CELLULAR INTERACTIONS BETWEEN LYMPHOCYTES AND RETINAL ENDOTHELIAL CELLS

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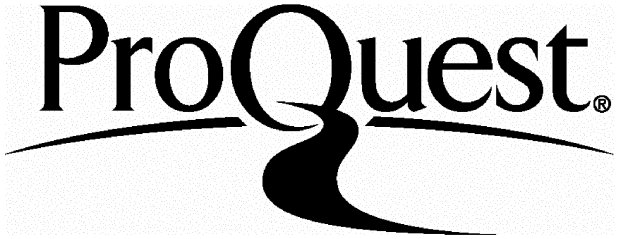

This thesis is dedicated to my wife Dan and my son Hao

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CONTENTS

ABSTRACT

Cellular interactions between lymphocytes and endothelial cells derived from retinal and brain microvasculatures are believed to play an important part in the pathogenesis of inflammatory diseases of the central nervous system. In this *in vitro* study we have investigated these interactions in two aspects; (1) lymphocyte adhesion to and migration across retinal endothelial cells, and (2) antigen presenting properties of retinal and brain endothelial cells.

Under normal conditions the level of lymphocyte adhesion to retinal endothelial cells was around 5%, but this low level of adhesion could be significantly upregulated following endothelial treatment with the cytokines, IFN-γ, IL-1 and IL-4, astrocyte conditioned medium and forskolin. Lymphocyte activation with Con A also increased the adhesion which could be further augmented by activating the endothelial cells with the cytokines, astrocyte conditioned medium and forskolin. Although $CD8^+$ T cells in normal and Con A activated lymphocyte populations adhered to endothelial cells more efficiently than $CD4^+$ T cells, the antigen-specific activated $CD4^+$ T-cell lines (S-Ag specific T cell lines) exhibited the greatest degree of adhesion and were also capable of migrating across retinal endothelial monolayers.

The role of adhesion molecules in lymphocyte adhesion and migration has been examined. Lymphocytes express both LFA-1 and VLA-4 and activation of lymphocytes with Con A and S-Ag can increase the expression of LFA-1, but not VLA-4. Resting cultured retinal endothelial cells express ICAM-1 and its expression was significantly increased by IFN-γ and IL-1. Treatment of lymphocytes with the monoclonal antibodies to LFA-1 and VLA-4 significantly inhibited the adhesion of Con A activated lymphocytes and S-Ag specific T cell lines to both resting and IL-1 activated retinal endothelial cells. Pre-incubation of retinal endothelial cells with the monoclonal antibody to ICAM-1 only showed an inhibitive effect on the adhesion of S-Ag specific T cell lines, but not Con A activated lymphocytes. Furthermore, the antibodies to LFA-1 and ICAM-1 also blocked the migration of S-Ag specific T cell lines across both resting and IL-1 activated endothelial monolayers and the antibody

to VLA-4 only inhibited the migration across IL-1 activated endothelial monolayers.

Cultured retinal and brain endothelial cells are capable of expressing MHC class II molecules following treatment of IFN-γ. With both sets of endothelial cells, only class II I-A, but not I-E molecules, could be induced significantly by day 3 with brain EC expressing lower levels of I-A. IFN-γ also caused a concomitant increase in the level of MHC class I molecules. In comparison with expression of adhesion molecules, induced expression of MHC class II molecules appeared to be slower than enhanced expression of ICAM-1 which occurred within 18 hours following treatment with IFN-γ.

Retinal and brain EC were capable of presenting S-antigen to S-antigen specific $CD4^+$ T-cell lines resulting in both T-cell proliferation and cytotoxicity. In contrast to the cytotoxic effect which was demonstrated with confluent endothelial monolayers, significant T-cell proliferation was only seen when subconfluent, rather than confluent endothelial cells were used as the antigen presenting cells. Optimal T-cell proliferation was observed at the ratio of T cells to the endothelial cells in 2:5. Subconfluent endothelial cells also resulted in a greater IL-2 production than confluent cells. Although retinal endothelial cells were capable of producing TGF-ß, removal of this factor with the anti-TGF-ß monoclonal antibody from the subconfluent proliferation assay attenuated the T-cell response. Contrary to these findings, the addition of exogenous TGF-ß to the media in the antigen presentation assays with either confluent or subconfluent endothelial cells did not affect the degree of T-cell proliferation and IL-2 production. Despite demonstrating significant T cell responses to retinal and brain endothelial cell antigen presentation, they remained less efficient than professional antigen presenting cells such as thymocytes.

ABBREVIATIONS

ACM	astrocyte conditioned medium
APC	antigen presenting cell
BBB	blood-brain barrier
BRB	blood-retinal barrier
BSA	bovine serum albumin
CNS	central nervous system
Con A	concanavalin A
CREAE	chronic relapsing EAE
CTL	cytotoxic T lymphocyte
EAE	experimental autoimmune (allergic) encephalomyelitis
EAU	experimental autoimmune uveoretinitis
EC	endothelial cell
ECGS	endothelial cell growth supplement
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GM-CSF	granulocyte macrophage colony-stimulating factor
GSA	griffonia simplifolia agglutinin
HBSS	Hanks' balanced salt solution
HEV	high endothelial venule
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
IFN-γ	interferon gamma
IL	interleukin
IRBP	interphotoreceptor binding protein
LCA	leucocyte common antigen
LFA	lymphocyte function-associated antigen
LPS	lipopolysaccharide
LT	lymphotoxin
mAb	monoclonal antibody
MBP	myelin basic protein
MHC	major histocompatability complex
NK	natural killer cell
NRS	normal rat serum
PDS	plasma derived serum
PHA	phytohemagglutinin
PLN	peripheral lymph node
PMA	phorbol myristate acetate
RPE	retinal pigment epithelium
S-Ag	retinal soluble antigen
TGF-ß	transforming growth factor beta
TNF	tumour necrosis factor
VAP	vascular adhesion protein
VCAM	vascular cell adhesion molecule

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CHAPTER 1
GENERAL INTRODUCTION

1.1. INTRODUCTION

Endothelial cells (EC) that form the inner vascular wall were once considered to be a passive, non-adhesive lining that functioned only to prevent blood coagulation and to separate the vascular space from tissues. Since this early perception of the EC research has expanded dramatically and produced a new image of the vascular lining as an active participant in a wide variety of physiological and pathophysiological processes. It is now evident that haemostasis, inflammatory reactions and immunity involve close interactions between immunocompetent cells and vascular EC (Cotran 1987; Pober 1988).

The role of the EC in the interaction with lymphocytes has recently been addressed and has revealed two major aspects of EC functions: antigen independent lymphocyte recruitment into the inflammatory site and antigen dependent T-cell activation (Pober and Cotran 1991). The recruitment of lymphocytes by EC is an initial step in the development of the immune reaction and lymphocytes enter the inflammatory site by adhering to and then migrating through the EC lining of the vascular wall. Central to these processes is the enhanced or induced expression of EC surface adhesion molecules which support both lymphocyte adhesion and migration, although other mechanisms may also be involved in this process. The potential role of antigen presentation by EC has also been suggested since EC can be induced to express MHC class II molecules by inflammatory factors, particularly interferon gamma (IFN-γ), and *in vitro* studies also show that EC can stimulate T cell-proliferation in an antigen-dependent manner. In association with these two functions, EC can produce a number of cytokines and other factors, such as IL-8 (which activates lymphocyte adhesion) and IL-1 (which co-stimulates T-cell proliferation).

The vascular EC of the retina and brain are highly specialized with unique features that result in a highly impermeable cellular barrier that is referred to as the

vascular blood-retinal and blood-brain barriers (BRB and BBB, respectively). In addition to restricting the non-specific passage of molecules, the BBB and BRB have the ability to limit immune cell movement across the EC and thereby potentially shield the central nervous system (CNS) from the immune system. Recent studies also indicate that these selective barriers not only play a role in separating the CNS parenchyma from the circulation, but are also implicated in the pathogenesis of diseases affecting the CNS. The role of these selective barriers in the induction and propagation of disease process is currently understood to include several separate but related phenomena. Firstly, the EC which form these barriers play a significant part in the recruitment of circulating immune cells. Secondly, they possess the potential to act as antigen presenting cells (APC) at the barriers and thirdly, lymphocyte extravasation and the release of cytokines and other inflammatory agents can lead to disruption of the barrier, formation of vasogenic oedema and other clinical problems.

In immune-mediated diseases the initial cellular interactions between lymphocytes and vascular barrier cells are believed to be crucial to the development of CNS inflammatory reactions and these cellular interactions can also lead to subsequent changes in the properties of the vascular barriers which may play an important role in the pathogenesis of the disease. The role of the CNS vascular EC in this cellular interaction, however is far less understood than that of non-CNS vascular EC. Although several approaches have been made on brain vascular EC in the past few years, the molecular mechanisms that control this interaction remain to be fully elucidated. Furthermore, in the retina which is a specialized site of the CNS the role of the vascular EC even less understood and investigations into lymphocyte interaction with retinal microvessel EC would provide an insight into the pathogenesis of autoimmune diseases that develop within the retina.

1.2. T-CELL MEDIATED INFLAMMATORY DISEASES OF THE RETINA

1.2.1. Posterior uveitis

Intraocular inflammatory disease (uveitis) is an important cause of visual

impairment in man. The aetiology of this disease is often unknown, and although it may occur in association with systemic disorders, it can occur alone as an organic specific autoimmune disease. It usually runs a chronic relapsing course that is difficult to manage clinically and may result in blindness. Infective agents are a cause in only few cases but diverse immunologic studies over the past years suggest that allergy or autoimmunity may be an important factor in many instances.

Several lines of evidence support a dominant role for T cells in mediating posterior uveitis (Lightman and Chan 1990; Lightman and Towler 1992). Histologic and immunohistochemical analysis of the retina from patients with sympathetic ophthalmia, Vogt-Koyanagi-Harada (VKH) disease, and birdshot chorioretinitis show a significant T-cell infiltration (Jakobiec et al., 1983; Chan et al., 1988a). In the case of sympathetic ophthalmia, it has been observed that $CD4^+$ T-cell subsets appear to be the major cells at the early phases of the disease, whereas a larger number of $CD8^+$ T cells are found in the chronic phases (Jakobiec et al., 1983). Few B-cells, neutrophils, little immunoglobulin and no evidence of immune complex deposition in the retina were detected (Lightman and Chan 1990), suggesting that antibodies and complement do not play a major role in the inflammatory process.

Currently it is not clear how T cells are responsible for the disease. Although the appearance of MHC class II molecules on retinal resident cells is often associated with T-cell infiltration, specific antigens have not yet been clearly identified. It has been suggested that retinal soluble antigen (S-Ag) and interphotoreceptor binding protein (IRBP) are the important retinal antigens in the induction of this immune response, since in the animal models of these diseases it has been demonstrated that these antigens are capable of inducing T-cell mediated uveoretinitis (Caspi 1989). In addition, peripheral blood lymphocytes from patients with posterior uveitis have been shown to recognize either S-Ag or IRBP resulting in a proliferative response (de Smet et al., 1990), and the peripheral blood $CD4^+$ cells have also been found to produce a significant amount of interleukin 2 (IL-2) following stimulation with S-Ag (Opremcok et al., 1991).

There is also some evidence to suggest that activated T cells may play an

important role in this disease. Treating uveitis patients with cyclosporin A (CsA), an inhibitor of activated T cells, can significantly reduce the inflammatory response in many cases. Furthermore, in patients with different forms of uveitis, there are increased numbers of circulating T cells expressing IL-2 receptors (Dechenes et al.,1988; Feron et al., 1992), and the level of soluble IL-2 receptors in their serum is also increased (Arocker-Mettinger et al., 1990; BenEzra et al., 1993).

1.2.2. Experimental autoimmune uveitis

Experimental autoimmune uveitis (EAU), the experimental animal model of posterior uveitis, has provided valuable information on the basic mechanisms involved in immune-mediated inflammatory diseases of the retina. EAU can be produced by active immunization of sensitive strains of animals with retinal antigens, such as S-Ag and IRBP. The disease first becomes apparent around 10-14 days after immunization with uveal inflammation. Histologically, lymphocyte infiltration and the damage of photoreceptor layers are the major pathogenic characteristics with the progression of the disease.

Various studies indicate that T cells play a crucial role in the initiation of the disease. Direct evidence implicating the mandatory role of T cells in EAU induction was provided by the finding that EAU could be adoptively transferred with long-term $CD4^+$ T-cell lines, derived from Lewis rats immunized with S-Ag. All the T-cell lines capable of adoptively transferring EAU, which have been developed by different investigators, are retinal antigen-specific and MHC class II-restricted. All are of the $CD4^+$ phenotype, mediate DTH and induce EAU. These MHC class II negative, IL-2 receptor positive $CD4^+$ T cells will induce disease that is histologically identical to that seen with active immunization (Caspi et al., 1986; Rozenszajn et al., 1986).

The immunological features of EAU are similar to those found in the animal model of another human CNS inflammatory disease, namely multiple sclerosis (MS; Calder and Lightman 1992). The animal model, experimental autoimmune encephalomyelitis (EAE) has also been shown to be a $CD4^+$ T-cell mediated disease which can also be induced either by active immunization of animals of certain strains

with site-specific antigen (myelin basic protein; MBP) or by adoptive transfer of activated MBP-specific $CD4^+$ T-cell lines.

Of fundamental importance to the pathogenesis of these human conditions and their experimental analogues is the infiltration of immunopathogenic T cells into the tissues. Morphological observations indicate that the immune response within the retina and brain progresses as the result of increasing number of inflammatory cells crossing vascular BRB and BBB (Lightman and Greenwood 1992; Raine et al., 1990). In association with this enhanced cellular traffic there is a concomitant breakdown of barrier integrity and subsequent edema formation. The blood-tissue barriers in controlling lymphocyte migration may therefore play an important role in orchestrating the development of these diseases.

1.3. THE ROLE OF VASCULAR EC OF BRB AND BBB IN INFLAMMATORY DISEASES OF THE CNS

1.3.1. Retinal and brain vascular EC

EC derived from the retina and brain are highly specialized and differ from the EC of other vascular beds. The fundamental property of retinal and brain EC is their ability to form a highly selective BRB and BBB between the blood and the retinal and brain parenchyma. The ultrastructural correlate of these barriers is formed principally by the presence of tight junctions which are comprised of points of fusion of the adjacent EC membrane. In addition to these specialized junctions retinal and brain EC also express low levels of pinocytosis, no fenestrae and no transendothelial pores. As a result of these properties retinal and brain microvessels have been shown *in vivo* to exhibit a very high transvascular resistance of the order of 1,500 $\Omega.cm^2$ (Butt and Jones 1992).

It is not completely clear what the controlling factors are that induce these specialized characteristics on the EC of the vascular barriers of the retina and brain. Current knowledge indicates that the specialization of CNS vascular EC is dependent on a number of accessory factors. Astrocytes, and in the case of the retina also

Müller cells, are probably most important in that it has been known for a long time that astrocytes extend to the EC basement membrane where they end in footprocesses. Recent findings suggest that this structural arrangement enables astrocytes to provide signals that are necessary for the formation of tight junctions of the EC (Brightman 1991). In *in vitro* studies it has been shown that coculturing astrocytes with EC can increase brain EC tight junctional complexity and the electrical resistance to 600 $\Omega.cm^2$. This effect can also be achieved by culturing EC in astrocyte conditioned medium (ACM) in a combination with an increase in cyclic AMP levels (Rubin et al., 1991).

Three major functions are implicated in the term of vascular CNS barriers: (1) the protection of the CNS from the contents of the blood as achieved by complex tight junctions between EC, preventing the non-specific passage of molecules; (2) the selective transport by specialized transport systems; and (3) metabolism or modification of blood- or CNS-borne substances (Risau 1991). However, these barrier functions may be terminated under many pathogenic conditions, and the breakdown of the vascular barriers can lead to the immediate problem of vasogenic oedema and the well defined clinical problems related to this condition.

1.3.2. Regulation of the immune response of the CNS by the BRB and BBB

The CNS has been characterized immunologically as a site of limited reactivity. This concept, termed immune privilege, was developed from classical studies which showed that allografts usually fare better in the brain than in conventional sites. The concept of the CNS being an immune privilege site has recently been redefined to include a limited access of immune cells during their normal role of tissue surveillance, and mechanisms that contribute to the immunologically isolated state of the CNS have been a subject of debate (Cserr and Knopf 1992). Traditionally, explanations include the absence of a conventional lymphatic drainage system, lack of constitutively MHC class II bearing cells and the presence of vascular barriers. More lately, it has also been suggested that the local production of immune suppressive factors, such as TGF-ß may also contribute to this

suppressive environment for immune reactivity in the CNS (Wilbanks and Streilein 1992).

The presence of the vascular barriers certainly plays a significant part in shielding the CNS parenchyma from the immune system. Under normal conditions the vascular barriers can limit the traffic of lymphocytes and other inflammatory cells into the CNS. In addition, these barriers also minimize the transfer of molecules such as immunoglobulins and complement from the blood to the nervous tissue extracellular fluid (ECF). Thus, the vascular barriers block the efferent arm by preventing the entrance of cellular and humoral elements of the immune system into the CNS.

Recent studies have revealed that the CNS vascular barriers are not completely impermeable to protein molecules (antigens). A significant fraction of protein injected into the intact CNS could be collected from the cervical lymphatics and also cause a systemic antigen specific antibody response which could be identified in deep cervical lymph nodes (Harling-Berg et al., 1989; Gordon et al., 1992). This continuous communication between the CNS and the draining lymph nodes is active and highly-regulated by the vascular CNS barriers and the immune system (Cserr and Knopf 1992). When damage to the CNS vascular barriers occurs, either through disruption of the tight junctions or vascular transport, transfer of molecules from the CNS to the circulation is markedly elevated and can lead to the immune system being exposed to CNS antigens.

The ability of the vascular barriers to limit lymphocyte traffic is also reduced when immune reactions develop within the CNS. Increased lymphocyte infiltration across these barriers has been found in many CNS inflammatory diseases, such as MS (Bellamy et al., 1985), posterior uveitis (Lightman and Chan 1990) and their animal models of EAE (Raine et al., 1990) and EAU (De Kozak et al., 1978; 1981; Lin et al., 1991; Lightman and Greenwood 1992), respectively. In these situations, the vascular barriers may play a role in recruiting lymphocytes from the circulation by increasing their ability to bind lymphocytes. Moreover, they could also be potentially

APC, activating T cells at the barrier sites, since they can express MHC class II molecules in an inflammatory lesion (Lightman et al., 1987; Chan et al., 1988b; Fujikawa et al., 1987; Sobel et al., 1984; Traugott et al., 1985).

1.3.3. The role of retinal and brain EC in inflammatory diseases of the CNS

Lymphocyte infiltration into the retina and brain is a hall mark of autoimmune diseases within the CNS, and this process inevitably involves lymphocyte interaction with CNS barrier cells. Examination of these cellular interactions have been made extensively in the animal models of these diseases, such as EAU and EAE, where it has been observed that there is a dramatic increase in lymphocyte traffic across the vascular EC. Migrated lymphocytes preferentially accumulate in the perivascular space often forming a lymphocyte cuff around the inflamed vessels particularly the postcapillary venules (Lightman and Greenwood 1992; Greenwood et al., 1994; Raine et al., 1990; Cross et al., 1990).

In contrast to the significant increase in lymphocyte infiltration in the development of EAE, the parenchymal pathology such as demyelination was not found to be a prominent feature of the lesion in the earlier stages despite clinical signs of EAE. These observations have lead to the suggestion that modification of vascular EC barriers by infiltrated lymphocytes are responsible for the initial symptoms (Kelero de Rosbo et al., 1985; Raine et al., 1990). Indeed, increased permeability of the BRB and BBB has been shown in both EAU and EAE, and this effect could result in oedema formation and the subsequent problems in the retina and brain. The increased permeability of the vascular EC is usually detected at the first sign of infiltrating inflammatory cells, although at this stage the barrier appears ultra-structurally intact with apparently normal tight junctions between the EC (Lossinsky et al., 1989; Lightman and Greenwood 1992).

Morphological observations indicate that the vascular BRB and BBB also undergo a number of alterations in association with lymphocyte infiltration. In EAE and EAU, the inflamed vessels show tall, plump EC with irregular pale nuclei

containing dense peripheral heterochromatin. Their cytoplasm shows prominent rough endoplasmic reticulum, a large number of ribosomes and active mitochondria. This EC activation has been suggested to be a consequence of increased EC metabolism and protein synthesis (O'Neill et al., 1991; Raine et al., 1990; Cross et al., 1990; Lossinsky et al., 1989; Lightman and Greenwood 1992; Dua et al., 1991). The raised, bulbous EC described in both EAE and EAU have led to the suggestion that these EC resemble the specialized EC lining the postcapillary venules in lymphoid tissues (referred as high endothelial venules; HEV) which have specifically evolved to recruit circulating lymphocytes.

One of the most striking changes observed in lymphocyte infiltration in the CNS is that the CNS vascular EC express addressins (Cannella et al., 1990; Raine et al., 1990) which were originally thought to be restricted to HEV for lymphocyte homing. The degree of expression of these molecules corresponds to the level of lymphocyte invasion particularly during the relapsing phase of chronic relapsing EAE (CREAE; O'Neill et al., 1991). Other adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) expressed on the vascular EC have also been implicated in lymphocyte extravasation. In EAE, ICAM-1 expression is upregulated from basal levels during the active stages of the disease (Rain et al., 1990; Cannella et al., 1990) and correlates with the induction of addressins during disease relapse.

In addition to the expression of addressins and ICAM-1, in the development of EAU and EAE, CNS vascular EC can aberrantly express MHC class II molecules. This expression of class II molecules has even been reported prior to the onset of inflammatory disease (Sobel et al., 1984) indicating that MHC class II molecules expressed on the EC could be involved in initiating the disease. The expression of MHC class II molecules suggests that these vascular barrier cells may play a role as APC, mediating an antigen specific class II restricted interaction with $CD4^+$ T cells, and that this interaction could potentially result in a clonal expansion of T cells and cytotoxic lysis of the integrity of the CNS barriers. Whether the expression of MHC class II molecules on EC can play a role in lymphocyte transendothelial infiltration

is not clear although some *in vitro* studies have shown that these molecules can mediate lymphocyte adhesion to the vascular EC (Masuyama et al., 1986; Goodall et al., 1992)

In studies with adoptively transferred EAE, injected antigen specific T cells (MBP specific $CD4^+$ T cells) have been found to accumulate preferentially in the perivascular space (Cross et al., 1990) although they appear only to form a small part of the total infiltrated cells. The major infiltrating cells were host inflammatory cells which migrate deep into the parenchyma. It has been suggested that the perivascular location of antigen-specific $CD4^+$ T cells can influence the vascular EC through the secretion of cytokines and regulate the influx of host origin inflammatory cells. Indeed, recent evidence indicates that host origin non-specific cells may play a vital role in the pathogenesis of both EAU (Caspi et al., 1993) and EAE (Kawai et al., 1993). Although antigen specific T cells infiltrate irrespective of antigen specificity, these initial migrated cells can interact with MHC class II bearing resident APC and this antigen specific interaction may lead to local T-cell proliferation and cytokine production. These locally produced cytokines would then be able to act on vascular EC by inducing or enhancing the expression of adhesion molecules, such as ICAM-1 and addressins (Raine et al., 1990, Cannella et al., 1990), and hence increase the ability of vascular EC to recruit other host origin non-activated inflammatory cells.

For initial lymphocyte infiltration, the activation state of antigen-specific T cells is crucial. It is known that antigen specific T cells stimulated to blast phase, either by their specific antigen or by non-specific mitogenic lectins, will produce EAU (Caspi et al., 1986) and EAE (Peters and Hinrichs 1982), whereas unstimulated cells will not. This experimental phenomenon has been elucidated by the demonstration that only activated, but not metabolically resting T lymphocytes are capable of crossing the BBB and penetrating into the CNS parenchyma (Hickey 1991). A further important aspect of the state of T-cell activation is that activated T cells are able to synthesize and secrete cytokines which can enhance non-specific inflammatory cell infiltration by activation of the vascular EC.

1.4. LYMPHOCYTE INTERACTIONS WITH CULTURED EC MONOLAYERS

1.4.1. Cultured EC monolayers

Cultured EC enable studies to be undertaken on the properties of these cells which are difficult to implement *in vivo*. Over previous decades cultures of human umbilical vein EC (HUVEC), which was introduced *in vitro* in the early 1970's (Jaffe et al., 1973), have made a significant contribution to these studies. Research concerning lymphocyte interactions with EC has also largely been carried out with HUVEC and these studies have provided a substantial amount of information towards our current understanding of the molecular mechanisms involved. In addition, there have also been several reports of *in vitro* studies on lymphocyte interactions with microvascular EC derived from dermal microvascular vessels (Haskard et al., 1987), high endothelial venules (Ager et al., 1988) and CNS microvascular vessels (Hughes and Lantos 1986; Hart et al., 1990), which more closely reflect these interactions that occur *in vivo* at the level of the microvasculature of individual organs and tissues. Despite an obvious anatomical difference, HUVEC are still most widely used for investigating the cellular interactions between lymphocytes and EC due to their convenience and yield. It has also been reported that HUVEC behave in a similar fashion to dermal derived microvascular EC in their interactions with lymphocytes (Haskard et al., 1987). Furthermore, since HUVEC can be affected by a number of cytokines produced at inflammatory sites, it has been suggested that such cytokine-induced HUVEC activation may reflect what is occurring at microvascular bed *in vivo* (Cavender 1989).

1.4.2. Lymphocyte adhesion to cultured EC monolayers

Lymphocyte adhesion to vascular EC is an initial step during lymphocyte infiltration into inflammatory tissues. The processes involved in adhesive interactions between lymphocytes and EC are a cell-activation dependent event and finely controlled by surface molecules expressed on both lymphocytes and EC. Studies have

shown that cytokines, produced in the inflammatory process, are an important stimulator of EC activation in that they induce or enhance the expression of adhesion molecules and thereby increase lymphocyte adhesion. Antigen or mitogen activated lymphocytes also exhibit an increased ability to bind EC.

Cytokine effects on lymphocyte adhesion

The role of the cytokines that might affect lymphocyte adhesion was initially studied by examining the effect of crude supernatants from mixed lymphocyte reaction and mitogen-stimulated cultures on the adhesion of human lymphocytes to confluent monolayers of HUVEC (Yu et al., 1985). In those study it was reported that such supernatants, when preincubated with the EC, increased the proportions of both B and T cells which adhered to the EC, but had no effect on red blood cell adhesion. The supernatants did not stimulate adhesion when preincubated with lymphocytes. The results of experiments in which such supernatants were treated with heat, acid or antibodies suggested that gamma interferon (IFN-γ) was one of the active factors. These results were confirmed by the demonstration that both affinity column-purified and recombinant IFN-γ were also active in stimulating EC adhesiveness for lymphocytes (Cavender et al., 1986). Stimulation of EC adhesion for lymphocytes by IFN-γ reached a maximum after 24 hours of incubation with a concentration of 100 U/ml (3 ng/ml).

A second cytokine interleukin-1 (IL-1) was also found to increase lymphocyte adhesion (Cavender et al., 1986). IL-1 is a pleiotropic cytokine produced by many cell types, most notably by mononuclear phagocytes (Durum et al., 1985) and has been shown to be an important costimulatory factor in T-cell activation (Weaver and Unanue 1990). IL-1 is also an important inflammatory factor which is found in a number of inflammatory diseases. It may participate in the inflammatory process by inducing EC eicosanoid metabolism, cytokine production, EC proliferation, by stimulating procoagulant activity by EC and by acting as a chemotactic agent for leucocytes (Mantovani and Dejana 1989).

EC treated with IL-1, both affinity column-purified human IL-1 and

recombinant IL-1α as well as IL-1ß provide a better substratum for the adhesion of both T and B lymphocytes (Cavender et al., 1986). Although fixation of the EC before incubation with IL-1 prevents the increase in adhesion, fixation after IL-1 incubation does not result in less lymphocyte binding suggesting that the IL-1-induced increase was the result of a change in the EC membrane, and was not due to the secretion of an EC-derived factor that acted on the T cells to stimulate their adhesiveness for EC. The effect of IL-1 appeared to be similar to the effect of IFN-γ in that it also required several hours to reach its maximum, and was only effective when preincubated with EC. However, some consistent differences were noted between IL-1 and IFN-γ (Cavender et al., 1986; 1988; 1989). First, the effect of IL-1 was more rapid than that of IFN-γ, reaching a peak within four hours and then declining slowly over time, even in the continued presence of IL-1. Secondly, relatively less IL-1 was required to induce a maximal increase in EC adhesiveness being effective at a concentration of 0.1 ng/ml for both recombinant IL-1α and recombinant IL-1ß. Thirdly, preincubation of the EC resulted in a much higher proportion of lymphocytes adherent to the EC than occurred with IFN-γ.

Tumour necrosis factor-α (TNF-α) has many of its bioactives similar to IL-1, including its effects on EC such as induction of procoagulant activivty (Bevilacqua et al., 1986) and the stimulation of EC adhesiveness for polymorphonuclear leucocytes and monocytes (Bevilacqua et al., 1985). Therefore, the effects of recombinant TNF-α and a closely related lymphokine, TNF-ß on EC adhesiveness for T cells has also been tested. By all criteria, the effects of TNF-α and TNF-ß were very similar to the effects of IL-1 both having strong stimulatory effects on EC adhesiveness for lymphocytes, reaching a maximum at 4 to 8 hours after exposure and slowly declining with time thereafter, and requiring the cytokines to be preincubated with EC and not lymphocytes (Cavender et al., 1987, Gamble et al., 1985).

Another cytokine to have an effect on EC is IL-4 which is a T cell-derived cytokine with a variety of biological activities. Originally described as a costimulator of B-cell proliferation, it also increases their expression of MHC class II, and low

affinity Fc receptors for IgE (Fc RII/CD23). IL-4 is an important regulator of immunoglobulin isotype production, promoting the synthesis of IgG1 and IgE isotypes and influencing thymic development. Furthermore, IL-4 receptors and activity have been demonstrated on a variety of haemopoitic cells, mast cells, macrophages, as well as fibroblasts and epithelial cells (Paul and Ohara 1987).

