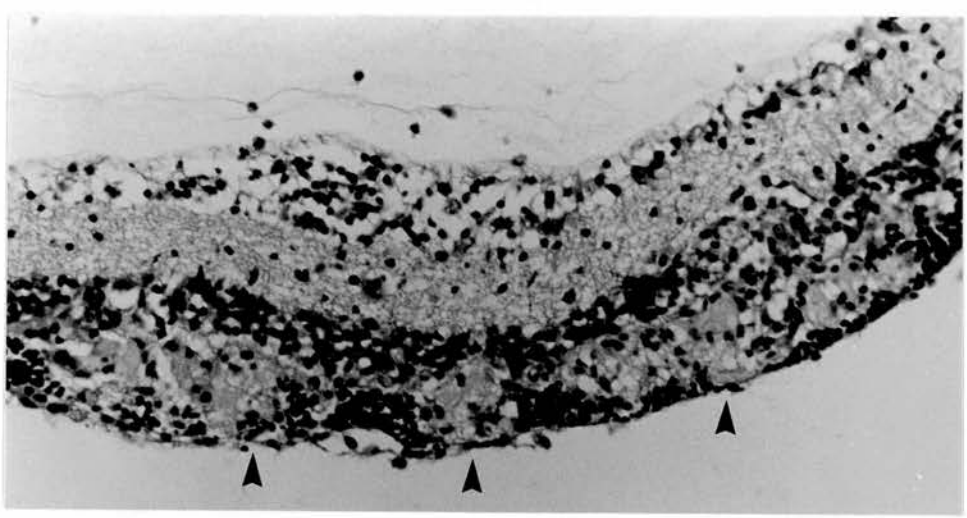


Figure 37 (b)

EAU - intermediate stage

Examples of areas of outer retinal destruction and oedema (arrowheads).

(a) & (b) x 200 H&E

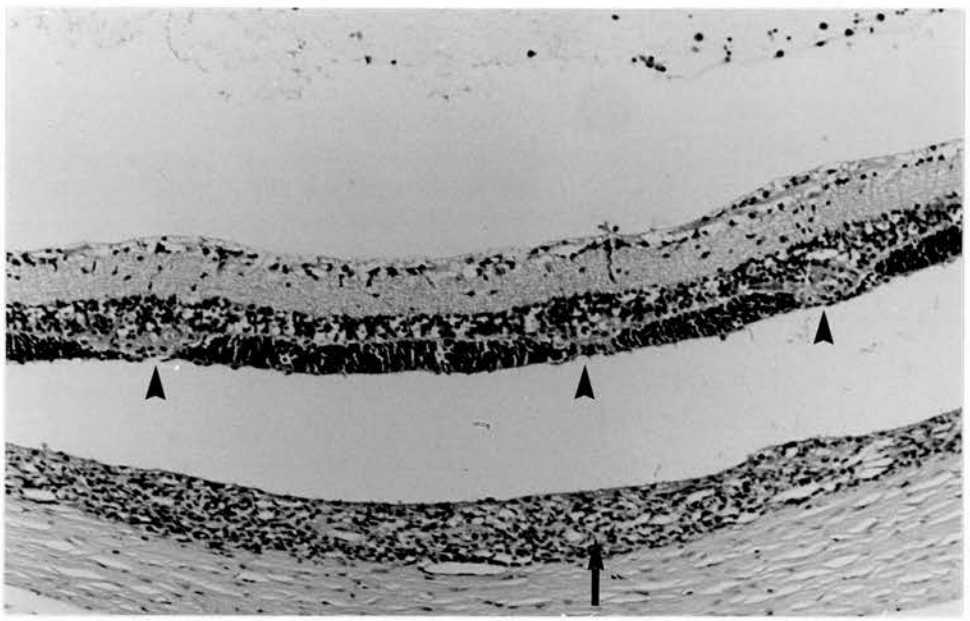


Figure 37 (c)

EAU - intermediate stage

Note that there is marked inflammatory cell accumulation in the choroid (arrow)

x 100 H&E

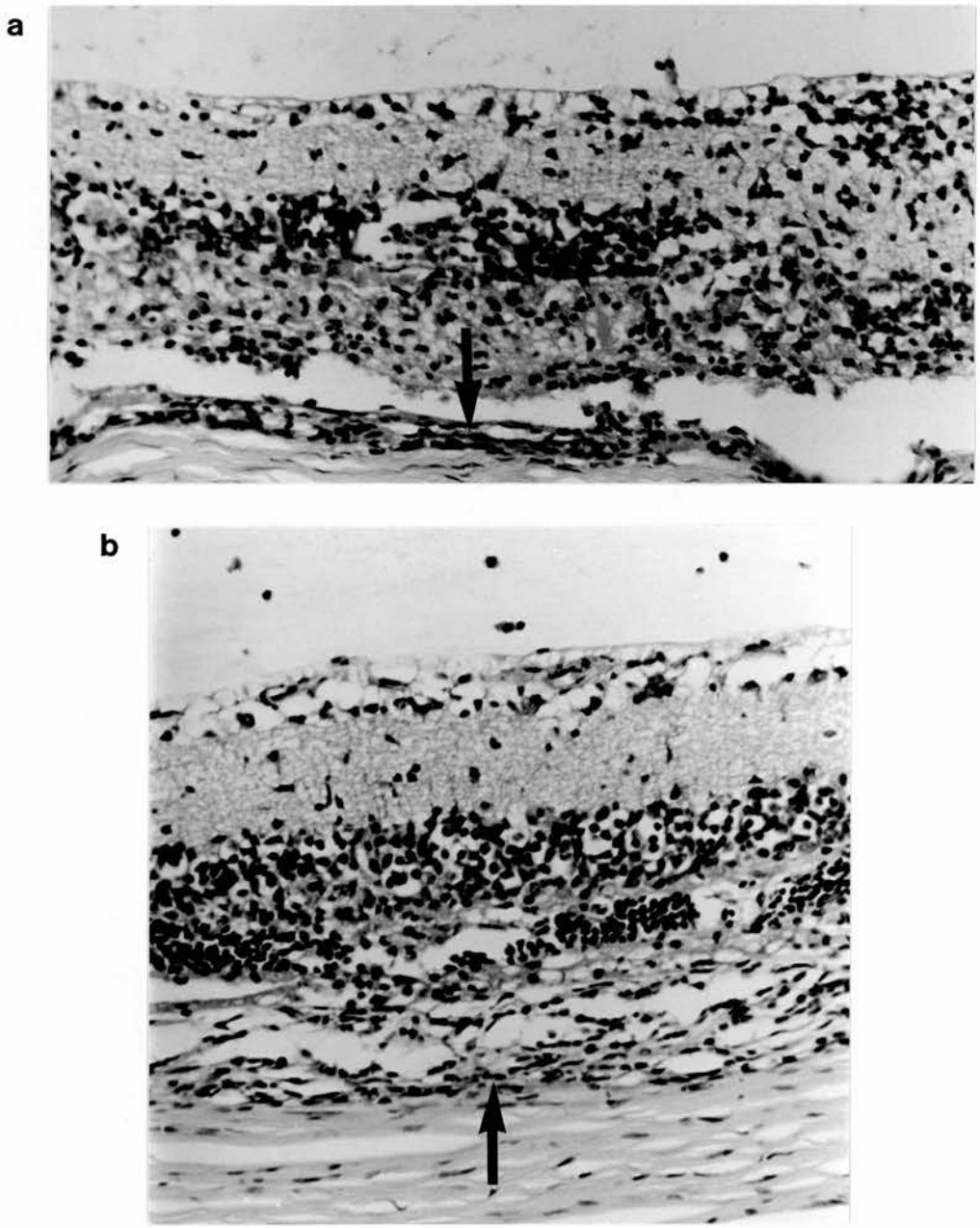


Figure 38

EAU - advanced disease

Retinal destruction and loss of architecture with an accumulation of mixed leukocytes in the choroid (arrows)

a) & b) x 200 H&E

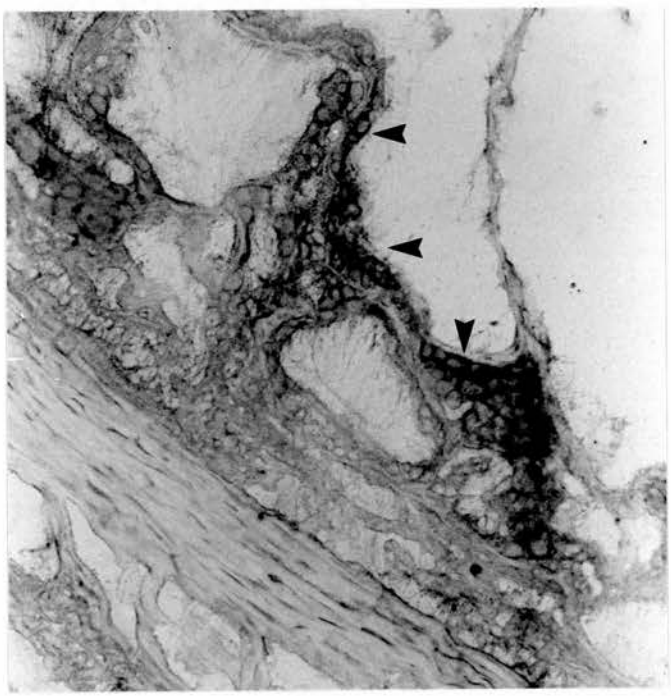


Figure 39

IL2 receptor positive T lymphocytes (arrowheads) infiltrating ciliary body. IL2 receptor monoclonal antibody, haematoxylin counterstain. x 250.

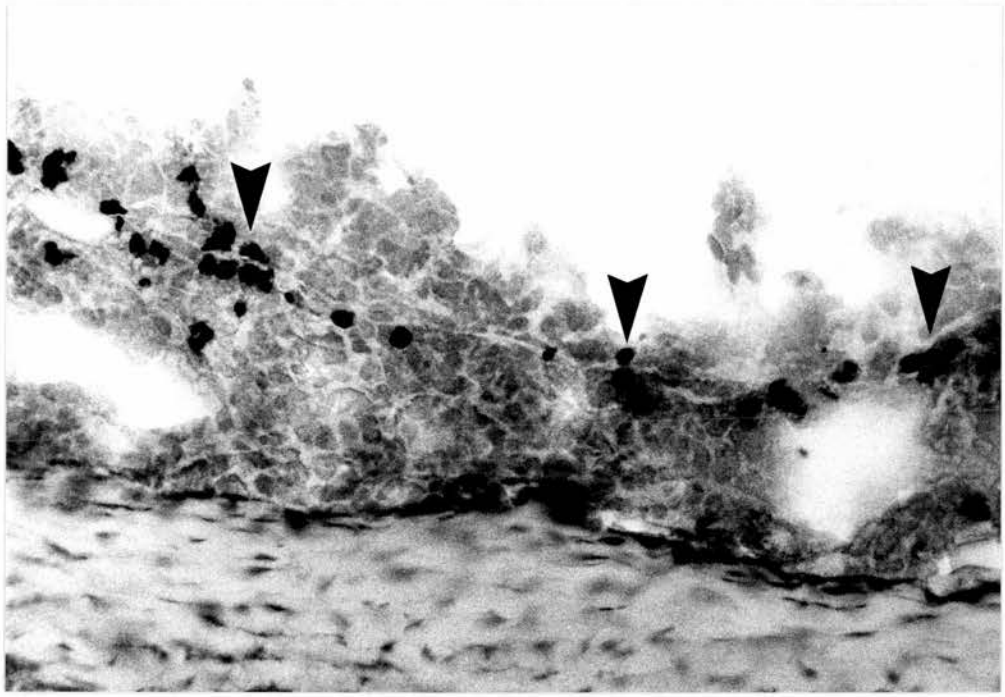


Figure 40

Positive immunohistochemical staining for interferon gamma (arrowheads) over cells in disorganised retina in advanced EAU. x360, haematoxylin counterstain.

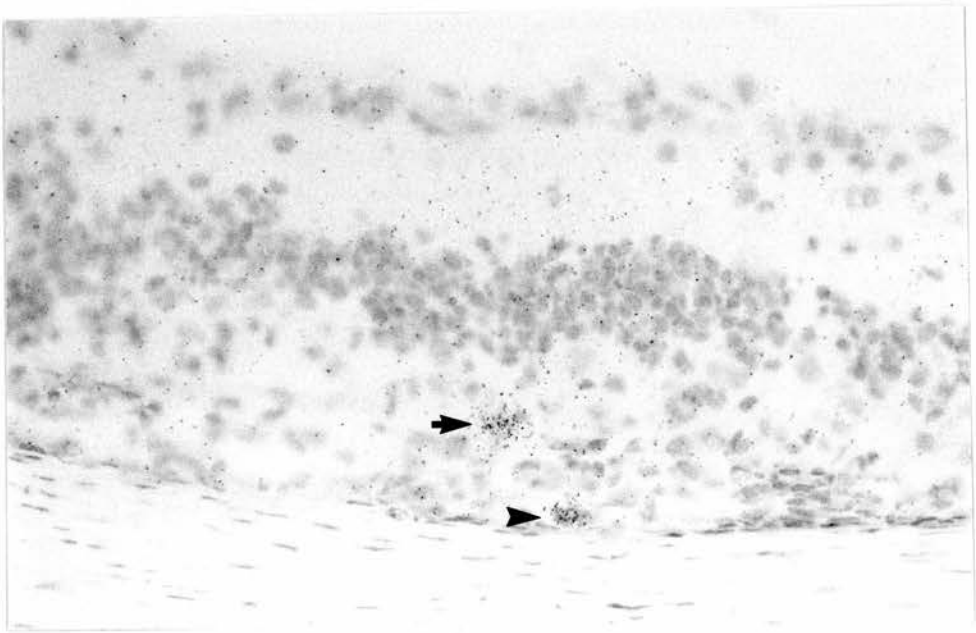


Figure 41

EAU : early stage of the disease process. Hybridisation signal from interferon-gamma probe over cells in outer retina (arrow) and choroid (arrowhead). x 450, haematoxylin counterstain.

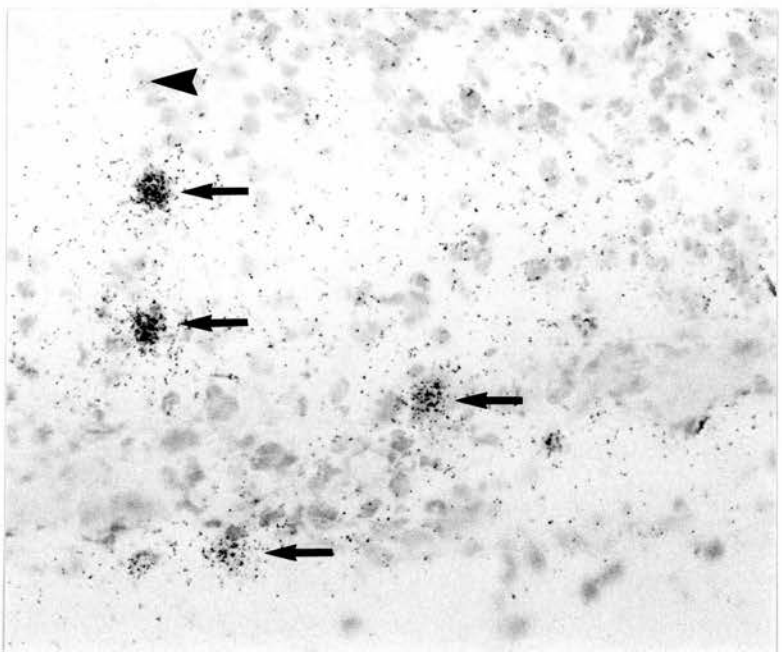


Figure 42

EAU : established disease. Hybridisation signal from interferon-gamma probe over cells in retina (arrows) adjacent to an area of retinal oedema (arrowhead). x450, haematoxylin counterstain.

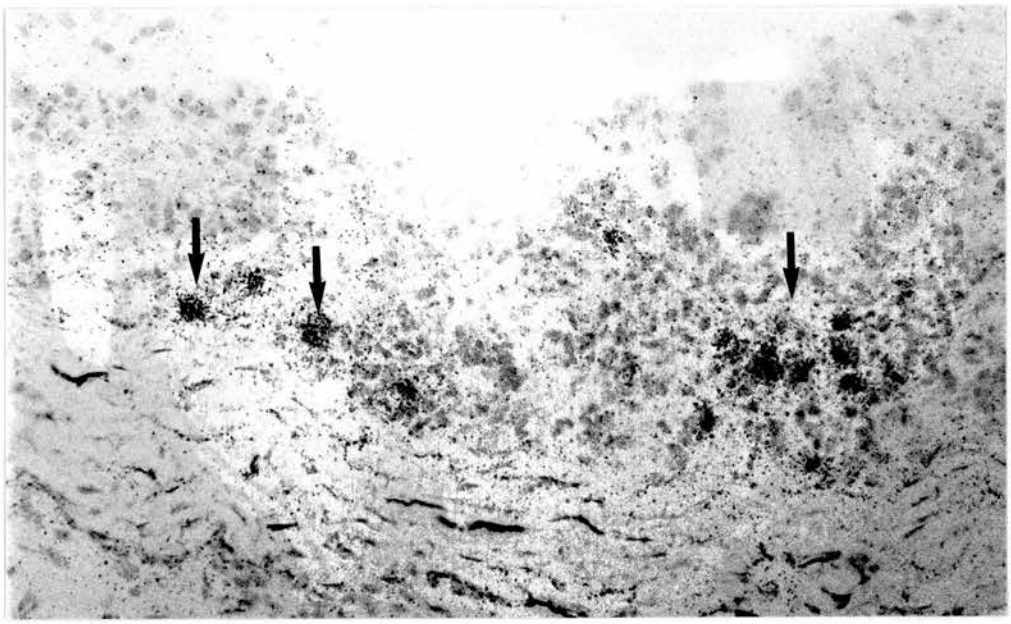
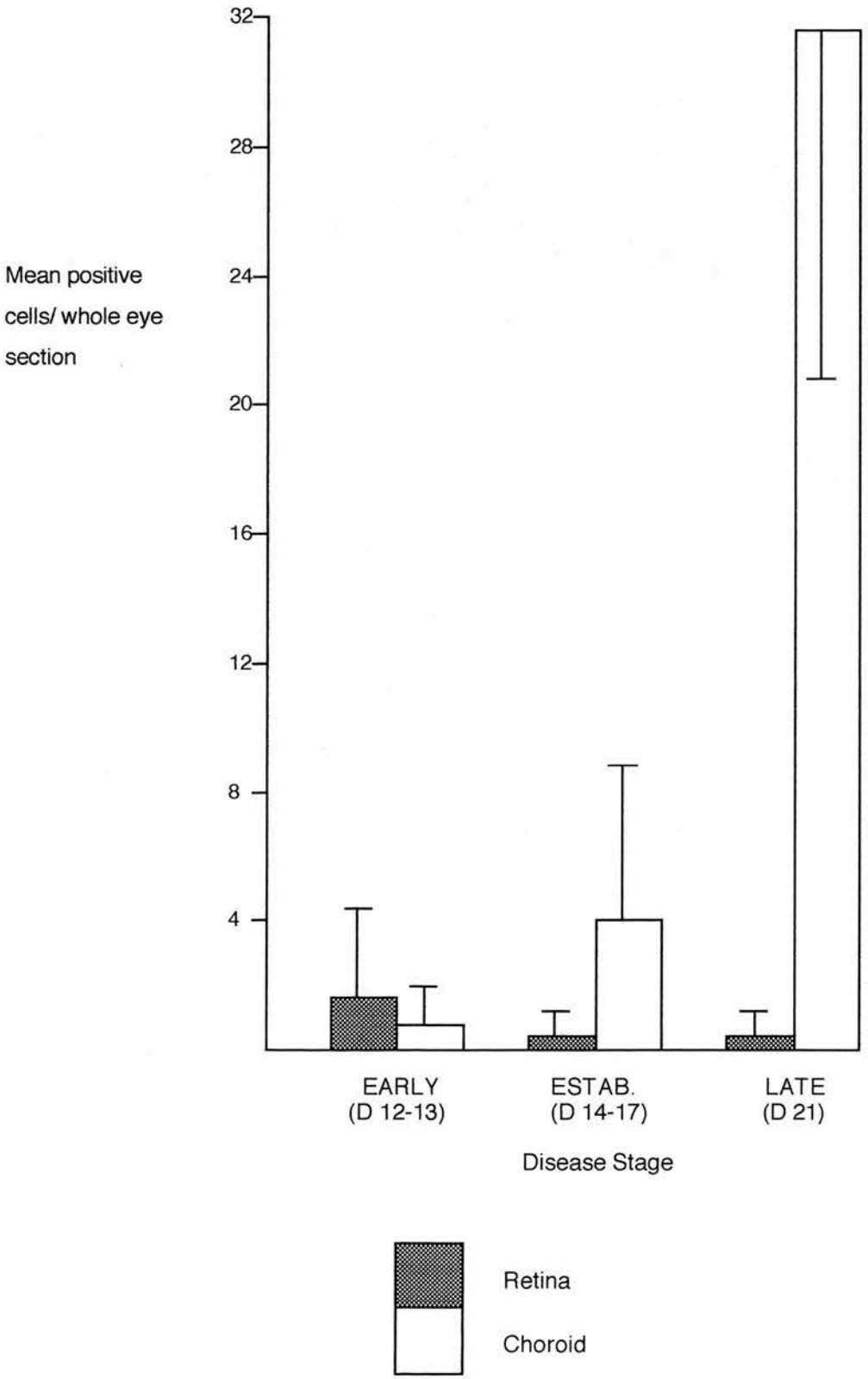


Figure 43

EAU : late disease. Multiple foci of hybridisation from interferon-gamma probe (arrows to examples) in an area of advanced retinal destruction. x 280, haematoxylin counterstain.

Figure 44 IFN-gamma probe:
Counts of positive foci of probe hybridisation throughout EAU disease process



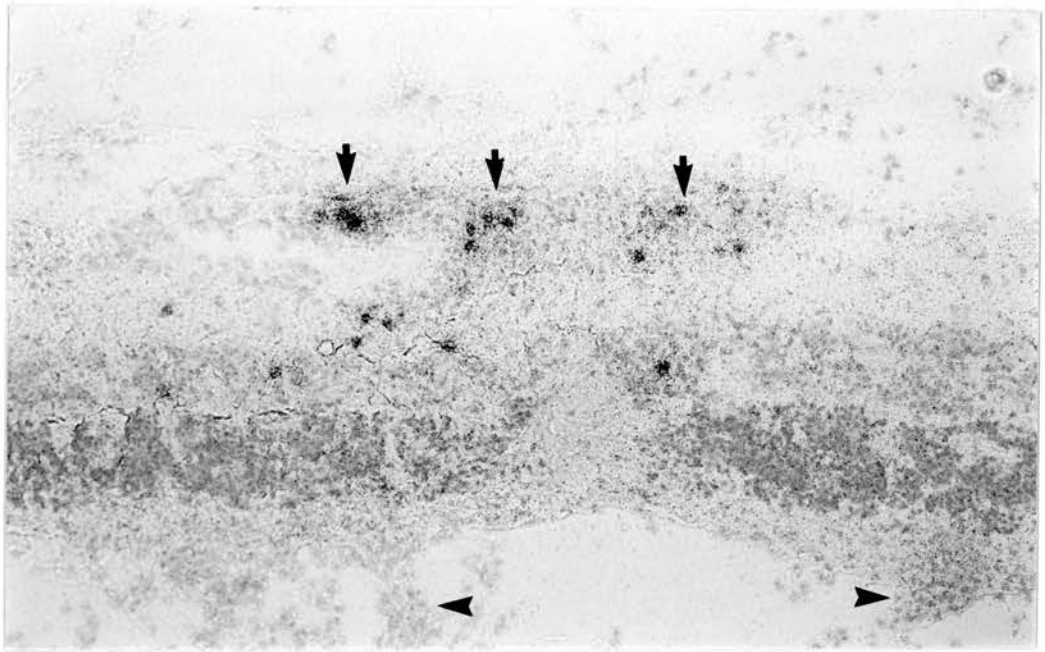


Figure 45 (a)

EAU : early stage of the disease process

Multiple areas of hybridisation to IL2 probe in inner retina (arrows to examples) around a focus of outer retinal oedema and destruction. The retina is detached with infiltration of leukocytes to the subretinal space (arrowheads) and vitreous.

x 180, haematoxylin counterstain.