One of the effects of IL-4 on EC is that it selectively increases leucocyte binding. Treating HUVEC or microvascular EC with IL-4 significantly increases the ability of EC to bind lymphocytes, but not neutrophils (Masinovsky et al., 1990), and this increased adhesion could be abrogated by neutralising the effect of IL-4 with an anti-IL-4 antibody (Thornhill 1990a). A significant increase in adhesion could be achieved with 3.3 U/ml of IL-4 with maximal adhesion occurring at 33 U/ml, and this effect occurred as early as 4 hours after induction, peaking at 24 hours, and was stable through to 72 hours of culture. Adhesion decayed to basal levels 72 hours after removal of IL-4 from the cultures (Masinovsky et al., 1990; Thornhill et al., 1990a).

The cytokines IL-1, IFN-γ, TNF and IL-4 have been widely investigated in their effect on inducing lymphocyte adhesion to EC and it has been proposed that they can be divided into two groups according to their properties of increasing adhesion. The first group of IL-1 and TNF increase adhesion rapidly after the initial stimulation, whereas IFN-γ and IL-4 have a delayed effect. Furthermore, a synergistic effect of TNF when added with either IL-4 or IFN-γ on lymphocyte adhesion to EC was also reported. In contrast, optimal concentrations of IL-1 combined with TNF or IL-4 with IFN-γ were no more effective than the individual cytokines alone (Thornhill et al., 1991).

Effects of lymphocyte activation on adhesion

It has been known for some time that lymphoblasts preferentially enter inflammatory sites and that this is probably due to an increased ability of the lymphocytes to bind to EC (de Bono 1979). This phenomenon has been extensively examined *in vitro* by studying lymphocyte adhesion to cultured EC monolayers following lymphocyte activation. It is now clear that not only antigen-specific

activation but also non-specific activation of lymphocytes can dramatically increase their adhesion to EC. These non-specific activators include the anti-CD3 monoclonal antibody (mAb; Shimizu et al., 1990), phorbol myristate acetate (PMA; Haskard et al., 1986a), calcium ionophores, mitogens such as phytohemagglutinin (PHA) and concanavalin (Con A; Burmester et al., 1987; Wysocki and Issekutz 1992), as well as IL-2 (Pankonin et al., 1992; Damle et al., 1987).

Stimulation of lymphocytes with PMA and anti-CD3 mAb increases adhesion to HUVEC rapidly within minutes, and therefore does not presumably require protein synthesis (Haskard et al., 1986; Shimizu et al., 1990). T cells activated with the mitogen Con A or a calcium ionophore also caused a rapid (1-4 hours post activation) and marked (7- to 12-fold) increase in the adhesion to both unstimulated and IFN-γ- or lipopolysaccharide (LPS)-treated EC (Wysocki and Issekutz 1992). When lymphocytes were activated over 24 hours with Con A, the increased level of adhesion started to return to basal levels (Male et al., 1990a). In contrast, the increase in adhesion of lymphocytes to EC following IL-2 treatment was delayed, taking 2 days to appear and continuing to day 7 post IL-2 activation (Pankonin et al., 1992), suggesting the existence of different mechanisms in mediating adhesion.

Upon antigen stimulation, lymphocytes undergo phenotype and functional changes and convert from ''naive'' cells to ''memory'' cells. Concomitant with these alterations, lymphocytes also exhibit an increased ability to bind to EC. This increased adhesion of antigen stimulated lymphocytes has been found in human peripheral blood lymphocytes where ''memory'' T cells adhered to EC more eficiently than ''naive'' cells (Shimizu et al., 1991). Antigen specific T-cell lines derived from animal models also had a greater ability to bind EC and their adhesion could be further upregulated by activation of EC with the cytokines, IL-1, TNF and IFN-γ (McCarron et al., 1993).

Molecular mechanisms of lymphocyte adhesion

The discovery of adhesion molecules on the surface of both lymphocytes and EC has been vital in understanding the mechanisms involved in the adhesion process

of these two cells. Adhesion molecules play an important role in cell-cell and cell-extracellular matrix interactions. They not only mediate lymphocyte adhesion, but also participate in antigen recognition, act as co-stimulatory signals in T-cell activation, and stimulate effector mechanisms of activated lymphocytes such as T-cell cytotoxicity and antibody-dependent cellular cytotoxicity (Springer 1990).

A number of studies have demonstrated that there are two major receptor-ligand pairs of adhesion molecules involved in lymphocyte-EC adhesion and migration. These include (1) the selectin-carbohydrate interactions and (2) the immunoglobulin superfamily cellular adhesion molecules (IgCAM)-integrin interactions (Bevilacqua 1993). Endothelial members of the immunoglobulin superfamily include intercellular adhesion molecules 1 and 2 (ICAM-1, ICAM-2) and vascular cell adhesion molecule 1 (VCAM-1) which bind to leucocyte cell-surface integrins, lymphocyte function associated protein 1 (LFA-1) and very late activation antigen 4 (VLA-4), respectively. Selectins, two of which are found on EC (E- and P-selectin) and one on lymphocytes (L-selectin) are believed to mediate adhesion through the recognition of carbohydrates.

The role of adhesion molecules in lymphocyte adhesion has been largely inferred from adhesion blocking experiments with the relevant mAb. During lymphocyte adhesion studies, it was observed that the mAb to LFA-1 almost completely blocked the adhesion of either untreated or PMA activated lymphocytes to resting HUVEC (Haskard et al., 1986b). A mAb to ICAM-1 also inhibited adhesion, but to a lesser degree than with the mAb to LFA-1 as ICAM-2 can also be a ligand for LFA-1 mediated lymphocyte adhesion (Dustin and Springer 1988). In contrast to the inhibitory effects of anti-LFA-1 mAb on the binding of lymphocyte adhesion to untreated HUVEC, it was reported that such mAb had almost no inhibitory effect on the increase in adhesion observed when the EC were activated with cytokines such as TNF or IL-1 (Haskard et al., 1986a), suggesting that EC express different adhesion molecules following cytokine activation.

Treating lymphocytes with a mAb against VLA-4 did not elicit a reduction in lymphocyte adhesion to resting HUVEC although it inhibited lymphocyte adhesion

to IL-1 activated EC (Shimizu et al., 1991a; Vennegoor et al., 1992), suggesting that VCAM-1, the ligand for VLA-4, is not constitutively expressed, but requires induction. It appears that the degree of VLA-4 mediated adhesion is less than that of LFA-1 (Shimizu et al., 1991a), but other conflicting results suggested that with activated EC, VLA-4 and VCAM-1 mediated adhesion become more prominent than LFA-1 (Vennegoor et al., 1992; Oppenheimer-Marks et al., 1991).

The selectins, including L-selectin and E-selectin, are important molecules in inducing leucocyte velocity in the blood by mediating transient interaction between leucocytes and the EC and causing lymphocytes and neutrophils rolling along the vascular wall, the first step in migration (Springer 1994). The role of selectins in lymphocyte adhesion has also been reported and it is shown that adhesion of a subset of lymphocytes, ''memory'' $CD4^+$ T cells, to activated EC can be via E-selectin (Shimizu et al., 1991b; Picker et al., 1991), and that this effect is independent of T-cell activation (Shimizu et al., 1991b).

Lymphocyte adhesion to CNS vascular EC

In contrast to the studies on HUVEC, few investigations into lymphocyte adhesion to EC derived from the CNS have been carried out. Current studies show that under normal conditions lymphocytes bind to brain EC to a lesser degree than that with non-CNS EC (Male et al., 1990a). Brain EC are able to respond to the cytokines, IFN-γ and TNF by increased adhesion for lymphocytes (Hughes et al., 1988). Activation of lymphocytes with mitogen or antigen also significantly increased lymphocyte adhesion, and this effect could be synergised by cytokine activated brain EC (Male et al., 1990a; McCarron et al., 1993).

Adhesion molecules are also believed to play an important role in mediating lymphocyte adhesion to CNS vascular EC although at present it is not yet known whether there are any CNS EC-specific adhesion molecules or whether these cells use the same molecules in a distinctive pattern and temporal sequence. Some preliminary studies have shown that lymphocyte adhesion to brain EC is similar to non-CNS EC and is mediated by LFA-1 and VLA-4 (Male et al., 1994). Cultured brain EC also

express increased levels of ICAM-1 following cytokine activation and its expression is well correlated with lymphocyte adhesion (Male et al., 1994; McCarron et al., 1993; Wong et al., 1992). However, ICAM-1 has been suggested not to be the ligand on EC for LFA-1 as blocking this molecule does not affect LFA-1 dependent lymphocyte adhesion to brain EC (Male et al., 1994; McCarron et al., 1993).

1.4.3. Lymphocyte migration through EC monolayers

Several *in vitro* studies have found that under normal conditions a proportion of human peripheral blood T cells are able to cross EC monolayers and that this migration could be upregulated by activation of both EC and lymphocytes (Oppenheimer-Marks et al., 1988, 1990; Masuyama et al., 1992). Preincubation of EC monolayers with cytokines, such as IL-1 and IFN-γ can dramatically enhance lymphocyte migration which was found not to be due to increased binding of T cells to EC, but rather to an action on the EC to facilitate subsequent migration (Oppenheimer-Marks and Ziff 1988). This increased migration was also found when lymphocytes were activated with IL-2 (Pankonin et al., 1992). In contrast, it has been reported that activation of lymphocytes with phorbol dibutyrate reduces the lymphocyte migratory capability, and this was suggested to be related to an increase in the avidity of lymphocyte binding to EC (Kavanaugh et al., 1991).

Examination of these migrated cells shows that they express significantly higher levels of IL-2 receptors, enhanced protein synthesis and increased expression of CD29 (VLA-4 ß1 chain) and are almost 80% positive for $CD45RO^+CD4^+$ (Masuyama et al., 1992; Pietschmann et al., 1992), indicating that these cells are likely to be long-term antigen activated T cells. A recent study on lymphocyte migration across retinal EC monolayers has also demonstrated that lymphocyte migratory ability is dependent on long-term antigen activation, whereas short-term mitogen activated lymphocytes are mostly incapable of migrating (Greenwood and Calder 1993).

Adhesion molecules are believed to play a pivotal role in mediating lymphocyte migration through EC monolayers although this has not yet been fully

elucidated. Currently it is known that LFA-1 and ICAM-1 receptor pairing, play an important part in mediating lymphocyte migration across HUVEC monolayers (Oppenheimer-Marks et al., 1991). Other adhesion molecules, such as VLA-4 and VCAM-1 were only found to play a partial role in the migration of CD18-deficient T-cell clones and this effect is insufficient to compensate for the lack of LFA-1 (Kavanaugh et al., 1991).

1.4.4. Antigen presenting properties of vascular EC

T cells remain in a quiescent state until they are stimulated by antigens complexed with a self MHC molecule. At this time the T cell may become either activated or rendered anergic, depending on the strength and nature of the antigen-specific signal as well as any secondary signals it may receive (Lamb and Feldmann 1984; Quill and Schartz 1987). Most cell types express MHC class I molecules and are able to present antigen to $CD8^+$ T cells. In contrast, only a certain subset of bone marrow derived cells (including mononuclear phagocytes, B cells, Langerhans cells and dendritic cells which are professional APC) express MHC class II antigens and are capable of presenting antigen to $CD4^+$ T cells, such that full activation will ensue. This limitation of antigen presentation to special cell types is thought to provide greater control over activation of $CD4^+$ T cells, the cells responsible for initiating many immune reactions. However, almost all cell types can be induced to express MHC class II molecules but whether these cell types are able to activate $CD4^+$ T cells is determined by their ability to provide additional costimulatory factors.

It has previously been suggested that antigen presentation by stromal cells may actually inactivate T cells. Although EC are anatomically found within the stroma, they differ from true stromal cells, such as fibroblasts and smooth muscle cells, in that they share a common embryological heritage with bone marrow-derived APC. Furthermore, there is clear-cut evidence from *in vitro* studies that EC can present antigen to $CD4^+$ T-cells in both primary (alloantigen; Hirschberg et al., 1975; Pober et al., 1986b) and secondary (nominal antigen; Geppert and Lipsky 1985) responses, as well as considerable circumstantial evidence to support a role for EC antigen

presentation *in vivo* (Pober et al., 1986b; Pober 1988; Pober and Cotran 1991). For a circulating T cell the most likely first point of contact with antigen is the luminal surface of the vascular endothelium of the tissue at the site of antigen exposure and this unique anatomical location of EC makes them a major candidate for initial presentation of antigen. Moreover, since lymphocyte traffic into peripheral tissues is usually low, antigen presentation by EC could also serve as a mechanism for recruiting specific lymphocytes to the site of antigen challenge.

Expression of MHC antigens on EC

HUVEC, under standard culture conditions, constitutively synthesize and express class I but not class II molecules (Pober and Gimbrone 1982). When EC are cocultured with lymphocytes, or lectin activated lymphocyte supernatant, expression of MHC class II can be induced (Pober et al., 1983a). Furthermore, $CD8^+$ and $CD4^+$ T cells in the presence of monocytes have also been shown to induce class II expression on EC (Pardi et al., 1987). All of the class II antigen-inducing activity can be attributed to one specific activated T-cell product, the cytokine IFN-γ. IFN-γ also causes a concomitant increase in the level of expression of class I MHC antigens (Pober et al., 1983a).

IFN-γ is the only cytokine found capable of inducing MHC class II antigen expression. Other cytokines, such as granulocyte/macrophage colony-stimulating factor (GM-CSF; Alvaro-Gracia et al., 1989) which acts on macrophages and IL-4 (Paul and Ohara 1987) which acts on B cells are without effect on EC. IFN-α, IFN-ß, IL-1 and TNF have been found to increase the effects of IFN-γ on MHC class I expression, but not class II expression (Collins et al., 1986; Leszczynski 1990). In contrast, IFN-α and IFN-ß strongly inhibit IFN-γ mediated induction of class II molecule expression (Lapierre et al., 1988; Leszczynsk 1990).

The role of IFN-γ in the induction of MHC class II molecule expression appears to be neither specific nor selective for EC. The same concentrations of IFN-γ that induce MHC class II expression on EC also acts on other cell populations such as fibroblasts, vascular muscle cells (Pober et al., 1983b, 1986c), or keratinocytes

(Basham et al., 1984). However, in a short-term exposure of cultured human skin tissue to IFN-γ, it was found that only EC, but not keratinocytes, fibroblasts, or smooth muscle cells were able to express MHC class II molecules (Messadi et al., 1988), suggesting that EC are more responsive to IFN-γ.

Antigen presentation by EC

A number of *in vitro* studies indicate that EC strongly stimulate allogeneic T-cell proliferation in a primary response (Pober et al., 1983b; Geppert and Lipsky 1985). The proliferative response to allogeneic EC is mediated by $CD4^+$ T cells and is largely directed by class II MHC molecules, especially HLA-DR (Salomon et al., 1991). It has also been demonstrated that EC are able to present nominal antigens to T cells in a class II restricted manner (Hischberg 1981; Wagner et al., 1985).

The capacity of EC to stimulate proliferation of resting T cells indicate that EC can provide full complement accessory signals for T-cell activation. This ability is in contrast to antigen presentation by some other cell types with inducible class II MHC molecule expression, including dermal fibroblasts, which can not present soluble antigens in a way that can stimulate resting T cells (Umetsu et al., 1986). However, fibroblasts have been shown to present antigen efficiently when monocytes or fixed DR negative EC are added (Geppert and Lipsky 1987). This suggests that unlike EC and monocytes, fibroblasts fail to provide the necessary costimulatory signals required for T cell activation and proliferation.

Antigen presentation by EC also results in cytotoxicity of allospecific T-cell clones (Pober et al., 1983b). This effect is dependent on the expression of MHC class II molecules on EC and shows an HLA-DR restricted alloreactive cytotoxic T-cell lysis. Pretreatment of EC with IFN-γ appeared to be necessary for this cytotoxicity since it did not occur on untreated EC. Furthermore, cytotoxicity could be blocked by the antibodies that recognize determinants on the allogeneic class II molecules on EC.

Accessory role of EC in T-cell activation

Several cytokines have now been described that can provide costimulatory signals to T cells in various experimental settings. The first such molecule was originally identified as lymphocyte-activating factor and is known as IL-1 (Gery et al., 1972). Two separate gene products, IL-1α and IL-1ß, share the ability to act as costimulators for T-cell activation (March et al., 1985). IL-4, 6, and 7 as well as TNF and possibly IFN-γ can also have costimulatory capability (Ceuppens et al., 1988; Balkwill and Burke 1989). Of these, EC have been found to synthesize IL-1 and IL-6. EC produce little IL-1 basally, but may be stimulated by lipopolysaccharide (LPS), IL-1 or TNF to produce IL-1α and to a lesser degree IL-1ß (Libby et al., 1986, Kurt-Jones et al., 1987). EC also synthesize IL-6, probably basally, but in large quantities in response to LPS, IFN-γ, IL-1 or TNF (Jirik et al., 1989, Leeuwenberg et al., 1990). However, whether a particular cytokine provides costimulation seems to depend on the system being studied, for example, although IL-1 has an enhancing role on T-cell activation (Mizel 1987), it does not provide the necessary signal to drive resting peripheral blood T-cell proliferation stimulated by class II-bearing allogeneic fibroblasts (Geppert and Lipsky 1985).

Surface molecules expressed on EC have also been suggested to play an accessory role for T-cell activation. Of these, the integrin LFA-3 has been particularly implicated as blocking this molecule with a mAb can significantly reduce EC MHC class II mediated stimulation of T-cell proliferation (Hughes et al., 1990). The surface expression of ICAM-1 and VCAM-1 may also function as costimulators since it was found that purified forms of these molecules, acting through their respective ligands LFA-1 and VLA-4, can provide costimulatory signals to trigger $CD4^+$ T-cell proliferation (Van Seventer et al., 1991).

Antigen presentation by CNS vascular EC

A major question that has been addressed extensively is the antigen presenting capabilities of EC of the CNS. As a result of their close proximity to circulating immune cells the EC have been the subject of attention in the search for a resident

APC within the CNS. The *in vivo* observations have indicated that both retinal and brain vascular cells can express MHC class II molecules in inflammatory lesions, and *in vitro* studies have also confirmed that brain EC are capable of expressing class II molecules following IFN-γ treatment (Male et al., 1987). The inducibility of MHC class II molecules suggests that CNS vascular EC may play a role as APC during inflammatory reactions.

In vitro studies demonstrate that MHC class II molecules expressed on CNS vascular EC can be recognized by $CD4^+$ T cells since brain EC can become the target for antigen-specific, MHC class II restricted cell-mediated cytotoxic damage (Risau et al.,1990; Sedgwick et al., 1991; McCarron et al., 1991). Although these CNS vascular EC appear to be capable of presenting antigen in a recognizable form to T cells, their ability to present the full complement of stimulatory signals to induce T-cell proliferation remains unresolved. Several studies have reported that brain EC are unable to stimulate T-cell proliferation (Pryce et al. 1989; Risau et al., 1990), and this has been suggested to be due to lack of a costimulator(s) provided by cerebral EC (Risau et al., 1990) although this may also equally be due to the presence of inhibitors, such as prostaglandins and cytokines like IL-10 and TGF-ß. In contrast to these results, others have shown that brain EC can present antigens to antigen specific T cells resulting in a significant degree of T-cell proliferation (McCarron et al.,1985; Wilcox et al., 1989; Myers et al., 1993). Explanations that have been postulated for such discrepancies include species differences and the degree of EC purity as in brain EC cultures, potential contaminating cells, such as smooth muscle cells and pericytes can express class II MHC and may themselves induce T-cell proliferation (Hart et al, 1987; Fabry et al, 1990).

1.5. PURPOSE OF THIS STUDY

The aim of this work is to investigate the molecular mechanisms involved in lymphocyte interactions with cultured rat retinal EC and to compare some of these aspects with brain EC. The study is divided into three parts:

1.5.1. Control of lymphocyte adhesion to and migration through cultured retinal EC

In this study, lymphocyte adhesion to retinal EC will be quantitated by using ^{51}chromium labelled lymphocytes. Some of the factors that may control lymphocyte adhesion to retinal EC will be examined. These include (1), the effect of the cytokines, IL-1, IFN-γ and IL-4 on lymphocyte adhesion; (2), the effect of lymphocyte activation with mitogen (Con A) and antigen (S-Ag) on adhesion; (3), the effect of forskolin (a cAMP stimulator) and ACM on lymphocyte adhesion; and (4) the role of the adhesion molecules, ICAM-1, LFA-1 and VLA-4 in lymphocyte adhesion.

Lymphocyte migration through retinal EC monolayers will also be investigated using time-lapse video microscopy. In this study, lymphocyte migration and the role of adhesion molecules in mediating this process will be studied.

1.5.2. Expression of MHC and ICAM-1 molecules on retinal and brain EC

The expression of MHC molecules on retinal and brain EC will be detected using enzyme-linked immunoassay, immunocytochemistry and flow cytometry. The inducibility of MHC class II molecules on retinal and brain EC will be examined by treating the EC with IFN-γ, and their induction compared. ICAM-1 expression on resting and IFN-γ treated retinal and brain EC will be analyzed using flow cytometry following immunofluorescence staining with an anti-ICAM-1 mAb (IA29).

1.5.3. Antigen presenting capability of retinal and brain EC

The antigen presenting capability of retinal and brain EC will be examined and compared by coculturing EC with S-Ag specific CD4$^+$ T-cell line lymphocytes. T-cell proliferation in response to S-Ag and mitogens (Con A and PHA) will be measured using ^{3}H-thymidine incorporation. Antigen specific cytotoxicity of retinal EC by antigen specific T cells will be also investigated.

The factors that affect EC stimulation of T-cell proliferation and IL-2 production will be analyzed. These include EC concentrations and the role of the cytokine TGF-ß.

CHAPTER 2
METHODOLOGY

2.1 ANIMALS

4-10 week-old specific pathogen-free female Lewis strain (RT1) were used throughout the experiments. The animals were obtained from Bantin and Kingman (Hull, UK) and Charles River (Kent, UK).

2.2. REAGENTS

2.2.1. Chemicals

Bovine serum albumin (BSA), collagen, concanavalin A (Con A, type V), deoxyribonuclease (DNase), endothelial cell growth supplement (ECGS), FITC labelled griffinia simplifolia agglutinin (GSA-FITC), forskolin, heparin, α-methyl mannoside, phytohemagglutinin-P (PHA), polyethylglycerol (molecule weight, 20,000 KD), Nα-p-Tosyl-L-lysine chloromethyl ketone (TLCK) and vitamin C were all purchased from Sigma (Poole, UK). Fetal calf serum (FCS), glutamine stock solution, Hams F-10, Hanks' balanced salt solution (HBSS), Ca^{2+} and Mg^{2+} free HBSS, non-essential amino acid, penicillin/streptomycin stock solution, RPMI-1640 and 1 mM sodium pyruvate were purchased from Gibco (Paisley, UK). Collagenase/dispase and Nutridoma-SP were purchased from Boehringer-Mannheim (Sussex, UK). Diamine ethane tetra-acetic acid disodium salt (EDTA) was obtained from BDH (UK). Sodium chromium-51 (^{51}Cr) and ^{3}H-thymidine were obtained from Amersham International plc (Buckinghamshire, UK).

Normal rat serum (NRS) was obtained from 4-7 week old female Lewis rats, and on the same day was heat inactivated (56°C, 30 min.). Bovine plasma-derived serum (PDS) was prepared from platelet-free clotted plasma, freed of platelet-derived growth factor by ion-exchange on CM-Sephadex (Sigma).

Astrocyte conditioned medium was obtained from type 1 astrocytes isolated from 0-2 day perinatal Lewis rats and grown to confluence as previously described

(Wolswijk and Noble 1989). Confluent type 1 astrocytes were cultured for 24 h in DMEM (Gibco) supplemented with 10% FCS. The supernatants were collected as ACM.

2.2.2. Antibodies

The monoclonal antibodies (mAb) used in experiments are listed in Tab.2.1.

Table 2.1. Monoclonal antibodies

Code	Specificity	Source
OX-6	anti-rat MHC class II I-A	* *
OX-7	anti-rat Thy 1.1	Serotec, Oxford, UK
OX-8	anti-rat CD8	* *
OX-17	anti-rat MHC class II I-E	* *
OX-18	anti-rat MHC class I	Serotec
OX-19	anti-rat CD5	* *
OX-21	anti-human C3b inactivator	* *
OX-22	anti-rat leucocyte common antigen	Serotec
OX-25	anti-rat IL-2 receptor	Serotec
OX-33	anti-rat CD45ABC, B-cell restricted	Serotec
W3/25	anti-rat CD4	* *
ED2	anti-rat macrophages	Serotec
ED7/ED8	anti-rat macrophages, dendritic cells etc.	Serotec
WT.1.	anti-rat CD11a	*
WT.3.	anti-rat CD18	*
IA29	anti-rat ICAM-1	*
NDS61	anti-rat IL-2 receptor (blocking)	Serotec
HIS52	anti-rat RECA-1 antigen	Serotec
P12520	anti-rat CD49d	AMS biotech. UK LTD.
BDA1	anti-porcine TGFß1 (neutralising)	R&D system, Oxford, UK.
BDA5	anti-porcine TGFß1 (detecting)	R&D system

** * The mAb were prepared by culturing the hybridoma cell lines (a generous gift from Dr. M. Puklavee, MRC, Oxford, UK). The hybridoma cell lines were cultured for 48 h in 10% FCS RPMI-1640 medium, and the supernatant collected as the sources of the mAb. The supernatants were kept at 4°C and used within 6 months.*
** These mAb are antibody containing supernatants which are a generous gift from Dr. I. Dranfield (Edinburgh, UK).*

In addition, the mouse anti-rat immunoglobulin antibody, fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse immunoglobulin G F(ab)2 fragments (RAMIG) were purchased from Serotec. Peroxidase conjugated rabbit anti-

mouse IgG was obtained from Sigma. Biotin-conjugated goat anti-rabbit IgG antibody and avidin labelled peroxidase were obtained from R&D system.

2.2.3. Cytokines

Rat recombinant IFN-γ was purchased from Holland Biotechnology bv (Leiden, Holland). Murine recombinant IL-1ß was obtained from Genzyme (Oxford, UK). Mouse recombinant IL-4 and porcine TGF-ß1 were purchased from R&D system. IL-2 was prepared by culturing the murine mIL-2 cDNA transfectant cell line obtained from ECACC (Salisbury, UK). The cells were cultured for 48 h in RPMI medium containing 10% FCS, and the supernatant was collected as a source of IL-2. Recombinant human IL-2 was purchased from Boehringer-Mannheim (Sussex, UK).

All cytokines were aliquoted and stored at -70°C, and the same batches were used throughout the experiments.

2.3. RETINAL SOLUBLE ANTIGEN (S-Ag)

S-Ag was extracted from bovine retina by using Ultra-gel filtration followed by Mono-Q ion-exchange according to the method described by Dorey et al., (1982).

Bovine retinas were removed, homogenized in PBS and then protein precipitated with saturated ammonium sulphate. The pellets were collected after 25,000g centrifugation and resuspended in PBS buffer. After 1 h centrifugation at 50,000g to remove contaminated nucleic acid (DNA and RNA), crude protein solution which contains S-Ag was further purified.

The S-Ag containing solution was loaded onto an Ultra-gel (Pharmacia, Sweden) column at a flow rate of 17 ml/h, and the column was eluted with 500 ml of 0.01 M NaCL buffer containing 0.2 mM of N-ethyl meleimide (Sigma) and 0.2 mM of phenylmethylsulfonyl fluoride (Sigma) at the same flow rate. The elution procedures were performed on a Superac system (LKB-Phamacia, Sweden). The elution curve is shown in Fig 2.1A and the S-Ag fraction appeared at the second elution peak which was detected by double-immune diffusion with anti-S-Ag serum.

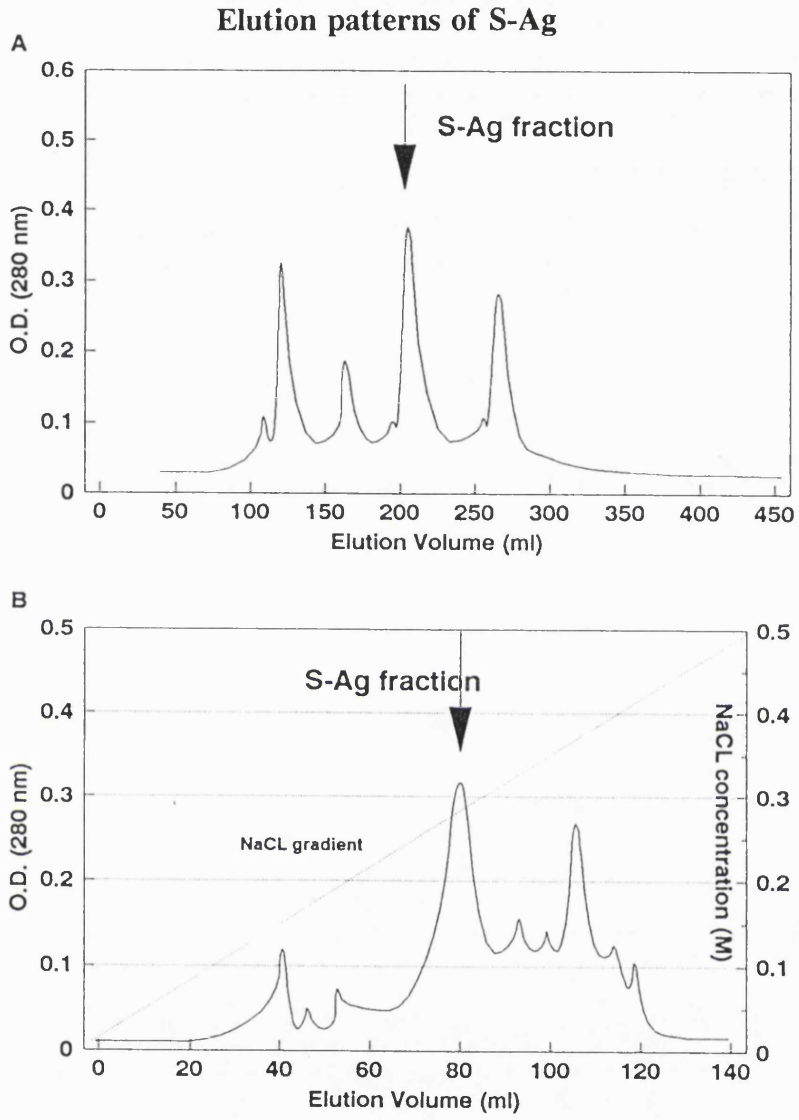

Fig.2.1. Elution pattern was measured by absorbance at 280 nm on an Ultra-gel filtration (A) and on the Mono-Q anion exchange column (B). S-Ag containing proteins were eluted on the Ultra gel column with 0.01 M NaCL at a flow rate of 17 ml/h, and the S-Ag fraction was then eluted on the Mono-Q anion exchange column with a continuing gradient of NaCL (0.01-0.5M) at a flow rate of 80 ml/h.

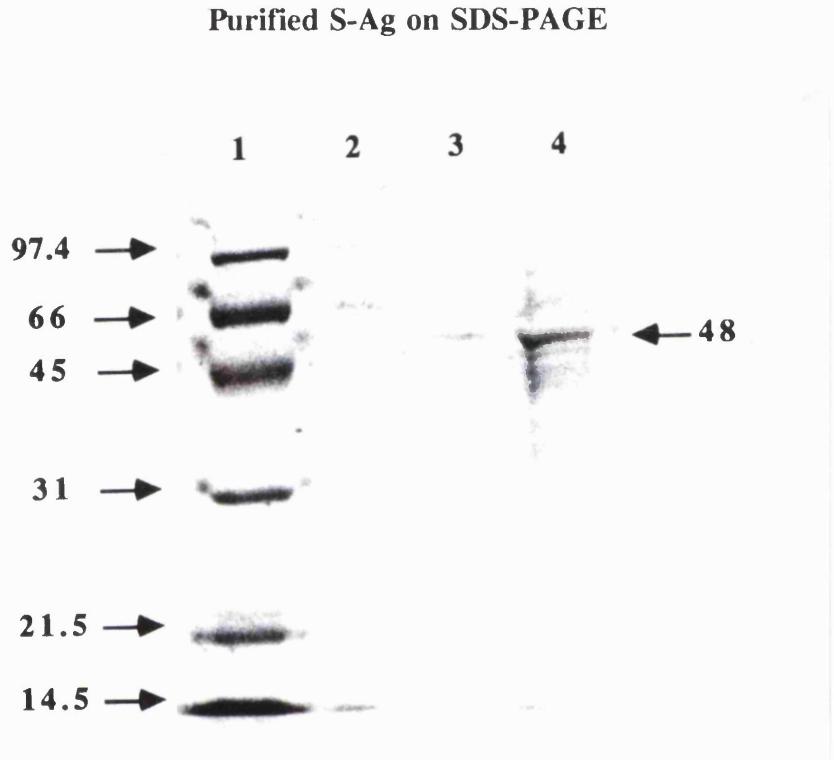

Fig.2.2. SDS-PAGE for S-Ag purified by Ultra-gel filtration followed by Mono-Q anion exchange column. Lane 1 and 2 has protein molecular weight standards (Pharmacia). Lane 3 and 4 were loaded with different protein concentrations of S-Ag. The proteins were stained with Coomassie blue R.

The S-Ag containing fraction was then loaded onto a Mono-Q column at a flow rate of 80 ml/h. Following washing off non-binding materials with 100 ml of 0.01 M NaCL, bound proteins were eluted with a continuous gradient buffer of 0.01 M to 0.5 M NaCL and S-Ag was eluted out at about 0.3 M NaCL (Fig.2.1B.).

Eluted S-Ag was collected and concentrated by dialysis in 35% polyethylglycerol (20,000 KD molecular weight; Sigma). The purity of S-Ag was identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Fig.2.2).

2.4 PREPARATION OF RAT RETINAL AND BRAIN MICROVESSEL EC

2.4.1. Buffers

25% BSA solution: 5 g BSA were added to 20 ml of Ca^{2+}, Mg^{2+} free HBSS, and kept at 4°C overnight to allow it to fully dissolve. The solution was sterilized by filtering through a 0.22 μm filter.

Handling buffer: 100 ml HBSS (Ca^{2+}, Mg^{2+} free) containing 10 mM HEPES and 200 U/ml penicillin/streptomycin was gased for 10 min with oxygen enriched gas (95% O_2 plus 5% CO_2), after which 2 ml of 25% BSA solution was added. pH was adjusted with 1 M NaOH to approximately 7.2 by eye with the indicator phenol red.

Digestion buffer: 5 ml of collagenase/dispase supplemented with 0.147 μg/ml TLCK and 200 U/ml DNase were gased for 5 min with oxygen enriched gas, and the pH was then restored to 7.2 with 1 M NaOH.

Percoll-gradient: After autoclaving, Percoll solution (Sigma) was prepared by mixing 50 ml of Percoll with 45 ml of HBSS, and 5 ml of 10x concentrated HBSS to restore the osmolarity.

High speed centrifuge tubes (Sorvall Instruments, Du Pont, USA) were sterilized with 75% ethanol. After washing, the tube was coated for at least 2 h with handling buffer and was then loaded with 7 ml of Percoll solution and centrifuged for 1 h at 25,000g at 4°C in a high speed centrifuge (Heraeus, Swiss) to establish a gradient.

2.4.2. Culture medium

HAMS F-10 medium supplemented with 17.5% PDS, 7.5 μg/ml of ECGS, 80 μg/ml of heparin, 2 mM glutamine, 0.5 μg/ml of vitamin C and 100 U/ml of penicillin/streptomycin was used for EC cultures.

2.4.3. Isolation of retinal microvessels

Removal of retinas: For each preparation 6 rats were sacrificed by CO_2 asphyxiation. The eyes were removed using sterilized scissors and placed into handling buffer cooled on ice. Under sterile conditions eyes were dissected using a

dissecting microscope; the cornea, lens and vitreous body were carefully removed and the retinas were lifted off gently from the posterior eye cup of the eyes and transferred into handling buffer at 4°C.

Digestion: The retinas were then incubated for 1 h in 5 ml digestion buffer at 37°C. The digested tissue was centrifuged for 15 min at 1000g in 25% BSA buffer. Small quantities of lipids were removed, and the pellet containing microvessels was resuspended and incubated for a further 1 h in 5 ml digestion buffer to separate pericytes from the microvessels. At the end of the incubation, the digestion buffer was removed after centrifugation and the tissue pellet was resuspended in 1 ml of handling buffer.

Isolation of microvessels: The suspension was loaded onto a Percoll-gradient and centrifuged for 15 min at 1000g. The microvessel layer (at about 1/3 from the bottom of Percoll solution) was aspirated and transferred into handling buffer. Following two washes, the microvessel fragments were resuspended in culture medium.

2.4.4. Isolation of cerebral microvessels

For each preparation two rat brains were used. The cerebrum was separated from the cerebellum and brain stem and after removal of meninges and white matter, the cortex was chopped into small pieces (approximately 1-2 m^3) in handling buffer. The subsequent steps were similar to the preparation of retinal microvessels, with the exception that the second digestion was for 3 h.

2.4.5. Cultures

96-well microtitre plates, 25 cm^2 flasks or 24 well plates (Nunclon, Gibco) were used for cell cultures. Prior to use, the plates or flasks were coated for at least 2 h at room temperature or overnight at 4°C with collagen solution (approximate 40 $\mu g/cm^2$). Excess collagen solution was removed and the plates or flasks were placed in ammonia vapour for 30 min to fix the collagen coating. After washing three times with HBSS, the plates or flasks were ready for use.

The isolated microvessel fragments were resuspended in culture medium and

placed onto tissue culture plates or flasks. After overnight culturing the plates or flasks were washed with Ca^{2+}, Mg^{2+} free HBSS, and fresh medium was added. The cultures were then maintained at 37°C in 5% CO_2 and fed every 3 days for 3-5 weeks until confluent EC monolayers had formed. Confluent cells at a purity of greater than 95% were used.

2.4.6. Dissociation of cultured EC monolayers

Retinal and brain EC monolayers cultured in 25 cm^2 flasks were washed in Mg^{2+}, Ca^{2+} free HBSS containing 0.02% EDTA followed by 1 h incubation with collagenase/dispase (0.1%) containing 200 U/ml of penicillin and streptomycin. The cell clumps were gently broken and the dissociated cells suspended in HBSS buffer prior to use.

2.4.7. Immuno-staining

Immuno-staining was carried out at 4°C. $5x10^4$ of dissociated retinal EC were incubated for 1 h with primary mAb of ED2, ED8, OX-7, OX-22 and HIS52 at the concentration of 5 μg/ml followed by a further 1 h incubation with FITC-RAMIG (1:50) in PBS containing 20% normal rat serum. Between each incubation cells were washed twice with PBS, and at the end of staining, cells were resuspended in PBS for flow cytometric analysis.

2.4.8. Griffinia simplifolia agglutinin (GSA)-FITC staining

10^6 dissociated EC per ml were incubated for 30 min with FITC conjugated GSA (GSA-FITC) at a final concentration of 20 μg/ml in PBS supplemented with 0.1 g/l of $CaCL_2$ (pH 7.4) and 10% FCS. As a control, retinal EC received no lectin. Cells were then washed three times with cold 10% FCS in PBS and GSA-FITC labelled cells were then resuspended in 20% FCS-PBS for flow cytometric analysis.

2.4.9. Flow cytometry

Flow cytometric analysis was performed on a FACScan (Becton-Dickinson, Oxford, UK). Background was eliminated by establishing gates to monitor live cells

only and not debris. Unstained cells were used to set the parameters. For immunofluorescence staining, cells stained with the second antibody alone (RAMIG-FITC) were used to set background control, while for GSA staining, unstained cells served as control.

2.5. ISOLATION OF S-ANTIGEN SPECIFIC CD4$^+$ T-CELL LINE LYMPHOCYTES

The procedure for preparation and maintenance of T-cell lines was based on a previously described method (Sedgwick et al.,1989) with minor changes.

2.5.1. Culture media

RPMI-1640 media supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml of penicillin/streptomycin sulphate, and $2.5x10^{-5}$ M of 2-mercaptonethanol was used. This is designated as complete medium.

2.5.2. Metrizoate-Ficoll (M-F) separation of cells

To separate live T cells from irradiated accessory cells, M-F at a specific density of 1.088 was used. Ficoll 400 was dissolved in distilled water at 14 g/100 ml. This was mixed with 32.9% (w/v) sodium metrizoate at a ratio of 10 vols of metrizoate to 24 vols of Ficoll, sterilized by autoclaving and stored at 4°C in the dark. On the day of use, the M-F was allowed to warm up to 20°C, then 5 ml of cell suspension (up to $5x10^7$ cells) in complete media-10% FCS or 1% NRS was overlaid onto 5 ml M-F. The cells were centrifuged at 1500 g for 30 min at 20°C and the live cells collected from the M-F/media interface.

2.5.3. Preparation of antigen presenting cells (APC)

Thymuses were removed from 4-6 week old Lewis rats and ground through a wire mesh to make a single cell suspension in complete medium-10% FCS. The cells were then resuspended in complete medium-10% FCS or 1% NRS and exposed to 2000 Rads gamma radiation (^{137}Cs, Gamma cell 40).

2.5.4. Preparation of T-cell lines

Lewis rats were immunized subcutaneously in a hind leg footpad and at the base of the tail with antigen emulsified in complete Freund's adjuvant. Each rat received 25-50 μg S-Ag and 100 μg pertussis. 12 days later, after onset of clinical EAU, popliteal lymph nodes were removed, prepared as a single cell suspension and resuspended at $2x10^6$ viable cells/ml in complete media containing 1% (v/v) NRS and S-Ag at 10 μg/ml. 50 ml volumes were added to 260 ml culture flasks (Nunc) which were placed horizontally in a 5% CO_2 incubator at 37°C for 5 days. Cells were centrifuged at 500g for 10 min, and the cell pellet was resuspended in 5 ml complete medium plus 10% FCS. Viable cells (which included blast cells) were separated on M-F by centrifugation. The viable cells at the interface between medium and M-F were carefully aspirated and placed into 10 ml complete medium-10% FCS. Cells were washed once and immediately centrifuged at 500g, and resuspended at 10^5/ml in complete media-10% FCS. IL-2 was added at a final concentration of 10 U/ml (recombinant human IL-2) or 1% IL-2 containing supernatant, and the cells added to flasks and placed in an incubator. After 4 days the cells were recovered, washed and resuspended at 10^5 viable cells/ml in medium-1% NRS containing S-Ag at 10 μg/ml together with $5x10^6$ irradiated thymocytes/ml (i.e. 1:50 ratio) and added to flasks for 3 days at 37°C in 5% CO_2.

The same procedure (i.e. M-F separation steps after antigen stimulation and propagation in IL-2 medium) was then repeated after the second round of antigenic stimulation. Thereafter (i.e. after the third and subsequent round of antigenic stimulation) blast cells were separated on M-F after the stimulation step and the period of resting of the cells extended up to 10-12 days to give a complete cycle time of around 14 days. Usually the IL-2-containing media became acid after 4 days and the cells were split at this point and left undisturbed until reactivation with antigen and thymocyte APC.

2.6. ISOLATION OF PERIPHERAL LYMPH NODE (PLN) AND SPLENIC LYMPHOCYTES

2.6.1. Resting PLN and splenic lymphocytes

PLN lymphocytes were obtained from cervical, inguinal and mesenteric peripheral lymph nodes on the day before the adhesion assay. The lymph nodes were teased apart with forceps into 10% FCS-RPMI medium, and the suspension passed through sterilized gauze to remove cell clumps. The cell suspension was washed two times with 10% FCS-RPMI medium and incubated overnight in the same medium in the 5% CO_2 incubator.

Splenic cell suspensions were prepared by grinding spleen through a wiremesh and mononuclear cells separated on M-F by centrifugation. Following a 2 h incubation of mononuclear cells on petridishes or flasks in 10% FCS RPMI medium, non-adherent cells were collected and used as splenic lymphocytes.

Subpopulations of lymphocytes from PLN and spleen were analyzed by flow cytometry following indirect immunofluorescence staining with the mAb OX-19 (CD5), OX-8 (CD8), W3/25 (CD4) and OX-33 (CD45ABC, B cell restricted).

2.6.2. Con A activated PLN lymphocytes

Before the adhesion assay, lymphocytes were treated for 18 h with optimal concentration of the mitogen Con A (5 μg/ml). In the kinetic studies of lymphocyte adhesion by Con A activation, lymphocytes were incubated for between 1 min and 48 h with 5 μg/ml of Con A. At the end of Con A activation, 0.1 M α-methyl mannoside was added to neutralize remaining Con A.

2.6.3. Enrichment of T-cell population

PLN lymphocytes or Con A activated PLN lymphocytes (5 μg/ml, 18 h) were placed onto anti-rat immunoglobulin antibody coated petridishes. After a 1 h incubation in a 5% CO_2 incubator non-adherent cells were gently washed with HBSS and collected. The cells were then resuspended in 10% FCS-RPMI medium and used as a enriched T-cell population.

2.7. ASSAY OF LYMPHOCYTE ADHESION TO RETINAL EC

2.7.1. EC

Retinal EC were grown in 96-well plates. After reaching confluence retinal EC were screened by inverse phase contrast microscope using morphological criteria. Confluent wells having more than 5% contaminating cells were not used. In addition, wells of less than 90% confluence were also rejected. The selected confluent wells were prepared by removing culture medium and washing 3 times with HBSS before the adhesion assay.

To determine the factors that effect the adhesive properties of retinal EC for lymphocytes, the EC were treated with cytokines, ACM, forskolin and antigen before the adhesion assay. The procedures are shown below.

Cytokine activation: Prepared confluent EC monolayers were incubated for 18 h with various concentrations of IFN-γ (5-200 U/ml), IL-1 (0.5-25 U/ml) and IL-4 (10-500 U/ml) in medium devoid of ECGS. After thoroughly washing with HBSS, retinal EC monolayers were then used for the adhesion assay.

Treatment with ACM and forskolin: ACM was added to the retinal EC culture medium (50:50) and placed onto retinal EC monolayers for 3 days, with or without 10 μM/ml forskolin for 18 h prior to the assays. The control group was incubated for 3 days with 50% of DMEM medium. In parallel experiments, optimal concentrations of cytokines (IL-1; 25 U/ml and IFN-γ; 200 U/ml) were added to retinal EC alone or combined with either ACM or forskolin, respectively, and incubated for 18 h.

Treatment with S-Ag: Retinal EC were treated for 3 days with 200 U/ml of IFN-γ, and 18 h prior to the assays 10 μg/ml of S-Ag was added. Control EC were treated with either medium alone, medium plus IFN-γ or medium and S-Ag.

2.7.2. Lymphocytes

PLN lymphocytes were prepared as previously described. Either resting or Con A activated lymphocytes were used for the adhesion assay.

S-Ag specific T-cell lines were used as antigen specific activated T cells. These cells were cultured for 3-4 days in IL-2 conditioned medium prior to the

adhesion assay.

2.7.3. ^{51}Cr labelling of lymphocytes

Lymphocytes were washed twice with HBSS. The cell pellets were incubated for 90 min at 37°C with ^{51}Cr at a concentration of 3 μCi per $1x10^6$ cells, after which the cells were spun down and washed twice with HBSS. ^{51}Cr labelled cells were resuspended in 10% FCS-RPMI medium at a concentration of $1x10^6$ cells/ml.

2.7.4. Adhesion assay

The lymphocyte adhesion assay was carried out by adding to retinal EC monolayers $2x10^5$ ^{51}Cr labelled lymphocytes per well in 200 μl of 10% FCS-RPMI medium in 96 well-plates according to the method previously described (Cavender et al., 1986; Male et al., 1990a). The cells were incubated for 2 h in a 5% CO_2 incubator at 37°C. As a background control the cells were added to collagen coated wells in the same plate. After a 2 h incubation the non-adherent cells were washed off with pre-warmed HBSS. The wash procedure was carried out by using a multichannel pipette. Each well was washed four times from each four poles of the wells. The adherent cells were then lysed with 200 μl of 10% SDS, and 100 μl of well-mixed lysis was taken for counting on a gamma emission counter (Dynatech, UK) to measure counts per minute (cpm) of adherent cells. The percentage of adherent cells was calculated from the following formula:

$$\% \text{ adhesion} = \frac{\text{cpm of 100 } \mu\text{l lysis - cpm of background}}{\text{cpm of } 1x10^5 \text{ cells}} x100\%$$

To assay the effect of duration of coculture on lymphocyte adhesion to the retinal EC, $2x10^5$ ^{51}Cr-labelled lymphocytes per well were added to the EC monolayers, and adhesion measured after 15 to 240 min of coculture.

To assess the effect of lymphocyte concentrations on adhesion, $5x10^4$-$2x10^6$ lymphocytes per well were added to retinal EC monolayers. Following a 2 h incubation, the percentage of adherent cells was measured as described above, or alternatively, the total number of adherent cells was calculated.

2.7.5. Staining of lymphocyte subsets among the adherent cells

The T-cell subsets adhering to confluent retinal EC grown on 4- or 8-well non-autofluorescent plastic Labtek chamber slides (ICN) were determined by immunofluorescence staining. Confluent retinal EC monolayers were treated for 18 h with 200 U/ml of IFN-γ before the adhesion assay. After washing four times with HBSS, $3x10^5$ of the enriched T-cell population were added per well and incubated for 2 h at 37°C in a 5% CO_2 incubator. Non-adherent cells were washed off with pre-warmed HBSS and adherent cells fixed for 15 min with 2% glutaradehyde in PBS-saline. The wells were then incubated with the primary mAb OX-19 (CD5), W3/25 (CD4) and OX-8 (CD8) followed by FITG-RAMIG as described above. Finally, the slides were washed and mounted with Citifluor (London, UK) and 200 adherent cells per sample counted by eye through a x40 neofluor objective on a fluorescence microscope (Polyvar, Leica Ltd., Bucks, UK). The adherent population of $CD5^+$, $CD4^+$ and $CD8^+$ cells were then expressed as a percentage of the total number of bound cells.

2.7.6. Antibody-blocking adhesion experiments

In order to investigate the role of adhesion molecules expressed on both lymphocytes and retinal EC in adhesion, antibodies against rat adhesion molecules were added to the EC or lymphocytes 1 h before the adhesion assay. 100 μl/well of anti-ICAM-1 supernatant was added to EC; 100 μl supernatant per $5x10^6$ cells of anti-LFA-1 (CD11a; WT.1 and CD18; WT.3) mAb, 10 μg per $1x10^6$ cells of anti-VLA-4 (anti-CD49d) mAb or a combination of anti-LFA-1 mAb and anti-VLA-4 mAb were added to lymphocytes. In control experiments, lymphocytes were incubated with an irrelevant antibody OX-21 (anti-human C3b inactivator mAb) as an isotype control or in the absence of mAb. Lymphocytes were then added to resting and IL-1 (10 U/ml, 18 h) activated retinal EC monolayers for the adhesion assay.

2.7.7. Flow cytometric analysis of adhesion molecules

VLA-4 and LFA-1 levels on resting PLN cells, Con A activated lymphocytes (18 h Con A activation) and S-Ag specific T-cell lines (3 days in IL-2 medium) were

measured following indirect immunofluorescence staining with anti-rat CD49d mAb and anti-LFA-1 mAb (both WT.1 and WT.3) respectively. ICAM-1 expression on resting and IL-1 (18 h, 5 U/ml) activated retinal EC was detected with the mAb IA29 following EC dissociation. Lymphocytes were labelled with the primary mAb of 1:4 diluted WT.1 and WT.3 supernatant or 2.5 μg per $1x10^6$ cells of anti-VLA-4 mAb and EC with 1:4 diluted IA29 supernatant. After 1 h incubation on ice, cells were washed twice and then incubated for a further 1 h with FITC-RAMIG (1:50). At the end of the staining, cells were washed, resuspended in PBS and analyzed on the FACScan as described in section 2.3.8.

2.8. LYMPHOCYTE MIGRATION ASSAY

The lymphocyte migration assay was carried out using time-lapse videomicroscopy according to the method previously described (Greenwood and Calder 1993). Antigen-specific T-cell line lymphocytes, prepared as described above (section 2.5.4.) and cultured in IL-2 conditioned medium 3-4 days before the assay, were added to retinal EC monolayers in 24 well plates at a concentration of $1x10^5$ lymphocytes/well and placed onto the stage of an inverse-phase contrast microscope housed in a 5% CO_2 and temperature controlled (37°C) environment. A field of 200 μm^2 was selected and recorded for 4 h using a video camera attached to the microscope and stored on a time-lapse video tape recorder. The recording was replayed at 160x normal speed and analyzed by enumerating the number of cells within the field that were (1) on the surface of retinal EC monolayer, and (2) underneath the EC monolayers, the latter group having migrated across the monolayers.

The migrated lymphocyte population could be clearly distinguished from those remaining on the surface by their distinctive morphology and refractive appearance. The data were then expressed as the percentage of lymphocytes within the field that had migrated beneath the monolayer.

In the antibody-blocking migration assay, the antibodies were added to EC or lymphocytes 1 h before the migration assay. Anti-ICAM-1 mAb was added to either

resting or IL-1 activated (5 U/ml, 18 h) retinal EC monolayers; anti-LFA-1 mAb and anti-VLA-4 mAb were added to lymphocytes. OX-21 treated lymphocytes were used as a control.

2.9. ASSAY OF MHC AND ICAM-1 MOLECULES ON RETINAL EC

2.9.1. ELISA for MHC molecules

The expression of MHC molecules was assayed on confluent EC monolayers cultured in 96-well plates following 5 days treatment with IFN-γ (0-500 U/ml) in Hams F-10 medium without ECGS. The kinetics of MHC induction was measured by taking cultures on days 0-5 after treatment with 200 U/ml of IFN-γ.

In parallel experiment, MHC class II expression on eluted peritoneal macrophages, which were used as a positive control, was measured following the similar treatment with IFN-γ. $1x10^5$ macrophages per well in 96-well plates were treated for 24 h with 0-50 U/ml of IFN-γ before the MHC class II antigen assay. In the time-course study macrophages were treated for 4-48 h with 50 U/ml IFN-γ.

At the end of the treatment retinal EC or macrophages were then fixed for 15 min in 0.1% glutaraldehyde in PBS. Following washing off the excess glutaraldehyde, cells were blocked for 20 min with 0.05 M Tris-HCL (pH 7.4), incubated for 1 h with the primary antibodies OX-18 (anti-MHC class I mAb), OX-6 (anti-class II I-A mAb), OX-17 (anti-class II I-E mAb) and OX-21 (anti-C3 inactivator mAb) followed by a further 1 h incubation with 1:500 diluted peroxidase-conjugated rabbit anti-mouse IgG. In all these procedures, cells were washed three times in 0.3% BSA-PBS between each step.

The plates were developed using 100 μg/ml tetramethylbenzine in 97 mM sodium acetate/3mM citric acid, containing 0.5 μl/ml 30% H_2O_2 for approximately 15 min. The reaction was stopped with 1 M H_2SO_4 and the plate read at 450 nm with an ELISA reader (WellcoZyme, UK). Levels of MHC expression on retinal EC are expressed as the optical density (O.D.) reading from the ELISA reader after subtracting the O.D. of the control (OX-21).

2.9.2. Immunocytochemical staining of MHC molecules

Confluent retinal EC cultured in 4 or 8 well plastic Lab-Tek chamber-slides (ICN) were treated for 5 days with 200 U/ml of IFN-γ. Following fixation with 0.1% glutaraldehyde, the wells were incubated for 1 h with the primary antibodies OX-18, OX-17 and OX-6, and then for a further 1 h with FITC-RAMIG. After washing, the slides were mounted with Citifluor (London, UK) and MHC expression observed by fluorescence microscopy (Polyvar).

2.9.3. Flow cytometric analysis of MHC and ICAM-1 molecules

Confluent retinal and brain EC cultured in 25 cm^2 flasks were treated for 0-5 days with 200 U/ml of IFN-γ. Following dissociation of the EC monolayers (see section 2.3.5), the single cell suspension was labelled with the primary antibodies OX-6, OX-17, OX-18 and IA-29 and then with FITC-RAMIG. The fluorescence labelled cells were analyzed by the FACScan (Becton-Dickinson) as described above (section 2.3.8).

2.10. ANTIGEN PRESENTATION ASSAY

2.10.1. Preparation of APC

EC: Confluent retinal EC of high purity (>95%) in 96-well plates were chosen for the antigen presentation assay according to previously described methods (Pryce et al., 1989; Risau et al., 1990). Prior to the assay the EC monolayers were treated for 3 days with 200 U/ml of IFN-γ.

In order to assess the effect of retinal and brain EC number on antigen presentation, the EC monolayers grown in 25 cm^2 flasks were dissociated with collagenase/dispase (see section 2.3.5.) and the single cell suspension plated onto collagen coated 96 well plates at concentrations of between $5x10^3$ and $1x10^5$ cells per well. After overnight incubation in EC culture medium, the cells were washed with HBSS to remove non-adherent cells prior to the antigen presentation assay. MHC class II induction of retinal EC were obtained by pre-treating retinal EC for 3 days with 200 U/ml of IFN-γ prior to dissociation.

Splenic adherent cells (SAC): Splenic mononuclear cells were placed onto petridishes (Gibco) and incubated for 2 h in 10% FCS-RPMI 1640 medium. The adherent cells were scrapped off with a plastic scraper and plated onto 96-well plates.

Thymocytes: Thymocytes for use as APC were prepared according to the method described previously (see section 2.5.3).

2.10.2. S-Ag specific T-cell lines

S-Ag specific T-cell lines were prepared as described as above (section 2.5). Prior to the antigen presentation assay, the cell lines were maintained in IL-2 medium for 10 days to obtain relatively rested cells.

2.10.3. Proliferation assay

The T-cell proliferation assay was performed in 96-well plates. $2x10^4$ S-Ag specific T-cell line lymphocytes per well were co-cultured with different concentrations of retinal or brain EC in Nutridoma-SP conditioned RPMI medium. The wells were treated with 5-20 μg/ml of S-Ag or the mitogen Con A (5 μg/ml) or PHA (10 μg/ml). In addition, 5 μg/ml indomethacin was added to prevent eicosanoid production by EC which is known to suppress lymphocyte division. T cells and EC were cocultured for 5 days in a 5% CO_2 incubator, at 37°C. On the third day of culture, exogenous IL-2 was added to each well and at 8 h prior to the termination of the proliferation assay, the wells were pulsed with 1 μCi ^{3}H-thymidine. The cells were then harvested onto nitrocellulose membranes with a cell harvester (Dynatech, UK) and incorporated ^{3}H-thymidine determined with a ß-scintillation counter (Tricarb, Canbera Packard).

Stimulation of S-Ag specific T cells by thymocytes or SAC was carried out in an identical manner. $5x10^4$ SAC or $1x10^6$ irradiated thymocytes per well were cocultured with $2x10^4$ S-Ag specific T-cell line lymphocytes. The wells were treated with 5-20 μg/ml S-Ag. Proliferation was measured by ^{3}H-thymidine incorporation as above.

2.10.4. IL-2 bio-assay

S-Ag specific $CD4^+$ T cells ($2x10^4$ cells/well) were stimulated for 3 days with either S-Ag (10 μg/ml) or PHA (10 μg/ml) in the presence of either confluent or subconfluent ($5x10^4$ cells/well) retinal EC. In separate wells and under identical conditions the assay was carried out in the presence of either 1 μg/ml of anti-TGF-ß1 neutralizing mAb or 10 ng/ml of exogenous TGF-ß1. These concentrations of the anti-TGF-ß mAb or TGF-ß were shown to be effective in a previous study in the same laboratory (Hu et al., unpublished data). At the end of the assay the supernatants were collected for subsequent analysis of IL-2 production. Proliferation of these cells was also measured as described above.

The IL-2 assay was carried out by incubating the IL-2 and IL-4 responsive HT-2 cell clone (ECACC, Salisbury, UK.) for 48 h with the supernatants, followed by measuring HT-2 cell proliferation by ^{3}H-thymidine incorporation. IL-2 dependent proliferation was confirmed by blocking HT-2 cell proliferation with an anti-IL-2 receptor mAb (Serotec,1:50 dilution). Proliferation of HT2 cells in response to IL-2 is shown in Fig.2.3.

2.10.5. ELISA for TGF-ß produced by retinal EC

Confluent retinal EC were cultured for up to 48 h in serum-free Hams F-10 medium supplemented with 1% Nutridoma in 24-well plates. For activation, retinal EC were treated with 10 ng/ml of lipopolysaccharide (LPS, Sigma). Supernatants were collected for the TGF-ß1 assay.