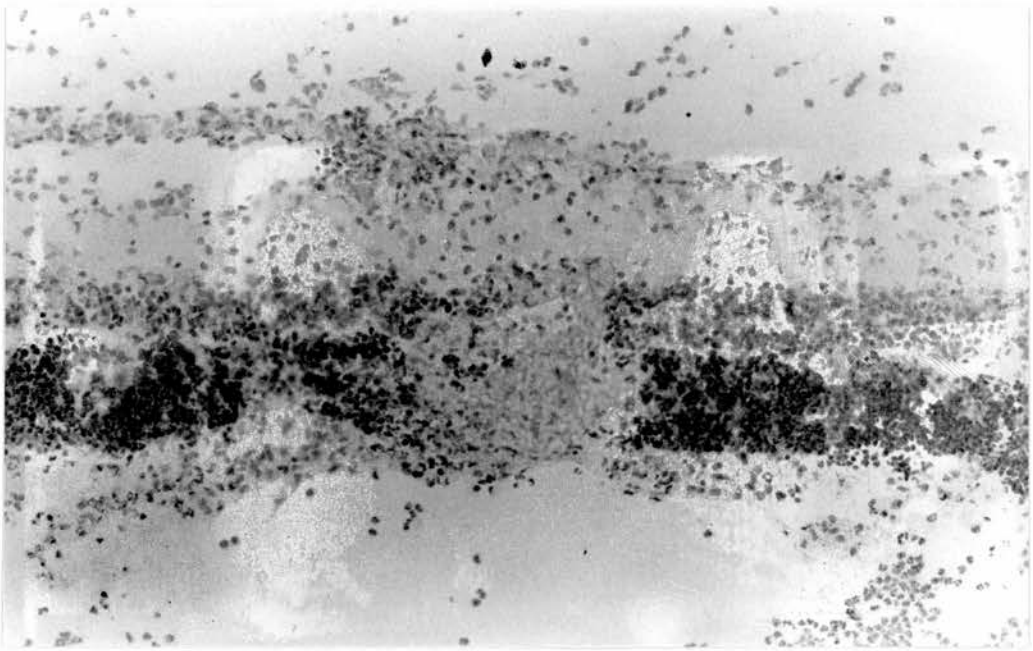


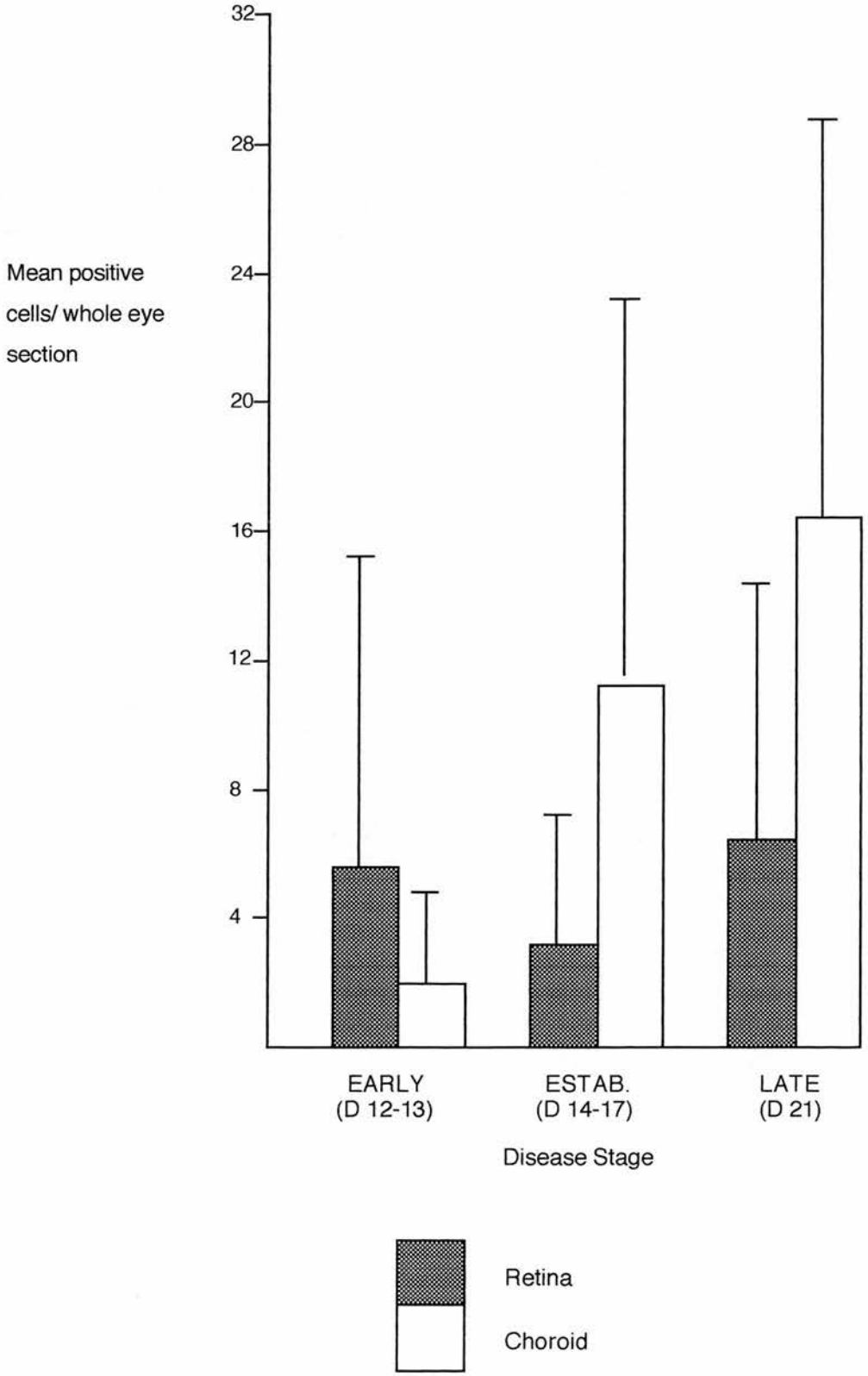
Figure 45 (b)

EAU : early stage of the disease process

RNAase treated control on an adjacent section to figure 45 (a) with no localising signal

x 180, haematoxylin counterstain.

Figure 46 IL2 probe:
Counts of positive foci of probe hybridisation throughout EAU disease process



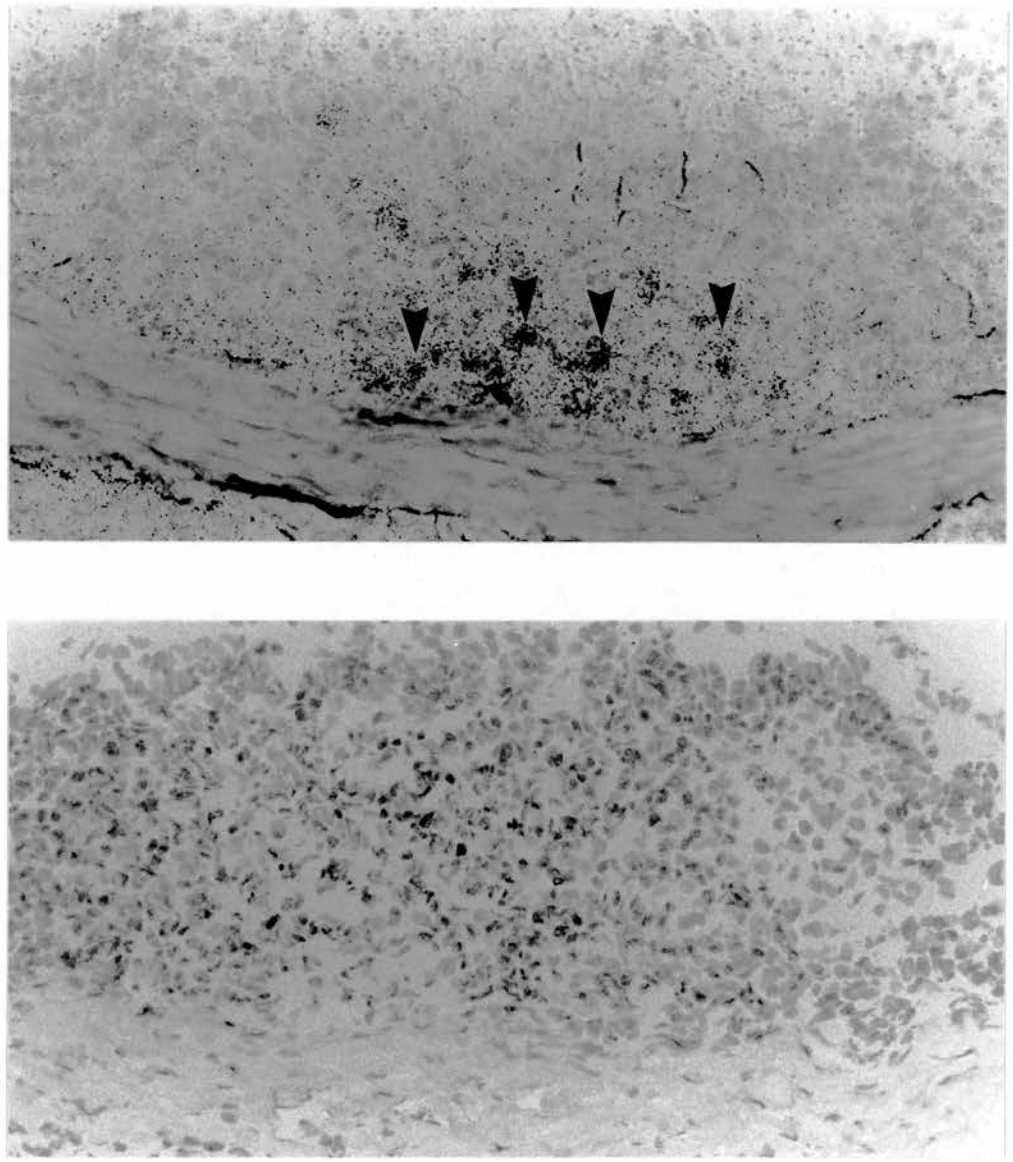


Figure 47

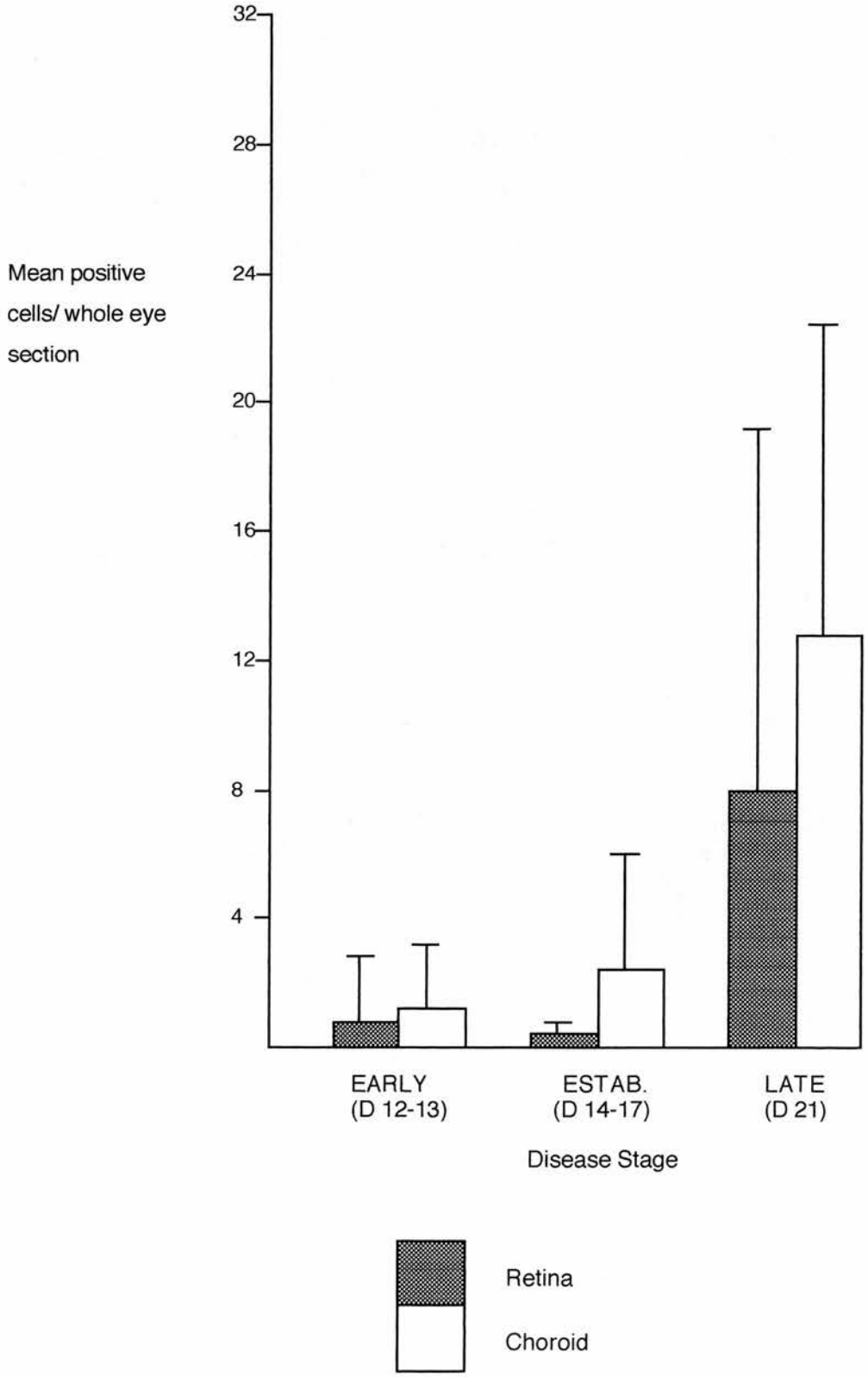
EAU : late stage

a) Marked retinal destruction with positive localisation of lymphotoxin mRNA (arrowheads).

b) RNAase control on adjacent section - no localising signal

x 200, haematoxylin counterstain.

Figure 48 Lymphotoxin probe:
Counts of positive foci of probe hybridisation throughout EAU disease process



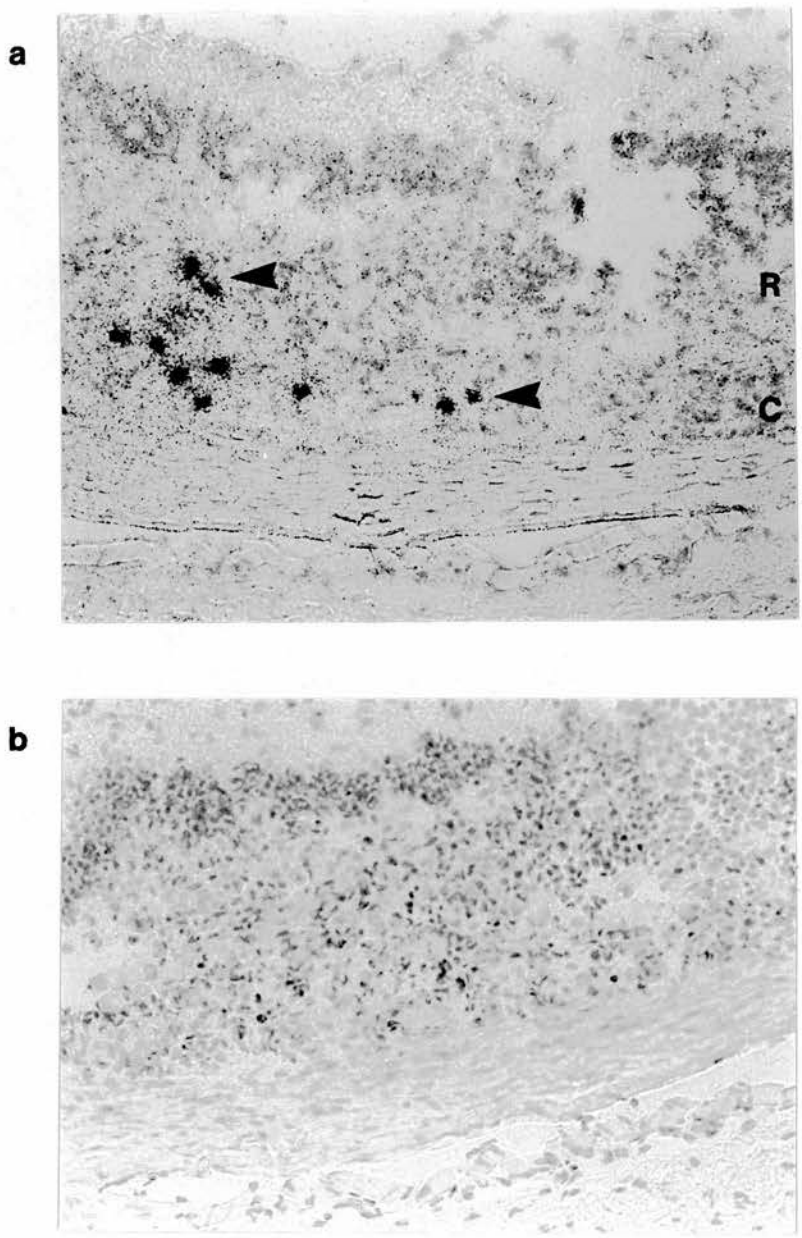


Figure 49

EAU : late disease

a) hybridisation signal from IL4 probe in choroid and retina

b) adjacent section to a) pretreated with RNAase - no localising signal

x 180, haematoxylin counterstain.

Figure 50 IL4 probe:
Counts of positive foci of probe hybridisation throughout EAU disease process

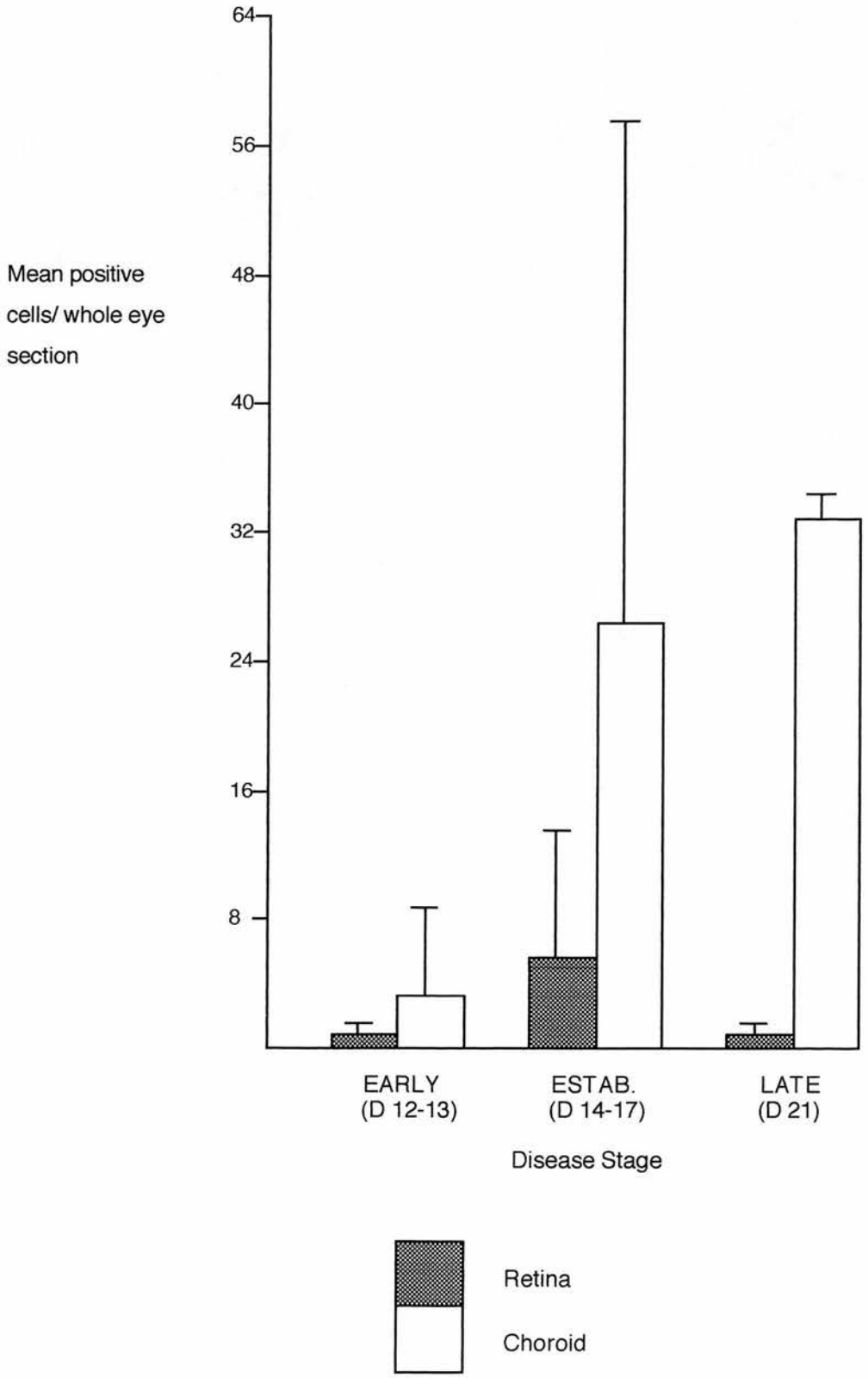


Figure 51 Comparison of lymphokine probes: Counts of positive foci of probe hybridisation throughout EAU disease process

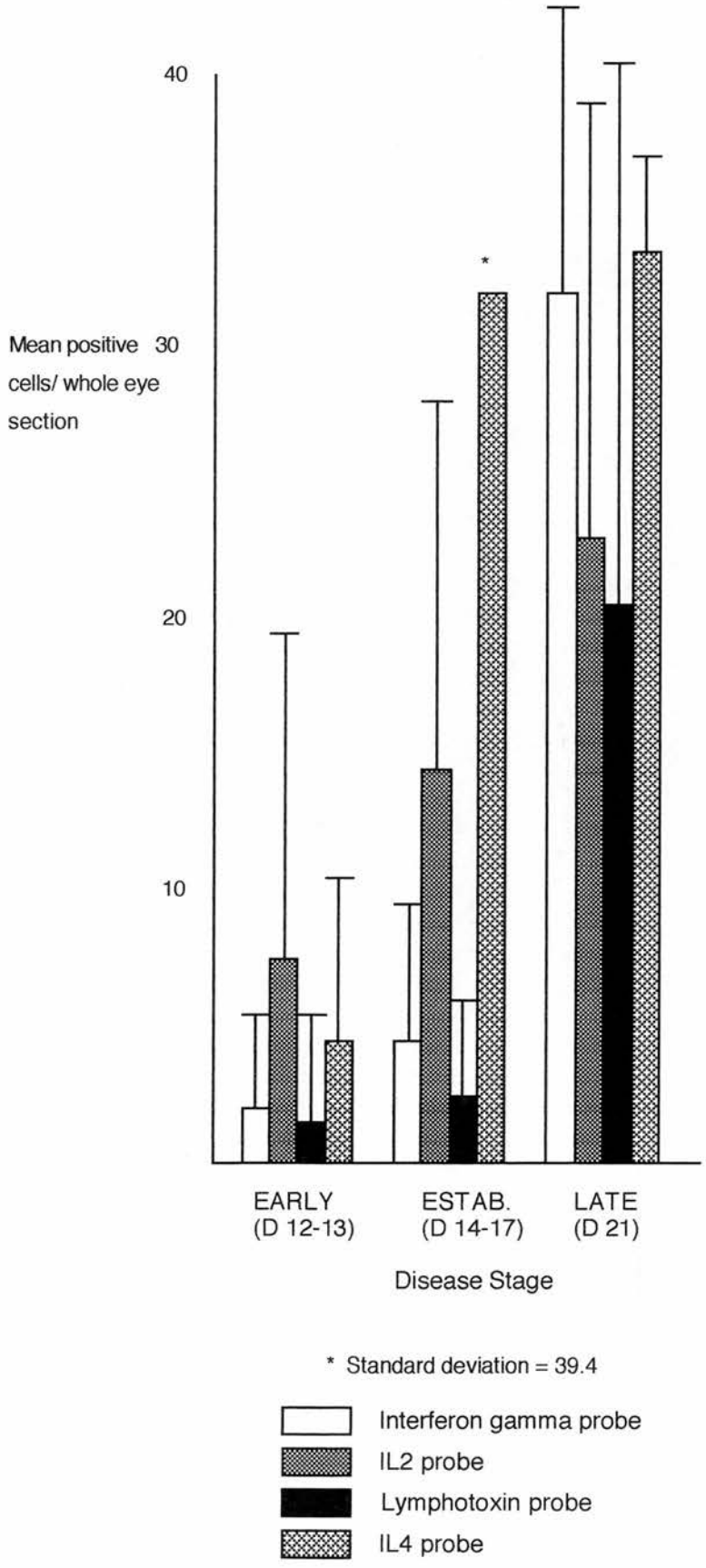


TABLE 9

EXPERIMENTAL AUTOIMMUNE UVEORETINITIS:

PRIMARY MONOCLONAL ANTIBODIES

ANTIBODY	SOURCE	DILUTION	SPECIFICITY
OX 19	Serotec	1:100	Pan T cells
W 3/25	Serotec	1:100	CD4 T cell subset
OX 8	Serotec	1:100	CD8 T cell subset
OX 39	Serotec	1:75	IL2 receptor
OX 6	Serotec	1:100	MHC class II antigen I-A
IFN-gamma	Holland Biotechnology	1:75	IFN-gamma protein

All antibodies are rat specific

TABLE 10 PATHOLOGICAL CHANGES IN EAU MODEL

	Clinical features	Anterior segment	Uveal tract	Retina/ vitreous
Disease onset (Day 12)	Conjunc-tiva hyper-aemic	Mixed PMN and mononuclear cells in anterior chamber	PMN and mononuclear cells in iris and ciliary body	Small numbers of lymphocytes infiltrating the retina
Early (Day 13)	Conjunc-tiva hyper-aemic Anterior chamber haze	Numerous mixed WBC	Mixed WBC infiltrate of iris and ciliary body	Early foci of photoreceptor necrosis with related infiltrate of monocytes
Inter-mediate (Days 14 - 17)	Hyper-aemia Media hazy Hypopyon in most eyes	Numerous mixed WBC	Mixed WBC infiltrate of iris and ciliary body Infiltrate of mononuclear cells in choroid	Foci of retinal oedema and necrosis with subretinal fluid Mixed WBC infiltrate in vitreous
Late (Days 18 -21)	Media hazy Hypopyon	Mixed WBC - smaller numbers	Marked infiltration of mononuclear cells in choroid	Retinal destruction: advanced in most eyes

TABLE 11

EAU: INTERFERON GAMMA PROBE
Mean positive hybridisation foci per whole eye section
n = sections counted
(standard deviation, standard error of mean)