The TGF-ß1 assay was performed using ELISA. 100 μl of the EC supernatants per well were added to 96-well plates and incubated overnight at room temperature. In order to quantitate the amount of TGF-ß1 in the supernatant, parallel experiments were conducted in which standard TGF-ß1 from 1 ng/ml to 100 ng/ml was added to 96 well plates for ELISA determination. The supernatant and TGF-ß1 coated plates were incubated for 1 h with rabbit anti-TGF-ß1 antibody (1:1000 dilution, R&D system) followed by 1 h incubation with biotin-conjugated goat anti-rabbit IgG antibody (R&D system) and then avidin labelled peroxidase (R&D system). Between each incubation, plates were washed 3 times with 0.5 M Tris-HCL (pH 7.4). The

Proliferation of HT-2 cells in response to IL-2

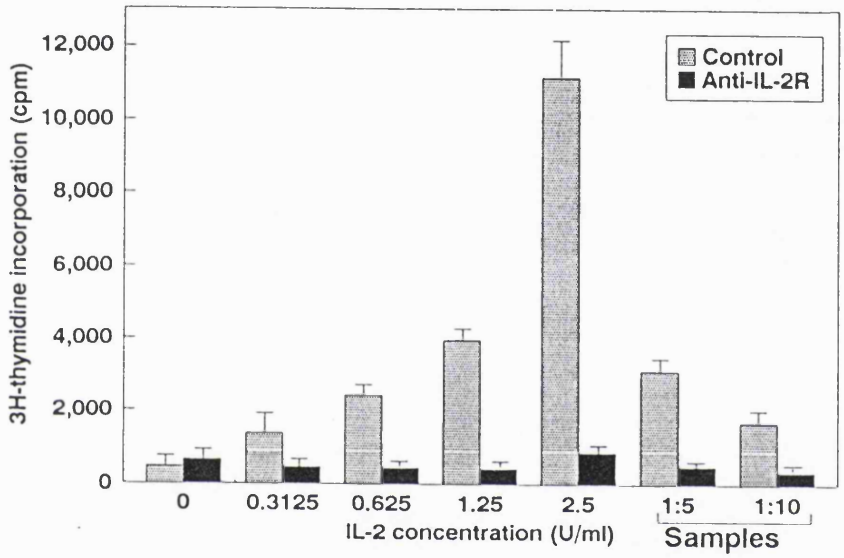

Fig.2.3. *HT-2 cell proliferation was measured by culturing $2x10^4$ HT-2 cells per well with purified recombinant IL-2 or diluted samples. The IL-2 dependent response was demonstrated by adding anti-IL-2R mAb.*

plates were developed with 0.04% O-phenylenediamine (Sigma) and 0.03% H_2O_2 and read on a ELISA reader at a wavelength of 492nm.

2.10.6. Cytotoxicity assay

Confluent retinal EC (>95% purity) cultured in 4 well plates were treated for 3 days with 200 U/ml IFN-γ before the cytotoxicity assay. $2x10^6$ S-Ag specific T-cell line lymphocytes per well were added to the EC monolayers and incubated for 24 h in the presence of 10 μg/ml of S-Ag. At the end of culture, the EC monolayers were observed by an inverse phase-contrast microscope.

The quantitative assay of cytotoxicity of retinal EC monolayers was carried out according to a previously described method (Risau et al., 1990). Confluent retinal EC grown in 96 well plates were incubated for 3 days with 200 U/ml IFN-γ prior to the cytotoxicity assay. The EC monolayers were labelled for 18 h with ^{51}Cr at a concentration of 1 μCi of ^{51}Cr per well in 50 μl of fresh medium (without ECGS) at 37°C, in a 5% CO_2 incubator. Non-bound ^{51}Cr was washed off with HBSS, and the ^{51}Cr labelled EC cocultured with $5x10^5$ cells/well of S-Ag specific $CD4^+$ T-cell line lymphocytes in 200 μl of 10% FCS RPMI medium per well. The wells were treated with either 10 μg/ml S-Ag or 10 μg/ml BSA. After 18 h of coculture the supernatants were taken and released ^{51}Cr measured on a γ-emission counter (Dynatech). Maximal release of ^{51}Cr was obtained by lysis of ^{51}Cr labelled retinal EC with 10% SDS. The specific release of ^{51}Cr was calculated by the following formula:

$$\text{Specific release (\%)} = \frac{\text{cpm of experiments-cpm of control}}{\text{maximal release.}} \text{ x100\%}$$

2.11. STATISTIC ANALYSIS

Each experiment has been repeated at least 3 times. Results if not described otherwise, are means$\pm$standard error of the mean (SEM). The significance between groups was assessed using the Student's *t*-test.

CHAPTER 3 RESULTS

3.1 CULTURES OF RETINAL EC

3.1.1. Morphological observations

The microvessel fragments isolated by this method normally adhered to collagen-coated plastic within 15 min (Fig.3.1a). After 1-2 days of culture, outgrowth of the EC from the attached microvessels could be seen (Fig.3.1b). The microvessel debris disappeared in a few days and the EC formed a small clump of cells. Retinal EC cultured under these conditions have a spindle-shape morphology. After 1-2 weeks, large monolayers of EC displaying contact-inhibition covered the collagen substrate (Fig.3.1c). Confluent monolayers in 25 cm^2 flasks could be obtained within 4-5 weeks. The purity of retinal EC observed by inverse-phase contrast microscopy was 95-100%. The contaminating cells appeared to be mainly pericytes which were identified by their typical morphology (flat, spread with many processes and no contact inhibition).

3.1.2. Characterization of cultured retinal EC

GSA staining: Dissociated cultured EC were labelled with the EC specific GSA conjugated with FITC and analyzed using flow cytometry. As shown in Fig.3.2., nearly all the cells in the culture stained positively for GSA although there was some variability in the level of the intensity of fluorescence but with the majority of cells expressing high levels (Fig.3.2).

Staining with mAb: The purity of cultured retinal EC populations was ascertained using indirect immunofluorescence staining with the mAb HIS52 which is specific to rat EC (RECA-antigen). With flow cytometry, we found that the majority of dissociated cells of EC cultures ($95\pm5.3\%$) expressed the RECA-antigen (Fig.3.2). We also stained cultured cells with the mAb against bone marrow derived cells, ED2 (dendritic cells), ED7 (macrophages) and OX-22 (leucocyte common antigens expressed on B-cells and T-cells). It was found that cultured cells were all negative for both ED2 and ED7 although there was a proportion of the cultured EC

Cultures of retinal EC

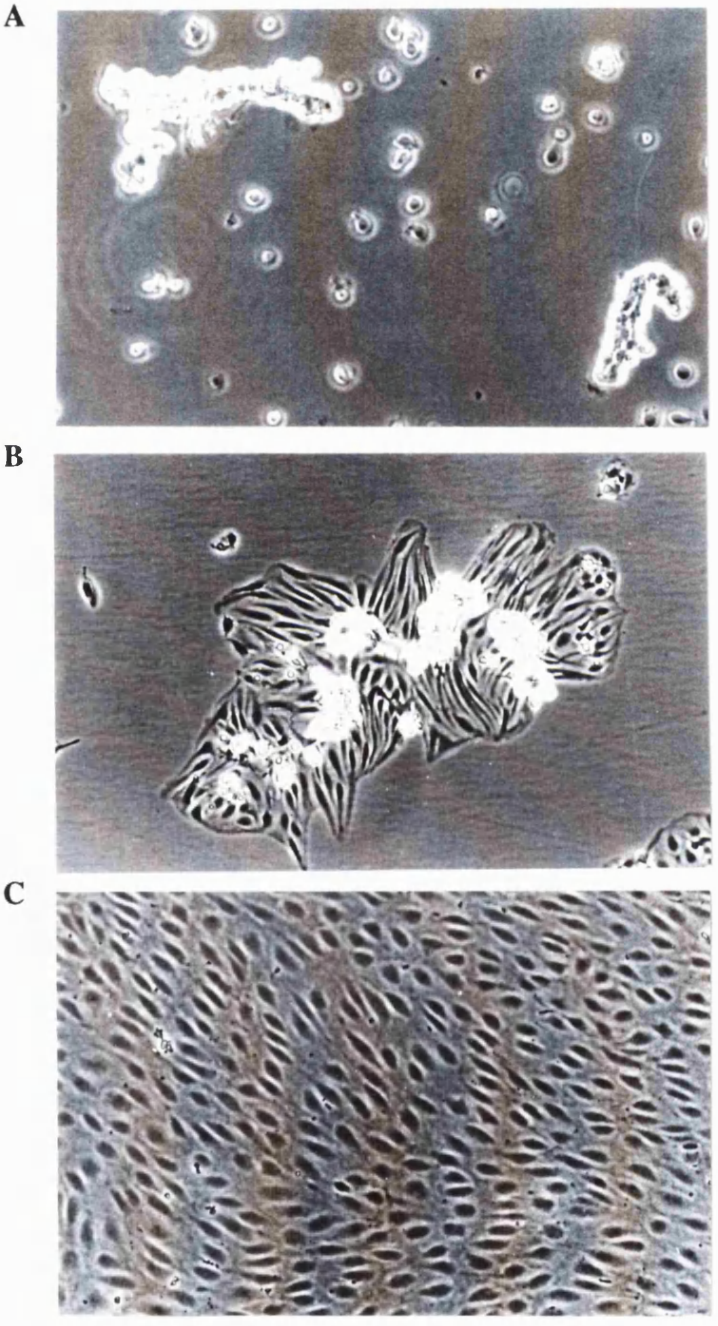

Fig.3.1. *Retinal microvessels were isolated from Lewis rats. a. retinal microvessels adhered to collagen coated substratum. b. 5 day old culture showing small colony of EC growing out from microvessel. c. 2 weeks old culture of retinal EC forming typical contact-inhibited monolayer of spindle-shaped cells.*

Flow cytometric analysis of retinal EC

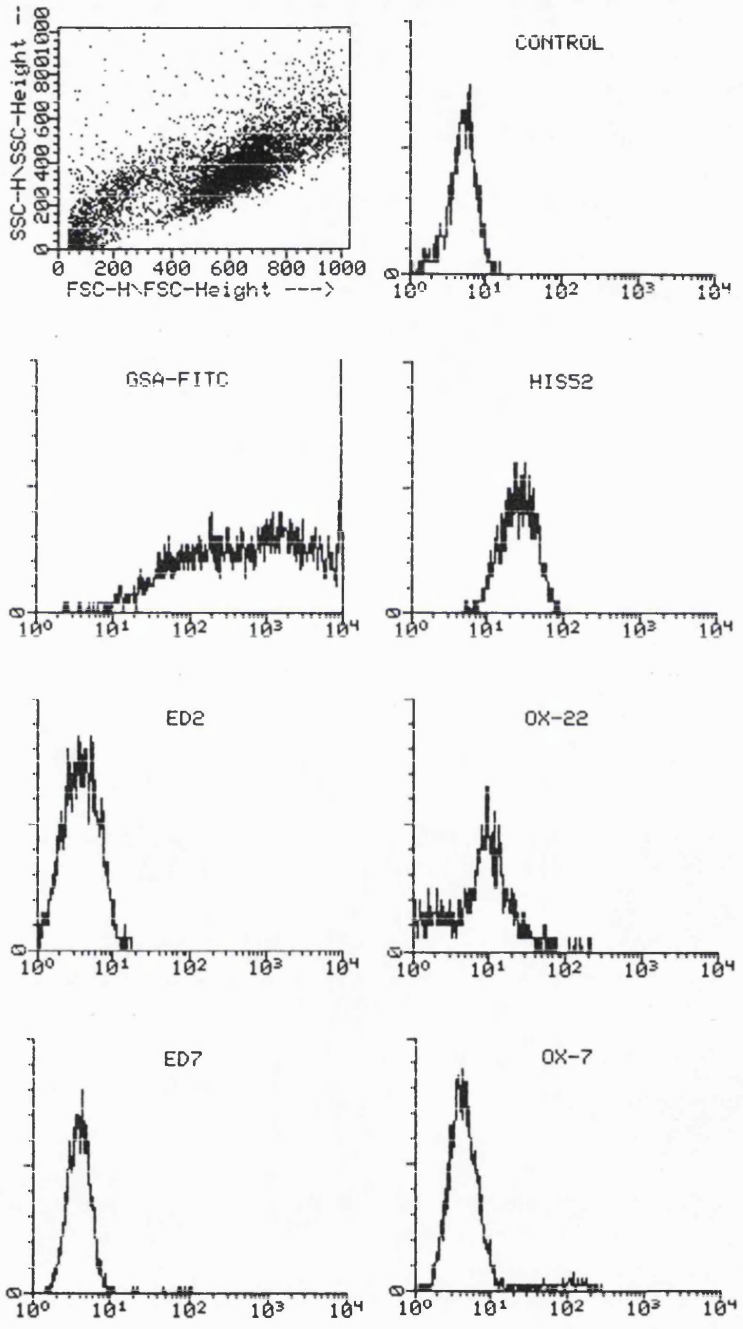

Fig.3.2. Enzyme-dissociated normal cultured retinal EC were stained with ED2, ED7, OX-22, HIS52 mAb and the lectin GSA-FITC. For antibody staining, the control was set by using FITC-labelled RAMIG. Non-stained retinal EC were used as the control for GSA-FITC staining.

(20.2±2.0 %) which were positive for OX-22 staining (Fig.3.2).

The cultures contained very few cells (< 5%) that were positive to the mAb OX-7, an anti-rat antibody raised against the Thy 1.1 antigen expressed on fibroblasts, pericytes and neuronal cells (Tab.3.1).

Table 3.1

Percentages of cultured EC stained with mAb and GSA

Probe	%
ED2	0
ED7	0
OX-7	5.0±0.3
OX-22	20.2±2.0
HIS52	95.0±5.3
GSA	95.0±4.0

Results show percentages of stained retinal EC analyzed by flow cytometry. Data represents means±SD.

3.2. LYMPHOCYTE ADHESION TO RETINAL EC

3.2.1. Characterization of lymphocyte adhesion to retinal EC

Morphological observations indicated that a small population of resting PLN lymphocytes were capable of adhering to retinal EC monolayers following 2 h coculture (Fig.3.3a). The adherent lymphocytes appeared to be unevenly distributed over the retinal EC monolayers with the majority of adherent lymphocytes preferentially located at the junctional sites of the EC monolayers. It was also observed that lymphocytes adhered only to the areas of the plate coated with EC. If any flaws were present in the retinal EC monolayers leaving the collagen substrate exposed, there was no adhesion to the substrate (Fig.3.3b).

The small population of adherent cells were quantitated using ^{51}Cr labelled lymphocytes collected from cervical, inguinal and mesenteric lymph nodes and it was found that approximately 5% of pooled PLN lymphocytes were able to adhere to resting cultured retinal EC (Fig.3.4). Analysis of the binding ability of lymphocyte populations from the individual sites revealed that lymphocytes from cervical

Lymphocyte adhesion to retinal EC

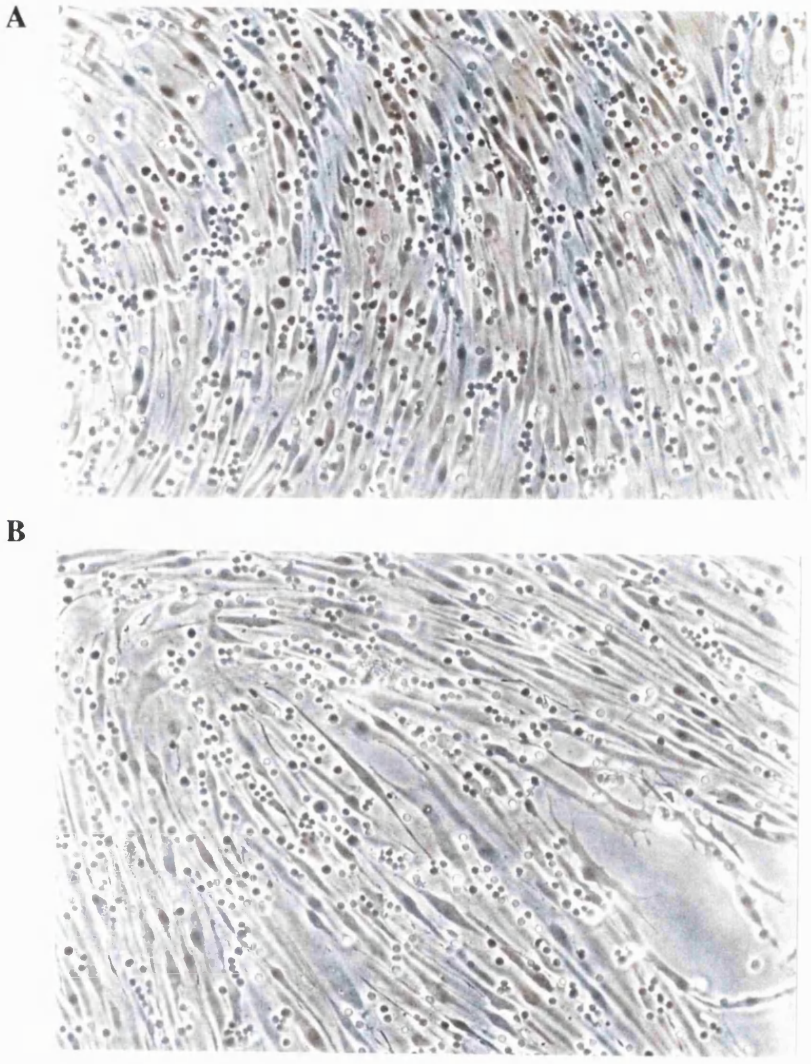

Fig.3.3. *Adhesive interactions between resting mixed PLN (cervical, inguinal and mesenteric PLN) lymphocytes and resting retinal EC monolayers was observed after 2 h of coculture following washing off non-adherent cells. a. lymphocyte adhesion to retinal EC monolayers (note: cells preferentially located at the sites of the EC junctions), b). lymphocytes only bind to the area covered by retinal EC, but not to the area of exposed collagen.*

Adhesion of lymphocytes from various sites.

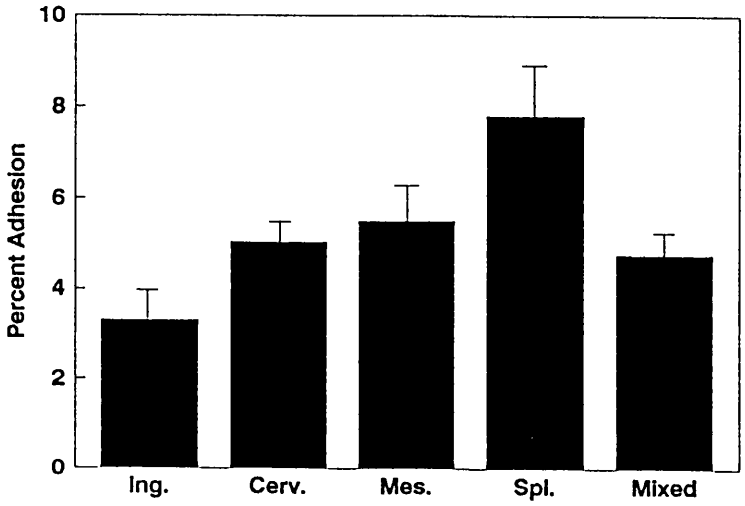

Fig.3.4. Adhesion of lymphocytes collected from inguinal (Ing.), cervical (Cerv.), mesenteric (Mes.), spleen (Spl.) and mixed lymph nodes.

Table 3.2

Distributions of lymphocyte subsets amongst various sites (%)

	CD5	CD4	CD8	CD45-ABC
Ing.	74.3±7.1	45.9±7.3	17.5±1.1	24.2±5.6
Cerv.	50.6±6.3	32.6±3.1	15.9±2.1	40.7±1.0
Mes.	60.0±6.6	42.8±2.5	15.6±0.8	30.5±1.7
Spl.	35.3±2.6	21.9±4.5	16.4±5.8	40.4±6.0
Mixed	68.2±8.7	38.8±0.9	17.5±1.0	31.5±3.2

Results show the percentages of lymphocyte subpopulations in cervical, inguinal, mesenteric, mixed PLN and spleen. The lymphocytes were stained with anti-CD5 (OX-19), CD4 (W3/25), CD8 (OX-8) and CD45-ABC (OX-33) and analyzed by flow cytometry. Data given as means±SD.

(5.4±3.2%) and mesenteric (5.0±0.8%) PLN exhibited slightly greater levels of adhesion than lymphocytes from inguinal PLN (3.3±0.6%, $p<0.05$) and splenic lymphocytes appeared to be more efficient in binding to retinal EC (7.8±0.4%) than PLN lymphocytes ($p<0.05$; Fig.3.4).

Subpopulations of lymphocytes from various PLN were analyzed by flow cytometry following indirect immunofluorescence staining with the mAb OX-19 (anti-CD5 mAb), W3/25 (anti-CD4 mAb), OX-8 (anti-CD8 mAb) and OX-33 (anti-CD45ABC mAb). The results (Tab.3.2) show that lymphocytes from inguinal PLN had higher percentages of T cells and $CD4^+$ T cells, and lower percentages of B cells than lymphocytes from other sites, whereas lymphocytes from cervical PLN had higher proportions of B cells than those from inguinal and mesenteric PLN. Splenic lymphocytes had a comparable level of B cells to cervical PLN lymphocytes, but a lower levels of T cells (particularly $CD4^+$ T cells) than PLN lymphocytes (Tab.3.2).

Subsets of adherent cells were measured using immunocytochemistry with the mAb OX-19, W3/25 and OX-8 following 2 h coculture of retinal EC with the enriched T-cell population. Analysis of these enriched T-cell populations showed that resting T-cell populations consisted of 60.4±1.8% $CD4^+$ and 31.7±5.3% $CD8^+$ in a ratio of 1.9:1. Con A stimulated populations had similar percentages of $CD4^+$ (58.2±2.3%) and $CD8^+$ (36.2±3.4%) T cells in the ratio of 1.6:1 (Tab.3.3). After a 2 h adhesion period with IFN-γ activated retinal EC, 34.9±4.5% $CD4^+$ and 53.5±8.1% $CD8^+$ T cells adhered to the EC at a ratio of 0.65:1 with the resting T-cell population, and 34.7±1.8% $CD4^+$ and 55.2±5.4% $CD8^+$ in a ratio of 0.63:1 with the Con A activated T-cell population (Tab.3.3).

Levels of lymphocyte adhesion were further studied by co-culturing retinal EC with pooled PLN lymphocytes for periods up to 240 min. It was found that a proportion of lymphocytes were able to adhere to retinal EC monolayers within 15 min and that activation of lymphocytes with Con A and the EC with IFN-γ, significantly increased the level of adhesion ($p<0.01$) with a further increase in adhesion following an increase in the duration of coculture. The percentages of adherent cells reached a peak by 2 h which was maintained for up to 4 h (Fig.3.5).

Table 3.3

Distribution of T-cell subsets in both pre-adhesion and adherent population (%)

	Pre-adhesion		Adherent cells	
	Resting	Con A	Resting	Con A
CD5	85.4±9.3	87.7±2.5	85.9±4.7	87.1±2.9
CD4	60.4±1.8	58.2±2.3	34.9±4.5	34.7±1.8
CD8	31.7±5.3	32.6±3.4	53.5±8.1	55.2±5.4

Percentages of lymphocytes in both pre-adhesion and adherent population were examined by fluorescent immunocytochemistry with the mAb OX-19 (CD5), W3/25 (CD4) and OX-8 (CD8). Cells were counted on a fluorescence microscope. Data given as means±SD.

The level of adhesion under normal conditions was typically low at approximately 5% at all time periods examined. Con A activated lymphocytes increased adhesion sharply by 15 min, whereas IFN-γ activation of EC increased adhesion gradually throughout 15-180 min of cocultures. Moreover, Con A activated lymphocytes demonstrate higher levels of adhesion than IFN-γ activated retinal EC but when both were activated, the levels of adhesion could be further increased.

In the concentration dependent adhesion assay we attempted to investigate whether the number of adherent cells was related to the number of lymphocytes applied. The results (Fig 3.6) show that under all conditions (normal, IFN-γ treated retinal EC, Con A activated lymphocytes and both activated) the greater the number of lymphocytes applied, the greater the number of adherent cells, with proportionally more adhesion when the lymphocytes and retinal EC were activated. However, a significant increase in percentages of lymphocyte adhesion by activation was only seen when less than $5x10^5$ lymphocytes per well were added, and the percentages of adhesion decreased sharply when more than $5x10^5$ lymphocytes were applied (Fig.3.6). Percentages of adhesion under normal conditions was maintained at similar levels over all the lymphocyte concentrations ($5x10^4$-$2x10^6$) studied.

Time-course of lymphocyte adhesion

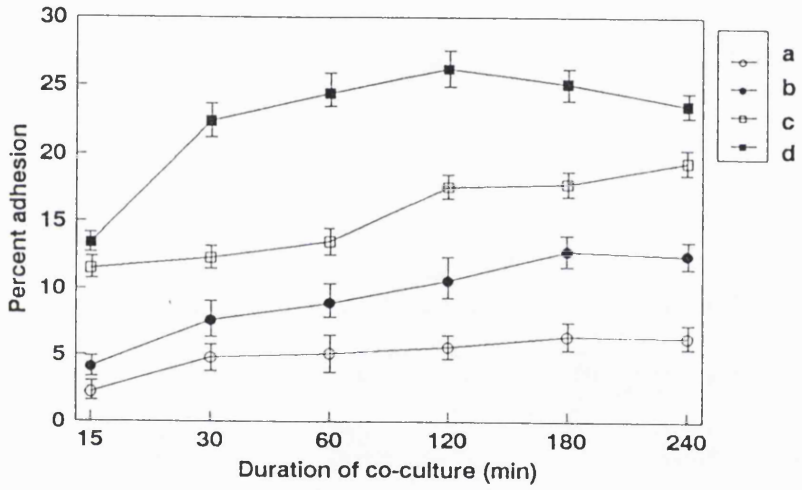

Fig.3.5. *Percent adhesion was assayed following 15-240 min cocultures of $2x10^5$ PLN lymphocytes per well with EC monolayers in 96-well plates. a) under normal conditions; b) IFN-γ activated retinal EC; c) Con A activated lymphocytes and d) both activated.*

Lymphocyte concentration dependent adhesion

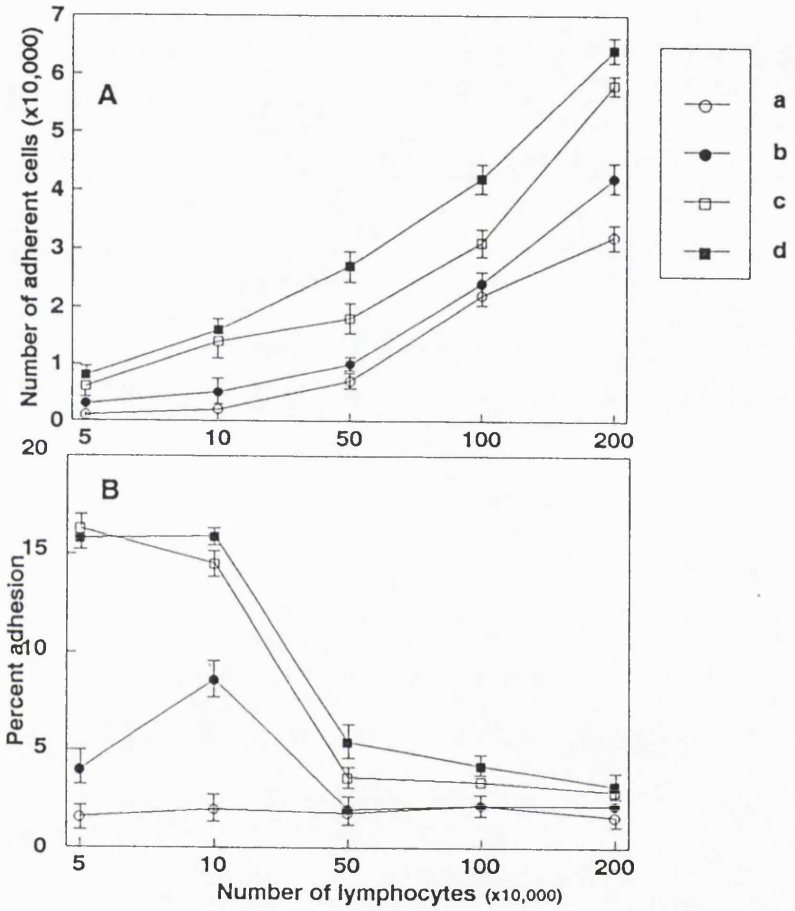

Fig.3.6. A range of $5x10^4$-$2x10^6$ lymphocytes per well were added to retinal EC monolayers. The number of adherent cells and percent adhesion were measured following 2 h incubation. a). under normal conditions; b). IFN-γ activated retinal EC; c). Con A activated lymphocytes and d). both activated.

3.2.2. Effect of cell activation on lymphocyte adhesion

Following treatment of EC monolayers for 18 h with varying concentrations of the cytokines IFN-γ, IL-1 and IL-4, there was a significant increase in the ability of retinal EC to bind lymphocytes (Fig.3.7). In the dose-response assay it was found that as little as 5 U/ml of IFN-γ significantly increased lymphocyte adhesion ($p<0.05$) and this increased adhesion peaked at a dose of 50 U/ml and remained constant to 200 U/ml. With IL-1, lymphocyte adhesion significantly increased at a dose of 0.5 U/ml ($p<0.01$), peaked with 5 U/ml and remained constant up to 25 U/ml. Lymphocyte adhesion to IL-4 activated retinal EC also exhibited a dose-dependent increase, which was significantly elevated with a dose of 10 U/ml ($p<0.01$) and peaked at 100 U/ml.

With optimal concentrations of the cytokines, lymphocyte adhesion could be increased up to $11.7\pm1.0\%$ ($p<0.001$) by IFN-γ, $12.9\pm1.2\%$ ($p<0.001$) by IL-1 and $12.1\pm2\%$, ($p<0.001$) by IL-4 (Fig.3.8A). Maximal levels of adhesion induced by IFN-γ (200 U/ml), IL-1 (10 U/ml) and IL-4 (100 U/ml) were comparable ($p>0.05$).

When lymphocytes were activated for 18 h with Con A (5 μg/ml), binding to resting retinal EC was increased up to $17.0\pm0.9\%$ ($p<0.0001$, Fig.3.8A) and this could be further increased when retinal EC were also activated with the cytokines IFN-γ ($22.3\pm1.0\%$), IL-1 ($24.0\pm1.0\%$) and IL-4 ($25.7\pm1.6\%$). In addition, it was found that Con A activated lymphocytes adhered to resting EC to a greater degree than with resting lymphocyte adhesion to IFN-γ ($p<0.05$), IL-1 ($p<0.05$) and IL-4 ($p<0.05$) activated retinal EC.

The kinetics of Con A activation-induced lymphocyte adhesion was determined by treating lymphocytes for between 1 min and 48 h with 5 μg/ml of Con A before the adhesion assay (Fig.3.8B). The results show that Con A activation caused a significant increase in the proportion of adherent cells within 10 min post stimulation to both resting and IFN-γ activated EC. Adhesion peaked in both groups at 6 h and remained elevated up to 18 h. After 24 h of Con A stimulation, when cells started to divide, their ability to adhere to retinal EC decreased, and this was still observed 48h

Effects of the cytokines on lymphocyte adhesion

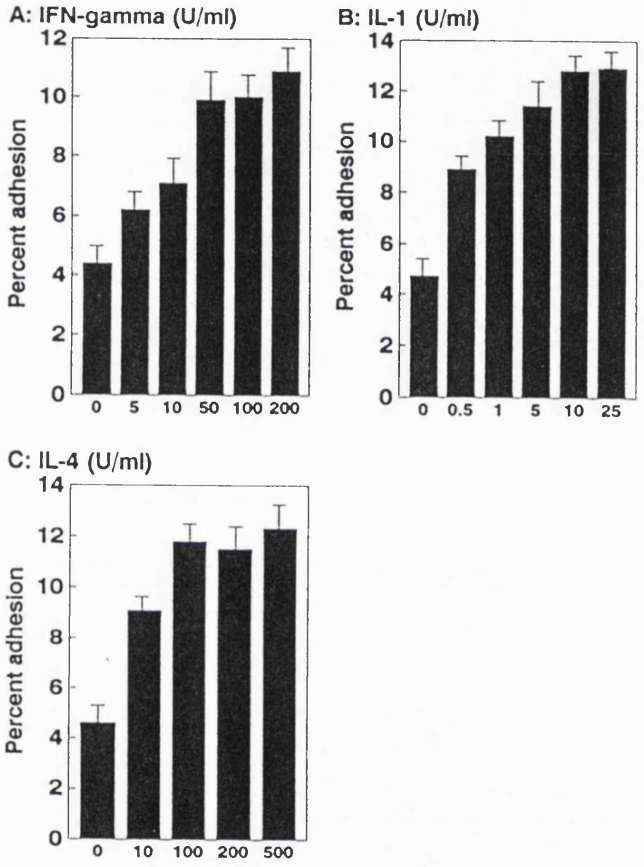

Fig.3.7. Lymphocyte adhesion to retinal EC monolayers following treatment with various doses of the cytokines, IFN-γ, IL-1 and IL-4.

Effects of cell activation on adhesion

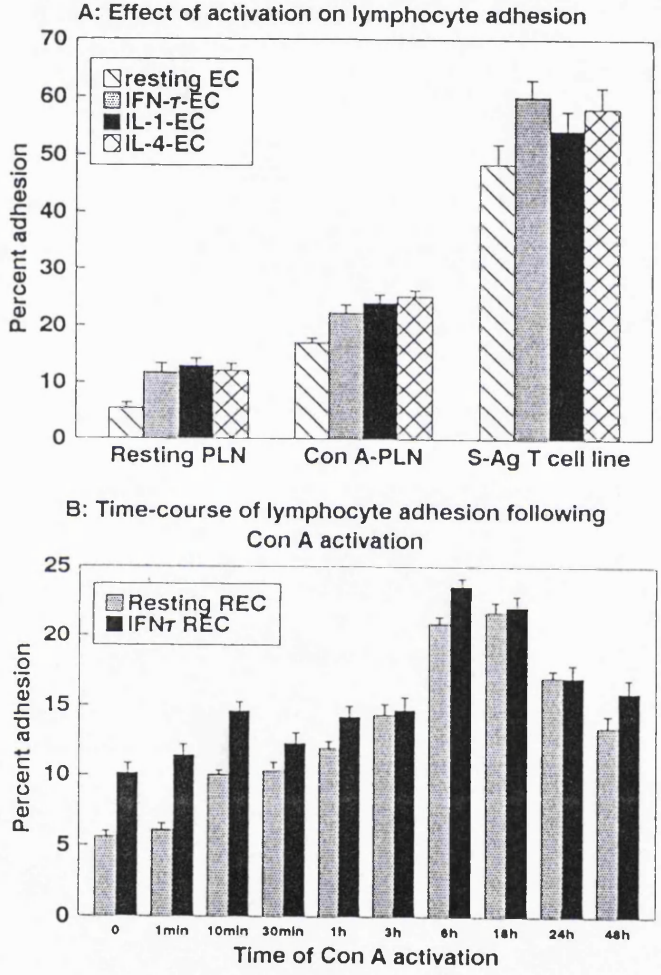

Fig.3.8. Graph A shows the adhesion of resting, Con A activated lymphocytes and S-Ag specific T-cell lines, to resting or IFN-γ (200 U/ml), IL-1 (25 U/ml) and IL-4 (200 U/ml) activated retinal EC. Graph B demonstrates the time-course of PLN cell adhesion to resting (shaded bars) and IFN-γ activated (solid bars) retinal EC following Con A activation.

after stimulation although the percent adhesion remained significantly higher than basal levels ($p<0.05$).

S-Ag specific T-cell lines, which are highly activated with S-Ag and IL-2, were also used in the adhesion assay. Prior to the assay these T cells were maintained for 3 days in IL-2 conditioned medium following specific antigen activation (Fig.3.8A). The terminally differentiated S-Ag specific T-cell line was found to be significantly more adhesive than Con A activated lymphocytes to resting retinal EC ($40.4\pm2.5\%$; $p<0.0001$), to IFN-γ activated ($60.3\pm3\%$; $p<0.001$), IL-1 activated ($54.3\pm1.4\%$; $p<0.001$) and IL-4 activated retinal EC ($58.0\pm3.2\%$; $p<0.001$). It was not possible to obtain a resting population of S-Ag specific T-cell lines for comparison since their growth is IL-2-dependent.

In order to determine whether the adhesion of antigen specific T-cell lines to retinal EC was affected by antigen specific interactions, terminally differentiated S-Ag specific T-cell lines were applied to either resting or IFN-γ activated retinal EC, in the presence or absence of the cognate antigen (Tab.3.4). In these experiments the adhesion of S-Ag specific T-cell lines was not significantly affected by the presence of S-Ag. Moreover, S-Ag specific T-cell lines adhered to IFN-γ activated retinal EC after 18 h ($60.3\pm3\%$; Fig.3.8A) and 3 days of treatment ($59.2\pm4.1\%$; $p>0.05$) to a comparable level despite 3 days of treatment with IFN-γ inducing EC to express MHC class II molecules (see section 3.4.1).

Table 3.4.

The effect of S-Ag on S-Ag specific T-cell adhesion (%)

	Control retinal EC	IFN-γ-retinal EC
[-] S-Ag	40.5 ± 2.4	59.2 ± 4.1
[+] S-Ag	40.1 ± 1.1	55.3 ± 1.4

S-Ag specific T-cell line lymphocytes were applied to retinal EC monolayers treated for 3 days with 200 U/ml IFN-γ or untreated retinal EC. S-Ag at a concentration of 10 μg/ml was pulsed for 18 h in some wells [+] prior to the adhesion assay.

3.2.3. Effect of ACM and forskolin on lymphocyte adhesion

After 3 days incubation of retinal EC monolayers with 50% ACM there was a significant increase in the adhesion of both resting PLN cells (12.7±0.9%; $p<0.0001$) and Con A activated lymphocytes (25.7±1.6%; $p<0.001$) (Fig.3.9). Forskolin treated retinal EC also showed an increased adhesiveness for resting PLN lymphocytes (13.9±1.2%; $p<0.001$) and Con A stimulated lymphocytes (19.2±1.5%; $p<0.05$). When both forskolin and ACM were used to stimulate the retinal EC, there was a further significant increase in the adhesion of resting lymphocytes (19.6±1.1%) over retinal EC treated with either ACM ($p<0.001$) or forskolin ($p<0.002$) alone. In contrast there was only a marginal increase in the adhesion of Con A activated PLN cells when the retinal EC were treated with both ACM and forskolin (27.4±1.3%) compared with ACM treatment alone. The degree of lymphocyte binding to ACM and forskolin treated retinal EC, however, was significantly greater than with forskolin treated retinal EC alone ($p<0.0002$).

On investigating the synergistic effect of ACM with cytokines it was found that the adhesion of resting lymphocytes could be further significantly increased by IL-1 (21.8±0.9%) and IFN-γ (20.0±1.4%) and this was significantly greater than with ACM ($p<0.01$), IFN-γ ($p<0.0002$) or IL-1 ($p<0.0005$) treatment alone. When Con A activated PLN cells were applied, stimulation of retinal EC with ACM and the cytokines, IL-1 and IFN-γ caused a significant increase in adhesion compared with IFN-γ and IL-1 on their own ($p<0.04$ and $p<0.05$ respectively) but this was not significant when compared with retinal EC treated with ACM.

An increase in adhesion of resting lymphocytes was also achieved by the combined treatment of EC with forskolin and either IL-1 or IFN-γ. When resting lymphocytes were added to retinal EC treated with a combination of both forskolin and either IFN-γ (16.9±0.6%) or IL-1 (18.9±1.1%) the level of adhesion was significantly above that for retinal EC treated with either forskolin ($p<0.02$ and $p<0.004$ respectively) or IFN-γ and IL-1 alone ($p<0.001$ and $p<0.04$ respectively). When Con A activated lymphocytes were applied to retinal EC treated with a combination of both forskolin and IL-1 (26.2±1.3%), the level of adhesion was significantly above that for retinal EC treated with either forskolin or IL-1 alone

Effects of ACM and forskolin on lymphocyte adhesion

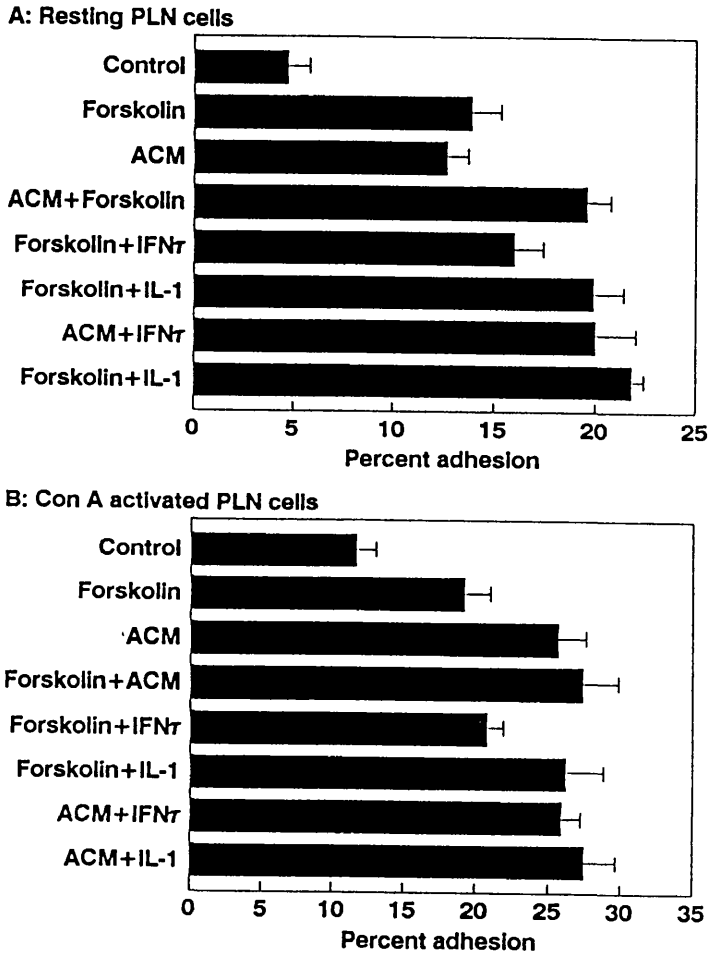

Fig.3.9. Retinal EC were treated for 18 h with combinations of ACM, forskolin, IFN-γ and IL-1. Following thorough washing, resting (A) and Con A activated (B) PLN cells were added to EC monolayers in an adhesion assay.

($p<0.05$ and $p<0.002$ respectively). Moreover, the combination of forskolin and IFN-γ increased adhesion significantly compared with IFN-γ alone ($p<0.05$), but only marginally when compared with forskolin treatment.

3.3. THE ROLE OF ADHESION MOLECULES IN LYMPHOCYTE ADHESION AND MIGRATION

3.3.1. Expression of adhesion molecules on lymphocytes and retinal EC

In order to determine the role of adhesion molecules in the interactions between lymphocytes and retinal EC, we examined the surface expression of LFA-1 and VLA-4 on resting and activated lymphocytes and ICAM-1 on resting and IL-1 activated retinal EC using flow cytometry following indirect immunofluorescence staining with WT.1 (anti-CD11a mAb), WT.3 (anti-CD18 mAb), P12520 (anti-CD49d mAb) and IA29 (anti-ICAM-1 mAb; Fig.3.10).

It was found that the majority of resting PLN lymphocytes expressed both LFA-1 (CD11a; 95.3±3.5% and CD18; 94.6±3.2%) and VLA-4 (CD49d; 90.8±7,3%) with a low intensity of fluorescence. Following activation of lymphocytes for 18 h with Con A, there were increased numbers of cells expressing high levels of the immunofluorescence intensity of LFA-1 (both CD11a and CD18) although the expression of VLA-4 molecules was found not to be significantly affected (Fig.3.10).

S-Ag specific T-cell lines expressed higher levels of LFA-1 than both Con A activated and resting lymphocytes. The enhanced expression of LFA-1 on S-Ag specific T-cell lines was due to an increase in the intensity of fluorescence and not to an increase in the percentage of LFA-1 expression. S-Ag activated T-cell lines, however, did not express increased levels of VLA-4 compared to resting or Con A activated lymphocytes (Fig.3.10).

The expression of the surface adhesion molecule ICAM-1 on retinal EC was analyzed following dissociation of the EC monolayer with collagenase/dispase. The majority of resting retinal EC were found to express ICAM-1 (60.2±3.2%), but following treatment with 5 U/ml of IL-1, the expression of this molecule was

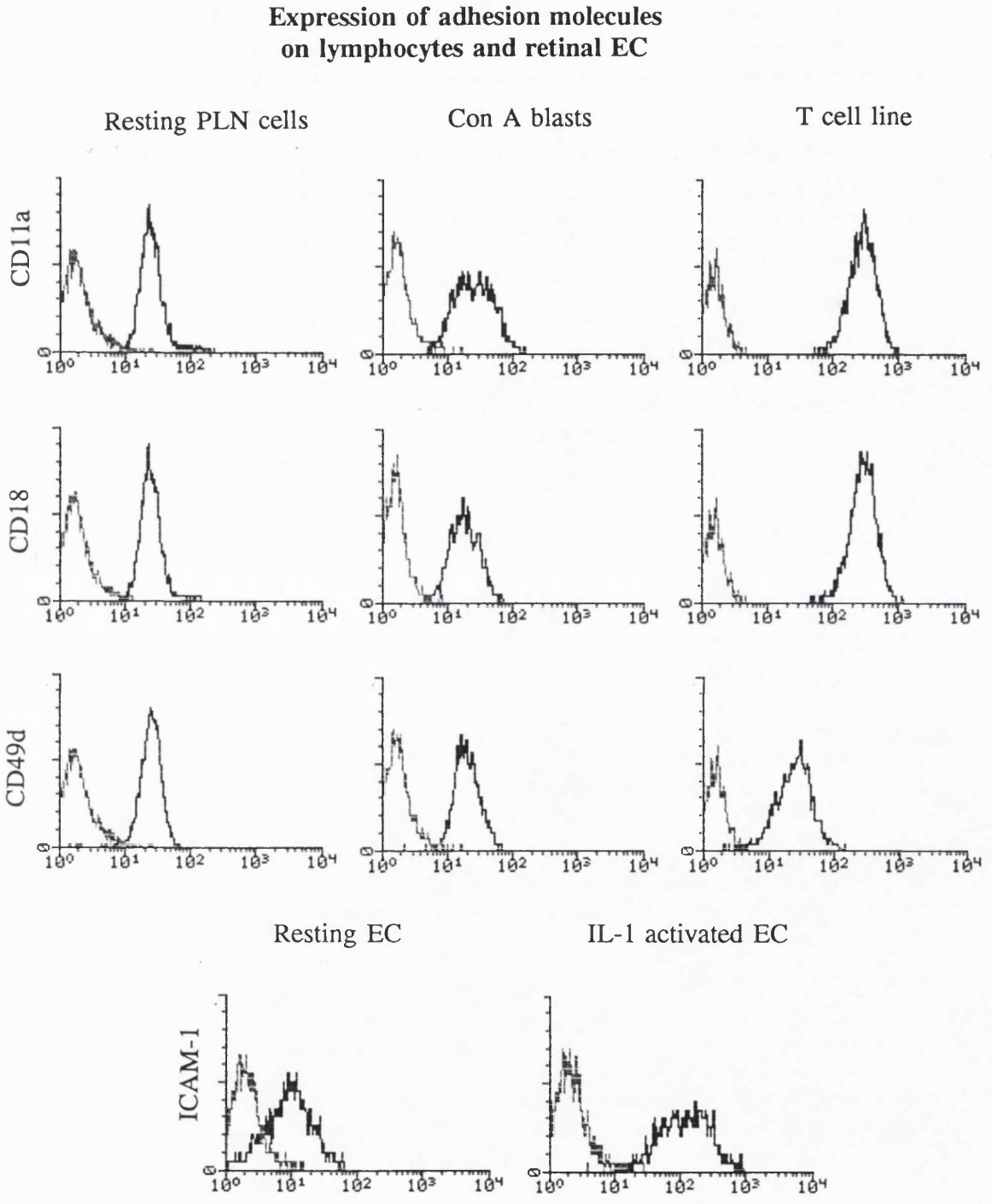

Fig.3.10. LFA-1 and VLA-4 expression on resting PLN cells, 18 h Con A activated cells and S-Ag specific T-cell lines, and ICAM-1 expression on resting and IL-1 activated retinal EC. OX-21 staining was used as the control.

significantly elevated both in the percentage of expression (93.4±3.5%) and in the fluorescence intensity (Fig.3.10).

3.3.2. The effects of anti-LFA-1, VLA-4 and ICAM-1 mAb on lymphocyte adhesion

The role of adhesion molecules in lymphocyte adhesion to retinal EC was investigated by an adhesion blocking assay with the antibodies to ICAM-1 (IA29), LFA-1 (WT.1/WT.3) and VLA-4 (P12520) which have been shown to block lymphocyte adhesion in previous studies (Issekutz 1991, Issekutz and Wykretowicz 1991; Tamatani et al., 1990, 1991). As resting lymphocytes adhered to retinal EC at very low levels, activated lymphocytes (either Con A blasts or S-Ag specific T-cell lines) were employed in the adhesion blocking assay. The results (Fig.3.11) show that both anti-α chain (CD11a) mAb (WT.1) and anti-ß chain (CD18) mAb (WT.3) of LFA-1, and anti-VLA-4 mAb blocked the binding of Con A activated lymphocytes and S-Ag specific T-cell lines to resting and IL-1 activated retinal EC. The anti-ICAM-1 mAb (IA29) had no effect on the adhesion of Con A activated lymphocytes to retinal EC, but it significantly blocked the apparent adhesion of S-Ag specific T-cell lines to both resting EC (47.5±3.3% of the control; $p<0.001$) and IL-1 activated retinal EC (55.0±4.9% of the control; $p<0.001$).

In the assay it was found that treatment of lymphocytes with either WT.1 or WT.3 resulted in similar levels of inhibition of adhesion of the S-Ag specific T-cell lines, although WT.3 blocked the adhesion of Con A activated lymphocytes more efficiently than WT.1 ($p<0.05$). In addition, WT.1, but not WT.3, blocked the adhesion of S-Ag specific T-cell lines to a higher level than with Con A activated lymphocyte to both resting and IL-1 activated retinal EC. In comparison with resting retinal EC, activation with IL-1 did not significantly affect the levels of inhibition by WT.1, WT.3 or IA29 although IL-1 did increase both the level of ICAM-1 expression on EC and the degree of lymphocyte binding.

The anti-VLA-4 mAb was found to be capable of inhibiting the adhesion of Con A activated lymphocytes and S-Ag specific T-cell lines to resting retinal EC monolayers ($p<0.01$). With S-Ag specific T-cell lines, the adhesion to resting EC was inhibited less efficiently with the anti-VLA-4 mAb (74.7±6% of the control)

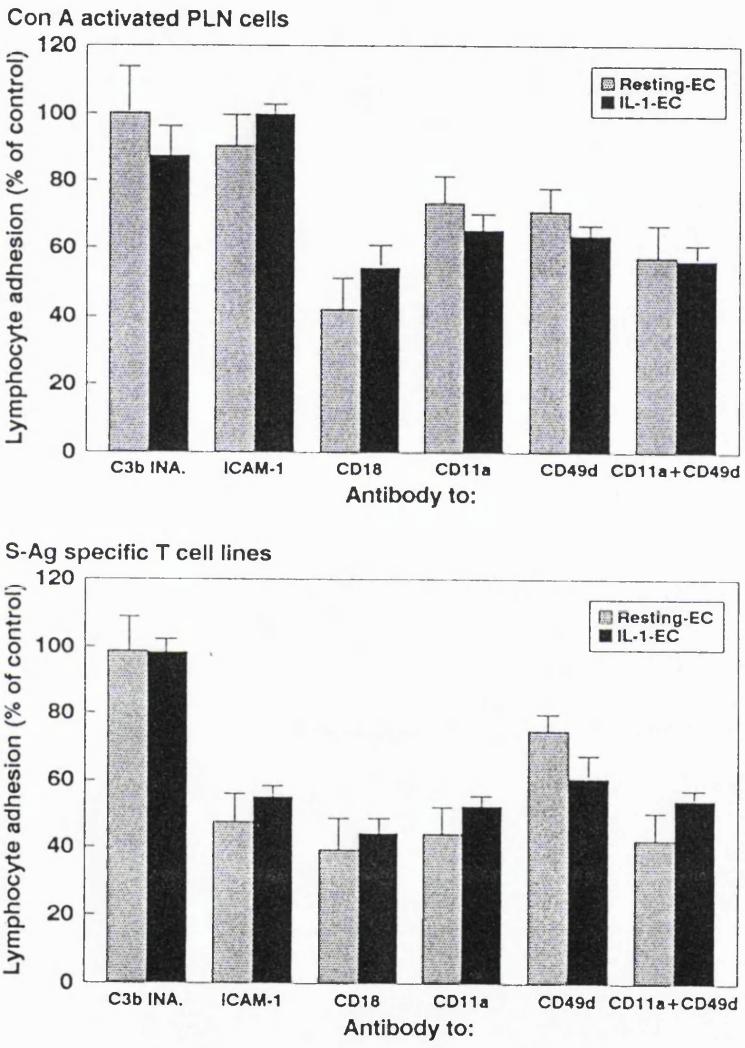

Fig.3.11. *Adhesion of Con A activated lymphocytes and S-Ag specific T-cell lines to resting (shaded bars) and IL-1-activated (solid bars) retinal EC in the presence of anti-ICAM-1, CD11a anti-CD18 and CD49d mAb and a combination of anti-CD11a and anti-CD49d mAb. For the control, lymphocytes were treated with anti-human C3b inactivator (C3b INA.) mAb (OX-21).*

than with WT.1 (43.9±4.6% of the control; $p<0.001$) or WT.3 (39.2±5.4 of the control; $p<0.001$), although activation of retinal EC with IL-1 significantly enhanced the blocking effect of anti-VLA-4 mAb (60.7±4.2% of the control;$p<0.05$). Conversely, with Con A activated lymphocytes, anti-VLA-4 mAb had a similar inhibiting effect on adhesion as WT.1 ($p>0.05$), but less than WT.3 ($p<0.001$), whilst IL-1 pretreatment of retinal EC only marginally increased the blocking effect of anti-VLA-4 mAb (63.6±4.8% of the control vs 70.7±4.9% of the control; $p>0.05$).

It was also attempted to block lymphocyte adhesion using a combination of both anti-CD49d mAb and WT.1. As shown in Fig 3.11, anti-VLA-4 mAb in association with anti-LFA-1 mAb (WT.1) did not produce a significantly higher level of blocking than the individual mAb applied alone.

3.3.3. The effect of anti-LFA-1, VLA-4 and ICAM-1 mAb on lymphocyte migration

Following the investigation into the blocking of lymphocyte adhesion by the antibodies we further examined the effects of these antibodies on lymphocyte migration. In a previous study it was demonstrated that S-Ag specific T-cell lines, but not Con A blasts were capable of migrating across retinal EC monolayers (Greenwood and Calder 1993) and therefore in this study we examined antibody-blocking migration of S-Ag specific T-cell lines. The migration blocking assay was carried out using time-lapse videomicroscopy in which cocultures of lymphocytes and EC monolayers were recorded for 4 h. In this system, under control conditions, a proportion of S-Ag specific T-cell line lymphocytes (approximately 50%.) readily migrate through the retinal EC monolayers irrespective of EC activation. Following treatment of S-Ag specific T cells with mAb against LFA-1 mAb and VLA-4, and the EC monolayers with an anti-ICAM-1 mAb (IA29) 1 h prior to coculture, the number of migrated cells were counted and compared with control migration. The results (Fig.3.12) show that mAb against LFA-1 and ICAM-1 significantly reduced lymphocyte migration across both resting and IL-1 activated retinal EC monolayers, and that anti-VLA-4 mAb blocked lymphocyte migration across IL-1 activated, but not resting, retinal EC monolayers.

The role of adhesion molecules in S-Ag specific T-cell line lymphocyte migration

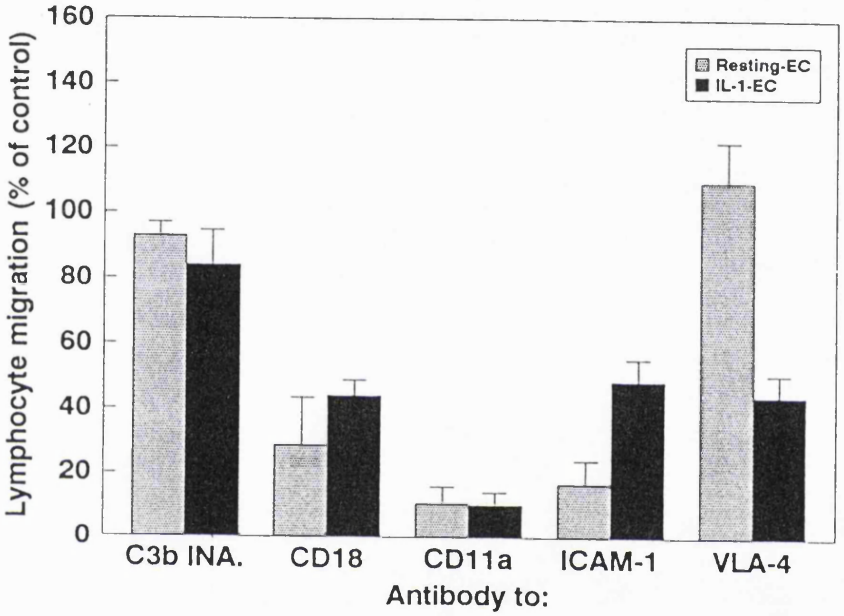

Fig.3.12. Migration of S-Ag specific T-cell line lymphocytes across resting retinal EC (shaded bars) and IL-1 activated retinal EC (solid bars) monolayers. Anti-CD11a, anti-CD18, anti-CD49d and anti-ICAM-1 mAb and anti-C3b inactivator (C3b INA.,OX-21) mAb were included in the assay system.

In this assay, it was found that WT.1 produced a greater degree of inhibition of migration than WT.3 on both resting (10.7±1.4% vs 28.7±8.7% of the control; $p<0.05$) and IL-1 activated retinal EC (10.3±3.2% vs 49.7±5.7% of the control; $p<0.001$). With resting retinal EC, IA29 caused a similar level of blocking to WT.1 (17.0±4.4% of the control; $p>0.05$), whereas following activation of the EC with IL-1, IA29 blocked migration proportionally less than with resting retinal EC (48.9±7.4% of the control; $p<0.001$). Furthermore, IL-1 activation of the EC did not significantly affect the level of inhibition caused by the mAb WT.1.

Although anti-VLA-4 mAb blocked migration across IL-1 activated retinal EC, the level of inhibition was significantly lower than that achieved with WT.1, but was comparable to that obtained with the mAb IA29 and WT.3 (Fig.3.12).

3.4. EXPRESSION OF MHC ANTIGENS ON RETINAL AND BRAIN EC

3.4.1. The expression of MHC antigens on retinal EC

Resting confluent retinal EC monolayers expressed MHC class I molecules detected by ELISA with the mAb OX-18. In contrast, there was no detectable levels of MHC class II on resting retinal EC monolayers stained with a combination of OX-6 and OX-17 antibodies. The expression of class II molecules could, however, be detected following IFN-γ activation and MHC class I antigen expression was also found to be significantly elevated by IFN-γ activation ($p<0.05$). The mAb OX-21, which was used as a control to detect non-specific binding of mouse immunoglobulin via Fc receptors, did not bind to either resting or IFN-γ (200 U/ml) treated retinal EC (Fig.3.13).