	Disease stage		
	Early Days 12-13 n = 16	Intermediate Days 14-17 n = 28	Late Day 21 n = 8
Uvea	0.86 (1.12, 0.28)	3.89 (4.9, 0.93)	31.37 (11.0, 3.89)
Retina	1.50 (2.98, 0.74)	0.54 (0.98, 0.19)	0.75 (1.09, 0.39)
Total	2.31 (3.25, 0.81)	4.43 (5.37, 1.02)	32.12 (10.4, 3.67)

Standard error of the difference between means

	Early v Established	Established v Late	Early v Late
Uvea	0.97 0.0027 > P > 0.001	4.00 P < 0.001	4.01 P < 0.001
Retina	0.77 0.317 > P > 0.1	0.43 NS	0.84 0.046 > P > 0.01
Total	1.3 0.317 > P > 0.1	5.3 P < 0.001	3.77 P < 0.001

NS = not significant

TABLE 12

EAU: IL2 PROBE

Mean positive hybridisation foci per whole eye section
n = sections counted
(standard deviation, standard error of mean)

	Disease stage		
	Early Days 12-13 n = 12	Intermediate Days 14-17 n = 15	Late Day 21 n = 6
Uvea	2.0 (2.70, 0.78)	11.0 (12.03, 3.11)	16.5 (12.6, 5.14)
Retina	5.5 (10.2, 2.94)	3.27 (4.02, 1.04)	6.33 (8.16, 3.33)
Total	7.5 (11.88, 3.43)	14.27 (13.61, 3.51)	22.83 (16.19, 6.61)

Standard error of the difference between means

	Early v Established	Established v Late	Early v Late
Uvea	3.14 0.01 > P > 0.0027	6.01 NS	5.16 0.01 > P > 0.0027
Retina	3.12 NS	3.49 NS	4.45 NS
Total	4.91 0.317 > P > 0.1	7.49 0.317 > P > 0.1	7.45 0.046 > P > 0.01

NS = not significant

TABLE 13
EAU: LYMPHOTOXIN PROBE

Mean positive hybridisation foci per whole eye section
n = sections counted
(standard deviation, standard error of mean)

	Disease stage		
	Early Days 12-13 n = 12	Intermediate Days 14-17 n = 15	Late Day 21 n = 6
Uvea	1.1 (1.75, 0.50)	2.27 (3.55, 0.92)	12.67 (9.98, 4.07)
Retina	0.92 (1.89, 0.54)	0.2 (0.54, 0.14)	8.0 (11.3, 4.61)
Total	2.0 (3.31, 0.95)	2.47 (3.64,0.94)	20.67 (19.82, 8.09)

Standard error of the difference between means

	Early v Established	Established v Late	Early v Late
Uvea	1.05 0.317 > P > 0.1	4.18 0.046 > P > 0.01	4.10 0.046 > P > 0.01
Retina	0.56 0.317 > P > 0.1	4.61 0.1 > P > 0.05	4.64 0.317 > P > 0.1
Total	1.34 NS	8.14 0.046 > P > 0.01	8.15 0.046 > P > 0.01

NS = not significant

TABLE 14

EAU: IL4 PROBE

Mean positive hybridisation foci per whole eye section
n = sections counted
(standard deviation, standard error of mean)

	Disease stage		
	Early Days 12-13 n = 4	Intermediate Days 14-17 n = 6	Late Day 21 n = 2
Uvea	3.5 (4.92, 2.46)	26.4 (31.72, 14.19)	32.5 (2.5, 1.77)
Retina	1.0 (1.22, 0.61)	5.4 (8.01, 3.58)	1.0 (1.0, 0.71)
Total	4.5 (6.10, 3.05)	31.8 (39.38, 17.61)	33.5 (3.5, 2.47)

Standard error of the difference between means

	Early v Established	Established v Late	Early v Late
Uvea	14.40 0.317 > P > 0.1	14.29 NS	3.03 P < 0.001
Retina	3.63 0.317 > P > 0.1	3.65 0.317 > P > 0.1	0.93 NS
Total	17.87 0.317 > P > 0.1	17.78 NS	3.93 P < 0.001

NS = not significant

CHAPTER 5

GENERAL DISCUSSION

5.1 Human intraocular inflammatory disease

The work in this thesis has covered both studies of tissue immunopathology in clinical intraocular inflammation and experimental work on an animal model of immune-mediated ocular inflammatory disease. Certain problems are inherent to the study of the eye specimens obtained from patients with intraocular inflammatory disease. Firstly the method of tissue fixation employed on pathological specimens determines the available investigational techniques. Fixation with glutaraldehyde gives optimal preservation of morphology and is suitable for electron microscopic examination. This method was used on one of the eyes available in the study of multifocal posterior uveitis reported in chapter two and resulted in the identification of swollen endothelial cells (seen on thin sections), small breaks in Bruch's membrane, cellular junctional attachments (for example the junctional complexes of RPE cells in the choroid) and cellular activity such as the phagocytosis of photoreceptor outer segments by exogenous macrophages. A limitation of glutaraldehyde fixation is that it makes immunohistochemical examination impossible because of its effect of cross-linking cellular proteins causing alterations of oligopeptide epitopes and exclusion of antibodies from penetrating tissue sections. Formaldehyde fixation gives good morphological preservation and also allows for immunohistochemical analysis as was carried out on the other eye in the study of multifocal posterior uveitis and on five of the eyes used in the study of Behcet's disease. This combination is useful for immunohistochemical identification of cell types involved in tissue pathology however the range of monoclonal antibodies suitable for use on formaldehyde fixed tissue is

limited (the monoclonal antibody UCHL1 for example is not absolutely specific for T lymphocytes (Norton et al.1986; Terry et al.1988)). In some cases antibodies may be less sensitive when used on formalin fixed tissue: the HLA DR α chain antibody may be less sensitive than other HLA DR antibodies which are suitable only for use on unfixed material. Unfixed, frozen material gives the best results when used for immunohistochemical analysis both in terms of antibody availability and sensitivity, however this is at the cost of morphological preservation. The optimal approach is to use the fixation method which will yield the maximal information from an individual specimen, which in some cases may mean using a combination of all the methods outlined above on a single specimen. In many cases this approach is not possible since the specimen may already be fixed when received. In retrospect it would have been advantageous to have frozen portions of all the eyes used for the studies of human immunopathology to allow for immunohistochemical analysis of cellular markers such as the interleukin 2 receptor and for more sensitive analysis of the MHC class II antigen expression.

A further problem is the end-stage nature of much of the material obtained for immunopathological analysis. The studies described in chapters 2 and 3 of this thesis have demonstrated the problems in determining the aetiology of many forms of intraocular inflammation. The initiating agent may no longer be present either systemically or locally at the time of clinical presentation of the ocular condition. This problem is common to other diseases thought to be of autoimmune aetiology such as insulin dependent diabetes mellitus where subclinical disease may be present for

months or even years (Bottazzo et al.1985) before the condition is manifest. The studies of human material in chapters 2 and 3 therefore primarily deal with the mechanisms of perpetuation of intraocular inflammation. Indirect evidence as to the aetiological factors involved may come from studies of peripheral blood such as those implicating Epstein Barr virus in multifocal choroiditis (Tiedeman, 1987) and herpes simplex in Behcet's disease (Eglin et al.1982; Bonass et al.1991; Hamzaoui et al.1990a; Young et al.1988; Hamzaoui et al.1990b) and it is likely that more information will be produced by studies of peripheral blood as the sensitivity of the tests available improves.

Study of ocular tissue does, however, give clues to the aetiology of these conditions: these may be negative such as the lack of any identifiable infectious agent (although this is hardly surprising since a transient viral infection may be sufficient to trigger an autoimmune disease) in any of the eyes studied or the lack of any evidence of previous vascular occlusion in multifocal posterior uveitis. Other findings are also relevant such as the vascular wall hyaline thickening and luminal narrowing seen in the Behcet's disease eyes (although not seen in all the end stage eyes again highlighting the need for suitable material) suggestive of previous vascular occlusion.

The search for aetiological agents may be of secondary importance since present therapies are aimed at a generalised control of the inflammatory immunopathology and treatment of an initiating factor is not, as yet, possible. This position may change, however, as newer and more specific therapies develop and identification of an initiating insult may then become of therapeutic significance.

Despite the limitations of studying human material the immunohistochemical analyses carried out on multifocal posterior uveitis and Behcet's disease do provide useful information on the infiltrating cells in the local pathology. These were characterised as predominantly T lymphocytes (CD4+ in the frozen Behcet's disease specimen) which is in line with the findings in eyes of other forms of posterior uveitis which have been examined (Jakobiec et al.1983; Chan et al.1986c; Wetzig et al.1988; Chan et al.1988b; Chan et al.1987).

5.2 T lymphocyte involvement in intraocular inflammation

The introductory discussion of EAU has stressed that this animal model may not be analogous to any one form of human disease. Furthermore, studies on patients with intraocular inflammation have given equivocal results as to whether the antigens commonly used in disease induction in animal models of EAU, that is S antigen and IRBP, are of significance in human disease (Doekes et al.1987; Froebel et al.1989; Forrester et al.1989; Chan et al.1985b; Froebel et al.1989; de Smet et al.1990). The EAU model does, however, provide a form of ocular inflammation induced and perpetuated by CD4 positive T cells.

The results of experimental work must be interpreted with caution in regard to human disease. In EAE, for example, treatment of actively immunised animals with cyclosporin A inhibits the development of disease as does in vitro conditioning of transfer active cells (Hinrichs et al.1983). In multiple sclerosis, however, the clinical response to cyclosporin therapy has been disappointing (Rudge et al.1989). In human intraocular inflammatory

disease there is often a favourable clinical response to therapy with cyclosporin A (Nussenblatt et al.1983a; Nussenblatt et al.1985; Graham et al.1985; de Vries et al.1990; Masuda et al.1989) similar to the response found in EAU (Nussenblatt et al.1981b). This T cell dependent aspect of the EAU model is therefore analogous to human disease. This finding, however, cannot necessarily be generalised to the interpretation of other aspects of the T cell mediated immunopathology found in EAU.

Despite its limitations the EAU model is of relevance to human intraocular inflammation. The infiltrating inflammatory cells in the posterior segment destructive foci in the Lewis rat model of EAU have been characterised as being predominantly T lymphocytes (with a high CD4:CD8 ratio in early disease) in studies from other centres (Chan et al.1985a) and in the model used for the experimental work described in chapter 4. This is analogous to the retinal and choroidal infiltrate of CD4 positive T lymphocytes found in forms of intraocular inflammatory disease where immunohistochemical studies have been carried out (Jakobiec et al.1983; Chan et al.1986c; Wetzig et al.1988; Chan et al.1988b; Chan et al.1987) and our studies of multifocal posterior uveitis and Behcet's disease. This aspect of the EAU model forms the basis of the experimental work since it is the lymphokine secretion by the T lymphocytes in the destructive foci in EAU which is under investigation. The results of the studies on in vivo lymphokine secretion in EAU may therefore be of significance in relation to human disease.

5.3 In vivo production of lymphokines in ocular inflammation

5.3.1. Cytokine mRNA expression

The demonstration by in-situ-hybridisation of cytokine mRNA on a tissue section indicates that individual cells have been stimulated to express the cytokine gene but does not necessarily mean that they are actively producing cytokine protein. This has been demonstrated for IFN-γ production by rat CD4+ T cells (McKnight et al.1991) where a greater frequency of CD4+ T cells positive for IFN-γ mRNA was found in the OX22 low group although the level of secreted IFN-γ protein is higher from OX22 high cells. This may be due to post transcriptional regulation of translation of IFN-γ mRNA or regulation of secretion of IFN-γ protein. Grabstein and coworkers demonstrated that stimulation of peripheral blood T cells resulted in expression of mRNA for IL2, IL2 receptor and IFNγ which correlated well with the levels of the respective proteins obrerved (Grabstein et al.1986). Likewise Carding and colleagues demonstrated good concordance between IL2 and IL4 mRNA and secreted lymphokine as detected by bioassay (Carding et al.1989). Recent work by Dallman et. al. (Dallman et al.1991) however, has shown that cellular IL2 mRNA expression is not necessarily reflected by production of bioactive IL2.

In view of these conflicting studies the results of experimental work demonstrating lymphokine mRNA must be interpreted with caution in regard to production of bioactive lymphokine. Where possible molecular studies should be supported by other findings such as the immunohistochemical localisation of cytokine protein (as with IFN-γ in the studies of EAU) or the presence of cellular cytokine receptors for example the IL2 receptor.

5.3.2. T cell activation

The expression of lymphokine mRNA by T cells in the inflammatory infiltrates in EAU implies that the T cells are activated within the eye or remain in a state of activation within the eye. It is uncertain, however, as to whether these cells are all S antigen specific arising from clonal expansion or a mixture of antgenic specificities resulting from T cell recruitment.

At present it is unknown whether the antigenic stimulus which T lymphocytes are subject to in the EAU model is similar in terms of quality or quantity to human disease. It has been demonstrated that the activation of cytokine genes in CD4 positive T cells is stimulus dependent in vitro (Carding et al.1989) and similar mechanisms may therefore also be important in vivo. The antigenic and soluble stimuli are likely to differ between the EAU model and the situation in human disease and may therefore affect the cytokine gene expression. It is also likely that these stimuli differ in the various clinical forms of introcular inflammation and may in part be responsible for the variation in the presentation and behaviour seen in clinical disease.

The IFN-γ, IL2 and IL4 producing cells were notably distributed in relation to foci of retinal oedema and destruction suggesting that a stimulus to produce these lymphokines is present in this area and their subsequent release may contribute to the focal destructive lesions seen in the early stages of EAU. Antigen contact is one potential stimulus, however it is notable that these cells were distributed almost entirely within the inner retina in contrast to the outer retinal distribution of S antigen. The stimulus for production of these lymphokines may therefore be another antigen or

inflammatory mediator.

Cells positive for all four lymphokines were also found in the accumulations of inflammatory cells surrounding the eyes, presumably trafficking to and from the sites of the intraocular pathology. This evidence raises the possibility that some T cells may be activated peripherally and "home" towards the eye. Lymphokine production in these extraocular sites would potentially be involved in the chemotaxis and local proliferation of inflammatory cells.

It is notable that despite a heavy mixed inflammatory cell infiltrate in the anterior chamber no cytokine production was found in this site suggesting that these lymphocytes in this location are not actively involved in the intraocular pathology. This may be a reflection of the absence of suitable antigen in the anterior chamber.

5.3.3. Lymphokine mRNA expression within the eye in EAU

As stated above expression of lymphokine mRNA is not absolute evidence of the presence of bioactive lymphokine within a tissue however it does provide evidence that a given lymphokine is potentially involved in the ongoing immunopathology being studied.

The finding of IFN-γ mRNA in actively inflamed EAU eyes was supported by the immunohistochemical demonstration of IFN-γ protein in similar locations. These observations can be correlated with the immunohistochemical finding of IFN-γ related to areas of activated T cells in human posterior uveitis (Hooks et al.1986). It was demonstrated that greater numbers of T cells had strong immunohistochemical staining for IFN-γ than

had a positive signal for IFN-γ mRNA. It is likely that this is due to the transient expression of mRNA for this lymphokine by T cells (McKnight et al.1991) compared to a longer time period when lymphokine protein can be demonstrated on cells, either localised to cytokine receptors or on cells no longer expressing cytokine mRNA.

It has been demonstrated that systemically administered IFN-γ downregulates the immune response in the mouse model of EAU (Caspi et al.1991). The demonstration of increased mRNA expression for IFN-γ at the later stages of the intraocular destructive process is evidence that its local production could play a role in the in vivo immune regulation of EAU. IFN-γ is known to induce or upregulate MHC class II antigen on a variety of cell types (Trinchieri et al.1985). It has been shown that IFN-γ can induce MHC class II expression in vitro in rat, guinea-pig (Liversidge et al.1988b) and human (Liversidge et al.1988a) retinal pigment epithelial (RPE) cells which form part of the outer blood-retinal barrier. RPE cells are known to express MHC class II antigens in vivo in human posterior uveitis (Chan et al.1986a) and in EAU (Chan et al.1986b) and it has been proposed that such aberrant expression of class II MHC may be important in the development of autoimmunity (Bottazzo et al.1983) by providing a mechanism of autoantigen presentation to autoreactive T cells. There is also evidence, however, that antigen presentation by cells lacking a co-stimulatory signal (this may apply to tissue resident cells such as RPE) acts to downregulate the immune response and results in T cell anergy (Schwartz, 1990; Gaspari et al.1988). In relation to these observations it is notable that iris and ciliary body cells treated in vivo with IFN-γ are able to suppress antigen driven T

cell responses in vitro (Streilein et al.1992). This suggests that the local IFNγ production found in EAU may be downregulatory to the disease process. In this context it is notable that IFN-γ has been shown to be inhibitory to the Th2 subset of CD4+ T cells (Mosmann et al.1989).

Intraocular injection of recombinant IFN-γ has been demonstrated to induce in vivo MHC class II expression in organ resident cells in the uveal tract and cornea and also in extraocular conjunctival epithelial cells. Subretinal injection of recombinant IFN-γ produces in vivo class II expression in rats (Hamel et al.1990). These changes were shown to be associated with an infiltrate of both neutrophils and monocytes to the iris and inner retinal layers suggesting that local IFN-γ plays an important role in the induction of autoimmune inflammatory disease.The studies of Lee and Pepose (Lee et al.1990) demonstrated that both mice and rats had induced expression of MHC class II antigens on intraocular injection of IFN-γ but only rats demonstrated an related ocular cellular infiltrate. This observation parallels the known susceptibility of mice and rats to EAU. It has also been demonstrated that intraperitoneal injection of IFN-γ leads to intraocular class II expression wheras topical administration does not (Kusuda et al.1989). Local production of IFN-γ has the potential to play a role in immune regulation in inflammatory eye disease although the studies outlined above demonstrate that the exact nature of this role is yet to be defined.

Interleukin 2 (IL2) is a lymphokine which is produced by helper T cells following activation by antigenic or mitogenic stimulation, it is required for the subsequent proliferation of T lymphocytes and therefore plays a key role in the initiation of an immune response (Smith, 1988). IL2 protein has been

demonstrated in human eyes with posterior uveitis (Hooks et al.1986) and an increase in circulating IL2 receptor positive (Deschenes et al.1988) T lymphocytes has been demonstrated in patients with active uveitis. The demonstration of IL2 mRNA and IL2 receptor positive lymphocytes in EAU is further evidence that this mediator is involved in the immunopathology of intraocular inflammatory disease.

Clinical and experimental studies of autoimmune disease have indicated abnormalities in the IL2-IL2 receptor system. Increased T lymphocyte IL2 production and enhanced IL2 responsiveness have been demonstrated in experimental models of autoimmunity (Kroemer et al.1989). IL2 mRNA has been demonstrated at the sites of tissue pathology in rheumatoid arthritis (Warren et al.1991) a condition thought to have an autoimmune aetiology. In vivo expression of IL2 mRNA by activated T cells at the site of tissue pathology in EAU is additional evidence for its role in autoimmune disease.

Lymphotoxin is produced by both CD4+ and CD8+ T lymphocytes and is thought to play a key role in T lymphocyte cytotoxicity (Paul et al.1988). IFN-γ is of importance in the maturation of cytotoxic T lymphocytes (Simon et al.1986; Chen et al.1986) and secretion of IFN-γ along with lymphotoxin appears to promote CD4+, class II restricted T cell cytotoxicity (Tite et al.1985). IL2 has also been demonstrated to produce differentiation of cytotoxic T lymphocytes (Simon et al.1986; Haworth et al.1989). The in vivo expression of mRNA for these lymphokines at the site of tissue pathology in EAU is evidence that tissue destruction may be produced by effector T lymphocytes.

T cell cytotoxicity can be mediated by several mechanisms (Tite et al.1985; Tite, 1990a; Tite, 1990b; Lancki et al.1991) and the relatively low numbers of cells in the retinal pathology expressing lymphotoxin mRNA would suggest that cytotoxic mechanisms other than those involving lymphotoxin are involved (Jongeneel et al.1988). The widespread cellular expression of MHC class II antigens in the eye in EAU would provide suitable target cells for cytotoxic CD4+ T cells which may subsequently produce more extensive damage through "innocent bystander" killing (Tite et al.1985). IFN-γ, however, also has the potential to activate macrophages (Nathan et al.1983) and it is possible that macrophages have an effector cell function at the sites of tissue destruction in EAU .

IL4 produces B lymphocyte activation, MHC class II expression and immunoglobulin (IgG_1 and IgE) production. It is also involved in T lymphocyte activation and growth (Paul et al.1987) and has also been shown to inhibit the action of certain T cell subsets (Peleman et al.1989). Although few B cells are present in the intraocular pathology in EAU, in vivo IL4 production would fascilitate antigen presentation by B cells to the infiltrating antigen-specific T lymphocytes.

In summary,with regard to the effector mechanisms operative in EAU the demonstration of in vivo production of IFN-γ, IL2, lymphotoxin and IL4 would allow CD4 positive and CD8 positive T lymphocytes and macrophages to act as effector cells and it may be that all these cell types are involved in the destructive process. Several issues remain to be resolved regarding the effector cells in EAU and inflammatory eye disease. Firstly the relative contributon of the potential effector cells present.

Secondly the mechanisms employed by these cells in vivo to produce the tissue pathology. Thirdly whether the T cells present are all antigen specific arising from local clonal expansion of small numbers of infiltrating cells or whether local cytokine production causes the attraction and infiltration of non-antigen specific T cells which may subsequently act as effectors.

5.3.4. T Cell Subsets

Subsets of T lymphocytes have been defined based on their pattern of lymphokine secretion and these subsets appear to correlate with the functional properties of the activated T cells. In general these studies have been carried out in vitro and it is uncertain if these patterns exist also in vivo. The experiments of Mossman and coworkers divided murine CD4+ T cell clones into two types on the basis of their lymphokine secretion (Mosmann et al.1986; Mosmann et al.1989). Th1 cells produce IL2, IFNγ and LT and are thought to function as cytotoxic or inflammatory effector cells and to mediate delayed type hypersensitivity (DTH) responses (Cher et al.1987). Th2 cells produce IL4, IL5 and IL6 and are considered to act as helper cells for example upregulating B cell and antibody responses and mediating many of the effects of allergic responses. Both cell types produce IL3, GM-CSF and TNF. In addition murine T cell clones have been described which do not fit these patterns of lymphokine secretion (Firestein et al.1993; Yokohama et al.1989; Mosmann et al.1991). It has been proposed that these cells, which in many cases have an unrestricted lymphokine secretion profile, have been termed T0 and it has been proposed that they represent a precursor stage in the development the restricted Th1 and Th2 patterns.

Th1 and Th2 helper T cell subsets have been shown to cross-regulate by the production of of lymphokine which are inhibitory or down-regulatory to the other subset. IL4 production by Th2 cells has been shown to suppress the production of IFNγ by Th1 cells (Peleman et al.1989) and IL10 (produced by Th2 cells) acts to inhibit the production of all lymphokines by Th1 cells (Mosmann et al.1991). Conversely IFNγ has been shown to inhibit the proliferative response of Th2 clones to IL2 and IL4 (Mosmann et al.1989).

T cell subsets have also been defined on the basis of their cell surface antigens, in particular the isotypes of the CD45 antigen (Leucocyte common antigen, LCA) (Mason, 1992). The monoclonal antibody OX22 binds to the product of exon C of the LCA gene on rat CD4+ T cells. These "OX22 high" (by flow cytometry) produce IL2 and IFNγ. Following antigen contact these cells have low OX22 binding and produce higher levels of IL4 (Powrie et al.1988; Powrie et al.1989; McKnight et al.1991). Recent work has suggested that these OX22 low CD4+ T cells have an inhibitory effect on the development of autoimmune disease in animal models (Mason et al.1992). Human and murine CD4+ T cells also appear to have functional properties defined by the expression of of differing isoforms of CD45, however the data on these subsets is as yet incomplete (Mason, 1992) and at present it is is difficult to fit the data relating to the CD45 isotype expression with any simple model of Th1 and Th2 subsets.

It would appear that the Th1/Th2 dichotomy represents an end-stage in T cell differentiation and there is now evidence that these stable patterns of lymphokine secretion may also be found in mice in vivo (Bass et al.1989). Initial work suggested that human T cell clones produced IL2, IL4 and IFNγ

and therefore did not subdivide as Th1 and Th2 (Paliard et al.1988). Recent studies have suggested that human T cell clones derived from peripheral sites of tissue pathology do fall into the Th1/Th2 subsets (Wierenga et al.1990; Maggi et al.1991; Kapsenberg et al.1991). These studies suggested that long term stimulation of T cells in atopic patients resulted in selection of Th2 cells. The previous finding of unrestricted lymphokine profiles in human CD4 positive T cells would therefore represent T cells not yet committed to a differentiation pathway and the chronically stimulated T cells found in atopic individuals have differentiated to a stable Th_2 secretion pattern (Kapsenberg et al.1991). In addition stable antigen-specific clones of Th_1 and Th_2 type have been derived from peripheral T cells from healthy individuals (Romagnani, 1991).

The presence of mRNA for the lymphokines IFNγ, IL2, lymphotoxin and IL4 was demonstrated in the areas of T lymphocyte infiltrate in actively inflamed eyes in EAU. The presence of these four cytokines has implications with regard to the subsets of CD4+ T cells (the predominant infiltrating cell type in EAU) present in the rat model used. In relation to the Th1/Th2 CD4+ T cell dichotomy the expression of mRNA for these four lymphokines implies the presence of a mixed infiltrate of both subsets. Alternatively the CD4+ T cells could be of the T0 subtype, not yet committed to to a Th1 or Th2 lymphokine secretion pattern. It is also possible that the infiltrating CD4+ T cells are an admixture of all three subsets (and potentially additional subsets which are as yet not fully defined).