Morphological observations of MHC expression on retinal EC were carried out using indirect immunocytochemistry. As seen in Fig.3.14, resting retinal EC are strongly positive for MHC class I antigen with the fluorescence distributed across the EC monolayer. The expression of MHC class II antigen, however, was negative. Following 5 days incubation of retinal EC monolayers with 200 U/ml IFN-γ, MHC class II molecules (detected with a combination with OX-6+OX-17) could be

Expression of MHC antigens on retinal EC monolayers

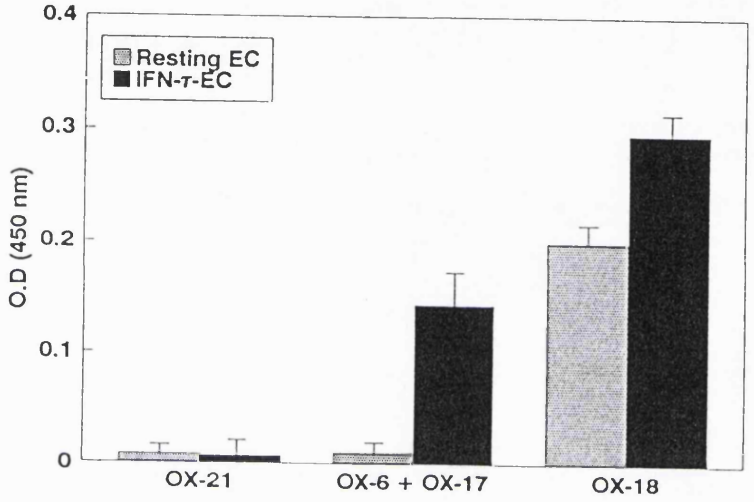

Fig.3.13. MHC antigens expressed on resting (shaded bars) and IFN-γ treated EC monolayers (200 U/ml, 5 days, solid bars) were measured using ELISA. Class I was detected with mAb OX-18, and class II with a mixture of OX-6 and OX-17. OX-21 was used as a negative control.

Immunocytochemistry staining of MHC molecules on retinal EC monolayers

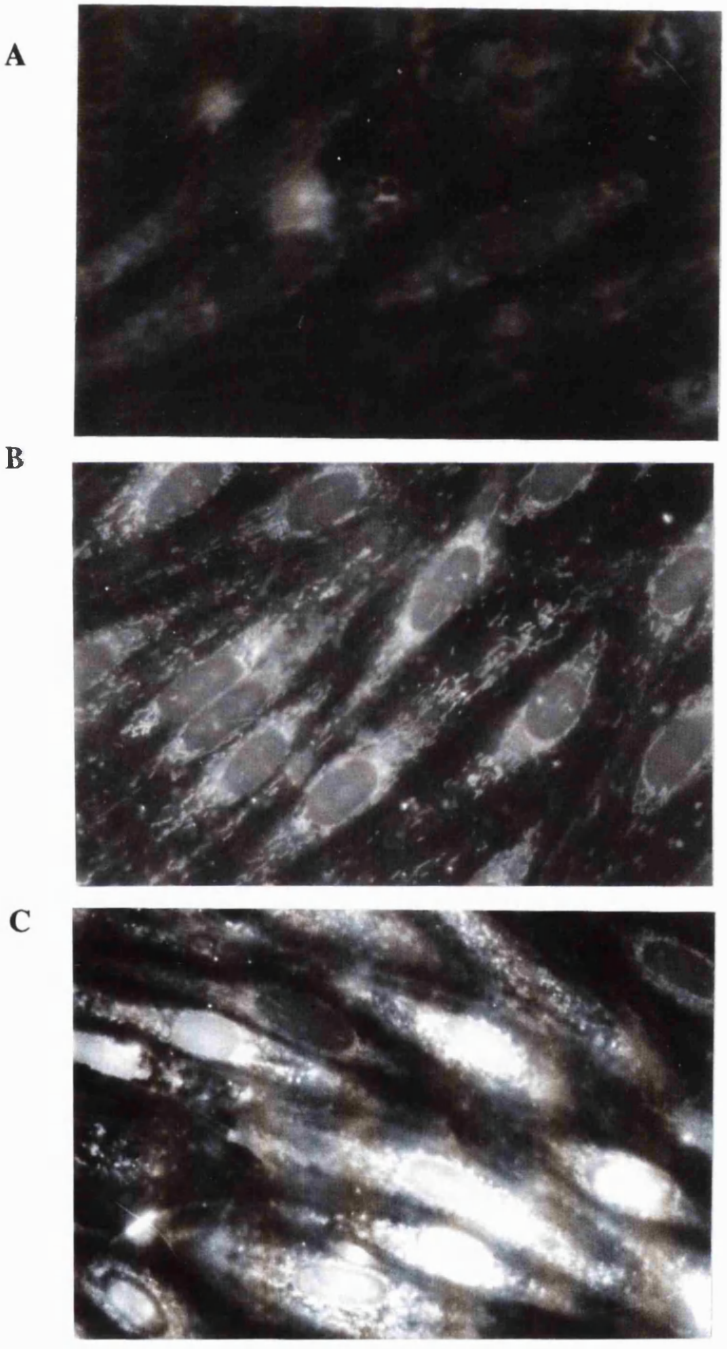

Fig.3.14. *Immunofluorescence labelling of MHC antigens on retinal EC monolayers. A. MHC class II antigens (OX-6+OX-17) on resting retinal EC; B. MHC class II on IFN-γ activated retinal EC; C. MHC class I (OX-18) expressed on resting retinal EC.*

demonstrated although the observed intensity of fluorescence of class II was much weaker than that of class I.

The capability of retinal EC to express MHC antigens in response to IFN-γ was further investigated in a dose-response and time-course study (Fig.3.15). Following 5 days treatment, the expression of MHC class I antigen was increased by as little as 10 U/ml of IFN-γ, with the levels of expression plateauing by 50 U/ml IFN-γ. The MHC class II I-A antigen was also significantly induced in a dose-dependent manner by 50 U/ml ($p<0.05$), reaching a peak at 100 U/ml although the induction of I-E was not observed even at concentrations as high as 500 U/ml IFN-γ.

The expression of MHC class I molecules significantly increased after 18 h treatment with the optimal concentration of IFN-γ (200 U/ml; Fig.3.15), and reached a plateau by 72 h. The induction of MHC class II I-A antigen could be detected after 2 days and increased up to 5 days after the retinal EC were exposed to 200 U/ml of IFN-γ. In contrast, there was no detectable expression of I-E antigen over the 5 days of the study.

In a comparative study, the expression of MHC class II molecules on macrophages was also investigated and normal residential peritoneal macrophages were found to express low levels of MHC class II antigens, both I-A and I-E. 5 U/ml of IFN-γ significantly increased the expression of I-A and I-E molecules ($p<0.05$) with no further elevation of expression with up to 50 U/ml of IFN-γ. With the optimal concentration of IFN-γ (50 U/ml), the expression of MHC class II I-A was significantly enhanced within 4 h ($p<0.05$) although the increase in I-E expression only reached significance by 16 h ($p<0.05$). The expression of both I-A and I-E peaked by 16 h after IFN-γ treatment.

3.4.2. Comparative study of MHC antigens and ICAM-1 on retinal and brain EC

In order to investigate the different patterns of surface antigens expressed on individual retinal and brain EC in response to the cytokine IFN-γ, the cells were analyzed by flow cytometry following indirect immunofluorescence staining with OX-18, OX-6, OX-17 and IA29 (Fig.3.17 and 3.18). The results show that the majority

Expression of MHC molecules on retinal EC

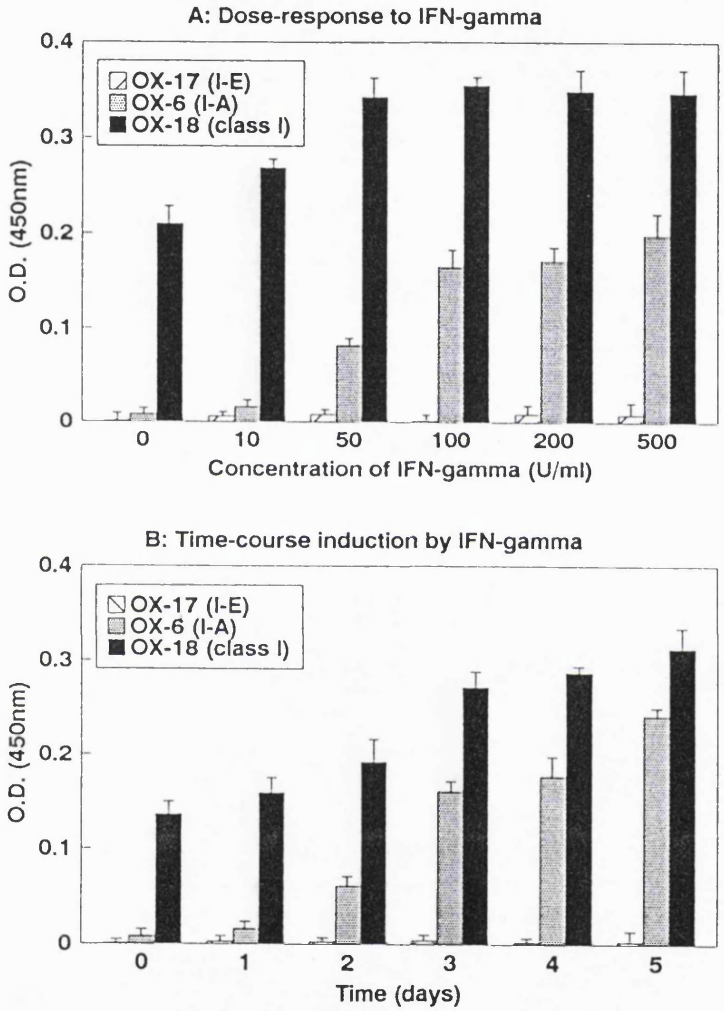

Fig.3.15. MHC molecule expression on retinal EC cultured in 96-well plates measured by ELISA. In the dose-response assay, MHC antigens were measured following the treatment of the EC for 5 days with various doses of IFN-γ (A); The time-course induction was carried out by treating EC for 0-5 days with 200 U/ml of IFN-γ (B). The O.D. levels shown were subtracted by the O.D. levels of the control mAb (OX-21)

Expression of MHC class II molecules on macrophages

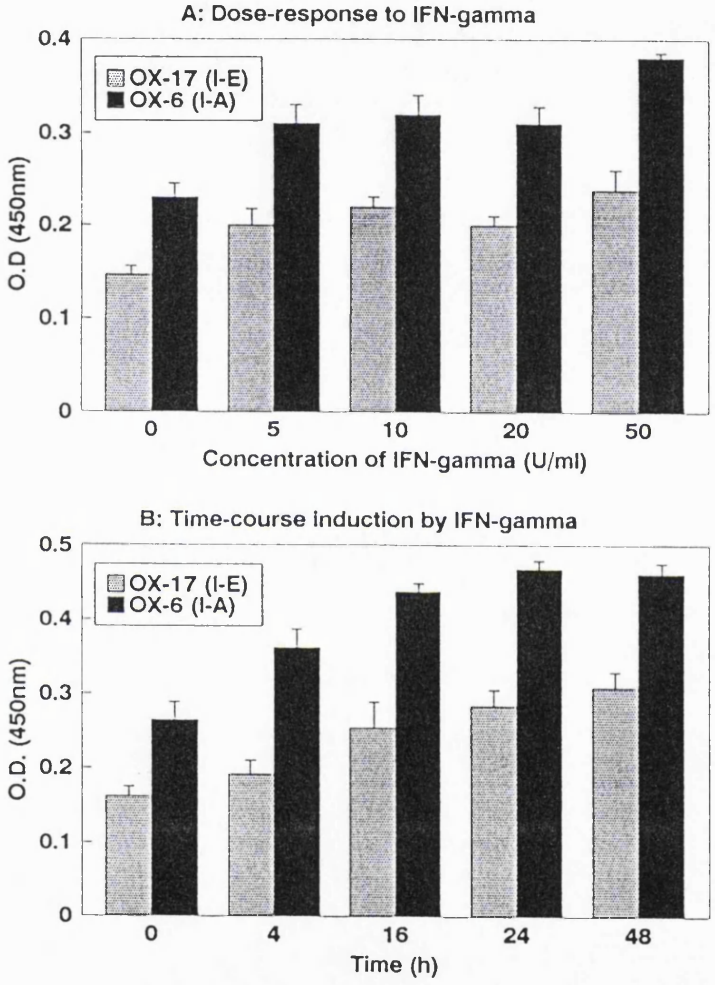

Fig.3.16. MHC class II expression on peritoneal macrophages was detected using ELISA following treatment with IFN-γ. The dose-response of MHC expression was assayed by treating macrophages for 24 h with 0-50 U/ml of IFN-γ (A); In the time-course study cells were treated for 0-48 with 10 U/ml of IFN-γ (B)

of cells in the retinal EC suspension prepared by collagenase/dispase dissociation expressed MHC class I molecules constitutively (90.43±5.3%) with slightly fewer of these cells expressing ICAM-1 (60.84±6.6%). In contrast, resting retinal EC were not found to express significant levels of class II I-A or I-E molecules.

Following incubation of retinal EC with 200 U/ml of IFN-γ, the fluorescence intensity of MHC class I staining was elevated after 18 h although the percentage of positive cells was not significantly increased (Tab.3.5). After 3 and 5 days treatment a further increase of MHC class I molecules was not observed. The expression of ICAM-1 was also significantly increased after 18 h of incubation (86.14±3.9%; $p<0.001$) although there was no further significant increase in the percentage of expression by days 3 and 5 (Tab. 3.6). However, it was observed that the intensity of staining was further elevated by day 5 following treatment. A significant expression of MHC class II I-A molecules was found at day 3 following incubation with IFN-γ (23.39±4.3%) with a further significant increase in the expression by day 5 (42.94±6.9%), whereas the expression of I-E molecules was minimal and unaffected by IFN-γ.

Resting brain EC were also found to constitutively express MHC class I molecules (83.23±7.3%) and ICAM-1 (66.86±7.3%), but not MHC class II molecules being similar to retinal EC. Treatment of brain EC for 18 h with 200 U/ml of IFN-γ increased the expression of MHC class I molecules by shifting the intensity of fluorescence but was not further elevated by days 3 or 5 of IFN-γ activation. The increased expression of ICAM-1 could be seen within 18 h of treatment with IFN-γ (81.3±2.5%; $p<0.001$) whereas no further significant increase in expression was observed at days 3 and 5. A significant expression of MHC class II I-A molecules on brain EC could be seen after 3 days IFN-γ treatment (13.28±4.7%) which was maintained up to day 5 (19.09±3.8%). By day 5 a low level of I-E expression was found on brain EC (11.25±5.7%), but this was not significantly different from non-treated and 3 days IFN-γ treated EC ($p>0.05$). It was notable that the levels of MHC class II I-A molecule expression following IFN-γ treatment of brain EC was significantly lower than on IFN-γ treated retinal EC ($p<0.05$).

Flow cytometric analysis of MHC antigen expression on retinal and brain EC

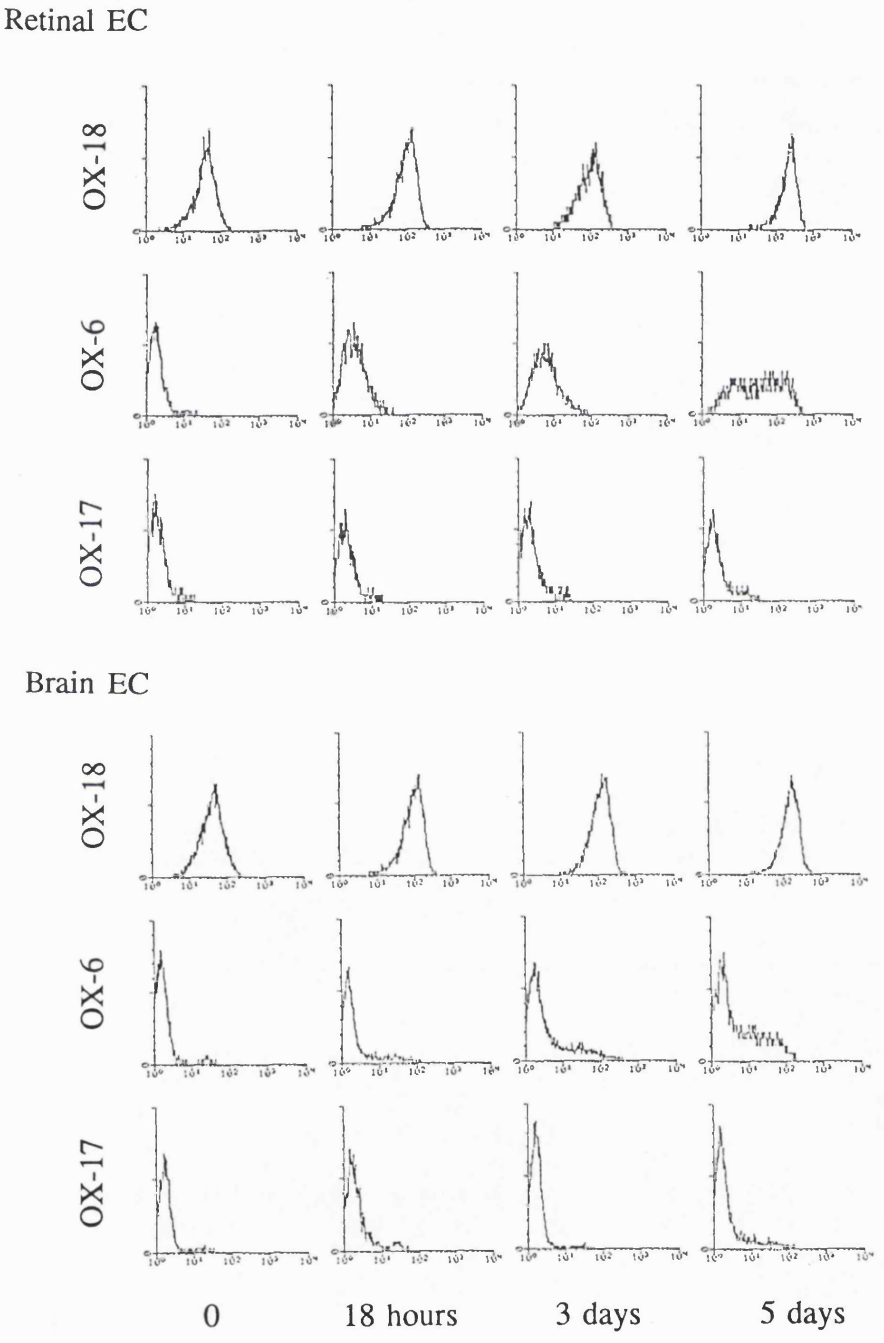

Fig.3.17. *Expression of MHC molecules on retinal and brain EC analyzed by flow cytometry. The single cell suspension was prepared following 0-5 days treatment of the EC monolayers with 200 U/ml IFN-γ.*

ICAM-1 expression on retinal and brain EC.

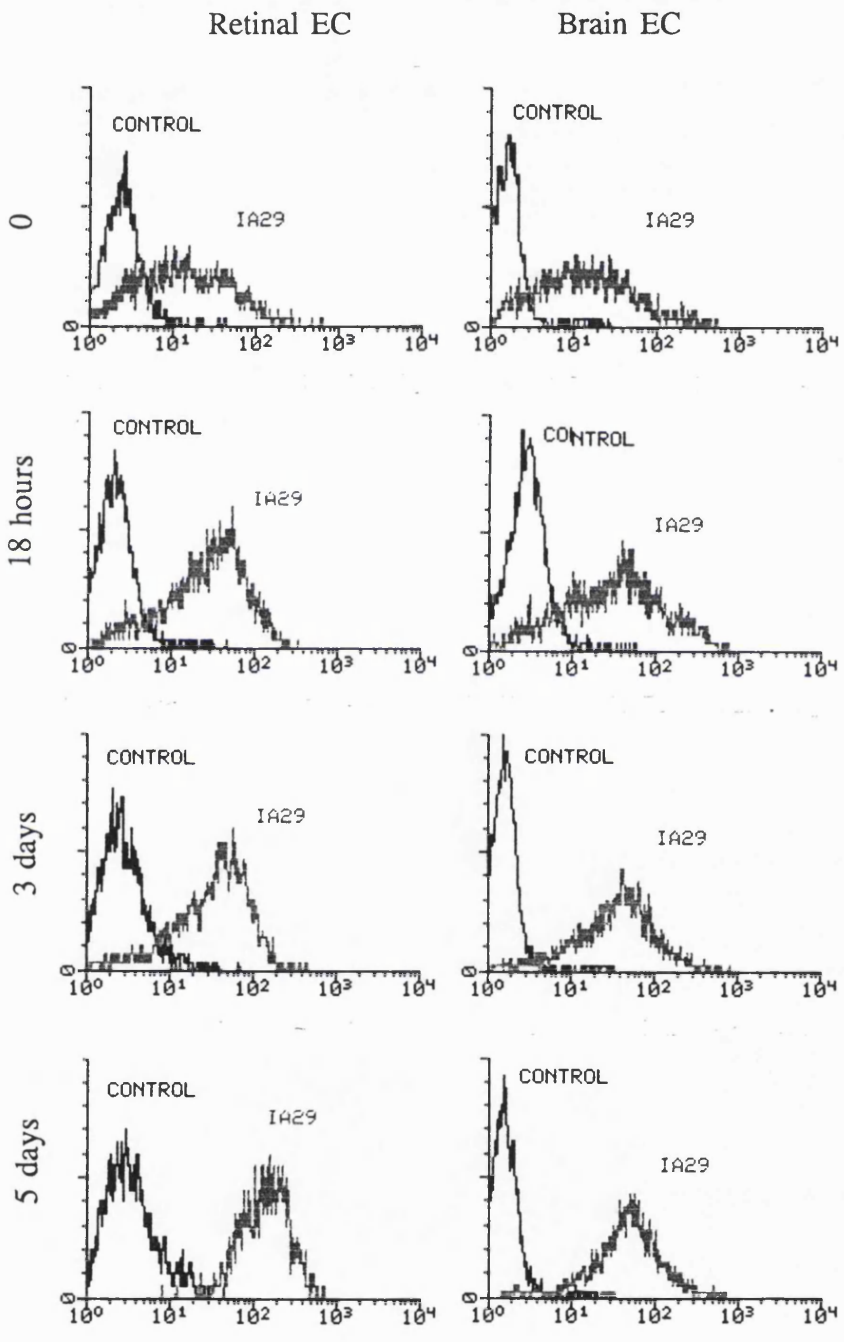

Fig.3.18. Retinal and brain EC monolayers were treated for 0-5 days with 200 U/ml IFN-γ prior to dissociation. ICAM-1 was detected with the anti-ICAM-1 mAb, IA-29 and analyzed by flow cytometry.

Table 3.5

Expression of MHC molecules on retinal and brain EC (%)

	OX-18		OX-6		OX-17	
	Retina	Brain	Retina	Brain	Retina	Brain
0	90.3±5.3	83.4±7.3	1.9±1.8	0.4±0.1	1.1±0.7	5.5±1.6
18 h	97.1±2.0	97.7±3.0	6.2±1.6	2.6±0.1	4.0±2.7	5.2±0.2
3days	96.0±3.4	96,5±4.1	23.3±4.3	13.2±4.7	5.8±1.1	6.8±3.1
5days	97.3±3.8	91.2±4.8	42.9±6.9	19.0±3.8	4.6±3.3	11.3±6.1

Percentages of MHC molecules on retinal and brain EC analyzed by flow cytometry following dissociation of the EC monolayers. Prior to the MHC assay, the EC monolayers were treated for 0-5 days with 200 U/ml of IFN-γ. Data given as means±SD.

Table 3.6

Expression of ICAM-1 on retinal EC and brain EC (%)

	Retinal EC	Brain EC
0	60.8± 6.6	66.8±9.7
18 h	86.1± 3.9	81.2±2.5
3 days	86.9± 4.5	83.7±2.5
5 days	93.9± 2.9	86.1±6.1

Percentages of ICAM-1 expression on retinal and brain EC were analyzed by flow cytometry following 0-5 days treatment of the EC monolayers with 200 U/ml of IFN-γ. ICAM-1 expression was detected with the mAb IA-29 following dissociation of the EC monolayer. Data given as means±SD.

3.5. ANTIGEN PRESENTATION BY RETINAL AND BRAIN EC

3.5.1. Support of T-cell proliferation by retinal and brain EC

The ability of retinal EC to present antigen to specific syngeneic $CD4^+$ T-cell line lymphocytes was investigated. T-cell lines ($2x10^4$ cells/well), derived from Lewis rats and specific for S-Ag, were incubated with retinal EC in the presence of either

S-Ag or mitogens. In the proliferation assay, confluent retinal EC monolayers were induced to express MHC class II molecules by treatment for 3 days with 200 U/ml IFN-γ prior to overlaying with T cells (Fig.3.19). The results demonstrated that under such conditions, confluent retinal EC monolayers were a poor stimulator of S-Ag specific $CD4^+$ T-cell proliferation in the presence of a range of concentrations of S-Ag (5-20 μg/ml) or mitogens. The levels of ^{3}H-thymidine incorporation by lymphocytes were generally less than 2000 cpm. The inclusion of indomethacin (5 μg/ml) in the coculture system to inhibit retinal EC producing eicosanoid only increased lymphocyte proliferation marginally.

A significantly greater increase in T-cell proliferation, however, was achieved when subconfluent retinal EC monolayers were used as the APC. The results (Fig.3.20) show that the magnitude of T-cell proliferation was related to the number of the EC per well with low numbers or confluent monolayers being poor stimulators of T-cell proliferation. Maximal T-cell proliferation was observed when retinal EC numbers were $5x10^4$, giving a T cell to EC ratio of 2:5. Maximal ^{3}H-thymidine incorporation was significantly higher than the controls ($p<0.001$), or than with either low numbers of EC ($5x10^3$, $p<0.001$) or at confluence ($>10^5$, $p<0.001$)

Brain EC were also able to present antigen to S-Ag specific T-cell lines and elicit a proliferative response in a manner similar to retinal EC (Fig.3.20). The magnitude of T-cell proliferation was thus found to be dependent on brain EC concentrations with low (less than $1x10^4$) or confluent EC monolayers ($>10^5$) being poor stimulators of T-cell proliferation. Maximal proliferation was also achieved with $5x10^4$ EC cells per well, in a T cell to EC ratio of 2:5.

3.5.2. Stimulation of T-cell proliferation by resting and IFN-γ activated retinal EC

Resting retinal EC were also found to stimulate significant T-cell proliferation in a ratio of T cells to EC of 2:5 retinal EC. They stimulated T-cell proliferation to the same degree as IFN-γ activated EC in the presence of range doses of S-Ag (5-20 μg/ml). The mitogens both Con A and PHA were also found to cause a similar proliferative response between resting and IFN-γ activated retinal EC (Fig.3.21).

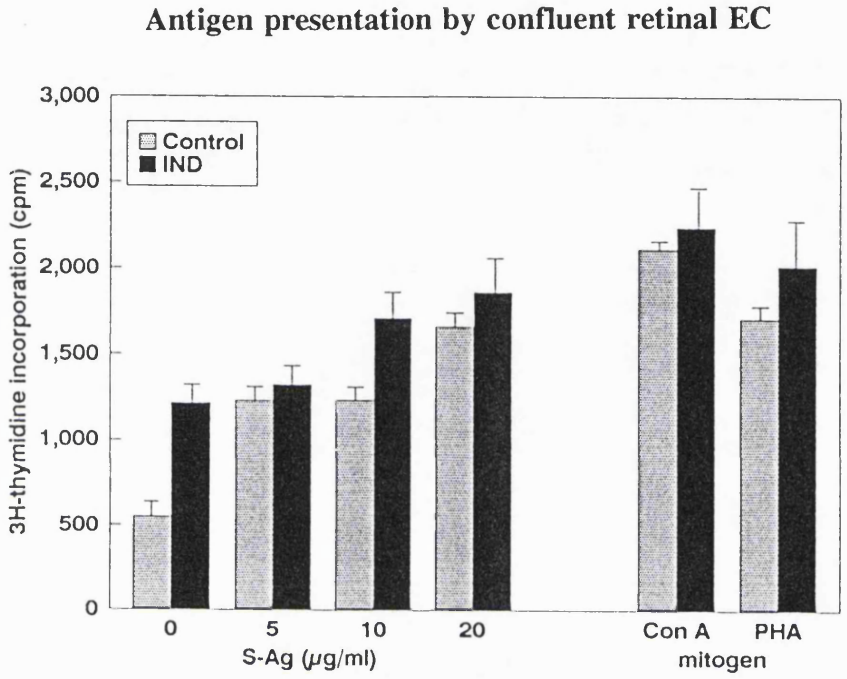

Fig.3.19. Stimulation of T-cell proliferation by confluent retinal EC monolayers was assayed by coculturing 20,000 S-Ag specific CD4$^+$ T-cell line lymphocytes with IFN-γ pretreated confluent EC monolayers. A range of doses of S-Ag, Con A (5μg/ml) and PHA (10μg/ml) were included in the cultures (shaded bars). In order to prevent eicosanoid production, 5 μg/ml of indomethacin (IND, solid bars) was added. T-cell proliferation was measured by ^{3}H-thymidine incorporation.

Antigen presentation by retinal and brain EC

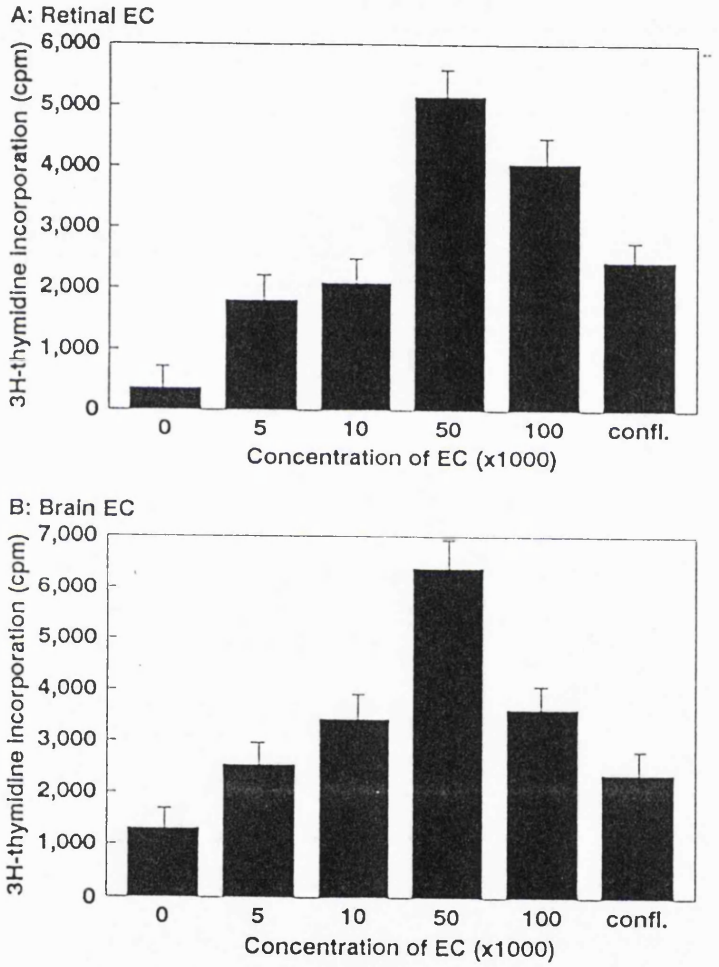

Fig.3.20. Various numbers of the EC were plated onto 96 well plates, and cocultured with 20,000 S-Ag specific CD4$^+$ T-cells per well in the presence of 10 μg/ml S-Ag. T-cell proliferation was measured by ^{3}H-thymidine incorporation. A, retinal EC; B, brain EC

In order to determine whether MHC class II molecules play a role in antigen specific T-cell proliferation stimulated by both resting and IFN-γ activated retinal EC, we attempted to block T-cell proliferation with anti-I-A and anti-I-E mAb (Tab.3.7). The results show that T-cell proliferation stimulated by either non-activated or IFN-γ activated retinal EC were MHC class II I-A restricted. Treatment of the EC with anti-I-A (OX-6) significantly inhibited S-Ag specific T-cell proliferation. In contrast, anti-I-E (OX-17) failed to block the induction of T-cell proliferation by both resting and IFN-γ activated EC.