A mixed Th1/Th2 response would result in the interaction of these two subsets producing mutual inhibition. This may be produced through several

mechanisms, for example through the action of the Th1 lymphokine IFNγ downregulating the Th2 response (Mosmann et al.1989) and the Th2 lymphokine IL4 acting to downregulate the Th1 response (Peleman et al.1989). These inhibitory responses may be important in the in vivo limitation of an immune response to any given antigen. Initial activation of the Th1 and Th2 subsets is thought to be dependent on the nature of the antigenic stimulus and the antigen presenting cell (Mosmann et al.1989) and manipulation of an immune response at this level could potentially provide a mechanism of downregulating ongoing in vivo immunopathology.

In relation to the CD45 antigen isoform expression of the T cells present (Powrie et al.1988; Faure, 1980; McKnight et al.1991; Mason, 1992), the expression of mRNA for these four lymphokines would imply the presence of both niave and memory T cells. This would seem logical since any section of an eye would be likely to contain a mixture of T cells involved in the dynamic process of trafficking to the eye followed by antigen contact and subsequent effector or helper function. It is also possible that additional subgroups of rat CD4+ T cells exist and the position may be considerably more complex. Analysis of the cytokine secretion by individual T cells from the inflammatory infiltrates would be necessary to further investigate the exact nature of the T cells present.

5.3.5. Cytokines in autoimmune disease

Several methods have been employed in the investigation of the mediators involved in autoimmune disease. Tissue fluids or cell extracts have been analysed using bioassays but this approach has not been

productive due to the lack of specificity of the assays used (although this can potentially be overcome by the use of blocking monoclonal antibodies) and the short half life of bioactive lymphokines. Immunohistochemical analysis of affected tissues can be employed but the range of suitable antibodies is at present limited. Analysis of the lymphokine mRNA by in-situ-hybridisation or mRNA extraction and estimation provides a further approach to the assessment of the production of cytokines in vivo. As argued above however the presence of cytokine mRNA does not necessarily correlate with bioactive cytokine (Grabstein et al.1986; Carding et al.1989; Dallman et al.1991).

Rheumatoid arthritis (RA) is a chronic destructive inflammatory disease involving the articular synovium which is thought to have an autoimmune aetiology. A wide spectrum of cytokines have been demonstrated to be represent in the destructive inflammatory lesions found in this condition. Buchan and colleagues have demonstrated the presence of mRNA for IL2, IL2 receptor, IFN-γ, IL1α, IL1β, LT and TNF in the mononuclear cells derived from the diseased joints of patients with rheumatoid arthritis (Buchan et al.1988a; Buchan et al.1988b). They also demonstrated increased levels of these cytokines were produced by mononuclear cells in vitro without stimulation for up to two weeks following removal from tissue implying a chronic abnormality in regulation of lymphokine production by these cells in the rheumatoid joint. In situ hybridisation analysis of the rheumatoid synovium has shown the in vivo expression of IL2 mRNA in inflammmatory foci where the majority of cells are CD4 positive, CD45RO positive T lymphocytes and IL2 protein is localised immunohistochemically (Warren et al.1991). Turner and coworkers demonstrated that T lymphocyte clones

derived from the sites of tissue pathology from patients with rheumatoid arthritis produced TNF, lymphotoxin and IFN-γ (Turner et al.1987). They also demonstrated mRNA for TNF and lymphotoxin in these cells. Feldmann has reviewed the results of cytokine research on rheumatoid arthritis joints showing that mRNA for IL1α, IL1β, IL2, IL6, IL8, TNF, LT, IFN-γ, TGFβ, PDGF(A/B), TGFα, EGF, G-CSF and GM-CSF is present in the joint and in most cases this correlates with the presence of cytokine protein although the relative concentration of protein is often lower than would be expected from the mRNA level (Feldmann et al.1990; Feldmann et al.1991). In addition Feldmann has documented that cytokine mRNA expression is not concordant with the presence of protein and that this is true especially for T cell derived cytokines. Several explanations have been offered for this finding including absorption by receptors, degradation by enzymes, inhibition by antagonists, and translational regulation by other cytokines.

The presence of cytokines at the sites of local tissue pathology in autoimmune thyroid disease has also been studied. This has demonstrated the presence of mRNA for IFN-γ, TNF, LT, IL6, IL1α and IL1β in both Graves' disease and Hashimoto's thyroiditis (Turner et al.1987; Feldmann et al.1991; Zheng et al.1991). The studies on rheumatoid arthritis and autoimmune thyroid disease have been supported by in vitro work which has explored the potential role of these cytokine in disease pathogenesis. For example it has been demonstrated that TNF and LT are strong inducers of IL1 in RA joint cell cultures in vitro (Feldmann et al.1991) and that anti-TNF (but not anti-LT) abrogated the production of IL1 bioactivity. Similarly in vitro studies have demonstrated that thyroid epithelial cells can produce IL1 and IL6

(Feldmann et al.1991) and this correlates well with previous work showing that such cells are capable of presenting antigen to cloned human T cells (Londei et al.1984).

In diabetes mellitus the localised autoimmune events take place in the pancreatic islets, however this tissue is generally inaccessible to direct investigation. A study of cytokine levels in the peripheral blood of diabetic patients at the time of diagnosis showed increased levels of TNF compared to controls but similar levels of IL1, IL2, IL6 and IFN-γ (Cavallo et al.1991). This highlights the difficulty in interpreting the results of investigations carried out on peripheral blood in the context of localised autoimmune pathology since all these cytokines may reasonably be expected to be involved in the local immunopathology.

Experimental work has been undertaken in transgenic mice to determine the potential role of cytokines in the immunopathology of diabetes mellitus. Sarvetnick and colleagues (Sarvetnick et al.1990) used transgenic mice in which the expression of IFN-γ is directed by the insulin promotor to demonstrate that development of diabetes with an influx of inflammatory cells to the pancreas and additionally that lymphocytes from these transgenic animals are cytotoxic to pancreatic islets in vitro. This implies a role for IFN-γ in the loss of tolerance to pancreatic islet tissue in DM.

In contrast mice expressing the IL2 transgene develop insulitis but not diabetes (Allison et al.1992). Double transgenic mice which express the MHC class I molecule H2-K^b and have specific anti K^b T cells do not develop diabetes (Heath et al.1992) however if these animals are crossed with the IL2 transgene mice diabetes does develop suggesting that autoreactive T

cells require the addition of IL2 to produce an autoimmune response.

Studies of cytokine involvement in multiple sclerosis have been supported by work carried out on the experimental allergic encephalomyelitis (EAE) animal model. Analysis of the infiltrating leucocytes at the sites of demyelinating lesions in the CNS have shown that both CD4+ and CD8+ T lymphocytes are present at the lesion margin and macrophages are found in the centre of the lesion (Traugott et al.1983). Investigations have attempted to define the TCR Vα and Vβ repertoire of the T cells involved. Initial work suggested that the TCR Vα and Vβ chain expression was restricted in both CSF and blood (Hafler et al.1988) however subsequent work has demonstrated a polyclonal population of TCR Vα and Vβ in localised brain lesions in MS (Wucherpfennig et al.1992). This may be due to non-specific recruitment of T lymphocytes and it is possible that the autoreactive T cells within these lesions do in fact have limited TCR heterogeneity (Bernard et al.1992).

Recent studies have also focussed on potential involvement of cytokines in the destructive CNS pathology in MS. Tumour necrosis factor and lymphotoxin have been identified immunohistochemically associated with both lymphocytes and glial cells within the lesions (Hofman et al.1989; Selmaj et al.1991). Wucherpfennig and coworkers demonstrated mRNA for IL1 in acute and subacute lesions but lower levels of IL2 and IL4 mRNA in only acute lesions (Wucherpfennig et al.1992). These studies have been paralleled by investigations on the EAE model which immunohistochemically demonstrated the presence of IL1, IL2, IL2 receptor, IL4, IL5, IL6, IFN-γ, TNF, LT and TGF-β protein in acute lesions leading to the

proposition that Th1 CD4+ T cell / macrophage interactions are responsible for the local acute pathology. This work has been developed by the demonstration that neutralising antibody to LT and TNF can reduce the severity of disease in EAE (Ruddle et al.1990).

The experimental work in this thesis has shown the expression of mRNA for IFN-γ, IL2, IL4 and LT in the areas of T cell infiltrate in EAU. This has been supported by the immunohistochemical demonstration of IFN-γ protein and IL2 receptor on lymphocytes. In common with the other forms of autoimmunity described above these results suggest that these cytokines can play a role in the development of intraocular inflammatory disease in the EAU model and potentially also in human disease. In the case of IFN-γ functional studies also defined an immunoregulatory role in EAU (Caspi et al.1991; Hamel et al.1990; Lee et al.1990). Additionally it has been shown that soluble IL1 receptor inhibits ocular inflammation in an animal model (Rosenbaum et al.1991). The results therefore provide a basis for the further investigation of the biological role these cytokines play in inflammatory eye disease.

5.4 Future developments in the treatment of intraocular inflammatory disease

Clinical therapy of intraocular inflammatory disease has been based on the non-specific suppession of the inflammatory and immune responses causing tissue damage. The efficacy of such treatment in severe intraocular inflammatory disease is limited by the non-specific nature of the drugs used, resulting in a generalised immunosuppression leading to susceptibility to

infectious disease and other systemic side-effects resulting from the prolonged high dosage of steroids and cytotoxic agents (Salaman, 1983; Kinlen et al.1979). The aim in developing new therapies is therefore to produce agents which will specifically target the elements involved in the detremental immunopathology found in intraocular inflammation while sparing the inflammatory and immune response components necessary for the normal function of systemic defence mechanisms. More specific agents would also potentially be free from other unwanted systemic side-effects.

A number of potential sites for specific immunotherapy in intraocular inflammatory disease can be identified. For example the interaction of the TCR with antigen and MHC, the costimulatory signals involved in antigen presentation and the interaction of T cells with vascular endothelium.

5.4.1. Anti T lymphocyte therapy

A first step towards selective clinical immunotherapy for inflammatory eye disease was the introduction of the anti T lymphocyte agent cyclosporin A (Nussenblatt et al.1983a; Nussenblatt et al.1985; Graham et al.1985; de Vries et al.1990; Masuda et al.1989), this was paralleled by experimental work demonstrating its anti T cell effect on the EAU model (Nussenblatt et al.1981b). Cyclosporin A produces a specific anti T cell action by inhibiting production of IL2 and IFN-γ and inhibiting expression of the IL2 receptor. FK 506 is a new immunosuppressive agent, at present used only on an experimental and trial basis, which has a mode of action similar to cyclosporin A although it is structurally distinct (Thomson, 1989). The primary immunosuppressive effect of FK 506 is an inhibition of antigen-

driven production of IL2 and IFN-γ, and in experimental models it produces this effect at much lower molar doses than cyclosporin A. It is hoped therefore that FK 506 will produce clinical immunosuppression at a lower dose than cyclosporin A and with fewer side effects. Initial work has suggested that FK 506 is relatively non-toxic although a vasculitis has been reported in dogs and further experimental and clinical studies are awaited. Furthermore, FK 506 appears to have a synergistic effect with cyclosporin A and combined therapy of low dose FK 506 and cyclosporin A has been shown to suppress EAU (Mochizuki et al.1991). Rapamycin is another new immunosuppressive agent which, although structurally related to FK 506 (both are macrocyclic lactones), appears to act by a different mechanism (Morris, 1991). Rapamycin blocks Ca^{2+} independent and cytokine-dependent T cell division and produces synergistic immunosuppression with FK 506. The clinical usefulness of FK 506 and rapamycin have yet to be evaluated but specific T cell immunosuppression has the potential to play a significant role in the future treatment of T cell mediated autoimmune disease.

5.4.2. Blocking of specific T lymphocyte responses

T lymphocytes have been shown to play a central role in the immunopathology in both human intraocular inflammation and EAU. Manipulation of T cell responses could therefore provide an effective means of suppressing the tissue damage seen in these conditions. Several approaches to the modification of T cell responses are currently under investigation and will hopefully prove useful in the treatment of human

disease. T cell vaccination involves inoculation with subpathogenic doses of disease promoting T cells which induce regulatory T cells that downregulate the immunopathological process (Cohen et al.1988). This approach has been shown to be effective in experimental animal models of autoimmune disease (Cohen, 1986) including EAU (Beraud et al.1990). Major limitations to its use in humans, however, are the need for antigen specific T cells since the autoantigen is unknown in most forms of human autoimmune disease and the need to use T cells specific for each patient. Therapeutic intervention using monoclonal antibodies specific for T cell surface receptors involved in antigen recognition may also be possible. In rheumatoid arthritis patients for example clinical improvement has been demonstrated using anti-CD4 monoclonal antibody (Kingsley et al.1991). The use of foreign immunoglobulins in humans can produce anti-globulin responses which can interfere with therapy or cause hypersensitivity reactions. To prevent this human antibodies should be used but it has been difficult to make such antibodies by hybridoma technology. A solution may be to produce "chimaeric" antibodies with mouse variable and human constant regions by the genetic engineering of lymphoid cells (Riechmann et al.1988). These antibodies have the potential to minimize adverse reactions.

Therapeutic intervention at the level of the interaction of the T cell receptor(TCR)/CD4 complex with the antigen/MHC complex may also be possible. Analysis of the restricted TCR and MHC gene expression and the protein structure of the inducing antigens in autoimmune disease models has led the development of specific therapies. In EAE a synthetic peptide analogous to part of the $V\beta 8$ TCR gene was found to be effective in

protecting against, and treating rats with EAE (Offner et al.1991). Alternatively antigen which interacts with MHC can be modified to produce a peptide which will block T cell activation and have enhanced MHC binding as has been demonstrated in EAE where a MBP peptide analog which binds to Ia but does not activate T cells was found to be effective in blocking T cell responses in vitro and in vivo (Urban et al.1989).

5.4.3. Blocking cellular interaction with blood vessel endothelium

The term "high endothelial venule" (HEV) is applied to the post-capillary venules of lymph nodes and also to vessels in inflamed tissue which morphologically have an increase in the height of their endothelial cells associated with an enlarged, ovoid nucleus and abundant cytoplasm (Freemont et al.1985; Freemont, 1988). The biological changes associated with endothelial cell activation such as MHC class I and II expression, secretion of IL1, increased ICAM-1 expression and increased lymphocyte adhesion have been shown to be associated with a change to "high endothelial venule" morphology (Pober, 1988; Duijvestijn et al.1989; Butcher, 1990). The binding of recirculating lymphocytes to endothelial cells plays an important role in the migration of lymphocytes to lymph nodes and to inflamed tissues. It has been demonstrated that lymphocytes can discriminate between HEV in different body tissues (Butcher, 1990) and it appears that these lymphocyte-endothelial interactions are mediated by tissue-specific homing receptors and non tissue-specific adhesion molecules (Duijvestijn et al.1989). The pathology described in the ultrastructural studies of multifocal posterior uveitis demonstrates that HEV-

like changes were found in the choroidal vessels (and the neovascularisation extending through Bruch's membrane to the retina) specifically related to the inflammatory foci. Interactions between these HEV and recirculating lymphocytes could result in the selective migration of lymphocytes to the sites of ongoing inflammation in uveitis.

In the Lewis rat model of experimental autoimmune uveoretinitis HEV like vessels have been reported to occur in the retinal vasculature but not in choroidal vessels (Dua et al.1991) suggesting that this model is similar to human retinal vasculitis rather than the predominantly choroidal disease described above.

The eye does not contain lymphatics and hence presentation of retinal antigens must occur at the sites of the blood-retinal barrier. The inner blood-retinal barrier is formed by the endothelial cells of the retinal vessels and the outer blood-retinal barrier is formed by the RPE cells, Bruch's membrane and the endothelium of the choriocapillaris. It has been demonstrated that when stimulated (for example by IFN-γ) vascular endothelial cells from a variety of organs can express MHC class II antigen (Pober et al.1986). In vitro investigations on cerebral vascular endothelial cells have produced conflicting results as to whether these cells can produce the co-stimulatory signals necessary for effective antigen presentation (Pryce et al.1989; Risau et al.1990; Hughes et al.1990). Endothelial cell activation may therefore be of importance in vivo for production of the co-stimulatory signals (cytokines or other mediators) required for effective presentation of antigen by vasular endothelium. The HEV-like vessels demonstrated above may therefore be the sites of both lymphocyte trafficking and antigen presentation.

The HEV-lymphocyte interaction is a further potential site of intervention in the disease process. Specific therapy aimed at inhibiting the interaction of T lymphocytes with the suface markers such as ICAM-1, ELAM-1 and MHC class II known to be present on activated endothelial cells (HEV) (Pober, 1988) could regulate the influx of T cells to sites of immunopathology within the eye. Furthermore, since it has been shown that lymphocytes can discriminate between HEV in different body tissues (Butcher, 1990) the differentiation markers for HEV in various tissues (although not yet definitively identified) are a potential target which would provide specificity for this therapy. ICAM-1 has been demonstrated to be involved in the pathological processes in asthma and therapy using an anti-ICAM-1 monoclonal antibody in a primate model of asthma produces an inhibition of the pathological responses (Wegner et al.1990) suggesting that therapy directed towards the interaction of lymphocytes and adhesion molecules may find widespread in vivo clinical application.

Although not yet fully identified, the costimulatory signals necessary for effective antigen presentation are another potential target for intervention. The demonstration that tolerance may result from antigen presentation without appropriate costimulation (Schwartz, 1990; Gaspari et al.1988) suggests the possibility of immunosuppression by blocking costimulatory signals.

5.4.4. Anti-cytokine therapy

The experimental work in this thesis has dealt with the in vivo T cell lymphokine production in EAU. Several strategies have been proposed to

produce specific anti-cytokine therapies and are currently under investigation. Monoclonal antibodies reactive with a cytokine or its receptor can act to interfere with the biological action of a particular cytokine. This approach has been employed in experimental autoimmune disease. For example, antibodies reactive with the p55 β chain of the IL2 receptor suppress the development of experimental autoimmune diabetes mellitus and systemic lupus erythematosus (Kelley et al.1988). Another method of interference with the cytokine-receptor interaction is the therapeutic administration of soluble cytokine receptors which bind to cytokine and provide a competitive antagonism of the cytokine action (Maliszewski et al.1990). Administration of soluble IL1 receptor has been shown to be effective in preventing experimental T cell-mediated graft failure (Fanslow et al.1991) and may also have wider applications in the treatment of autoimmune disease. Specific cytokine inhibitors are also being developed, for example the IL1 antagonist IL1ra (Ohlsson et al.1990), although the therapeutic role of these is at present uncertain.

Targetting a specific cytokine receptor could also downregulate an immune response. Such an approach has been demonstrated to be successful in the NOD (non-obese diadetic) mouse model of autoimmune diabetes using a diphtheria toxin related IL2 fusion protein (Pacheco-Silva et al.1992).

Lymphokines exert their effects in the local microenviroment of the secreting cell. This may lead to problems in the systemic administration of anti-lymphokine therapy requiring very high dosage to achieve sufficient concentrations in the areas of the local immunopathology. A further problem

is whether immunotherapy aimed at a specific cytokine would be effective in the complex cytokine networks which mediate severe immunopathology. and further experimental work will be required to resolve this issue.

5.5 Conclusion

This thesis has for the first time demonstrated the involvement of T lymphocytes in the intraocular immunopathology in Behcet's disease and multifocal posterior uveitis. The experimental work in the thesis has shown the expression of lymphokine mRNA in the active stages of experimental autoimmune uveoretinitis. Overall these findings are evidence for the central involvement of T lymphocytes in the immunopathology of ocular inflammatory disease. The EAU studies are the first published investigations to address the local ocular immunopathology using in situ hybridisation. The results of these studies provide the basis for future investigations of human and experimental intraocular inflammation at the molecular level. The knowledge gained from such work will contribute to the development of specific and effective future treatment for human intraocular inflammatory disease.

Appendix

Notes on the statistical methods used in chapters 3 & 4

$$\text{S.E.M.} = \frac{\text{S.D}}{\sqrt{n}}$$

$$\text{S.E. Diff.} = \sqrt{\frac{SD_1^2}{n_1} + \frac{SD_2^2}{n_2}}$$

S.E.M. = standard error of the mean
S.D. = standard deviation
n = number of counts
S.E. Diff. = standard error of the difference between two means

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Multifocal posterior uveitis: clinical and pathological findings

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CASE REPORTS

Multifocal posterior uveitis: clinical and pathological findings

David G Charteris, William R Lee

Abstract

A pathological study was performed on the necropsy eyes of a 59-year old-woman who had suffered for nine years from multifocal posterior uveitis. The disease had been controlled by steroid therapy with good preservation of visual function. Extensive investigation did not reveal the aetiology. On macroscopic examination numerous focal lesions with various degrees of pigmentation were observed scattered across the fundi. These lesions were studied by light and electron microscopy and immunohistochemistry. There was ongoing chorioretinal inflammation in the foci, producing destruction of Bruch's membrane, the retinal pigment epithelium (RPE), and the outer retina. The focal scars showed migration of RPE and glial cells and neovascularisation. Capillary and venule endothelial cells were swollen at the inflammatory sites. Attempts to establish a cause for this condition were unsuccessful.

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The pathogenesis of many forms of multifocal chorioretinal inflammatory disease remains unknown, and a lack of histopathological material has in many cases prevented an understanding of the aetiology of these conditions. In this report we describe the unexpectedly active and destructive inflammatory changes found in a case of multifocal choroiditis which clinically was thought to be quiescent.

Case report

In 1978 a 50-year-old woman presented to the eye casualty department with a one-month history of aching pain and blurring vision in both eyes. Her vision was 6/12 in the right and 6/18 in the left eye. She had mild iritis and mild optic disc oedema in both eyes. Two weeks later her vision deteriorated to counting fingers right and hand movements left. In addition to moderate anterior chamber inflammatory activity she had bilateral total exudative retinal detachments. She had no systemic illness, was on no medication, and extensive investigation including viral serology gave negative results.

Treatment with systemic prednisolone 80 mg/day resulted in rapid regression of the exudative detachments; cream coloured round patches then became evident throughout the whole extent of both fundi (Fig 1). Fluorescein angiography showed patchy late hyperfluorescence. The electro-oculogram (EOG) was reduced to 140% in the right eye and 120% in the left. A provisional diagnosis of acute posterior multifocal placoid pigment epitheliopathy (APMPPE) was made. The patient's vision gradually improved, and the steroid dose was reduced, though two transient episodes of increased activity required temporary increases in steroid dosage. One year after presentation her visual acuity was 6/9 right and 6/6 left, and there was patchy depigmentary chorioretinal scarring, with mottled pigmentation of the maculae (Fig 2).

Low dose systemic steroids were continued for a total of five years. There was mild residual anterior uveitis following systemic steroid withdrawal. The patient died in 1987 from an acute myocardial infarct. Six weeks prior to death her vision was 6/9 right and left, there was a mild bilateral anterior uveitis, and there were patches of chorioretinal scarring which did not show any clinical evidence of active inflammation.

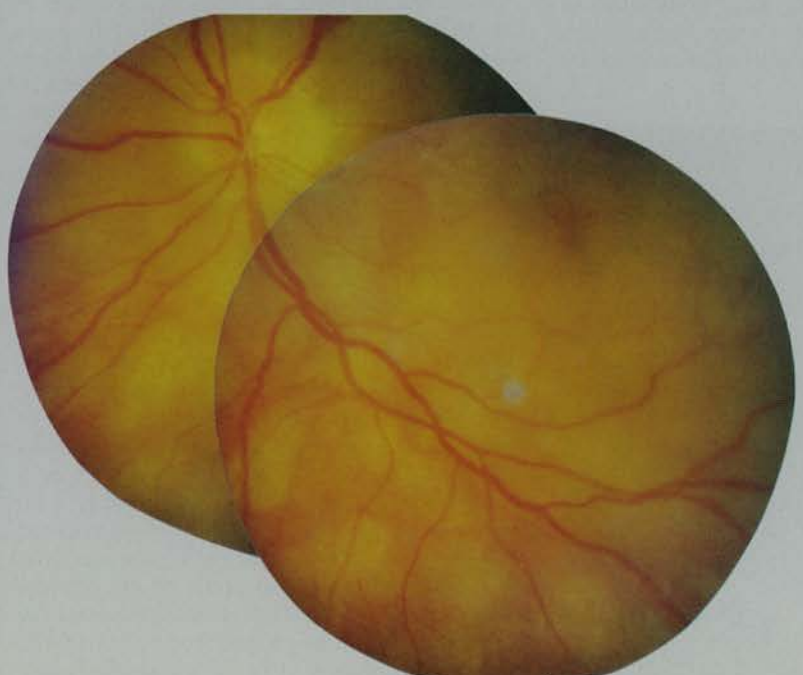

Figure 1 The appearance of the left fundus at the time of presentation in 1978. Note the multiple pale lesions scattered across the central retina.

Material and methods

The eyes were removed approximately 12 hours after death. The left eye was fixed in formal

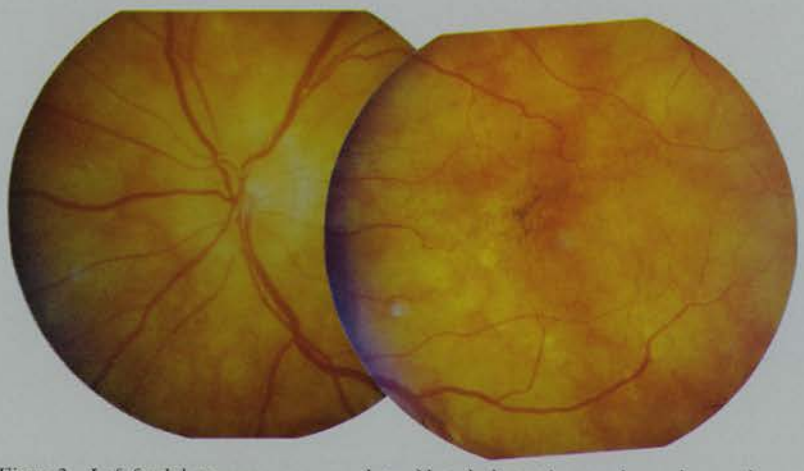

Figure 2 Left fundal appearance one year later. Note the hyperpigmentation at the macula and the loss of pigmentation elsewhere.

saline and the right in 2% cacodylate buffered glutaraldehyde. Both eyes were of normal dimensions and were divided horizontally above and below the macula.

Paraffin-histology. After paraffin embedding serial sections were taken from both paraffin blocks and stained with haematoxylin-eosin (H-E), periodic acid Schiff (PAS), and stains for axons (Bodain), myelin (Loyez), and iron (Perls). Additional blocks were taken from the calottes for immunohistochemistry and electron microscopy.

Immunohistochemistry. Sections from the formal saline fixed tissue were stained by the peroxidase-antiperoxidase (PAP) technique for B and T lymphocytes, macrophages (muraminidase, α-ACT, α-1AT), and immunoglobulins (IgA, IgG, IgM, κ and λ). In-situ hybridisation was employed for identification of herpes simplex viral antigen.

Electron microscopy. Macrophotographs were taken of the superior and inferior calottes from the right eye, and designated foci of hypo and hyperpigmentation were excised for study (Figs 3 and 4). Fifteen blocks were embedded in Araldite and 1 μm sections were stained with toluidene blue. Eight blocks were studied by semiserial sectioning (at 50 or 100 μm steps), and, where appropriate, ultrathin sections were taken and stained conventionally for examination in a Philips 301 electron microscope.

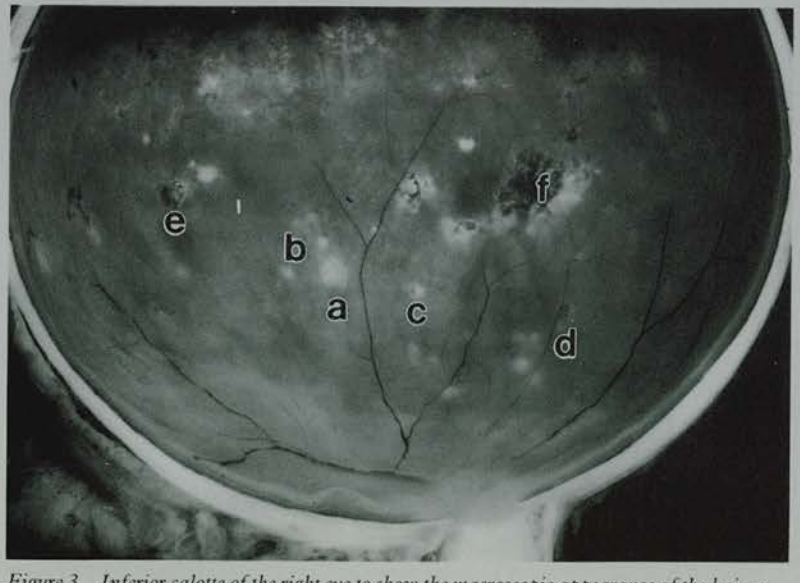

Figure 3 Inferior calotte of the right eye to show the macroscopic appearance of the lesions. EM blocks were taken from areas designated a-f (letters located below lesions).

Results

MACROSCOPIC EXAMINATION

The findings in both globes were essentially the same. The anterior segments appeared normal, and the vitreous was clear. There were meridional folds on the pars plana, but no evidence of pars planitis. Focal lesions, varying in size between 0·5 and 1·5 mm, were scattered throughout the fundus, predominantly in the mid periphery. The smallest lesions were pale and free from pigmentation, while the more obvious contained specks and clumps of pigment (Figs 3 and 4). The largest lesions were heavily pigmented with lacunae of depigmentation. The appearances of the maculae, the peripapillary retinae, and the optic discs were within normal limits for post-mortem material.

MICROSCOPIC EXAMINATION

Paraffin sections

The conjunctiva, the cornea, the outflow system, and the lens were of normal architecture. The iris stroma contained scattered lymphocytes which formed aggregates at the pupil and at the root. The ciliary body contained a few clusters of lymphocytes, and there were non-specific proliferative changes in the epithelial layers of the pars plana. The vitreous was free from inflammatory cell infiltration.

At the far periphery the retinal pigment had non-specific age-related degenerative changes, and occasional drusen were observed. The inner retina was well preserved, and the nerve fibre layer and optic nerve contained axons of normal density.

At the macula there was depletion of nuclei in the outer nuclear layer; here the photoreceptors showed autolytic changes. Clusters of lymphocytes were noted in the posterior choroid and in the adventitia of some of the posterior ciliary vessels and nerves. A study of designated representative lesions (Figs 3 and 4) was made from plastic embedded matrial.

Plastic embedded histology

The following description is derived from blocks taken from the sites designated in Figs 3 and 4. Where appropriate light microscopy was supplemented by electron microscopy. The descriptions relate to the centre of the lesions.

The small non-pigmented lesions were of variable histological appearance. In three blocks (Fig 4, lesions P, T, and W) there was a lymphocytic infiltration in the choroid, with some cellular infiltration in the hypopigmented retinal pigment epithelium (Fig 5); the overlying retina showed minor atrophy of the photoreceptor layer. Electron microscopy showed the cells within the retinal pigment epithelium (RPE) to be macrophages (Fig 5C) and the outer segments to be adequately preserved (Fig 5b). By contrast in a somewhat similar macroscopic

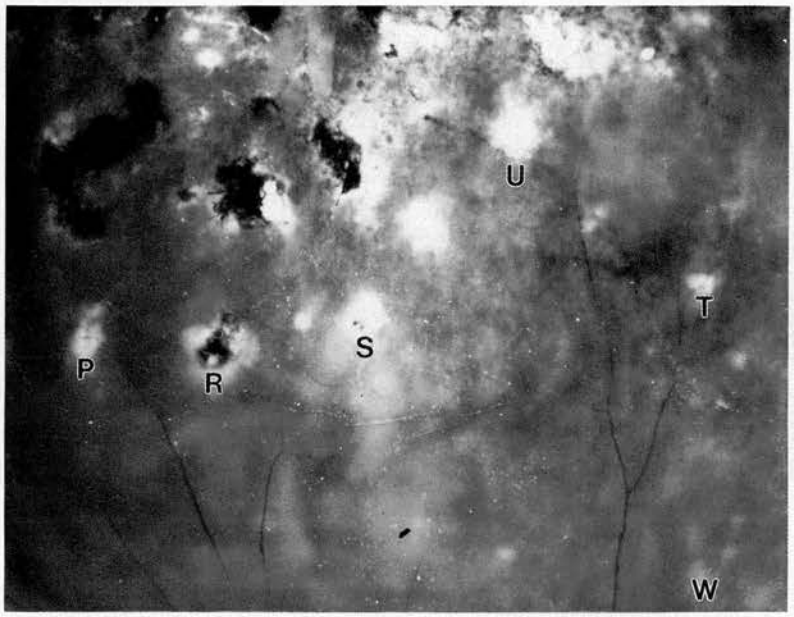

Figure 4 The appearance of the superior calotte to show in more detail the characteristic lesions. EM blocks were taken from the areas designated P-W.

lesion (Fig 3, lesion b) the RPE was replaced by glial scar tissue which had penetrated Bruch's membrane and had destroyed the choriocapillaris. Beneath this lesion there was a cluster of lymphocytes adjacent to a myelinated nerve bundle. It was noteworthy that in almost every block examined branches of ciliary nerve were observed in association with the lymphocytic clusters within the choroid.

Partially pigmented lesions also varied in their histological appearance. In three blocks (Fig 3, lesions a, c, and d) Bruch's membrane was interrupted by small breaks but was easily identifiable. In lesion c the photoreceptor layer was replaced by glial cells which abutted on to Bruch's membrane (Fig 6a). In lesions a and d (Fig 3) the RPE was multilayered and infiltrated by macrophages and lymphocytes (Figs 6b, c). Over these areas of RPE disturbance there was outer retinal atrophy of varying degrees. The choriocapillaris was either normal, infiltrated by inflammatory cells, or replaced by collagenous

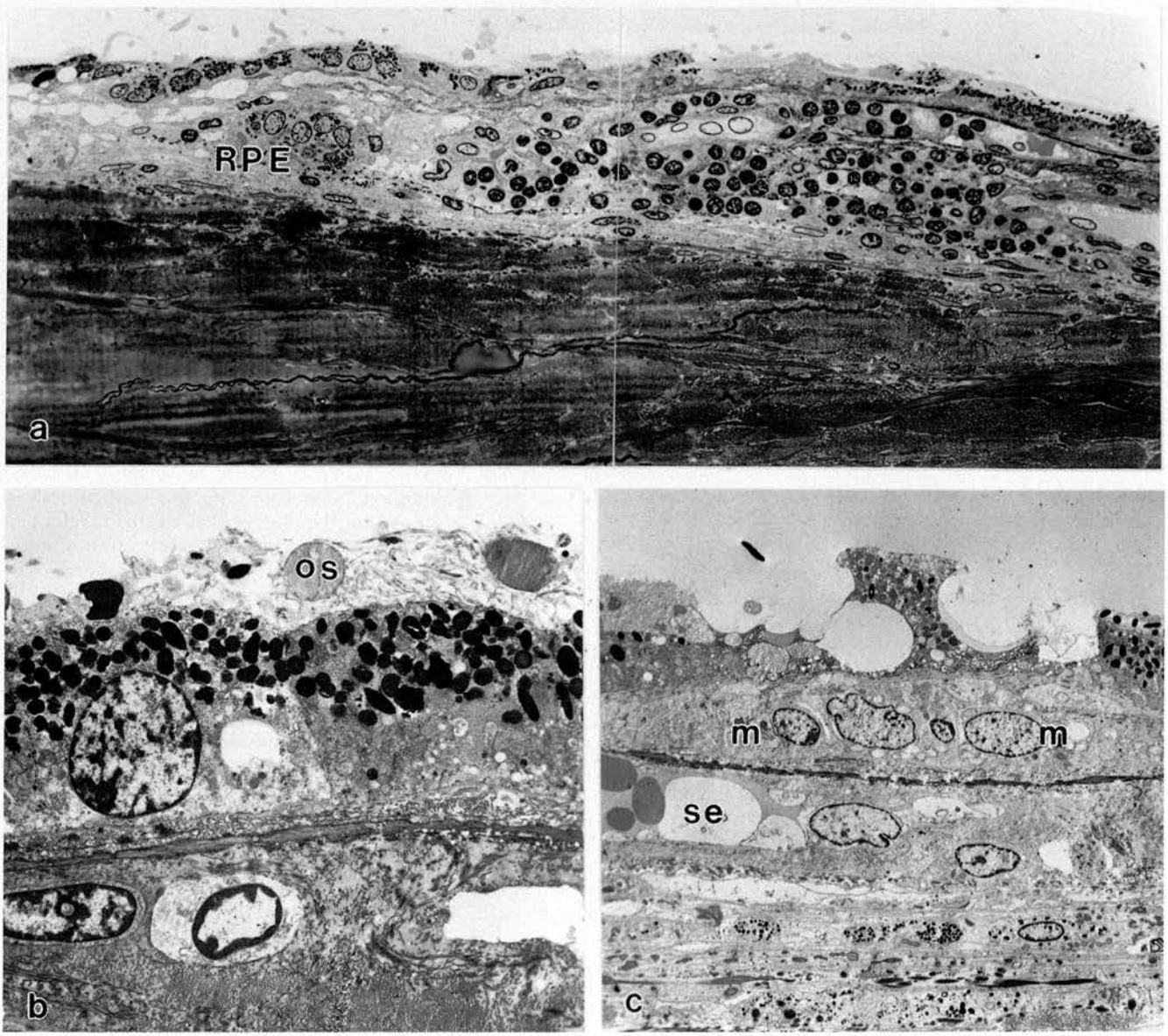

Figure 5 The choroid in small pale lesions. (a) The stroma contains lymphocytes (which are also present in the choriocapillaris) and RPE clusters (RPE). (b) Outer segments (os) are present on the surface of the RPE. (c) Macrophages (m) are infiltrating the pigment epithelium. The choriocapillaris is partially replaced by fibrous tissue and elsewhere the endothelial cells are swollen (se). (a, ×250. b, ×3000. c, ×1700.)

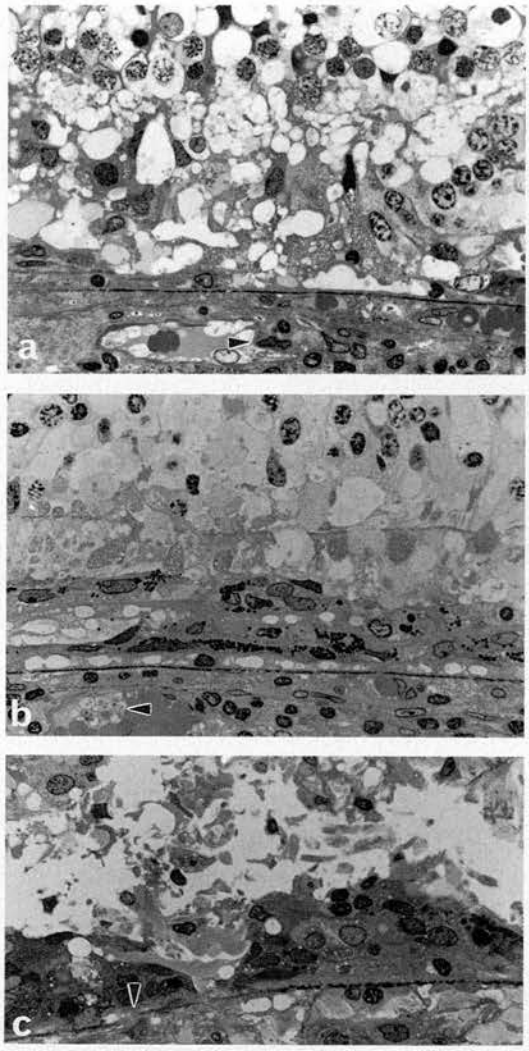

Figure 6 Light micrographs of lesions c (top), d (middle), and a (below) shown in figure 3. Bruch's membrane is intact in all three examples. (a) Inflammatory cells are sparse and gliotic retina abuts on to the membrane. (b) There is multilayering of the RPE but there is some preservation of the inner segments. (c) The photoreceptors are fragmented over clusters of RPE cells, macrophages, and lymphocytes. Note inflammatory cells in swollen endothelium in (a), a platelet aggregate in (b), and a macrophage within Bruch's membrane in (c) (arrow heads). (×375.)

tissue. Invariably there were clusters of lymphocytes in the choroidal stroma.

In other partially pigmented lesions the inflammatory process was more destructive (Fig 3, lesion e, Fig 4, lesion s). In these it was difficult to identify Bruch's membrane. The outer retina was invaded by lymphocytes, macrophages, RPE cells, and capillaries (Fig 7).

Swelling and cytoplasmic rarefaction of endothelial cells in choroidal capillaries and venules was a constant feature irrespective of the degree of inflammatory destruction (Figs 5, 6a, and 7). At the ultrastructural level the junctions between these endothelial cells were intact, but lymphocytes were often found in close relationship with the endothelial cells (Fig 6a). In some vascular lumina there were platelet clusters (Fig 6b), but neither fibrin, thrombi, nor immune complex deposits were identified. There was no evidence of previous choroidal vascular occlusive disease as shown by hyalinisation of the intima or media. Migration of RPE cells into the choroid was seen in lesions of varied appearance and degree of destruction (Figs 5 and 7).

Two heavily pigmented foci were studied (Fig 3, lesion f, and Fig 4, lesion R). These consisted of atrophic retina in all layers over a band of multilayered and hyperplastic RPE cells with an admixture of macrophages. There was a dense choroidal inflammatory infiltrate. At the periphery of even advanced lesions the photoreceptor outer segment architecture was preserved, and evidence of photoreceptor phagocytosis by exogenous macrophages was obtained (Fig 8).

In none of the sections examined at the light or ultrastructural level was it possible to identify structures with protozoal, viral, or bacterial characteristics.

Immunohistochemistry

Within the choroidal infiltrate 70–80% of the lymphocytes were identified as T cells, less than 20% were B cells. Small numbers of macrophages were noted in all parts of the inflammatory foci. There was no significant deposition of immunoglobulin. In-situ hybridisation for identification of herpes simplex virus gave negative results.

Discussion

The case was initially diagnosed as acute posterior multifocal placoid pigment epitheliopathy (APMPPE)[1] because of the multiple pale inflammatory lesions seen across the posterior fundi. Exudative retinal detachments have been described as an unusual presenting feature of APMPPE,[2] and more recent reports have stressed that APMPPE can be chronic or recurrent.[3–5] However, the presentation and subsequent course of the disease are more typical of Harada's disease.[6–8] Similarities between these two conditions have been noted, and it has been suggested that they represent a continuous spectrum of disease.[9] This case has the ocular picture of Harada's disease without the systemic manifestations and therefore best fits an intermediate group as defined by Wright, Bird, and Hamilton.[9] Because of the difficulties in categorising the case we have elected to refer to it in the broader clinical context of multifocal posterior uveitis.

The histopathological characteristics of the lesions identified in the choroid and retina include a deep choroidal lymphocytic infiltrate (usually associated with a myelinated nerve), with extension to the choriocapillaris and variable breaks in Bruch's membrane. Thickening of the RPE was due to proliferation of the native RPE cells (with and without melanin depletion) and to infiltration by macrophages and lymphocytes. The restricted focal nature of the lesions was impressive, as was the sparing of the adjacent retina. Indeed it appeared that involvement of the photoreceptor layer could be regarded as the destruction of an innocent bystander. The pattern of cellular infiltration is similar to that found by Perry and Font in cases of Harada's disease,[7] although we found few plasma cells within the infiltrate.

It has been suggested that both APMPPE and multifocal choroiditis may be initiated by choroidal ischaemia.[9–13] With reference to this

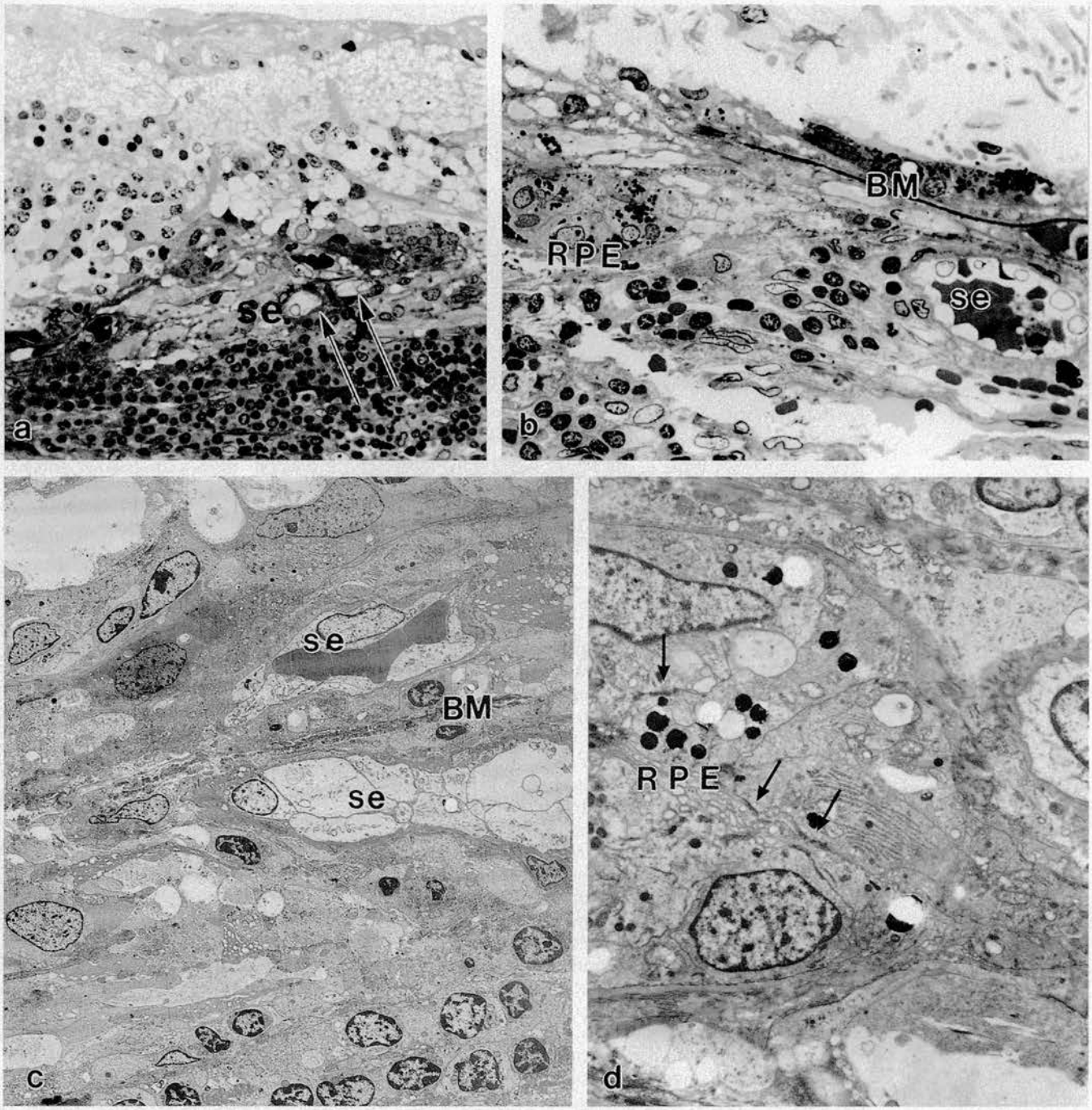

Figure 7 Lesion S in Fig 4. The inflammatory reaction had penetrated Bruch's membrane. (a) The outer retina is destroyed by an infiltrate which contains lymphocytes, macrophages, and capillaries (arrows), with swollen endothelial cells (se). (b) The choroid adjacent to the focus contains RPE cells (RPE), and the venule is lined by swollen endothelial cells (se). The capillaries present on both sides of Bruch's membrane are shown in more detail in (c). (d) This demonstrates the junctional attachments between RPE cells (RPE) within the choroid (arrows). (a, ×250. b, ×630. c, ×1500. d, ×4300.)

hypothesis we did not find immune complexes or fibrinoid necrosis in blood vessels, nor was there any evidence to suggest a previous inflammatory vasculopathy which had produced choroidal vascular hyalinisation and luminal narrowing. There was no evidence of current bacterial, fungal, viral, or protozoal infection.

The evolution of the destructive process appeared to depend on the preservation of Bruch's membrane. If the membrane survived the initial inflammatory insult, the RPE responded by proliferation and formation of layers of collagen. An intact Bruch's membrane did not necessarily protect the RPE and photoreceptor layer. RPE loss and photoreceptor destruction were seen despite the preservation of the underlying membrane, and we demonstrated that inflammatory cells may gain access to the outer retina through small breaks in an otherwise intact Bruch's membrane (Fig 6).

There was notable photoreceptor survival despite ongoing inflammation within the foci. The long term survival of photoreceptors contrasts with the early lysis of rod outer segments reported in experimental autoimmune uveitis.[14] Our evidence suggests that photoreceptor outer segments are not the primary autoimmune target in this form of human disease.