Table 3.7

Inhibition of T-cell proliferation by anti-MHC class II I-A mAb

	Resting EC	IFN-γ-EC
Control	5732±517	5467±367
OX-6(I-A)	943±119	1037±395
OX-17(I-E)	5516±282	5174±319

Antigen presentation by retinal EC in the presence of the mAb OX-6 (anti-I-A mAb) or OX-17 (anti-I-E mAb). T-cell proliferation was stimulated with 10 μg/ml S-Ag and was measured by ^{3}H-thymidine incorporation.

3.5.3. Stimulation of T-cell proliferation by splenic adherent cells and thymocytes.

In parallel experiments we also used professional APC (thymocytes and splenic adherent cells) to stimulate S-Ag specific T-cell proliferation and to compare them with retinal and brain EC. In those experiments in which an optimal number of irradiated thymocytes and splenic adherent cells were used to stimulate S-Ag specific T-cell proliferation in the presence of a range of S-Ag concentrations (5-20 μg/ml), it was found that the levels of T-cell proliferation stimulated by splenic adherent cell were comparable to that obtained with retinal EC and brain EC (Fig.3.22). Thymocytes stimulated T-cell proliferation, however, to a much higher degree than either the EC or the splenic adherent cells (Fig.3.22). The levels of proliferation stimulated by thymocytes reached up to 20,000 cpm, giving a 10 fold proliferation.

Antigen presentation by resting and IFN-γ activated EC

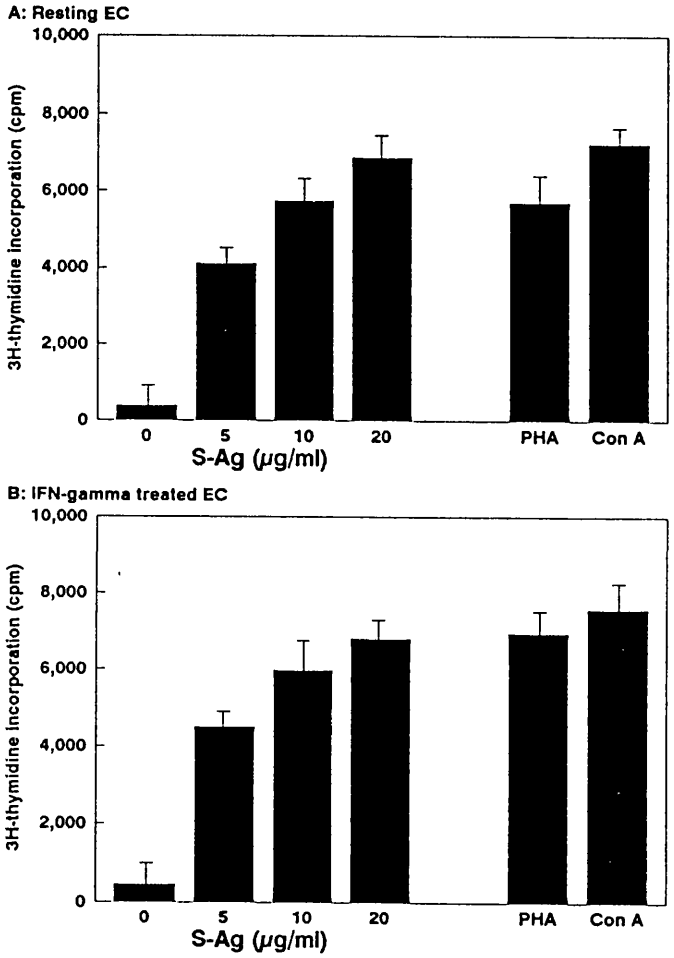

Fig.3.21. Subconfluent retinal EC (5x10⁴/well), resting (MHC class II negative EC) or pre-treated for 3 days with 200 U/ml IFN-γ (MHC class II positive EC), were cultured with 20,000 S-Ag specific CD4⁺ T cells in the presence of S-Ag or mitogens. T-cell proliferation was measured by ³H-thymidine incorporation.

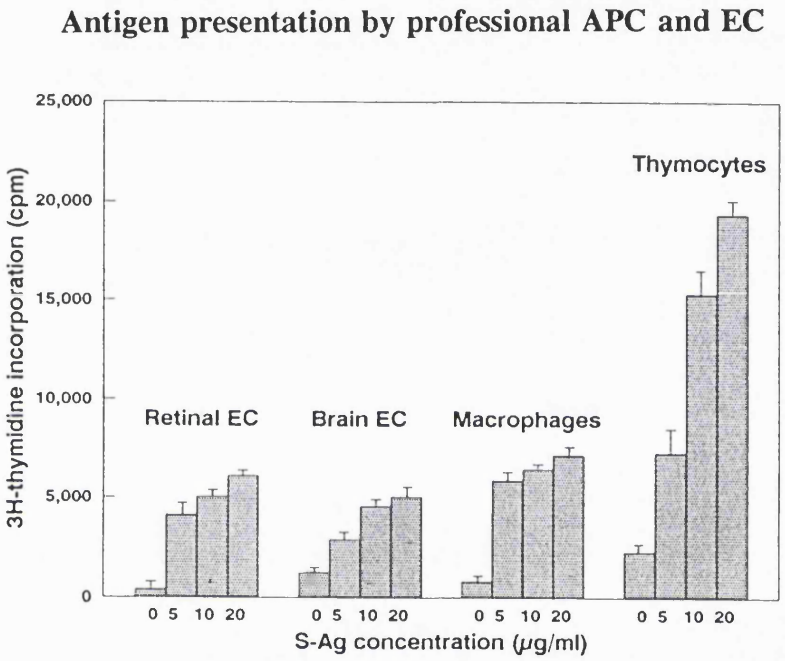

Fig.3.22. Retinal EC, brain EC, splenic adherent cells (enrichment of macrophages) and thymocytes were cultured with 20,000 T cells per well in the presence of a range of doses of S-Ag (5-20 μg/well). T-cell proliferation was measured by ^{3}H-thymidine incorporation.

3.5.4. T-cell IL-2 production

S-Ag specific T-cell lines were cocultured with either confluent ($>1x10^5$ cells/well) or subconfluent retinal EC ($5x10^4$/well). In this coculture, T cells were stimulated for 3 days with either 10 μg/ml of S-Ag or 10 μg/ml of PHA, and IL-2 production and T-cell proliferation was subsequently measured with the IL-2/IL-4 response HT2 cell line. Confirmation that the HT-2 cell line response was due to an effect of IL-2 and not IL-4 production was provided by successful blocking of the proliferative response with the IL-2 receptor antibody (see methodology, Fig.2.3). The results (Fig.3.23) show that in the presence of confluent EC, T cells produced considerably more IL-2 ($p<0.05$) than T cells cultured alone although there was no significant T-cell proliferative response (Fig.3.23). However, when S-Ag specific T-cell line lymphocytes were cocultured with subconfluent retinal EC, their IL-2 production was significantly higher than with T cells cultured with confluent retinal EC ($p<0.001$), and T cells also showed significant proliferation ($P<0.001$)

3.5.5. The role of TGF-ß

One possible reason that confluent EC were poor stimulators of T-cell proliferation could be the presence of TGF-ß produced by EC. We therefore examined the ability of confluent retinal EC to produce TGF-ß. By using ELISA, we found that there were detectable levels of TGF-ß in the EC supernatants. The amount of TGF-ß produced by EC increased with time reaching levels of between 10-15 ng/ml in 48 h. Stimulation of EC with LPS led to an increase in TGF-ß production over resting EC (Fig.3.24).

Following this finding we attempted to neutralize TGF-ß in the antigen presentation assay with a neutralizing mAb to TGF-ß. The results showed that the addition of the anti-TGF-ß mAb did not effect T-cell IL-2 production or proliferation stimulated by confluent retinal EC. However, the presence of anti-TGF-ß mAb significantly inhibited subconfluent retinal EC stimulation of both T-cell IL-2 production ($p<0.01$) and proliferation ($p<0.001$; Fig.3.23). Conversely, the addition of exogenous TGF-ß did not significantly affect these parameters with either confluent or subconfluent EC.

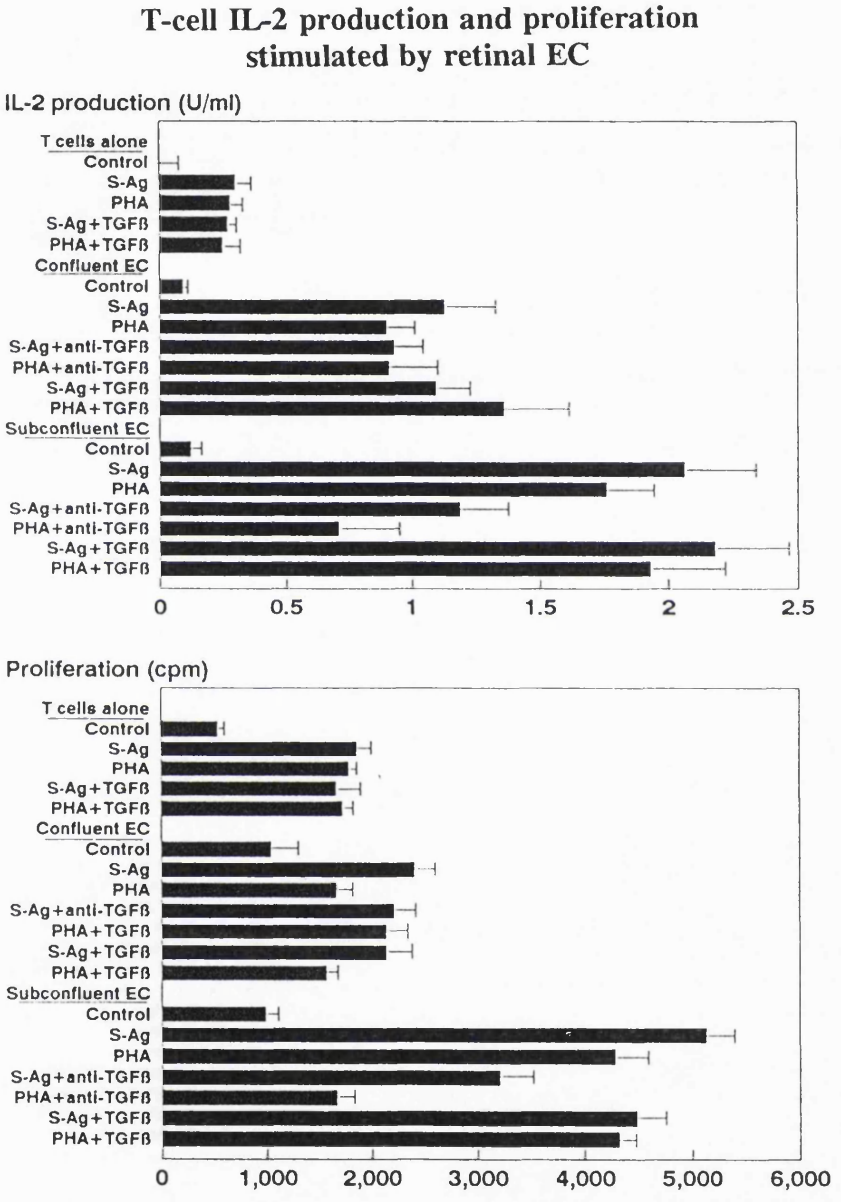

Fig.3.23. Stimulation of S-Ag specific $CD4^+$ T-cell proliferation and IL-2 production by subconfluent ($5x10^4$ cells/well) and confluent ($>10^5$ cells/well) EC in the presence of either S-Ag (10 µg/ml) or PHA (10 µg/ml). The effect of 10 ng/ml of TGF-ß or anti-TGF-ß mAb (5µg/ml) in the wells was investigated

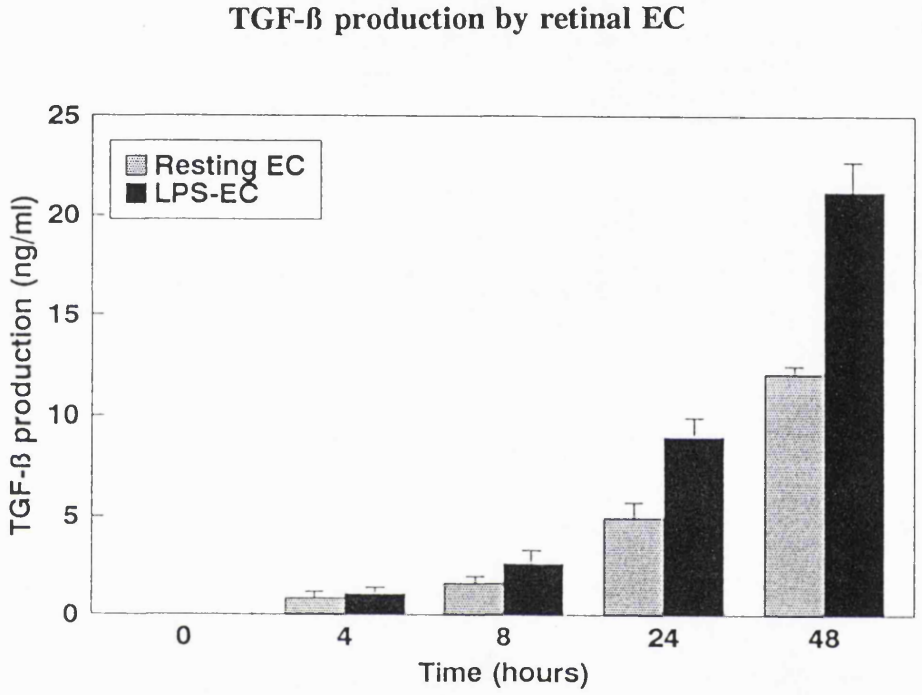

Fig.3.24. TGF-β production by confluent retinal EC monolayers was measured by ELISA. Retinal EC were treated with or without LPS (10 ng/ml), and the supernatants of 0-48 h culture taken for the assay

3.5.6. Cytotoxicity of retinal EC monolayers by S-Ag specific T-cell lines

The cytotoxicity of cultured retinal EC monolayers was carried out by co-culturing IFN-γ activated retinal EC and S-Ag-specific T-cell line lymphocytes. The antigen specific T-cell lines were maintained for 7-10 days in IL-2 medium prior to the cytotoxicity assay. When the EC monolayers were cocultured with these T-cell lines in the absence of S-Ag, the monolayers appeared morphologically normal as observed on an inverse-phase contrast microscope (Fig.3.25a). Cytotoxic cell damage to the EC monolayers could be seen when S-Ag was included in the coculture system (Fig.3.25b) and this effect often resulted in the breakup of the intact retinal EC monolayers. The morphology of the remaining retinal EC appeared to be normal.

In the quantitative assay of cytotoxicity using ^{51}Cr labelled EC it was found that there was a low level of ^{51}Cr release (approximately 15%) after 18 coculture of S-Ag specific T-cell lines with retinal EC although apparent morphological disruption of the EC monolayers was not observed. Inclusion of S-Ag or Con A in the coculture significantly increased the level (in the region of 40%) of specific ^{51}Cr release ($p<0.01$), whereas the presence of an irrelevant antigen (BSA) did not result in an increase in cytotoxicity of retinal EC caused by S-Ag specific T-cell lines ($p>0.05$, Fig.3.26).

S-Ag specific CD4$^+$ T-cell cytotoxicity to retinal EC monolayers

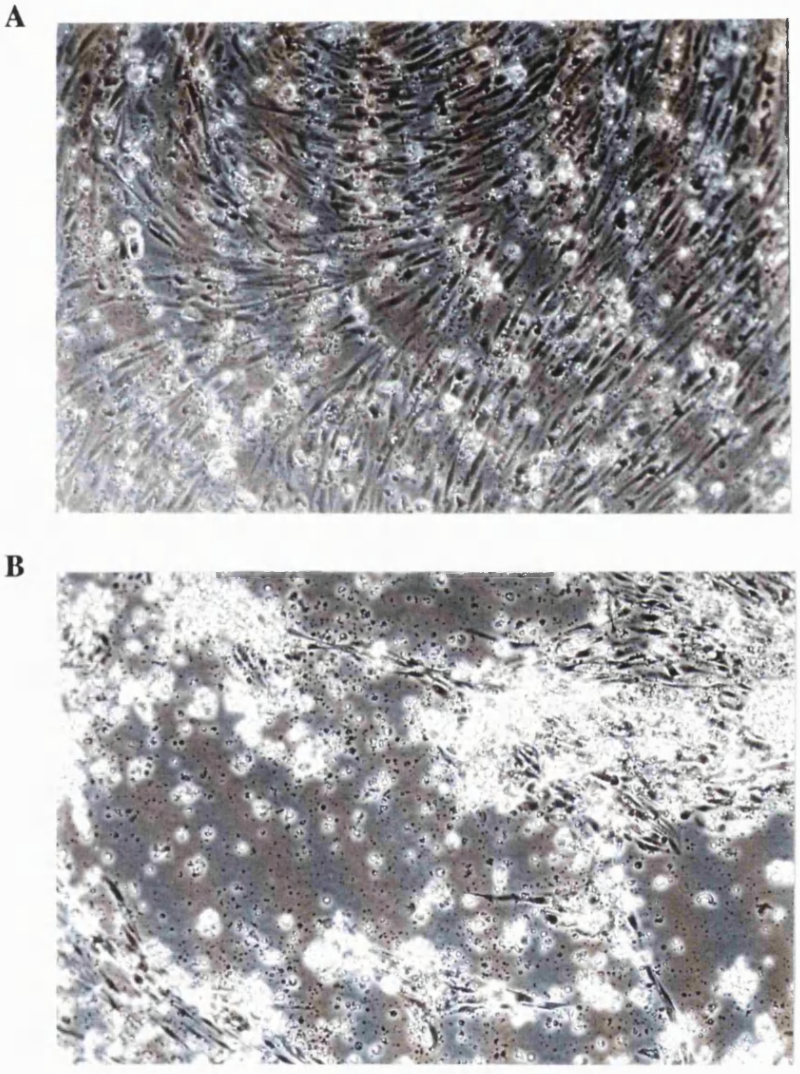

Fig.3.25. *Confluent EC monolayers pre-treated for 3 days with 200 U/ml IFN-γ were cocultured for 18 h with 1x10^6 S-Ag specific T-cells in the absence (A) or presence (B) of S-Ag (10 μg/ml).*

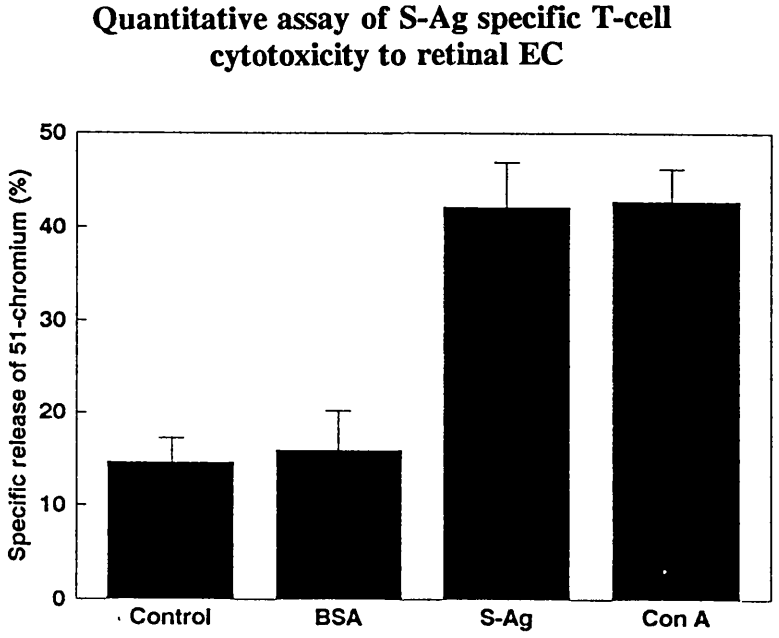

Fig.3.26. Retinal EC monolayers, following 3 day treatment with 200 U/ml of IFN-γ, were cocultured for 18 h with $2x10^5$ S-Ag specific T-cells. 10 μg/ml S-Ag, 5 μg/ml Con A and an irrelevant antigen (10 μg/ml BSA) were included. Cytotoxicity was measured by specific ^{51}Cr release.

CHAPTER 4
GENERAL DISCUSSION

Investigations into the pathogenesis of inflammatory reactions of the CNS have indicated that direct interactions between cellular elements of the CNS and immune system are important in the induction of CNS inflammatory diseases. Of these interactions, antigen presentation to T cells within the CNS has been suggested to be a key step. Previous studies have suggested that microglia and astrocytes may play an early part role in the development of the immune response by presenting antigen to T cells under pathogenic conditions. Within the retina RPE and Müller cells have also been implicated in the development of inflammatory conditions of the eye. The anatomical arrangement, however, determines that lymphocytes must interact with CNS vascular EC prior to the development of immune reactions within the CNS parenchyma, and that this interaction apparently controls the trafficking of T cells into the CNS. In addition, the vascular EC of the CNS may also play a role as APC and thus in lymphocyte activation within the retina and brain.

In this *in vitro* study, we have demonstrated that activation of rat lymphocytes and retinal EC are important for both lymphocyte adhesion and migration, which are essential steps in lymphocyte trafficking into the retina. Adhesion molecules expressed on lymphocytes and retinal EC have been shown to support both lymphocyte adhesion and lymphocyte migration. Furthermore, retinal and brain EC can play a role as APC causing antigenic activation of antigen specific T cells.

4.1. CULTURE OF RETINAL EC

Over the past decade cultures of HUVEC have provided a reliable and convenient source for investigating the cellular interactions between lymphocytes and EC, and are believed to be a useful model of lymphocyte-EC interactions occurring *in vivo* at inflammatory sites (Cavender 1989). However, it is known that microvascular EC are the major site of lymphocyte emigration *in vivo* under inflammatory conditions, and recent studies have revealed that these EC are different

from large vascular EC in their expression of surface antigens, such as adhesion molecules (Page et al., 1992; Steinhoff et al., 1993) and in their cytokine responsiveness (Hauser et al., 1993; Petzelbauer et al., 1993). Furthermore, it is well known that CNS microvascular EC are significantly different from non-CNS vascular EC, in that cerebral and retinal EC possess a number of specialized characteristics. It is also believed that there is a degree of heterogeneity between CNS and non-CNS vascular EC in their interactions with immune component cells and in their response to inflammatory factors, such as cytokines (Male et al., 1990b). Thus, investigation into the interactions between lymphocytes and EC derived from the CNS is necessary for understanding of the role of the EC in the development of the immune response within these organs.

In contrast to cultures of HUVEC, isolation and culture of microvessel EC from sites, such as the retina and brain has been far less reported. Despite some reports of culturing brain EC there are very few studies in which retinal EC have been cultured. In this study, using a modified method of that used to culture brain EC (Hughes and Lantos 1986), we isolated rat retinal microvessels and cultured retinal EC of high purity which were identical to cultured brain EC with typical spindle-shape morphology and exhibiting contact inhibition (Fig.3.1).

By using cultured retinal EC, it is possible to conduct detailed investigations into their interactions with lymphocytes which would otherwise be difficult to implement *in vivo*. Despite the obvious advantages of using *in vitro* preparations, the stability of the CNS vascular EC phenotype in culture has been questioned. Recently, however, several studies have shown that by using primary cultures, by coculturing with astrocytes, or by using ACM, many of the *in vivo* characteristics of these EC can be maintained *in vitro* (Rubin et al., 1991, Greenwood, 1991). In our experiments, we have used primary cultures of EC grown from retinal microvessels that have previously been demonstrated to express tight junction-associated polypeptide (ZO-1), the transferrin receptor and P-glycoprotein (Greenwood 1992), indicating that these primary cultures maintain some major *in vivo* characterization.

The purity of these primary culture has also been assessed by demonstrating the expression of von Willebrand factor (factor VIII related receptor) which is widely

used as a marker of EC derived from different sites including the retina (Greenwood 1992), but excluding high endothelial venule EC (HEV, Duijvestijn et al., 1992). Due to the intracellular location of this specific protein marker, however, von Willebrand factor cannot be used for identifying living EC in culture.

The binding of griffonia (formly Bandeiraea) simplifolia agglutinin (GSA), a lectin with high affinity for α-D-galactopyranosyl residues on the cell surface has been shown to be a specific histochemical surface marker for EC from selected organs including mouse brain (Alroy et al., 1987; Fetehi et al., 1987; Sahagun et al., 1989). In this study we found that 95% of cultured EC stained positively for GSA-FITC by flow cytometry. This result is consistent with a previous report in which the purity of the retinal EC was determined morphologically in the same system (Greenwood 1992) and indicates that binding of GSA can be used as a surface marker to identify cultures of rat retinal EC.

The high purity of cultured retinal EC was further confirmed by staining with the mAb RECA-1 (Fig.3.2), a specific mAb reacting with the surface protein (RECA-1 antigen) on all rat vascular EC, but not other species. Although the functions and properties of this protein are largely unknown, RECA-1 antigen was shown to be an enzyme collagenase and trypsin resistant (Duilvestijn et al., 1992). Thus, RECA-1 offers possibilities for identification and purification of retinal EC by techniques such as analysis by fluorescence flow cytometry.

A major problem associated with using primary cultures is the difficulty in obtaining 100% purity of cells. In previous studies, mechanical methods were used to remove contaminating cells which usually could damage retinal EC monolayers of the primary cultures (Buzney et al., 1983). In this study, in order to minimise the growth of other cells, the microvessel isolation procedure employed a two step enzyme digestion which restricts the number of non-EC growing in culture (Hughes and Lantos 1986). Moreover, by using a culture medium containing PDS instead of FCS to supplement the medium, EC growth are promoted whilst inhibiting the growth of contaminating cells such as pericytes (Vogel et al., 1978). By using these procedures a high degree of purity (95%) of primary cultures of retinal EC can be achieved.

Few contaminating cells in primary cultures of EC isolated from the retinal microvessels appeared to be mainly pericytes (5%) although other cell types such as astrocytes, smooth muscle cells and fibroblasts could also be potential contaminating cells. Pericytes could be distinguished by their typical morphology being flat and spread with many processes and no contact inhibition as well as expressing the Thy1.1 antigen (Risau et al., 1990).

Cultures of EC may also be contaminated by MHC class II positive cells, such as dendritic cells, and this has been suggested as an important factor in the apparent EC induction of T-cell proliferation (Nunez et al., 1983). The possibility that primary cultures of retinal EC can be contaminated by macrophages and dendritic cells exists, as these cells are present in choroidal tissues (Forrester et al., 1994). However, in primary cultures of retinal EC we demonstrated that neither macrophages nor dendritic cells were present in detectable numbers.

OX-22 positive cells detected in a proportion of cultured retinal EC was unexpected. OX-22 recognizes the leucocyte common antigen (LCA) expressed on both T cells and B cells, and is also used to characterise a subset of $CD4^+$ T cells (assumed to be ''memory'' cells; $CD4^+OX\text{-}22^{low}$). In this study we found that about 20% of retinal EC appeared to be positive for OX-22 staining. This may be due to EC sharing a common embryonic heritage (mesoderm) with bone marrow derived cells although the significance of this expression on cultured retinal EC requires further investigation.

4.2. LYMPHOCYTE ADHESION TO RETINAL EC

The *in vitro* assay of lymphocyte adhesion to a cultured EC monolayer has previously been described for investigating lymphocyte trafficking into a number of different tissues. The adhesion assay measures the proportion of settled lymphocytes that are able to withstand an applied sheer force and it is assumed that this reflects the strength of the adhesive interactions between lymphocytes and EC, and therefore the probability of adhesion occurring *in vivo*.

By using a similar adhesion assay method, basal levels of lymphocyte binding

have been found to be largely dependent on the source of the EC although these levels could vary between individual investigators. Under normal conditions, levels of lymphocyte adhesion to HUVEC were reported to be between 20-40% (Cavender et al., 1986; Yu et al., 1985), whereas lymphocytes adhered to brain EC to a lesser degree with binding at around 5% (Male et al., 1990a). We found that the basal levels of lymphocyte adhesion to retinal EC were similar to that reported for lymphocyte adhesion to brain EC. This low level of lymphocyte adhesion was found consistently throughout all experiments of the adhesion assay, and it was also not affected by prolonging the cellular contact between lymphocytes and retinal EC (Fig.3.5). Since we employed the same method as used in previous studies on HUVEC and brain EC for the adhesion assay, the difference between lymphocyte adhesion to CNS and non-CNS vascular EC probably reflects the special properties of the former which are thought to restrict lymphocyte traffic into the CNS. The molecular mechanisms responsible for this low level of lymphocyte binding to CNS vascular EC may be reduced expression of adhesion molecules (Lassmann et al., 1991) which thus limit the ability of the EC to bind lymphocytes.

The basal level of adhesion, however, could be rapidly upregulated when lymphocytes were activated with the mitogen Con A and retinal EC with the cytokine IFN-γ. With Con A the significant increase in adhesion could be detected by 15 min after the addition of lymphocytes to the EC monolayers although with IFN-γ activation the increase in adhesion was significant after 1 hour of coculture. The rapid increased adhesion found with Con A activated lymphocytes (Fig.3.5) suggests that Con A activation increased not only the number of lymphocytes that are capable of adhering to the EC but also their binding avidity.