Glial cell migration from the retina to the choroid has been described in syphilis[15] and other inflammatory conditions.[16] The occurrence of this abnormality in multifocal posterior uveitis

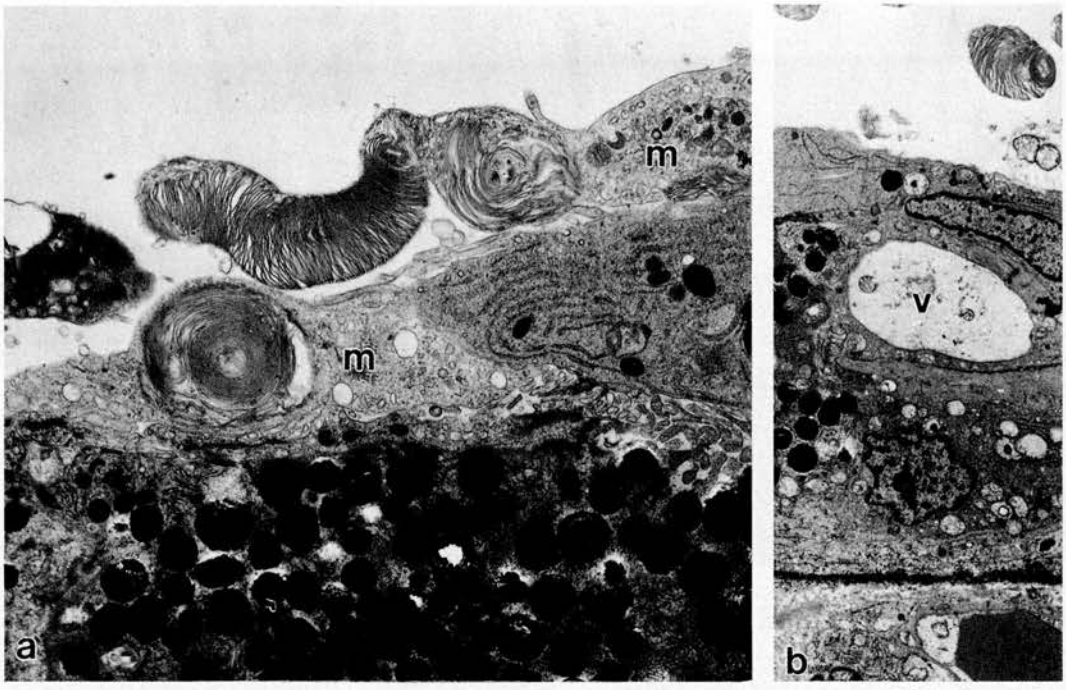

Figure 8 Electron micrographs to illustrate the preservation of the outer segments of the photoreceptors, which in this example are phagocytosed by macrophages (m). Some RPE cells contain vacuoles (v). (a, ×6000. b, ×2480.)

is further evidence of the non-specific nature of the glial cell activity. Similarly migration of RPE cells into the choroid is now regarded as non-specific,[16] as is neovascularisation in a glial scar.[17]

Recent work has highlighted the importance of high endothelial venule (HEV) like vessels in the recruitment of lymphocytes into inflamed tissues.[18,19] The term 'high endothelial venule' is applied to the post-capillary venules of lymph nodes and to vessels in inflamed tissue which morphologically have an increase in the height of their endothelial cells. These endothelial cells have histochemical alterations relating to lymphocyte-endothelial interactions and are believed to play a role in the uptake of lymphocytes to lymphatic and inflamed tissues. We frequently found this response to dense focal accumulations of lymphocytes, and we also demonstrated the presence of lymphocytes within the vessel wall. Endothelial cell swelling was demonstrated in both the choroidal venules and the choriocapillaris. The presence of such changes is of added significance in view of the recently reported association of class II major histocompatibility complex (MHC) antigen expression on endothelial cells with lymphocyte recruitment in experimental ocular inflammation.[20]

In conclusion, we have demonstrated that active, ongoing focal inflammation can occur in a case of clinically quiescent posterior uveitis. Despite detailed study we were unable to correlate the macroscopic appearance of the lesions with specific patterns of pathological change. No ischaemic or infective cause of the disorder was found.

There was migration of glial and RPE cells within the inflammatory foci and neovascularisation of the glial scars; these changes are seen in other forms of posterior uveitis. We believe that this is the first study to demonstrate the presence of high endothelial venule-like vessels at the sites of inflammation in human ocular tissue.

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CD4$^+$ LYMPHOCYTE INVOLVEMENT IN OCULAR BEHÇET'S DISEASE

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Despite extensive study, the pathogenic mechanisms of Behçet's disease remain uncertain. The ocular inflammation caused by this disease is severe, often causing significant visual loss and, although the nature of the cellular infiltrate has been examined in many of the involved organs, the infiltrating cells in inflamed eyes have not.

To investigate the mechanisms involved in perpetuating the ocular inflammation, five enucleated eyes from patients with Behçet's disease were examined by immunohistochemical staining using a panel of monoclonal and polyclonal antibodies. Control eyes from patients with chronic intraocular inflammation from other causes were also examined.

Cellular infiltrates were a consistent finding in choroid and periretinal scar tissue, formed almost entirely by mononuclear cells. T lymphocytes were found to predominate (largely the CD4$^+$ subset). B lymphocytes and NK cells were infrequent findings but macrophages were present in significant numbers. No complement or immunoglobulin deposits were found. Infiltrating lymphocytes and macrophages were HLA DR positive. Retinal vacular and retinal pigment epithelium were only occasionally positive.

Our findings suggest that cell mediated immunity, rather than immune complex deposition is responsible for the perpetuation of the ocular inflammation in Behçet's disease and that CD4$^+$ T lymphocytes play a central role in this.

KEY WORDS: CD4$^+$ T lymphocytes, Behçet's disease, uveitis, MHC class II antigens.

INTRODUCTION

Behçet's disease is a multisystem disorder characterised by aphthous mouth ulcers, genital ulcers and ocular inflammation. Other manifestations are erythema nodosum, cutaneous thrombophlebitis, arthropathy, gastrointestinal disturbances and less commonly central nervous system involvement and major vessel thrombosis. The underlying pathological mechanism is thought to be an immune-mediated occlusive vasculitis[1].

The ocular manifestations of Behçet's disease, which occur in 70–85% of patients are recurrent inflammation of the iris, ciliary body and choroid with occlusive retinal vasculitis. These may be complicated by oedema of the central retina, ischaemic atrophy of the optic nerve and visual loss. The visual prognosis is poor for untreated disease but systemic steroids and cyclosporin have been used successfully to control the inflammation[2,3].

Studies of aqueous humour[4] and peripheral blood[5,6] showing alterations in levels of complement components, led to the proposition that immune complex deposition resulted in the immunopathology of ocular Behçet's disease. Herpes simplex virus[7] and streptococcal infections[8,9] have been implicated in the initiation of the pathogenic mechanism of Behçet's disease. These theories, however, remain unproven and the aetiology of this disease is still uncertain.

Immunohistological studies of erythema nodosum like lesions[10,11], oral ulcers[11,12] and terminal ileum[13] have revealed infiltrates of predominantly T lymphocytes suggesting a central role for cell mediated immunity in the pathogenesis of these lesions in Behçet's disease. Alterations in peripheral blood T cell populations have been found[14] and decreased suppressor activity noted in the pre-active stage of Behçet's disease patients[15].

The beneficial effect of cyclosporin in systemic and ocular Behçet's disease emphasizes the importance of T cells in this condition[2,3].

Histological examination of enucleated eyes from Behçet's disease patients reveals a non-granulomatous uveitis, retinal vasculitis and infarction associated with retinal detachment[16,17]. Immunohistological studies from ocular tissue from patients with other forms of chronic immune-mediated uveitis such as sympathetic ophthalmia[18] and sarcoidosis[19] demonstrate that the predominant infiltrating cells in the retina and choroid are T lymphocytes and that the majority of these T cells are of the CD4 positive subset. The aim of this study was to examine eyes, enucleated from patients with Behçet's disease in which active inflammatory foci were present, to determine

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the type of cells involved in perpetuation of the inflammatory response.

MATERIALS AND METHODS

Material

Five eyes, enucleated for the complications of ocular inflammation characteristic of Behçet's disease, were obtained from the archives of the Institute of Ophthalmology. All cases fulfilled the major diagnostic criteria for Behçet's disease confirmed by contacting the clinician involved in 4 of the 5 cases. The average age of the patients at enucleation was 27 years (range 18–37 years). All had severe panuveitis, 2 with recurrent hypopyon. Three eyes were enucleated after recurrent aggressive episodes of acute panuveitis within 3 years of developing symptoms, all of which were still active at enucleation. The other 2 eyes were enucleated after more chronic disease with persistent activity, 13 years after symptoms developed in one case.

The indication for enucleation in each case was that the eye was blind and painful. In 3 cases this was due to glaucoma (1 chronic closed angle and 2 rubeotic). All the patients had been treated with systemic steroids and one with additional azathioprine. None had received cyclosporin or chlorambucil.

The eyes were formalin fixed and paraffin embedded. Horizontal sections of the whole eye were cut at 6–8 μm thickness. Initial examination was made using haematoxylin and eosin stained sections. Sections were then examined immunohistochemically using a panel of antibodies (Table 1). Control studies were made on eyes with: (a) active sympathetic ophthalmia—to compare the findings in Behçet's disease with another form of chronic immune mediated uveitis, and (b) neovascular glaucoma—these cases have minimal inflammation and secondary complications of the type seen in Behçet's eyes but are due to non-inflammatory causes.

Immunohistochemistry

The immunohistochemical staining was carried out as follows: Sections were dewaxed through toluene and graded alcohols and washed in phosphate buffered saline (PBS) pH 7.3. Endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide in 50% methanol. The slides were washed in PBS and background staining blocked with serum appropriate to the secondary antibody at a concentration of 10% in PBS with 2% ovalbumin for 30 min. The primary antibody was applied at optimum concentration ranging from 1:10 to 1:1500 for 45 min. Slides were washed and secondary antibody applied at a dilution of 1:50 for 30 min. After further washing peroxidase antiperoxidase complex (PAP) (Dako, High Wycombe, UK) was applied for 45 min at a dilution of 1:75 in PBS. After washing again, amino ethyl carbazole (BDH, Poole, UK) was used as the reaction substrate. Sections were counterstained with haematoxylin and coverslips applied using aqueous mountant—Glycergel (Dako, High Wycombe, UK). Negative control sections were processed in identical fashion omitting primary antibody. Known positive control slides were also stained using each monoclonal antibody.

Cell counting

To quantify numbers of cells staining with the monoclonal antibodies to leucocyte common antigen, CD43 (T lymphocytes), OPD4 (CD4 positive T lymphocytes)[20] and L26 (B lymphocytes) cell counts were made from twenty high power fields (×400) in (a) the choroid and in (b) the retina (and periretinal scar

Table 1

Antibody	*Antibody Type*	*Source*	*Specificity*
Leucocyte Common Antigen (LCA)	Mouse monoclonal	Dako	Leucocytes
ICHL1	Mouse monoclonal	Dako	T lymphocytes Monocytes
DFT1 (CD43)	Mouse monoclonal	Dako	T lymphocytes Monocytes
OPD4	Mouse monoclonal	Dako	CD4 positive T lymphocytes
L 26	Mouse monoclonal	Dako	B lymphocytes
Leu 7	Mouse monoclonal	Becton Dickinson	NK cells
MAC 387	Mouse monoclonal	Dako	Monocytes Granulocytes
HLA DRα	Mouse monoclonal	Dako	Alpha chain of HLA-DR Antigen
IgG	Mouse monoclonal	Serotec	IgG heavy chain
IgM	Mouse monoclonal	Serotec	IgM heavy chain
C1q	Sheep polyclonal	Serotec	C1q component of complement
C3c	Sheep polyclonal	Serotec	C3c component of complement

Dako, High Wycombe, U.K.; Becton Dickinson, Oxford, U.K.; Serotec, Oxford, U.K.

tissue). Cells were considered positive if they exhibited a ring of pink to red stain around their cellular membrane.

Neither UCHL1 nor CD43 are specific for CD3 positive lymphocytes. The UCHL1 antibody recognises a peptide of the CD45 family[21] which is present on 72% of CD4 positive and 35% of CD8 positive T cells[22] although it may stain small numbers of macrophages and myeloid cells. The CD43 antibody (Dako DFT1) stains a high percentage of T cells but also some B cells, neutrophils and macrophages[25]. Since we found very few B lymphocytes (see Results) and since neutrophils and macrophages could be distinguished morphologically, we used counts of the CD43 positive cells to represent the T cell population.

RESULTS

Pathology

The five eyes varied in spectrum and severity of intraocular inflammation but all had typical pathological features of Behçet's disease with retinal detachments and periretinal fibrovascular tissue of varying degrees. Two of the eyes had evidence of neovascular glaucoma complicating the inflammation. The retinae were markedly thinned and degenerate. Inflammatory cell infiltrates were noted and around the episcleral vessels, within the optic nerve, within the iris, ciliary body and choroid, around and within the walls of the retinal vessels (Figure 1) and within the fibrovascular scar tissue. These infiltrates were composed almost entirely of mononuclear cells. Neutrophils were infrequent within the infiltrates. In the sections studied there was no evidence of thrombus in any of the retinal vessels.

The eye enucleated for sympathetic ophthalmia, another cause of chronic intraocular inflammation (used as a positive control), had moderate inflammation in the iris and ciliary body and marked mononuclear cell infiltrate in the choroid, retina and preretinal scar tissue. The eyes with neovascular glaucoma from a non-inflammatory cause had a moderate lymphocytic infiltrate in the iris but no detectable inflammation in the choroid or atrophic retina.

Immunohistochemistry

Throughout the eyes, the majority of infiltrating cells were positive for the leucocyte common antigen (LCA) marker. T lymphocytes, identified by the UCHL1 and CD43 markers were a consistent finding within the infiltrates. T lymphocytes ($CD43^+$ sensitive) formed 26–95% of the LCA^+ cell population in the choroid (mean 53.8%) and 9–60% in the retina and periretinal fibrovascular scar tissue (mean 30%). T cells were prominent in the perivascular infiltrates in the retina, fibrovascular scar tissue and uveal tract and in the choriocapillaris below disrupted retinal pigment epithelial cells (Figure 2). $CD4^+$ T cells, identified by the OPD4 monoclonal antibody, constituted 33–90% (mean 77%) of the T cell populations (Figure 3).

B lymphocytes were an infrequent and variable finding. In the LCA positive population between 0 and 5% of cells in the choroid and less than 1% of cells in the retina and epiretinal scar tissue were positive for the B cell marker. In two of the eyes no B cells were seen in the choroid and in three eyes no B cells were found in the retina and fibrovascular scar tissue. B cells did occur in small clusters within the choroidal and perivascular infiltrates. NK cells, identified by the Leu 7 marker, were absent in three of the globes and were a very infrequent finding within the leucocyte infiltrates in the other two.

Macrophages were a consistent finding within the T lymphocyte containing infiltrates being prominent in the fibrovascular scar tissue where they constituted 20–40% of the leucocyte population in some areas. Significant numbers of lymphocytes and macrophages within the leucocyte infiltrates showed positive staining for HLA DRα chain. In four of the five eyes small numbers of retinal pigment epithelial cells (separating the choroid and retina) were weakly positive for HLA DRα but minimal numbers of vascular endothelial cells in the choroid, retina and periretinal scar tissue also showed positive HLA DRα staining.

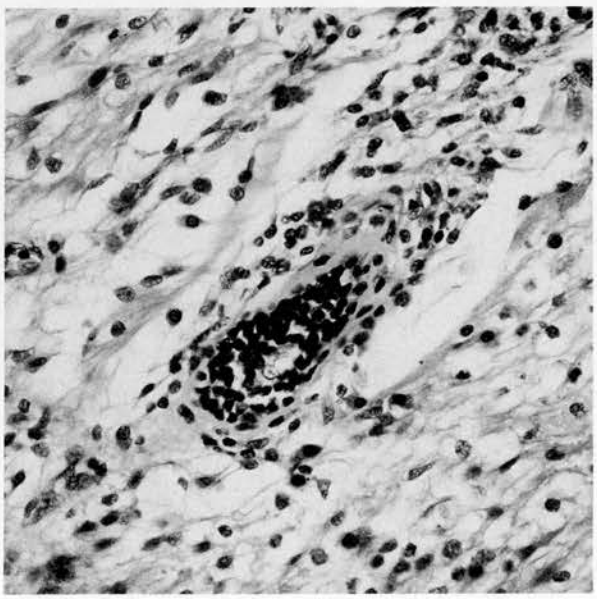

Figure 1 Perivascular infiltrate of lymphocytes in the retina. H&E, ×360.

Markers for IgG and IgM immunoglobulins generally gave a faint stain of the serous fluid within the subretinal space and within vessels. This staining was observed to be no greater than the non-inflamed neovascular glaucoma control eye. In a total of three areas in two eyes there were small collections of cells which showed cell membrane staining for immunoglobulins; two for IgG and one for IgM. These cell collections were closely related to the small B cell clusters seen with the L26 marker. There was no evidence of immunoglobulin deposits within blood vessels or elsewhere within the inflamed tissue. Both the C3c and C1q markers gave a faint stain of the serous fluid within blood vessels and in the subretinal fluid. There was no evidence of any solid deposition of complement components. Within the choriocapillaris of the inflamed eyes the C3c and C1q staining of serous fluid was more prominent suggesting some concentration of complement components in this region.

In the control eye with sympathetic ophthalmia numerous T and B lymphocytes were found in the choroidal and retinal cellular infiltrates consistent with previous reports[18]. The lymphocytic infiltrate in the iris of the eye with neovascular glaucoma consisted of minimal numbers of T and B lymphocytes but no inflammation was seen in the retina or choroid demonstrating that our findings in the retina and choroid in the Behçet's eyes are not secondary to the complicating pathology which has developed.

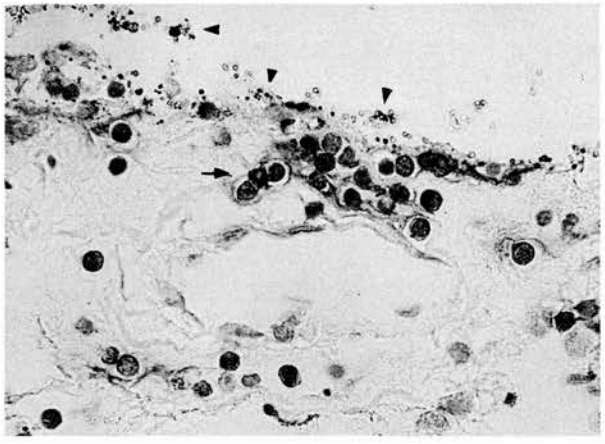

Figure 2 CD43 positive T lymphocytes (arrow) infiltrating choriocapillaris below pigment granules released from disorganised retinal pigment epithelium (arrowheads). Haematoxylin counterstain, ×450.

DISCUSSION

This investigation demonstrates that T lymphocytes are the principal infiltrating cell type in inflammatory foci in ocular Behçet's disease. B Lymphocytes, neutrophils, and NK cells were not a prominent finding. There were no deposits of immunoglobulin or complement components suggesting that deposition of immune complexes do not contribute to the pathology. The high proportion of CD4 positive T cells implies a central role for these cells in the perpetuation mechanism and helps explain the favourable response of ocular Behçet's disease to cyclosporin[2,3]. It should be emphasised that these results do not provide any information on the mechanism of initiation of the inflammatory process but purely on the cells involved in its perpetuation.

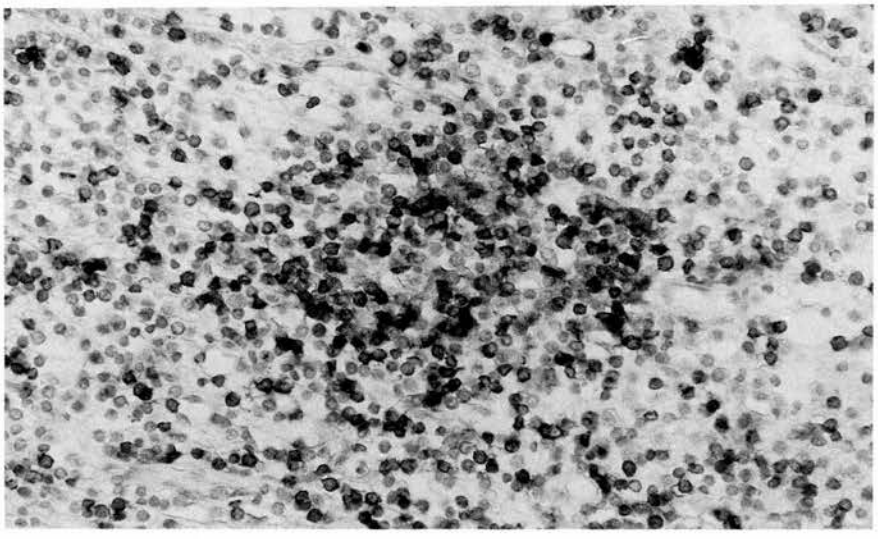

Figure 3 Infiltrate of CD4 positive T cells in epiretinal scar tissue. (OPD 4 monoclonal antibody). Haematoxylin counterstain, ×385.

The implication that cell mediated immunity is responsible for the tissue damage within the eye in Behçet's disease is in accordance with the findings in tissue from other affected organs[10–13]. It is notable that the findings of many of the peripheral blood studies in Behçet's disease do not reflect the tissue immunopathology. This is a similar situation to the findings in sarcoidosis[19,24] where normal or low levels of circulating $CD4^+$ T cells are found in peripheral blood in contrast to the large numbers of T cells in the affected tissues.

Aberrant expression of HLA class II by endogenous tissue cells *in vivo* may be important in the development and perpetuation of autoimmunity[25] and may play a role in the recruitment of antigen specific CD4 positive T lymphocytes from the blood to inflamed tissues[26]. This study has demonstrated that minimal aberrant HLA Class II expression is present on the retinal pigment epithelium and retinal vascular endothelium, cells which constitute the tight blood–retinal barrier and which are in contact with circulating lymphocytes. This is in contrast to the marked HLA Class II expression found in retinal pigment epithelial cells (RPE) and retinal vascular endothelial cells (RVE) in other forms of human posterior uveitis[27]. It may be that early class II expression does not persist during late disease. However, in the animal model experimental allergic encephalomyelitis, cerebral vascular endothelial cells which demonstrate aberrant expression of HLA class II antigens lack the co-stimulatory signal required to activate myelin basic protein (MBP) autoreactive T cells *in vitro*[28] and are thought to result in T cell anergy. Some of the infiltrating T cells in our specimens were also HLA DR positive but whether these cells are able to present antigen to each other *in vivo* is unclear.

One proviso to this argument is that we have used paraffin sections for immunohistochemistry whereas studies carried out on other inflamed eyes were performed using frozen sections. It is possible that aberrantly expressed class II antigens are better preserved in frozen tissue or that the monoclonal antibodies to HLA DR used on frozen sections are more sensitive than those we used. Some of the infiltrating T cells in our specimens were also HLA DR positive but whether these cells were able to present antigen to each other *in vivo* is unclear. With the large number of macrophages present with the T cells in the inflammatory infiltrate, it is likely that these are the major cells presenting antigen to the $CD4^+$ T cells.

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Behçet's disease: activated T lymphocytes in retinal perivasculitis

David G Charteris, Caroline Champ, A Ralph Rosenthal, Susan L Lightman

Abstract

A 38-year-old man who died from systemic Behçet's disease had previously suffered from severe, recurrent bilateral retinal vasculitis, and anterior uveitis for 10 years. Immunopathological examination of the eyes postmortem revealed marked hyaline thickening of the retinal and optic nerve vessels. The vessels had an intramural and perivascular infiltrate of T lymphocytes which stained positively for CD4 and IL2 receptor surface markers. Small numbers of cells in the optic nerve head, retinal vascular endothelium, and retinal pigment epithelium were HLA DR positive.

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Behçet's disease is a multisystem inflammatory disorder mediated by an occlusive vasculitis.[1] The disease is characterised by aphthous mouth ulcers, genital ulcers, and ocular inflammation. Ocular manifestations of Behçet's disease, which occur in 70–85% of cases, are a recurrent iridocyclitis, retinal vasculitis, and retinal ischaemia. These frequently lead to cystoid macular oedema and optic atrophy resulting in profound visual loss.

Alterations in complement components in aqueous humour[2] and peripheral blood[3 4] led to the theory that immune complex deposition caused the immunopathology in Behçet's disease. Studies of tissues involved in the disease process[5–8] have however shown that it is predominantly T lymphocytes which infiltrate the affected tissues. This suggests a central role for cell-mediated immunity in the disease process.

Histopathological examination of enucleated eyes from patients with Behçet's disease reveals a non-granulomatous uveitis, retinal vasculitis, and infarction.[9 10] This paper documents the findings of a pathological and immunohistochemical study on eyes obtained postmortem from a patient who died from Behçet's disease.

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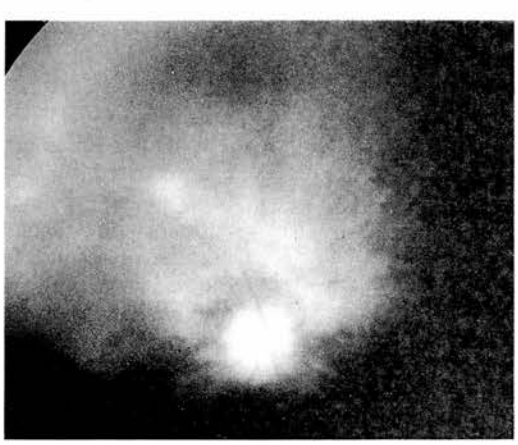

Figure 1 Right optic fundus 3 months prior to death. The optic disc is oedematous with surrounding retinal haemorrhages and patches of retinal oedema.

Case report

A 29-year-old man was diagnosed as having Behçet's disease in 1981 on the basis of a 3 year history of recurrent retinal vasculitis, anterior uveitis, and oropharyngeal aphthous ulcers. These manifestations were controlled initially by high dose oral prednisolone. Between 1985 and 1990 his systemic condition deteriorated with the development of erythema nodosum, multiple joint symptoms, and meningoencephalitis which resulted in epilepsy. Treatment with chlorambucil, cyclosporin, cyclophosphamide, and azathioprine failed to halt the progression of the disease. The patient died in October 1990 from pneumonia secondary to raised intracranial pressure due to central nervous system involvement which failed to respond to treatment.

From 1981 until his death he had recurrent episodes of anterior uveitis (with hypopyon) and retinal vasculitis in both eyes. This resulted in retinal ischaemia and visual loss despite systemic immunosuppression and orbital steroid injections. A dense cataract developed in the left eye early in the disease process, but cataract extraction was not considered justified because of the advanced retinal ischaemia. At his last ophthalmic review 3 months prior to death he had no light perception in either eye. The right optic disc was swollen and surrounded by multiple retinal haemorrhages and retinal oedema (Fig 1). The retinal vessels in the right eye were sheathed. There was no view of the left fundus due to the lens opacity. Anterior uveitis was minimal in both eyes. Intraocular pressures were normal throughout the course of the disease. Immunosuppressive treatment at the time of death was high dose systemic prednisolone, azathioprine, and cyclophosphamide.

Materials and methods

Consent was obtained for full postmortem examination including examination of the eyes. Both eyes were removed, the right globe was fixed in 4% glutaraldehyde and the left globe was frozen in OCT (Shandon, Runcorn). Horizontal sections, through the pupil and optic nerve, of both globes were cut at 6 μm thickness. Initial staining was carried out using haematoxylin and eosin. Sections of the frozen (left) eye were stained immunohistochemically using the avidin-biotin-complex (ABC) method (Vector, Peterborough). Briefly, slides were fixed in acetone, endogenous peroxidase activity quenched and blocked using normal serum. Primary monoclonal antibodies (Table 1) were applied for 30 minutes at appropriate concentration, biotinylated secondary antibody and ABC complex were subsequently applied following washing in phosphate-buffered saline.

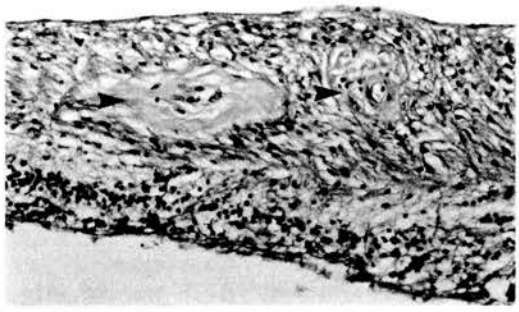

Figure 2 Disorganised, detached retina of the left eye showing marked hyaline thickening and luminal narrowing of retinal vessels (arrowheads) with intramural inflammatory infiltration. H and E ×140.

Slides were then developed in amino ethyl carbazole to give a red final reaction product and counterstained in haematoxylin.

CONTROLS

Sections of each level examined were stained immunohistochemically as above with the omission of the primary monoclonal antibody to provide negative controls. Frozen sections from a normal eye were stained with each monoclonal antibody to demonstrate the distribution of each cell type in uninflamed ocular tissue. Known positive tissues (inflammatory eyelid or orbital lesions) were stained simultaneously using each primary monoclonal antibody to verify the effectiveness of the staining procedure.

Results

Pathological examination of the brain revealed widespread cerebral oedema with gyral flattening but no evidence of coning. Histologically, there was no active cerebral vasculitis.

The pathological appearances were similar in the right and left eyes. There was a mild lymphocytic infiltrate of the iris and ciliary body; this extended into the choroid where there were scattered lymphocytes in all sections examined. The retinas showing loss of ganglion cells and

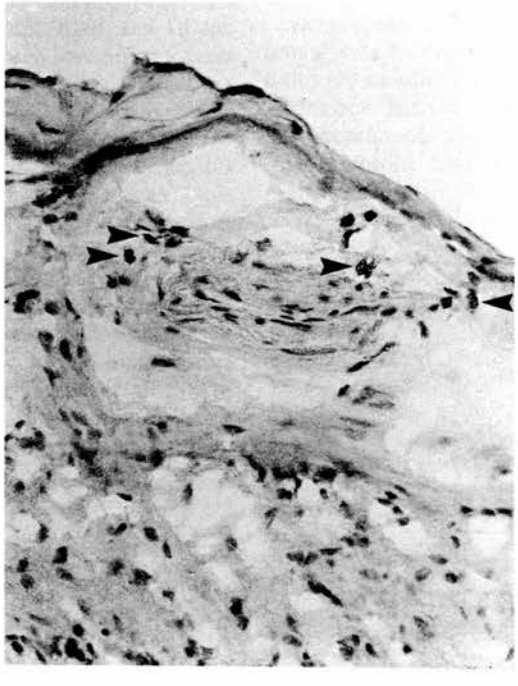

Figure 3 Optic nerve head of left eye showing CD3 positive T lymphocytes (arrowheads) around hyalinised vessel. CD3 (pan-T cell) monoclonal antibody, haematoxylin counterstain. ×260.

Table 1 Primary monoclonal antibodies

Antibody	*Source*	*Specificity*
T3	Dako*	Pan T lymphocytes
T4	Dako	CD4+ T lymphocytes
T8	Dako	CD8+ T lymphocytes
L 26	Dako	B lymphocytes
IL2R	Dako	Interleukin 2 receptor
MAC 3	Dako	Monocytes/macrophages
HLA DR	Dako	HLA DR antigen expressio

*High Wycombe, UK

photoreceptors. The retinal vessels were paten but showed marked hyaline thickening of thei walls (Fig 2). There were infiltrating lymphocyte within this hyaline tissues. The retinas wer focally detached with underlying patches c subretinal fluid. In the right eye the optic nerv head was oedematous and there was a lympho cytic perivasculitis of the central retinal vessel extending into the nerve. The left optic nerv head was not oedematous but had a perivasculitis Both optic nerves had moderate loss of neura tissue.

Positive immunohistochemical staining for T lymphocytes was seen in cells in the retinal vesse walls and surrounding the optic nerve hea vessels (Fig 3). These cells also stained positivel with the CD4 monoclonal antibody; however th small number of cells present precluded count of T cell subset ratios. The perivascular T cell also stained positively with the IL2 recepto monoclonal antibody. Lymphocytes in the iris ciliary body, and choroid were also positive fo the T lymphocyte monoclonal antibody althoug they were generally IL2 receptor negative. N CD8+ T lymphocytes or B lymphocytes wer found. A few cells in the hyalinised vessel wall were positive for the macrophage primary anti body. No neutrophils were identified in th vasculitic lesions.

A small number of stromal cells of fibroblasti morphology in the optic nerve head and retina vascular endothelial cells were HLA DR positive There were also numerous HLA DR positiv cells in the retinal pigment epithelium.

CONTROLS

There was minimal background staining i sections where the primary antibody was omitted In the normal eye there were very occasional cell in the choroid which were positive for the T lymphocyte and CD4 monoclonal antibodies There were also small numbers of cells in th choroid which were HLA DR positive. All othe antibodies were negative.

Discussion

The ocular inflammation in Behçet's disease i characterised by a severe occlusive vasculitis Most of the eyes examined pathologically fron patients with Behçet's disease have been remove because of secondary complications and reflec the end stage pathology of the condition.[9 10] Th eyes we have studied were obtained postmorter and were not removed because of complicatin pathology. This tissue therefore, althoug subject to modification by immunosuppressiv therapy, gives a better picture of the ongoin

immunopathology of the condition. A perivascular lymphocytic infiltrate and marked thickening of the vascular walls was seen in the retinal and optic nerve vessels of both eyes and the lymphocytes in and around the vessel walls were identified as T cells. The perivascular lymphocytes in ocular sarcoidosis[11] and pars planitis[12] have also been identified as T cells.

A proportion of the T cells found in the perivascular infiltrates and within the vessel walls stained positively with CD4 and IL2 receptor monoclonal antibodies. The IL2 receptor expression of these cells is remarkable since the patient was heavily immunosuppressed at the time of death and demonstrates the difficulty in treating adequately the severe vasculitis seen in Behçet's disease.

The role of T lymphocytes in the tissue pathology of Behçet's disease has been demonstrated in immunohistopathological studies of tissues from other affected sites.[5-8] The identification of T cells as the predominant cell type in the ongoing vascular lesions in the eye supports the view that cell-mediated immune mechanisms are responsible for the tissue damage seen in this condition. The absence of B lymphocytes and neutrophils is evidence that humoral immune mechanisms do not play a major role in the ocular immunopathology.

The expression of the MHC class II antigen HLA DR by retinal pigment epithelial cells and vascular endothelial cells has been described in other forms of intraocular inflammatory disease.[11-14] Aberrant expression of MHC class II antigens has been demonstrated on endogenous tissue cells in conditions thought to be of autoimmune aetiology,[15 16] and it has been suggested that these cells may play a role in antigen presentation to infiltrating CD4+ T lymphocytes[17] such as those demonstrated in this study.

Successful immunotherapy of intraocular disease will depend on targeting specific cellular components involved in the pathogenesis of the tissue destruction. The demonstration in this study activated CD4+ T cells in the vasculitic lesions in ocular Behçet's disease suggests that future treatment directed at the down-regulation of the effects of CD4+ T cells would be beneficial in controlling the severe ocular inflammation.

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erferon-gamma (IFN-γ) production *in vivo* in experimental autoimmune uveoretinitis

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SUMMARY

Experimental autoimmune uveoretinitis (EAU) is a well-characterized model of immune-mediated intraocular inflammation. The intraocular infiltrate in EAU consists predominantly of T lymphocytes. The *in vivo* production of interferon-gamma (IFN-γ) by these T cells was investigated immunohistochemically and by *in situ* hybridization using a cDNA probe to rat IFN-γ mRNA. Positive localization of IFN-γ mRNA began simultaneously with disease onset and increased as the inflammatory tissue destruction progressed. The positive signal was seen on cells in the retina, uveal tract and extraocular region where collections of inflammatory cells contained many T lymphocytes. Numerous cells in these locations also stained positively immunohistochemically for IFN-γ. These results indicate that the *in vivo* production of IFN-γ within the eye could play a role in the immune regulation of intraocular inflammatory disease.

INTRODUCTION

onic posterior uveitis and retinal vasculitis are ocular mmatory conditions which often result in significant visual despite treatment. The aetiology of these diseases is largely own but autoimmune mechanisms involving ocular anti- have been implicated in their pathogenesis. Experimental immune uveoretinitis (EAU) is an animal model of autoim- e intraocular inflammation[1] induced by systemic immuni- n with a purified retinal antigen in adjuvant. In the rat el of EAU there is a mixed infiltrate of polymorphonuclear ocytes and lymphocytes to the anterior and posterior ents of the eye with $CD4^+$ T lymphocytes predominating in destructive retinal lesions.[2]

EAU can be adoptively transferred by activated, ocular gen-specific $CD4^+$ T-cell lines[3] demonstrating that these ated T cells are capable of initiating the disease process. The ominant cell infiltrating the retina in the inflammatory e is also the $CD4^+$ T cell implicating this cell in the uctive inflammatory process.

Murine $CD4^+$ T cells have been divided into two groups d on their lymphokine secretion pattern *in vitro*.[4] Th1 cells uce interleukin-2 (IL-2), interferon-gamma (IFN-γ) and hotoxin and are thought to act as primary effector cells. cells produce IL-4, IL-5 and IL-6 and act as helper cells, for ple providing specific B-cell help. Human T-cell clones do necessarily follow this pattern.[5] The OX22 monoclonal body has been used in the rat to characterize $CD4^+$ T cells as (OX22 high) and memory (OX22 low).[6] This division appears to have functional significance related to cytokine production, the OX22 high cells producing greater amounts of IL-2 and IFN-γ and the OX22 low cells producing more IL-4.[6–8] Rat uveitogenic T-cell lines have been demonstrated to produce IL-2, IL-4 and IFN-γ when activated *in vitro*[9] but the *in vivo* production of lymphokines in EAU by infiltrating activated T cells is not known.

IFN-γ is a lymphokine produced by activated T lymphocytes and natural killer (NK) cells which has multiple actions in immune response regulation.[10] In addition to its roles in major histocompatibility complex (MHC) class II induction and cellular differentiation and activation it has recently been demonstrated to play an important role in the development of effector cell function in cytolytic T lymphocytes.[11,12] In addition IFN-γ has been shown to induce macrophage activation.[13] IFN-γ is therefore likely to have a key role in the autoimmune inflammatory process.

The aim of this study was to determine if IFN-γ was produced *in vivo* by the infiltrating T lymphocytes in the Lewis rat model of EAU. IFN-γ protein was localized immunohistochemically using a rat IFN-γ-specific monoclonal antibody and a cDNA probe was used to detect IFN-γ mRNA.

MATERIALS AND METHODS

Animals

Female Lewis rats [bred under specific pathogen-free (SPF) conditions, St Thomas's Hospital Medical School, London, U.K.], 100–150 g weight, 6–8 weeks old were used for all experiments.

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Induction of uveitis

Rats were immunized in a hind footpad with 50 μg of purified bovine S-antigen [14] in a 1:1 emulsion in complete Freund's adjuvant (Sigma, Poole, Dorset, U.K.) supplemented with *Mycobacterium tuberculosis* organisms (Sigma) to a final concentration of 1·5 mg/ml. Animals were also given 5×10^9 heat-inactivated *Bordetella pertussis* organisms (Wellcome Laboratories, Beckenham, Kent, U.K.) in 150 μl phosphate-buffered saline (PBS) intraperitoneally. A total of 17 eyes from individual animals were studied. Two eyes from Days 10, 11, 12, 13, and 21, and three eyes from Days 14 and 17 after disease induction, as well as control eyes from a non-immunized animal were removed, embedded in OCT (Shandon, Runcorn, U.K.) and snap frozen in acetone and dry ice. Specimens were stored at −70°.

Probe preparation

A cDNA probe to rat IFN-γ mRNa in the pPC3 plasmid vector was kindly supplied by Dr Ton Kos, TNO, Rijswijk, The Netherlands. The probe consisted of a 528-base pair (bp) fragment comprising two identical repeats of a 264-bp sequence corresponding to the last 21 residues of exon 2, all of exon 3 and the first 60 residues of exon 4 as deduced from the rat IFN-γ gene structure.[15] The plasmid was amplified in HB 101 *Escherichia coli* and the cDNA probe was extracted, purified and labelled with ^{35}S-dCTP alpha (Amersham International, Amersham, Bucks, U.K.) using the random primer technique (Boehringer Mannheim, Lewes, U.K.) and adjusted to 2×10^5 c.p.m./ml in hybridization buffer [600 mM sodium chloride, 50 mM sodium phosphate pH 7·0, 5 M EDTA, 0·02% Ficoll, 0·02% bovine serum albumin, 0·02% polyvinylpyrolidene, 0·1% salmon testis DNA and 50% deionized formamide (all chemicals supplied by Sigma)].

In situ *hybridization*

Whole-eye sections of 12 μm thickness were cut on a cryostat and mounted on specially prepared gelatin (300 bloom swine, Sigma) coated slides. Sections were fixed for 5 min in 4% glutaraldehyde in 0·1 M Sorensens phosphate buffer pH 7·2 with 20% ethylene glycol, rinsed twice in hybridization buffer and soaked in hybridization buffer for 1 hr, rinsed in ethanol and dried. The ^{35}S-labelled probe in hybridization buffer was heated to 90° for 10 min, cooled and 100 μl applied to each slide under a parafilm (Sigma) coverslip. Sections were left to hybridize in a humidified chamber at room temperature for 72 hr. Post-hybridization, slides were immersed in 2 × SSC until the coverslips dislodged, rinsed in 2 × SSC and washed at 40° for 30 min in 1 × SSC. Slides were then rinsed briefly in distilled water, in 70% ethanol for 5 min, in 95% ethanol for 5 min and allowed to dry.

Autoradiography

The slides were dipped in K5 (Ilford, Mobberley, U.K.) photographic emulsion diluted 1:1 in 0·5% glycerol and left to expose at 4° for 21–24 days over silica gel. Slides were then developed for 3·5 min in D19 (Kodak, Hemel Hempstead, U.K.) developer, fixed in Unifix (Kodak), washed in distilled water and counterstained with haematoxylin.

Controls

The control studies outlined below were carried out simultaneously with the *in situ* hybridization.

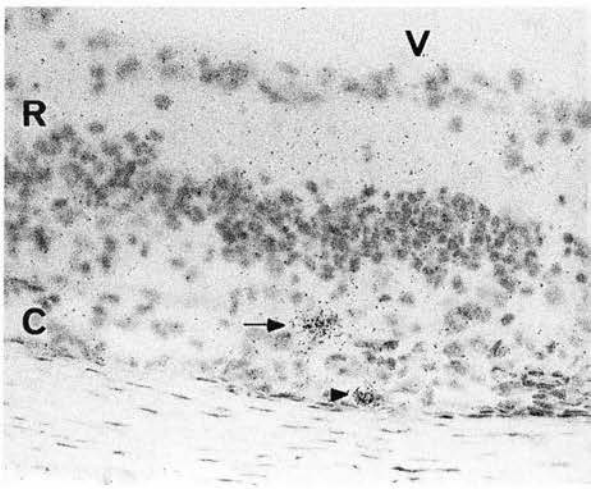


Figure 1. Cells showing positive hybridization signal in the outer r
(arrow) and choroid (arrowhead) at the early stage of inflamma
Retina (R), choroid (C), vitreous (V). Haematoxylin counterstain ×

RNAase. Sections from each eye were fixed for 5 m
glutaraldehyde buffer, washed once in 2 × SSC with 5% T
and three times in 2 × SSC. One hundred microlitres of RN
A (Boehringer Mannheim) 1 mg/ml in 2 × SSC was applie
each slide under a parafilm coverslip and incubated at 37°
hr, slides were then washed in 2 × SSC and hybridization ca
out as above.

P53 probe. To determine the specificity of the IFN-γ p
hybridization, each section was also hybridized with a
labelled probe to the p53 oncogene which was considered
irrelevant to the EAU disease process.

Non-immunized animal. Eyes from normal, non-immun
Lewis rats were studied both by *in situ* hybridization for I
mRNA and by immunohistochemistry.

Monoclonal antibodies

For immunohistochemistry primary monoclonal antibodi
rat IFN-γ (Holland Biotechnology, Leiden, The Netherla
pan T cells (OX19, Serotec, Oxford, U.K.), IL-2 rece
(OX39, Serotec) and MHC class II expression (OX6, Ser
were utilized.

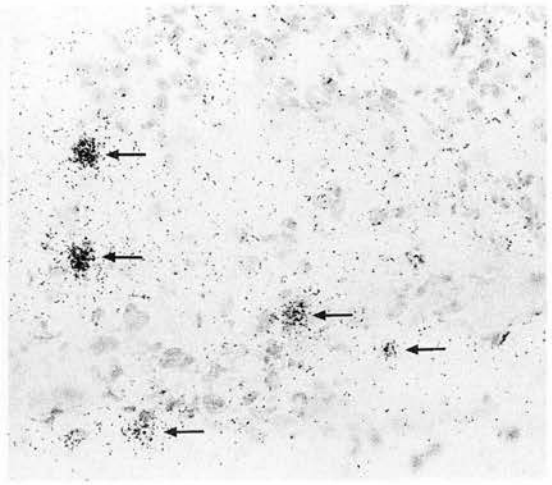
Immunohistochemistry

Sections of 6 μm thickness were cut from each eye and mou
on 3Amino Propyl Triethoxysilane (APES) (Sigma) co
slides. Slides were fixed in acetone for 7 min and endoge
peroxide activity blocked with 3% hydrogen peroxide in
methanol. Sections were stained by a standard avidin-bi
complex method (Vector, Peterborough, U.K.) using a
immunoglobulin adsorbed biotinylated secondary antib
(Vector) and amino ethyl carbazole to provide a red
reaction product.

RESULTS

Induction of disease

Animals developed histological evidence of disease on Da
post-induction. The histological changes were characteric o



e 2. Positive cells (arrows) adjacent to a patch of retinal oedema in disease. Haematoxylin counterstain ×485.

J.[1] There was a marked mixed leucocyte infiltrate of the rior and posterior segments of the eyes with patches of al oedema progressing to total retinal destruction in which phocytes were the predominant infiltrating cell.

alization of IFN-γ mRNA

tive hybridization signals, observed as collections of dark ns in the photographic emulsion, were seen over cells from 12 post-immunization onwards. In the early stages of the ase process positive cells were seen in the outer retina and oid (Fig. 1) and related to patches of retinal destruction and ma (Fig. 2). As the retinal destructive process progressed easing numbers of positive cells were seen in these regions 3a) as well as in accumulations of inflammatory cells in the ry body and the anterior extraocular region. Later in the ase when there was marked retinal destruction, positive cells found predominantly in the choroid.

unohistochemical localization of IFN-γ

IFN-γ monoclonal antibody gave positive membrane ing of cells from Day 12 post-induction onwards. Mono- ear cells in the extraocular tissues, anterior chamber, ciliary , choroid, retina (Fig. 4), vitreal cavity and subretinal fluid owed positive staining. The staining intensity increased as nflammatory process progressed.

ular immunohistochemistry

9 (pan T cell) positive cells were found in inflammatory rates throughout the extraocular tissues, anterior chamber, ry body, choroid and retina, including the areas of IFN-γ NA localization (not shown). Cells in these regions also ved positive staining with the OX39 antibody for IL-2 otor expression. MHC class II expression was found on cells Day 10 post-induction onwards; this increased markedly e disease progressed and was seen both on organ-resident infiltrating cells (not shown).

Controls

The eyes from non-immunized animals showed no localization of IFN-γ mRNA or evidence of infiltrating inflammatory cells. Pre-treatment with RNAase abolished all localizing hybridization signal (Fig. 3b) and hybridization with the p53 oncogene probe gave only a vague background signal in the inflamed tissue without any cellular localization (not shown).

DISCUSSION

We have demonstrated the presence of IFN-γ mRNA localized to cells in areas of T-lymphocyte infiltration in the rat model of experimental autoimmune uveoretinitis. Furthermore we have shown that IFN-γ is present throughout these eyes using a monoclonal antibody specific for rat IFN-γ. These results correlate with the immunohistochemical finding of IFN-γ related to areas of activated T-cell infiltration in human posterior uveitis.[16] IFN-γ mRNA was found in two areas within the eyes of rats with EAU, the retina and the uveal tract (choroid and ciliary body) and also in extraocular inflammatory cell infiltrates. No IFN-γ mRNA was found in cells in the anterior chamber or vitreous cavity although many T lymphocytes were present in these sites. This would imply that IFN-γ production takes place in the retina, choroid or ciliary body where activation of the T lymphocytes may take place or in the extraocular region where cells are trafficking to and from the eye. It follows that once T cells are in the anterior chamber or vitreous cavities they no longer produce IFN-γ.

Greater numbers of T cells had strong immunohistochemical staining for IFN-γ than had a positive signal for IFN-γ mRNA. It is likely that this is due to the transient expression of mRNA for this cytokine by T cells[8] compared to a longer time period when cytokine protein can be demonstrated on cells, either localized to cytokine receptors or on cells no longer expressing cytokine mRNA.

It has recently been demonstrated that systemically administered IFN-γ down-regulates the immune response in the mouse model of EAU.[17] The demonstration that there is increased mRNA expression for IFN-γ in the later stages of the intaocular destructive process is evidence that its local production could play a role in the immune regulation of this disease *in vivo*.

IFN-γ is known to induce or up-regulate MHC class II antigen expression on a variety of cell types.[10] It has been shown that IFN-γ can induce MHC class II expression *in vitro* in rat, guinea-pig[18] and human[19] retinal pigment epithelial (RPE) cells, a layer of cells lying between retina and choroid forming part of the blood-retinal barrier. RPE cells are known to express MHC class II antigens *in vivo* in human posterior uveitis[20] and in EAU[21] and it has been proposed that such aberrant expression of class II may be important in the development of autoimmunity.[22] Local production of IFN-γ provides a mechanism whereby aberrant expression of class II antigens on organ resident cells can occur in uveitis.

Intraocular injection of recombinant IFN-γ into the vitreous cavity of rat eyes has been demonstrated to induce *in vivo* MHC class II expression on organ-resident cells in the uveal tract and cornea and also on extraocular conjunctival epithelial cells. Subretinal injection of recombinant IFN-γ produces *in vivo* class II expression on RPE cells in rats.[23] These changes were shown to be associated with an infiltrate of both neutrophil polymorphonuclear leucocytes and monocytes to the iris and inner

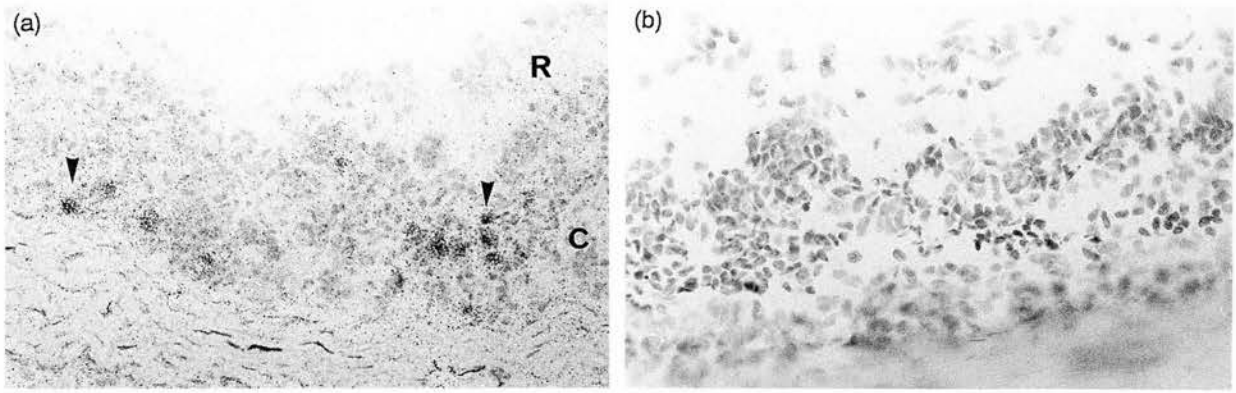


Figure 3. (a) Numerous positive cells (arrowheads to examples) within disorganized retina (R) and choroid (C) in advanced inflammation. Haematoxylin counterstain ×300. (b) Retina from adjacent section to (a) pre-treated with RNAase. No localizing hybridization signal. Haematoxylin counterstain ×300.

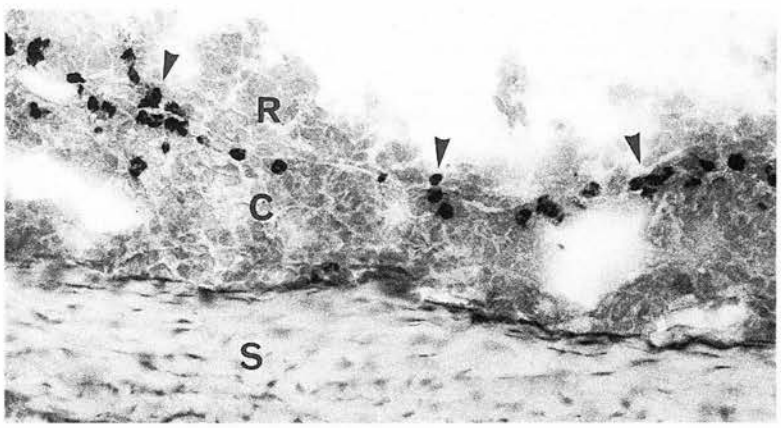


Figure 4. Immunohistochemical localization of cells staining positively for IFN-γ within the retina (R) and choroid (C) at a stage of advanced destruction (arrowheads to examples). Sclera (S). Haematoxylin counterstain ×300.

retinal layers suggesting that local IFN-γ plays an important role in the induction of autoimmune inflammatory eye disease. This finding is contrary to the aforementioned down-regulation of murine EAU by systemic IFN-γ[17] and the exact role of locally produced IFN-γ in EAU requires further elucidation.

The investigation of patterns of cytokine production has given information on the roles of T-cell subsets in the mouse,[4] rat[6,7,8,24] and in humans.[5,7,25] The murine subgroups Th1 and Th2 do not appear to be consistent in rats and humans. IFN-γ is produced predominantly by the OX22 high subgroup of rat CD4+ T cells which are thought to represent naive cells prior to antigen contact.[7,24] This finding is in conflict with human CD4+ T cells where CD45RO (memory) T cells produce IFN-γ. It has been proposed that a further subset of human CD4+ T cells may exist and that rat and human CD4+ T cells may therefore have similar patterns of lymphokine secretion.[7] Analysis of rat CD4+ T cells for IFN-γ mRNA has shown that there is a greater frequency of positive cells in the OX22 low group although the level of secreted IFN-γ protein is higher from OX22 high cells.[8] This may be due to post transcriptional regulation of translation of IFN-γ mRNA or regulation of secretion of IFN-γ protein since continued stimulation of OX22 low T cells results in a down-regulation of expression of IFN-γ mRNA. The IF mRNA expressing cells found in EAU may be from eithe OX22 high or low groups and further analysis of the infiltrating the retina in EAU by flow cytometry woul necessary to determine their exact phenotype.

Although the identity of the effector cells in the destru retinal lesions in EAU is not known, T lymphocytes macrophages have been shown to be present in large numbe these lesions as the disease progresses.[1] IFN-γ is capabl producing development of cytotoxic T-lymphocyte precu into cytotoxic effector cells[11,12] and also has the potentia activate macrophages,[13] its *in situ* production at the site retinal destruction in EAU would potentially enable both t cell types to act as effector cells. The further investigation o pattern of cytokine production in these destructive foci will us to understand the roles of these cells in the disease proc

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In vivo lymphokine production in experimental autoimmune uveoretinitis

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SUMMARY

Experimental autoimmune uveoretinitis (EAU) is a well-characterized model of immune-mediated intraocular inflammation. The intraocular infiltrate in EAU consists predominantly of T lymphocytes. The *in vivo* production of interleukin-2 (IL-2), lymphotoxin and IL-4 by these T cells was investigated by *in situ* hybridization using cDNA probes to lymphokine mRNA. Localization of lymphokine mRNA was found simultaneous with disease onset in areas of T-cell infiltration. Positive signal was seen over cells in the uveal tract, retina and extraocular region. Less than 10% of the population of T cells defined immunohistochemically had positive localization of mRNA for these lymphokines. The number of positive cells was similar for each of the three probes and increased as the disease progressed. The findings suggest that these lymphokines are produced *in vivo* in immune-mediated intraocular inflammation and may play a role in the immunopathology seen in these conditions.

INTRODUCTION

erimental autoimmune uveoretinitis (EAU)[1] is a model of an ocular inflammatory disease, a range of conditions ch can result in significant visual loss despite treatment. The ology of human intraocular inflammation is unknown but immune mechanisms involving ocular antigens have been icated in its pathogenesis. EAU can be induced in various nal species by systemic immunization with a retinal antigen djuvant. In the Lewis rat model of EAU, immunization is owed 10–12 days later by a mixed infiltrate of polymorpho-ear leucocytes and lymphocytes to the anterior and pos-or segments of the eye, with $CD4^+$ T lymphocytes predomi-ng in the destructive retinal lesions.[2] It has been onstrated that activated, retinal antigen-specific $CD4^+$ T are capable of adoptively transferring EAU to non-unized animals.[3] $CD4^+$ T lymphocytes are therefore impli-d in both the initiation of the disease process and the ructive retinal pathology.

Murine $CD4^+$ T cells have been divided into two types based heir pattern of lymphokine secretion[4] and this has been elated with their function. Th1 cells secrete interleukin-2 2), interferon-γ (IFN-γ) and lymphotoxin and are thought nction as effector cells, whereas Th2 cells produce IL-4, IL-d IL-6 and are considered to be helper cells, for example viding specific B-cell help. Both cell types produce IL-3, ulocyte–macrophage colony-stimulating factor (GM-CSF) tumour necrosis factor-α (TNF-α). Rat $CD4^+$ T cells do not appear to follow this stable pattern of lymphokine secretion and have been subtyped according to their binding of the OX22 monoclonal antibody. Naive $CD4^+$ T cells (OX22 high) have a high binding affinity for this antibody and produce IL-2 and IFN-γ, following antigen contact these cells become OX22 low and produce IL-4.[5–7]

Rat uveitogenic T-cell lines have been shown to produce IL-2, IL-4 and IFN-γ when activated *in vitro*.[8] We have previously demonstrated that there is *in vivo* expression of IFN-γ mRNA and the presence of IFN-γ protein in areas of T-lymphocyte infiltrate in the destructive tissue pathology throughout the time-course of EAU.[9] In this study we have extended these investigations to the lymphokines IL-2, lymphotoxin and IL-4 with the aim of further characterizing the *in vivo* pattern of lymphokine production in the destructive lesions seen in EAU. We have identified infiltrating cells immunohistochemically and have used cDNA probes to detect lymphokine mRNA by *in situ* hybridization.

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MATERIALS AND METHODS

Animals

Female Lewis rats [bred under specific pathogen-free (SPF) conditions; St Thomas' Hospital Medical School, London, U.K.], 100–150 g weight, 6–8 weeks old were used for experiments and as controls.

Induction of uveitis

Rats were immunized in a hind footpad with 50 μg of purified bovine S-antigen[10] in a 1:1 emulsion in complete Freund's adjuvant (CFA) (Sigma, Poole, U.K.) supplemented with

Mycobacterium tuberculosis organisms (Sigma) to a final concentration of 2·5 mg/ml. Animals were also given 5×10^9 heat-inactivated *Bordetella pertussis* organisms (Wellcome Laboratories, Beckenham, U.K.) in 150 μl phosphate-buffered saline (PBS) intraperitoneally. A total of 24 immunized and four control (non-immunized) eyes from individual animals was studied. Two animals were studied on days 10 and 11 post-immunization, and four animals were studied on days 12, 13, 14, 17 and 21 post-immunization. Eyes were rapidly removed, embedded in OCT (Shandon, Runcorn, U.K.) and snap frozen in acetone and dry ice. Specimens were stored at −70°.

Probe preparation

Three cDNA probes were used for the *in situ* hybridization. The rat IL-2 probe was a full-length 740 base pair (bp) fragment corresponding to rat IL-2 mRNA[11] and the rat IL-4 probe consisted of the first 406 bp of the specific rat IL-4 gene structure.[7] Both these were probes kindly supplied by Dr A. McKnight (MRC Cellular Immunology Unit, Oxford, U.K.) The lymphotoxin probe was a 1·42 kbp coding sequence derived from the murine lymphotoxin gene[12] (kindly given by Dr N. Ruddle, Yale University Medical School, New Haven, CT). The probe-containing plasmids were amplified in HB 101 *Escherichia coli* and the cDNA probes were extracted, purified and labelled with ^{35}S dCTP alpha (Amersham International, Amersham, U.K.) using the random primer technique (Boeringer Mannheim, Lewes, U.K.) and adjusted to 2×10^5 c.p.m./ml in hybridization buffer [600 mM sodium chloride, 50 mM sodium phosphate pH 7·0, 5 mM EDTA, 0·02% Ficoll, 0·02% bovine serum albumin (BSA), 0·02% polyvinylpyrolidene, 0·1% salmon testis DNA and 50% deionized formamide (all chemicals supplied by Sigma)].

In situ *hybridization*

Whole eye sections of 12 μm thickness were cut on a cryostat and mounted on specially prepared gelatin (300 bloom swine, Sigma) coated slides. Sections were fixed for 5 min in 4% glutaraldehyde in 0·1 M Sorensens phosphate buffer pH 7·2 with 20% ethylene glycol, rinsed twice in hybridization buffer and soaked in hybridization buffer for 1 hr, rinsed in ethanol and dried. The ^{35}S-labelled probe in hybridization buffer was heated to 90° for 10 min, cooled and 100 μl applied to each slide under a parafilm (Sigma) coverslip. Sections were left to hybridize in a humidified chamber at room temperature for 72 hr. Post-hybridization, slides were immersed in 2 × SSC until the coverslips dislodged, rinsed in 2 × SSC and washed at 40° for 30 min in 1 × SSC. Slides were then rinsed briefly in distilled water, in 70% ethanol for 5 min, in 95% ethanol for 5 min and allowed to dry.

Control sections from each eye were fixed for 5 min in glutaraldehyde buffer, washed once in 2 × SSC with 5% Tween and three times in 2 × SSC. One hundred microlitres of RNAase A (Boehringer Mannheim) 1 mg/ml in 2 × SSC was applied to each slide under a parafilm coverslip and incubated at 37° for 1 hr, slides were then washed in 2 × SSC and simultaneous hybridization with each probe carried out as above.

To determine the specificity of the lymphokine probe hybridization, each section was also hybridized with a ^{35}S-labelled probe to the P53 oncogene which was considered to be irrelevant to the EAU disease process.

Autoradiography

The slides were dipped in K5 (Ilford, Mobberley, U photographic emulsion diluted 1:1 in 0·5% glycerol and le expose at 4° for 21–24 days over silica gel. Slides were developed for 3·5 min in D19 (Kodak, Hemel Hempstead, U developer, fixed in Unifix (Kodak), washed in distilled wate counterstained with haematoxylin.

To compare the number of cells producing each lymphc the mean number of positive cells at each stage of the dis process was estimated for each probe.

Monoclonal antibodies

For immunohistochemistry primary monoclonal antibodi pan rat T cells (OX19; Serotex, Oxford, U.K.), IL-2 rece (OX39, Serotec) and (MHC) class II expression (OX6; Ser were utilized.

Immunohistochemistry

Sections of 6 μm thickness were cut from each eye and mou on 3-amino propyl triethoxysilane (Sigma) coated slides. S were fixed in acetone for 7 min and endogenous pero activity blocked with 3% hydrogen peroxide in 50% metha Sections were stained by a standard avidin–biotin com method (Vector, Peterborough, U.K.) using a rat immu globulin adsorbed biotinylated secondary antibody (Vec and amino ethyl carbazole to provide a red final reac product.

RESULTS

Induction of disease

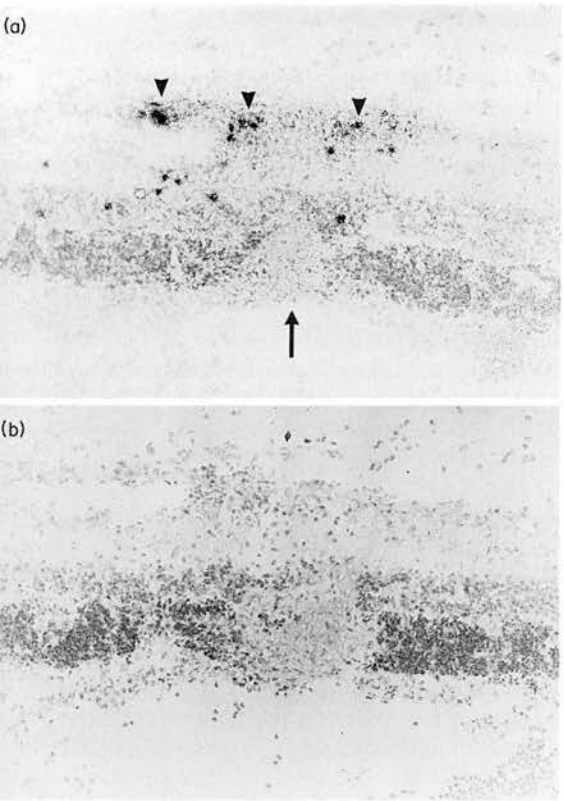
Animals developed histological evidence of disease on da post-induction. The histological changes were character EAU in rats immunized with S antigen[1] and it is well descr that this does not occur in animals immunized with C alone.[13–15] There was a marked mixed leucocyte infiltrate o anterior and posterior segments of the eyes with patche retinal oedema progressing to a total retinal destructio which lymphocytes were the predominant infiltrating cell.

Localization of IL-2 mRNA

Positive signal for IL-2 mRNA, observed as collections of grains in the photographic emulsion, was found over cells f day 12 post-immunization onwards. In the early phase of disease there was an accumulation of positive cells around areas of retinal oedema and destruction (Fig. 1). These pos cells were localized to the inner retina despite the focal nec of the photoreceptors. At the early disease stage there were minimal numbers of positive cells in the choroid and vitreou the eyes with established and advanced disease there numerous positive cells in the retina and vitreous, and mark increased numbers of positive cells scattered throughout ciliary body and choroid. No positive signal was seen on cel the anterior chamber. Numerous positive cells were seen in extraocular inflammatory infiltrates in both anterior and terior extraocular regions at all stages of the disease proce

Localization of lymphotoxin mRNA

Positive hybridization signal from the lymphotoxin probe generally less strong than the other probes utilized. In the e



1. (a) Detached retina in early (day 12 post-immunization) EAU. in inner retina have positive hybridization signal to IL-2 probe vheads to examples) around a focus of outer retinal oedema and ction (arrow). Haematoxylin counterstain × 120. (b) Adjacent n to (a) pre-treated with RNAase. No localization of IL-2 mRNA. atoxylin counterstain × 120.

of the disease positive cells were most numerous in the ocular inflammatory cell infiltrates but a few scattered ve cells were also found in the choroid and very occasional ve cells in the retina at this stage (Fig. 2). Numbers of ve cells increased as the disease progressed and were inent in the choroidal infiltrate in the later phase of the se. In general very few lymphotoxin positive cells were d in the retina. No positive cells were found in the anterior ber.

ization of IL-4 mRNA

idizations with the IL-4 probe revealed positive cells from 2 post-immunization onwards. These were seen in the ned retina (mainly the inner retina, related to patches of l necrosis and destruction), choroid and extraocular ates in the early phase of the disease. In the established and tages of the disease (Fig. 3) there was a marked increase in umbers of positive cells found in the ciliary body and oid. Small numbers of cells in the retina and subretinal fluid positive in the later stages of the disease process. Again, no ve signal was seen on any of the numerous inflammatory n the anterior chamber.

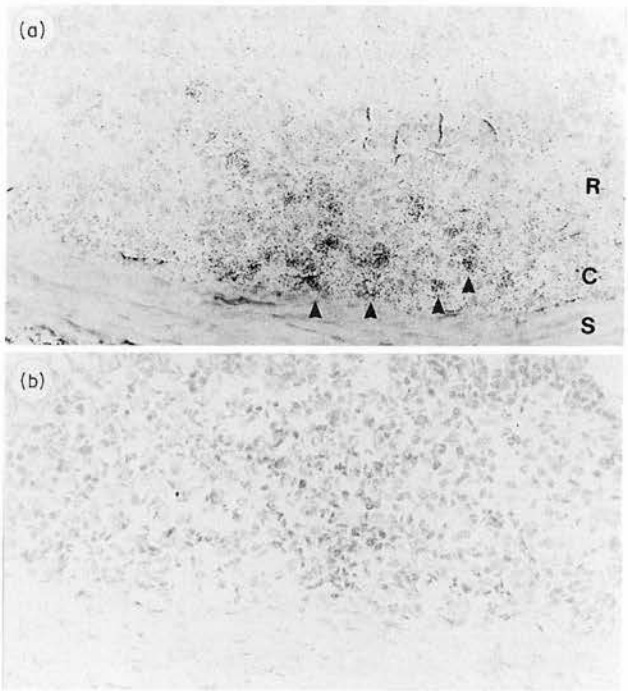


Figure 2. (a) Foci of localization of lymphotoxin mRNA (arrowheads to examples) in outer retina (R) and choroid (C) day 14 post-immunization. S = sclera. Haematoxylin counterstain × 120. (b) Adjacent section to (a) pretreated with RNAase. No localizing signal. Haematoxylin counterstain × 120.

Overall results of *in situ* hybridization

Positive cells for each probe were found simultaneous with the onset of disease on day 12 post-immunization, increased in numbers during the established phase of the disease and remained constant during the recovery phase. A similar number of cells were positive for each of the three probes at all stages of the disease. On the frozen sections examined immunohistochemically it was not possible to make accurate cell counts of the T-cell numbers because of the difficulty in defining individual cells with positive immunohistochemical stain in areas of marked accumulation of infiltrating mononuclear cells. It was estimated that 5–10% of the T-cell population had positive localizing signal for each probe.

No autoradiographic signal suggestive of localization of lymphokine mRNA was found using any of the probes in any of the four non-immunized animals studied.

Analysis of sections pretreated with RNAase at a concentration of 1 mg/ml for 1 hr showed that this abolished all localizing signal on the sections of all the positive eyes for each probe (Figs 2b, 3b). Sections hybridized with the P53 probe revealed a generalized background signal over areas of inflamed tissue without any focal cellular localization (not shown).

Immunohistochemistry

OX19 (pan T cell) positive cells were found in inflammatory infiltrates throughout the extraocular tissues, anterior chamber, ciliary body, choroid and retina, including the areas of lymphokine mRNA localization (not shown). Cells in these regions also

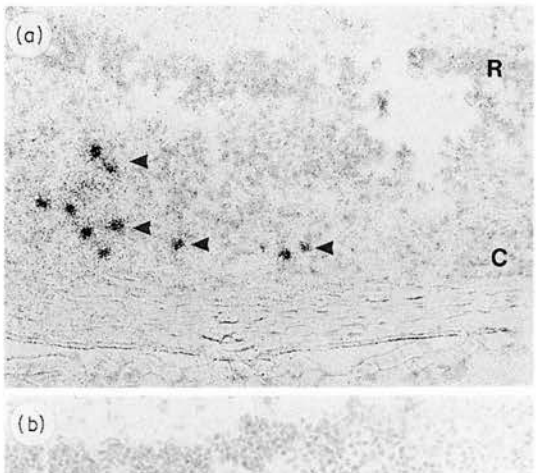


Figure 3. (a) Localizing foci of IL-4 mRNA localization in retina and choroid (C) (arrowheads) in advanced (day 21 post-immunization) EAU. Haematoxylin counterstain × 180. (b) Adjacent section to (a) pretreated with RNAase. No localizing signal with IL-4 probe. Haematoxylin counterstain × 180.

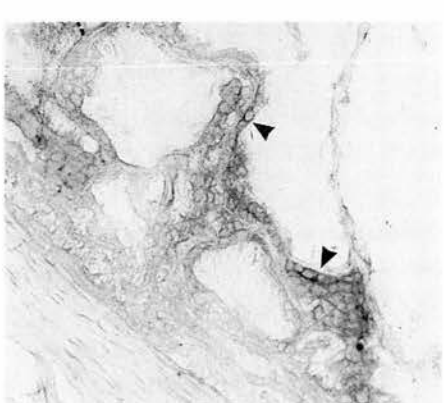


Figure 4. IL-2 receptor (OX39) positive T lymphocytes (arrowheads) infiltrating the ciliary body in advanced (day 17) disease. Haematoxylin counterstain × 150.

had positive staining with the OX39 antibody for IL-2 receptor expression (Fig. 4). MHC class II expression was found on cells from day 10 post-induction onwards, this increased markedly as the disease progressed and was seen both on organ-resident and infiltrating cells.

DISCUSSION

The presence of mRNA for the lymphokines IL-2, lymphot and IL-4 was demonstrated in the areas of T-lymphc infiltrate in actively inflamed eyes in EAU. We have previc demonstrated that IFN-γ mRNA and protein are present similar distribution.[9] At present no specific monoclonal bodies are available for the immunohistochemical detectic rat IL-2, lymphotoxin and IL-4 protein but the other inves tions have shown that levels of IL-2, IL-2 receptor and IF protein[16] and bioactive IL-2 and IL-4[17] reflects the level of respective cellular mRNA.

The presence of these four cytokines has implications regard to the subsets of CD4+ T cells (the predomi infiltrating cell type in EAU) present in the rat model used. I CD4+ T cells conform to the pattern of subsets found in mu CD4+ T-cell clones, that is the Th1 and Th2 types,[4] the follows that both subsets are present in the T-cell infiltrat this model. Alternatively it is possible that the T cells presen of the Th0 subset which are thought to be a product of an a or short-term stimulation and produce IFN-γ, IL-2 and IL There is evidence, however, that rat CD4+ T cells do not fit i the stable pattern of lymphokine secretion seen in murine CI T-cell clones. Rat CD4+ T cells have been subtyped accordin their binding of the OX22 monoclonal antibody, naive CD4 cells (OX22 high) produce IL-2 and IFN-γ and follov antigen contact cells become OX22 low and produce IL- According to this scheme both naive and memory CD4+ T would be present in the infiltrates found in EAU. This wc seem logical since any section of an actively inflamed eye wc be likely to contain a mixture of T cells involved in the dyna process of trafficking to the eye followed by antigen contact subsequent effector or helper function. It is also possible additional subgroups of rat CD4+ T cells exist and the posit may be considerably more complex. Analysis of the cytok secretion by individual T cells from the inflammatory infiltr would be necessary to investigate further the exact nature of T cells present. Additionally non-ocular antigen-specific T c may be involved in the destructive process following che attraction produced by activated ocular antigen-specific T c

The IL-2 and IL-4 producing cells were notably distribu in relation to foci of retinal oedema and destruction suggest that a stimulus to produce these lymphokines is present in area and their subsequent release may contribute to the fc destructive lesions seen in the early stages of EAU. Anti contact is one potential stimulus; however, it is notable these cells were distributed almost entirely within the in retina in contrast to the outer retinal distribution of S antig The stimulus for production of these lymphokines may th fore be another antigen or inflammatory mediator.

IL-2 is a lymphokine which is produced by helper T c following activation by antigenic or mitogenic stimulation; required for the subsequent proliferation of T lymphocytes therefore plays a key role in the initiation of an imm response.[19] IL-2 mRNA has been demonstrated at the site tissue pathology in autoimmune disease such as rheumat arthritis.[20] IL-2 protein has been demonstrated in human e with posterior uveitis[21] and an increase in circulating I receptor positive[22] T lymphocytes has been demonstrated patients with active uveitis. The demonstration of IL-2 mR and IL-2 receptor positive lymphocytes in EAU is furt

·nce that this mediator is central to the immunopathology traocular inflammatory disease.

.ymphotoxin is produced by both $CD4^+$ and $CD8^+$ T hocytes and is thought to play a key role in T-lymphocyte oxicity.[23] Secretion of IFN-γ along with lymphotoxin ars to promote $CD4^+$, class II-restricted T-cell cytotox-[24] IL-2 has also been demonstrated to produce differentia- of cytotoxic T lymphocytes.[25,26] The *in vivo* expression of NA specific for these lymphokines at the site of tissue ology in EAU is evidence that the tissue destruction may be iuced by effector T lymphocytes.

T-cell cytotoxicity can be mediated by several mechan-[24,27-29] and the relatively low numbers of cells in the retinal ology producing lymphotoxin would suggest that cytotoxic nanisms other than those involving lymphotoxin may be lved.[30] The widespread cellular expression of MHC class II gens in the eye in EAU would provide suitable target cells cytotoxic $CD4^+$ T cells which may subsequently produce e extensive damage through 'innocent bystander' killing.[24] L-4 produces B-lymphocyte activation, MHC class II ession and immunoglobulin (IgG1 and IgE) production. It so involved in T-lymphocyte activation and growth.[31] ough few B cells are present in the intraocular pathology in , *in vivo* IL-4 production would facilitate antigen presen- n by those B cells present to the infiltrating antigen-specific mphocytes.

L-4 has been shown to increase the cytotoxic capacity and C class II expression of macrophages.[31] IFN-γ also has the ntial to activate macrophages[32] and the *in vivo* production ese two lymphokines could therefore promote an effector unction in the numerous macrophages present at the sites of e pathology in EAU.

t has been demonstrated that mRNA for multiple cytokines esent in affected joints in rheumatoid arthritis,[33] a disease ght to have an autoimmune aetiology. Our results indicate this may also be the case in intraocular inflammatory se and the relative contribution of each individual cytokine e tissue pathology will require further study.

Targeting the lymphokines involved in the pathogensis of une-mediated pathology may limit the subsequent tissue uction. Several such approaches are currently under inves- ion, for example the use of monoclonal antibodies to a kine or its receptor,[34] the administration of soluble cytokine otors[35] to interfere with the cytokine–receptor interaction or se of specific cytokine antagonists.[36] Further investigation e cytokine mechanisms involved in EAU will be useful in evelopment of more specific therapies for human intraocu- nflammatory disease.

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