The number of adherent cells can also be upregulated by increasing the number of lymphocytes applied (Fig.3.6). This effect of lymphocyte number on adhesion may reflect unsaturated binding sites for lymphocytes on the EC monolayers. In the adhesion assay it was observed that lymphocytes at concentrations of less than $2x10^6$ per well in 96-well plates do not completely cover the endothelial monolayers. Therefore, an increase in the number of lymphocytes can cause more cells to come into direct contact with the EC monolayer and thus may increase the

number of adherent cells. There is also the possibility of lymphocyte migration across the EC monolayers which could vacate the binding sites on the upper surface of EC monolayers allowing re-occupation by other lymphocytes as migrated lymphocytes rarely return to the surface (Hourihan et al., 1993; Greenwood and Calder 1993). As a result of this, the total number of adherent cells could be increased following increased numbers of lymphocytes plated onto the EC monolayers. Furthermore, a larger population of lymphocytes would also contain proportionally more activated lymphocytes which may be sufficient to activate the retinal EC via the release of the cytokines thus further increasing adhesion.

4.3. CONTROL OF LYMPHOCYTE ADHESION TO RETINAL EC

4.3.1. Control by retinal EC

A. The cytokines

Activation of EC with cytokines *in vitro* and thereby upregulation their ability to bind lymphocytes have been demonstrated on HUVEC (Yu et al., 1985; Cavender et al., 1986, 1987), non-CNS microvascular EC (Haskard et al., 1987) and cerebral EC (Hughes et al., 1988). In this study, cultured retinal EC have also been shown to respond to the cytokines IL-1, IL-4 and IFN-γ by increasing their adhesion for lymphocytes above resting levels. This effect occurred at relatively low doses of the cytokines (Fig.3.7) indicating that retinal EC are sensitive to low physiological levels of cytokines. Furthermore, the upregulation of lymphocyte adhesion appeared to be independent of lymphocyte activation as IL-1, IL-4 and IFN-γ can increase the adhesion of both resting and activated lymphocytes (Fig.3.8).

Although treatment of retinal EC with IL-1 (as little as 0.5 U/ml) increased lymphocyte adhesion significantly, this was not observed in brain EC (Hughes et al., 1988). The reason for this difference is unclear but may be due to the source of IL-1, as in the brain studies human recombinant IL-1 was used, whereas mouse recombinant IL-1 was employed in the retinal EC. The ability of IFN-γ to increase lymphocyte adhesion to retinal EC is similar to the finding on brain EC (Male et al.,

1990a), whereas IL-4 activation of retinal EC increasing lymphocyte adhesion has not previously been reported with CNS vascular EC.

It is of interest that the cytokines IL-4 and IFN-γ, which are able to increase lymphocyte adhesion, are produced by two different subpopulations of lymphocytes (at least as clearly defined in the mouse). IFN-γ is produced by Th1, whereas IL-4 is produced by Th2 lymphocytes (Mosmann and Coffman 1989). As a result, each population may independently have the ability to increase EC adhesiveness for circulating lymphocytes and so recruit T cells to the inflammatory site.

In previous studies it has been found that the cytokines IL-4 and IFN-γ can increase the ability of non-CNS EC to bind lymphocytes but not polymorphonuclear leucocytes. IL-1, however, has been shown to preferentially increase polymorphonuclear leucocyte adhesion, with the increase in lymphocytes binding to EC, occurring to a lesser degree (Thornhill et al., 1990b). This selective increase in EC adhesiveness for different leucocytes by individual cytokines suggests that IL-1 is probably more important in neutrophil mediated conditions such as acute inflammation, playing a lesser role in the more chronic lymphocyte mediated inflammation, whereas IFN-γ and IL-4 may be of greater importance in the latter.

It is clear that the cytokines IL-1, IL-4 and IFN-γ directly activate EC by up-regulating their expression of adhesion molecules and thereby increasing lymphocyte adhesion, although the distinct patterns of adhesion molecule expression depend on the identity of the cytokines used. IL-1 has been shown to induce the expression of ICAM-1, VCAM-1 and E-selectin, while IL-4 was reported to induce VCAM-1 alone (Pober and Cotran 1991; Bevilacqua 1993). IFN-γ has been found only to increase ICAM-1 expression on vascular EC, although it was also capable of inducing VCAM-1 expression on HEV (May et al., 1993). Thus, the cytokines IL-1, IL-4 and IFN-γ may operate by different mechanisms to increase lymphocyte adhesion to retinal EC.

In addition to the ability of IFN-γ to increase the expression of ICAM-1 on EC and thus increase lymphocyte adhesion, it has also been suggested that the induction of MHC class II molecules on EC may assist in lymphocyte adhesion (Masuyama et al., 1986; Goodall et al., 1992). However, in this study we have demonstrated that the increase in lymphocyte adhesion brought about by the cytokine

IFN-γ occurred at a low doses (5 U/ml; Fig.3.7) and within a short time period (18 hours), whereas higher doses of IFN-γ (50 U/ml) and longer times (48 hours; Fig.3.15) are required to induce MHC class II expression on the EC. This suggests that the induction of MHC class II molecules by IFN-γ is unlikely to play an important role in increasing lymphocyte adhesion. Other mechanisms by which IFN-γ may increase adhesion have also been suggested. It has been found that IFN (including IFN-γ) are able to induce the EC to produce a protein, named IFN inducible protein 10 (IP-10, a chemokine) which can stimulate T-cell adhesion to EC (Taub et al., 1993). The role of this molecule at CNS vascular EC remain to be elucidated.

The increase in EC ability to bind lymphocytes *in vitro* following stimulation by cytokines, such as IL-1, IL-4 and IFN-γ is considered to be an important mechanism whereby lymphocytes are recruited from the circulation to the sites of inflammation. *In vivo* studies have also demonstrated that these cytokines, when they are injected into tissues, can increase the ability of the EC to bind inflammatory cells and thereby induce inflammatory cell infiltration (Issekutz et al., 1988; Colditz and Watson 1992). Therefore, increased lymphocyte adhesion to retinal EC *in vitro* following cytokine activation suggests that cytokines play an important role *in vivo* in the EC recruitment of inflammatory cells into the inflamed retina.

B. Forskolin and ACM

Forskolin, which is a stimulator of adenylate cyclase, elevates intracellular cAMP, which is thought to play a role in maintaining intercellular junctional integrity in EC monolayers, leading to the restriction of movement of macromolecules (Hoek 1992). ACM is believed to play a role in promoting the specialized characteristics of CNS vascular EC. It has been reported that coculture of cerebral EC with ACM influences the degree of tight junction formation (Dehouck et al., 1990; Rubin et al., 1991; Greenwood, 1991), and that this could be further enhanced by upregulating intracellular cAMP.

In unrelated experiments, forskolin treatment of HUVEC has also been reported to mimic the action of IL-1 by activating second messenger pathways and

inducing lymphocyte adhesion and migration (Rekonen et al., 1990; Turunen et al., 1990). In this study we have demonstrated that pretreatment of retinal EC monolayers with forskolin, which elevates cAMP, also influences the degree of lymphocyte adhesion. Furthermore, with resting lymphocytes, the apparent synergistic effect of both IFN-γ and IL-1, suggests that forskolin may operate by mechanisms that are, in part, independent of those activated by these cytokines. Therefore, it is unlikely that the induced or enhanced expression of adhesion molecules is the mechanism through which forskolin increases adhesion. Rather, it has been reported that forskolin treatment inhibits the expression of E-selectin and VCAM-1 on TNF-treated EC (Pober et al., 1993), suggesting that there are other mechanisms such as alternations in affinity and avidity, may be involved in forskolin induced lymphocyte adhesion.

The preliminary observation that ACM treatment of retinal EC can also influence the level of adhesion has not previously been reported and, like forskolin, its action appears to be partially independent of that induced by IFN-γ and IL-1, as these cytokines augment ACM induced lymphocyte adhesion. The synergistic effects of these cytokines are unlikely to be due to ACM and forskolin optimising the concentration of these cytokines in the system as the amounts used were shown to produce maximal adhesion. Unlike forskolin, which may act by bypassing the receptor-mediated signal transduction stage of activation, ACM may induce the appearance of adhesion molecules on the EC surface. The differences in the adhesive response of Con A activated lymphocytes to their resting counterparts suggests that significant changes occur in the relative importance of particular adhesion molecules following mitogen activation. Whether ACM can induce adhesion molecules responsible for lymphocyte attachment remains to be demonstrated although in cultured brain EC it does enhance the expression, and influence the distribution of the cell adhesion molecule E-cadherin (Rubin et al., 1991).

In the CNS, astrocytes are considered to be an important accessory cell in the development of CNS inflammation since they are capable of expressing MHC class II molecules and presenting antigen *in vitro* (Fontana et al., 1987; Hertz, et al., 1990). In addition, astrocytes can also participate in the inflammatory processes by synthesis and secretion of a number of inflammatory factors, such as cytokines (Aloisi

et al., 1992). The ability of ACM to increase lymphocyte adhesion implies that interactions between astrocytes and CNS vascular EC may play a role in supporting retinal EC to recruit inflammatory cells from the circulation into the retina under pathogenic conditions.

4.3.2. Control by lymphocytes

A. Lymphocyte activation

In this study we demonstrated that lymphocyte adhesion to retinal EC could be dramatically increased above low resting levels when lymphocytes were activated with either mitogen (Con A) or antigen (S-Ag). This increase in adhesion is mediated by mechanisms that are independent of the cytokine induced adhesion of retinal EC as activation of both the EC and lymphocytes produced maximal levels of lymphocyte adhesion. This demonstrates that both lymphocytes and retinal EC play an important part in the control of lymphocyte adhesion.

The rapid increase in adhesion of PLN cells following Con A treatment (10 min; $p<0.001$; Fig.3.8b) suggests that this is unlikely to be due to *de novo* synthesis of adhesion molecules by the lymphocytes. This rapid increase in adhesion was also not due to excess lectin causing binding as α-methyl mannoside, which neutralizes Con A, did not decrease the level of adhesion. It is likely that Con A activated lymphocytes alter the affinity of the adhesion molecules (presumably LFA-1 and VLA-4), and hence increase lymphocyte binding ability. In previous studies on human peripheral blood lymphocytes it was also demonstrated that the increase in binding of mitogen-activated lymphocyte integrins, such as LFA-1 and VLA-4, to their EC ligands is due to qualitative changes in their surface expression and not to an increase in the number of lymphocyte membrane receptors (Dustin and Springer 1989; Shimizu et al., 1990).

The mechanisms that upregulate the affinity of adhesion molecules by Con A activation are not fully understood. Treatment of lymphocytes with the protein kinase C activator, PMA, has been shown to increase the affinity of adhesion molecules, possibly by a phosphorylation mechanism, and thus to an increase in lymphocyte

binding (Buyon et al., 1990; Haskard et al., 1986a). Whether Con A initiates a similar signalling system in lymphocytes, however remain to be elucidated.

In addition to a possible change in the affinity of adhesion molecules, prolonging mitogen activation of up to 18 hours led to maximal lymphocyte binding to retinal EC and also resulted in an increased expression of LFA-1 on lymphocytes (Fig.3.10). It is not clear, however, whether this increased expression of LFA-1 is important in mediating Con A activated lymphocyte adhesion. In a recent similar study Con A was shown to increase the expression of LFA-1 on lymphocytes at the later stages of activation (Male et al., 1994), and this is in contrast to the effect of the time-course of Con A activation on adhesion, suggesting that increased LFA-1 expression is not important for Con A activated lymphocyte adhesion.

Lymphocyte adhesion started to decrease after 24 hours of Con A activation, suggesting that there are certain stages of the cell cycle in which lymphocyte adhesion is particularly efficient. Although the molecular mechanisms involved in this change of binding ability are not clear, it was observed that when lymphocytes became blastogenic without any significant proliferation, they adhered to retinal EC more efficiently. When cells started dividing after 24 hours of activation, their ability to bind to EC monolayers decreased. Before being plated onto the EC monolayers, the cell clumps which formed after 24 hours of Con A activation were carefully broken up into a single cell suspension to prevent them affecting the adhesion assay and thus the decreased adhesion at the later stages of activation is most likely to be due to functional changes in the lymphocyte binding molecules.

The adhesion of S-Ag specific $CD4^+$ T-cell line lymphocytes activated with S-Ag to retinal EC were found to be significantly greater than that of Con A activated lymphocytes. This difference indicates that the mode of lymphocyte activation is important in determining the degree to which lymphocytes bind to EC. Retinal EC are also able to actively participate in the highly adhesive interaction with S-Ag specific T-cell lines, since adhesion could be augmented by activation of retinal EC with the cytokines IL-1, IFN-γ and IL-4. The high level of adhesion of S-Ag specific T-cell lines demonstrates that antigen activation is an important factor in increasing the capability of lymphocytes to cross the vascular BRB.

The enhanced adhesion by these highly activated, terminally differentiated T-cell lines could be explained by studies in which so-called ''memory'' cells have been shown to adhere more efficiently than ''naive'' cells (Shimizu et al., 1991a). Despite the difficulty in identifying rat ''memory'' T cells, it has been suggested that low expression of OX-22 is indicative of the rat ''memory'' cell phenotype ($OX\text{-}22^{low}$), and this low level of expression has been demonstrated on S-Ag specific $CD4^+$ T-cell lines (Calder et al., unpublished data). In addition, ''memory'' cells have a greater ability to migrate across EC monolayers than ''naive'' cells (Pietschmann et al., 1992), and this migratory capability was also found with S-Ag specific T-cell lines, but not with Con A activated lymphocytes (Greenwood and Calder 1993). The increased level of migration of S-Ag specific T-cell lines through retinal EC monolayers compared with Con A activated lymphocytes can result in an apparent increase in the number of lymphocyte binding, where a significant proportion are not in fact adhering to the surface but are under the monolayers.

Human "memory" cells ($CD4^+CD45RO$) have been shown to express higher levels of the integrins than "naive" cells and a corresponding increase in their capability to bind to the relevant ligands (Shimizu et al., 1990). Thus, increased adhesion of these cells may be partly due to a genuine increase in the binding proportion. In this study we found that expression of LFA-1, but not VLA-4 on S-Ag specific T-cell lines derived from Lewis rats was substantially increased. These quantitative changes in LFA-1 on S-Ag specific T-cell lines are likely to be long-term unlike the qualitative and transient changes induced by Con A activation in which the expression of LFA-1 was much lower and whose adhesion began to reverse by 48 hour post-stimulation.

The finding that there was an increased expression of LFA-1 rather than VLA-4 on S-Ag specific T-cell lines contradicts the findings in human studies in which "memory" T cells express higher levels of both VLA-4 and LFA-1 integrins than the "naive" cells (Sanders et al., 1988). This result is also in contrast to a previous study in rat in which it has been shown that rat $CD4^+CD45R^-(OX\text{-}22^{low})$ cells express higher levels of VLA-4 than $CD4^+CD45R^+$ (Issekutz and Wykretowicz 1991). This difference in the increase in integrin expression could be due to the T cells derived

from different species, the antigen specificity of T cells, or the mAb used.

In addition to the increased expression of adhesion molecules, it has been demonstrated that "memory" T cells can also induce EC to express the adhesion molecules E-selectin and VCAM-1 (Damle et al., 1991), and thereby enhance the degree of adhesion. Although it is not clear how ''memory'' cells induce EC to express the adhesion molecules, this effect was shown to be dependent on direct cellular contact and was not due to cytokines released from "memory" cells (Damle et al., 1991). This, therefore, may be an additional way in which our T-cell lines achieved significantly greater adhesion than the Con A activated lymphocytes.

Finally, it has been reported elsewhere that ovalbumin-specific T-cell lines adhered to cerebral EC to a lesser degree than Con A stimulated PLN cells (Male et al., 1990a). This discrepancy could be explained either by functional differences between the cells, the amount and type of cytokines produced or their state of activation.

B. Lymphocyte subsets

In this study we found that $CD8^+$ T cells preferentially bind to retinal EC and that this is similar to that previously reported for lymphocyte subset adhesion to brain EC (Pryce et al., 1991), demonstrating that there is a differential interaction of lymphocyte subpopulations with CNS vascular EC. It has previously been suggested that the mechanisms by which $CD8^+$ T cells bind preferentially to EC monolayers is that MHC class I molecules expressed on EC may play a role in mediating their binding. However, a recent study shows that treatment of brain EC with anti-MHC class I mAb does not affect lymphocyte binding (Male et al., 1990a), suggesting that MHC class I and CD8 molecule interaction is unlikely to be the explanation for this increased binding capacity. The greater binding of $CD8^+$ T cells, however, may be explained by studies in which a subset of human circulating $CD8^+$ T cells expressed higher levels of LFA-1 and VLA-4 than $CD4^+$ ''naive'' T cells ($CD45RA^+$) (Pardi et al., 1989).

The greater $CD8^+$ T-cell binding to the EC seemed to contradict most *in vivo* studies in which $CD4^+$ T cells are usually the dominant cells amongst infiltrated

lymphocytes during the development of EAU, whereas $CD8^+$ T cells infiltrate into the retina at much later stages of the disease (Chan et al., 1985). This difference, however, may also be explained by the finding that although $CD4^+$ T cells bind to retinal EC less effectively than $CD8^+$ T cells, they are able to migrate through the EC monolayers to a much greater degree (Pitzalis et al., 1991; Pryce et al., 1994).

The different ability of lymphocyte subpopulations to bind to EC has also been previously reported. Of these, B lymphocytes appear to bind more efficiently than T cells (Pryce et al., 1991), and natural killer (NK) cells preferentially adhere to TNF-α activated, but not normal HUVEC (Ikuta et al., 1991). Furthermore, under normal conditions, lymphocytes adhere to EC to a greater degree than polymorphonuclear cells (PMN), whereas IL-1, TNF or LPS activation of EC cause a large and transient increase in PMN adhesion but a small and gradual increase in lymphocyte adhesion (Thornhill et al., 1990b). The significance of this differential binding of leucocyte subsets to EC is not fully understood, but may contribute to the fine control of leucocyte extravasation into inflammatory tissues. It is also thought that the switch in adhesion and migration of certain cell types may correspond to the switch between the development and remission phases of acute inflammatory disease.

This difference in the ability of lymphocyte subsets to adhere is also likely to explain differences found in lymphocyte adhesion of lymphocytes from various lymph nodes, as it was shown that these containing higher percentages of B cells and $CD8^+$ T cells yielded greater percentages of adhesion. Therefore, although lymphocytes from cervical lymph nodes bind to retinal EC to a greater degree than those from inguinal lymph nodes (Fig.3.3), this higher level of adhesion is likely to be due to a greater number of $CD8^+$ T cells distributed in these lymph nodes (Pryce et al., 1991).

4.3.3. Role of adhesion molecules

As it has been described in a previous chapter, lymphocytes and EC possess a repertoire of adhesion molecules that can potentially mediate interactions between the two cell types. It appears likely that adhesion ligands, such as ICAM-1 and VCAM-1, that are expressed by EC and which can be induced by cytokines, might play a role in facilitating the entry of lymphocytes into inflammatory tissues. The

activation status of lymphocytes may also determine the ability of adhesion molecules, such as VLA-4 and LFA-1, to bind to their ligands on EC. These ligand-receptor interactions are also likely to be involved in lymphocyte adhesion to the vascular BRB although few studies investigating these interactions at this site have been reported. Thus, the focus of this study was to determine if, or how, the adhesion molecules function during lymphocyte adhesion to retinal EC.

A. ICAM-1

In studies with human non-CNS EC, ICAM-1 has been demonstrated to be an important adhesion molecule which mediates EC binding to lymphocytes, neutrophils, macrophages and NK cells (Bevilacqua 1993). It is also a crucial molecule in mediating lymphocyte migration through EC monolayers (Oppenheimer-Marks et al., 1991). However, in the rat, the role of ICAM-1 in lymphocyte-EC interactions is less clear. Previous studies on rats have shown that the anti-ICAM-1 mAb (IA29) could inhibit the adhesion of mitogen-activated lymphocytes to HEV (Tamatani et al., 1990), although it was incapable of inhibiting IL-2 activated lymphocyte adhesion to either HEV or microvascular EC (Pankonin et al., 1992). In this study we demonstrated that this same anti-ICAM-1 mAb (IA29) was able to inhibit the adhesion of S-Ag specific T-cell line lymphocytes to resting and IL-1 activated retinal EC, but failed to inhibit the adhesion of Con A activated lymphocytes. This may suggest that ICAM-1 plays a different role in support of retinal EC interactions with Con A activated lymphocytes and S-Ag specific T cells although other explanations are more likely (see below). Nevertheless, it is important to note at this point that there is a significant functional difference between Con A activated PLN cells and S-Ag specific T-cell lines. Con A activated lymphocytes are highly adherent but have negligible levels of migration through retinal EC monolayers. The antigen-specific T cells however are both extremely adherent and migratory (Greenwood and Calder 1993) and thus this functional difference of these two populations allow us to examine the mechanisms of the separate processes of adhesion and migration (diapedesis).

The lack of any effects of anti-ICAM-1 mAb on Con A activated PLN cell adhesion indicates that LFA-1 dependent lymphocyte adhesion may occur via an

alternative ligand of LFA-1. This alternative ligand could be ICAM-2, which has been shown to be constitutively expressed by HUVEC and may mediate Con A activated lymphocyte adhesion to the resting EC. Although it has been found that activation of retinal EC with the cytokine IL-1 upregulates ICAM-1 expression (Fig.3.10) and enhance its ability to bind lymphocytes (Shimizu et al., 1991), we also failed to demonstrate that anti-ICAM-1 mAb could inhibit the adhesion of Con A activated lymphocytes to IL-1 activated retinal EC. This ICAM-1 independent pathway of LFA-1 binding to IL-1 activated retinal EC is not clear.

An alternative explanation for this failure of adhesion blockade with IA29 is that the mAb does not block the functional site of ICAM-1. However, IA29 did lead to an apparent inhibition of the adhesion of S-Ag specific T-cell line lymphocytes to both resting and IL-1 activated retinal EC. This difference may be explained by the effect of IA29 on migration of S-Ag specific T-cell line lymphocytes. Previous studies with HUVEC have demonstrated that ICAM-1 is the dominant molecule in mediating lymphocyte transendothelial migration (Oppenheimer-Marks et al., 1991). This is confirmed by our migration assay in which IA29 dramatically reduced T-cell line migration across retinal EC (Fig.3.12). Thus, the apparent inhibition of S-Ag specific T-cell adhesion by anti-ICAM-1 mAb is likely to result from a reduction of subsequent migration and not adhesion. This would be consistent with the lack of an effect of this mAb on the adhesion of Con A activated lymphocytes which are highly adhesive but are mostly incapable of migrating across retinal EC monolayers (Greenwood and Calder 1993).

B. LFA-1 and VLA-4

LFA-1 and VLA-4 are members of the integrin family, perhaps the most versatile members of the adhesion molecule family. The common feature of the integrin family is that each integrin molecule comprises an α- and ß-subunit. LFA-1 is composed of a ß2 (CD18) and αL (CD11a) subunit. Its counter receptors on EC are ICAM-1 and ICAM-2 (Figdor et al., 1990). VLA-4 is composed of a ß1 (CD29) and α4 (CD49d) subunit and binds to VCAM-1 expressed on EC (Hynes 1992). The important role of these adhesion molecules has been demonstrated in various studies

on lymphocyte adhesion to HUVEC (Haskard et al., 1986; Shimuzu et al., 1991a, 1992; Oppenheimer-Marks et al., 1991; Vennegoor et al., 1992).

The expression of LFA-1 and VLA-4 was found with resting lymphocytes and an enhanced expression of LFA-1 with Con A activated lymphocytes or S-Ag specific T-cell lines. Whether the increased expression of LFA-1 correlates with the increased adhesion has been discussed above. Resting retinal EC express ICAM-1 and its expression can be upregulated following cytokine activation. The expression of VCAM-1, the ligand for VLA-4, on retinal EC has not been described, but it is believed that this molecule is the ligand for VLA-4 in lymphocyte binding to rat brain EC (Male et al., 1994).

In antibody blocking experiments we have demonstrated that LFA-1 plays an important role in mediating the adhesion of both Con A activated lymphocytes and S-Ag specific T cells to retinal EC. The two subunits of the LFA-1 molecule have been shown to be critical to the function of lymphocyte binding after inhibition of lymphocyte adhesion by anti-CD11a and anti-CD18 mAb treated cells. In this study we also found that the blocking of the $\alpha 4$ subunit of VLA-4 molecule with the mAb (P12520) also has an inhibitive effect on lymphocyte adhesion. This effect was observed with both resting and IL-1 activated retinal EC although it is generally thought that VCAM-1 is an inducible molecule on EC (Osborn et al., 1989; Vennegoor et al., 1992).

The anti-LFA-1 mAb (both WT.1 and WT.3) showed significant inhibition of lymphocyte adhesion to resting retinal EC and this effect was not affected by IL-1 activated EC, indicating that the ligand(s) for LFA-1 on the EC are constitutively expressed. Although we found that ICAM-1 is expressed on resting cultured retinal EC and that its expression could be increased following IL-1 activation, blocking this molecule did not reduce lymphocyte binding. In another study with brain EC, it was also observed that anti-LFA-1 mAb, but not anti-ICAM-1 mAb (IA29) inhibited lymphocyte adhesion (Male et al., 1994). An alternative explanation to the mAb not binding to the functional sites is that ICAM-1 expressed on CNS vascular EC may not be the important ligand for LFA-1 mediated lymphocyte binding.

Although the ligand VCAM-1 does not appear to be expressed on resting EC,

we found that anti-VLA-4 mAb could inhibit lymphocyte adhesion to non-activated retinal EC. The reason for this is not fully clear although it has been observed that anti-VLA-4 mAb treated lymphocytes can cause cell clumping (Male et al., 1994). The clumping of lymphocytes could interfere in the interaction between lymphocytes and EC monolayers leading to an apparent inhibition of lymphocyte adhesion resulting from the reduced ability of clumped lymphocytes to withstand a sheer force applied for washing off non-adherent cells.

In addition, there are some reports showing that resting cultured EC may express VCAM-1 under certain culture conditions such as the presence of ECGF and xenogeneic serum in cultured medium (Schwartz et al., 1990; Kavanaugh et al., 1991). Whether resting cultured retinal EC express this molecule is not clear, but the study (see below) on lymphocyte migration suggest that the expression of VCAM-1 on retinal EC probably required cytokine induction. Another possible explanation for VLA-4 binding to resting retinal EC is that mitogen or antigen activated lymphocytes may be capable of inducing VCAM-1 expression during the adhesion assay via either cytokine release or direct cellular contact (Thornhill et al., 1991; Wellicome et al., 1990; Damle et al., 1991).

In this study we found that a combination of the anti-LFA-1 mAb and anti-VLA-4 mAb did not further increase the blocking of lymphocyte adhesion (Fig.3.11). This result is, however in contrast to the finding in brain studies in which a synergistic effect was observed when a combined anti-LFA-1 and anti-VLA-4 mAb was used (Male et al.,1994). A likely explanation of this difference is that in the brain studies, the synergistic effects were achieved by combining suboptimal concentration of anti-VLA-4 mAb, whereas in our study we employed undiluted supernatant of anti-LFA-1 and 10μg per 1x10^6 cells of anti-VLA-4 mAb and at these concentrations individual mAb are likely to produce a maximal effect on blocking. An alternative explanation is the source of the anti-VLA-4 mAb, as the anti-VLA-4 (CD49d) mAb we used was derived from rats, whereas an anti-human VLA-4 (CD49d) mAb which cross-reacts with rat VLA-4 molecules, was employed in the brain studies.

The incomplete inhibition of adhesion by anti-LFA-1, anti-VLA-4 or a combination of these mAb suggests that other processes are likely to be involved in

lymphocyte adhesion to retinal EC. E-selectin, which has been demonstrated to play an important role in mediating the adhesion of ''memory'' T cells (Picker et al., 1991; Shimuzu et al.,1991b) may be an important alternative molecule in mediating S-Ag specific T-cell line lymphocyte adhesion. Furthermore, P-selectin, L-selectin, CD44, CD31 (PECAM) and CD2/LFA-3 may also be important in the adhesion of both Con A activated and antigen activated lymphocytes (Bevilacqua 1993). Recently lymphocyte vascular adhesion protein (VAP) 1 and 2 have also been found to be expressed on EC which can bind lymphocytes via unknown ligands (Salmi and Jalkanen 1992; Airas et al., 1993).

4.3.4. Antigen specific T-cell adhesion

It is known that in autoimmune disorders, such as EAE or EAU, $CD4^+$ T cells are predominant among the infiltrated inflammatory cells. It has been proposed that the presence of MHC class II molecules expressed on inflamed EC and the presence of cognate antigen may play an important role in selectively guiding $CD4^+$ T-cell extravasation (Pals et al., 1989). Several *in vitro* studies have also shown that either anti-CD4 mAb or anti-MHC class II (DR) mAb can inhibit or reduce the binding of $CD4^+$ T-cells to IFN-γ activated EC (Masuyama et al., 1986; Goodall et al., 1992).

In contrast, a previous study on brain EC demonstrated that the adhesion of an ovalbumin specific T-cell line was not affected by the presence of cognate antigen or antibody blocking MHC class II molecules, suggesting there is no antigen specific interaction involved in mediating cell adhesion. In this study where we employed an antigen specific $CD4^+$ T-cell line, specific to antigen derived from the retina, to investigate this antigen specific interaction. Our experimental observations indicated that antigen specific interactions are also not important in the adhesion of S-Ag specific T-cell lines to retinal EC. Firstly, retinal EC treated for 3 days with IFN-γ, which not only induces adhesion molecules (such as ICAM-1) but also MHC class II molecules, did not significantly increase antigen specific $CD4^+$ T-cell adhesion compared to cells binding to 18 hours IFN-γ treated retinal EC (where only ICAM-1 expression was induced). In addition, the presence of S-Ag in the MHC class II induced retinal EC, which can be recognized by S-Ag specific T-cell lines resulting

in a T-cell proliferative response and cytotoxicity, did not affect the levels of adhesion of S-Ag specific T-cell lines to retinal EC.

4.4. CONTROL OF LYMPHOCYTE MIGRATION THROUGH RETINAL EC

Following adhesion to EC, lymphocytes may then migrate across the vascular wall. In studies on non-CNS vascular EC, it is clear that, like the adhesive interactions, lymphocyte migration is also controlled by adhesion molecules although they may depend on the differential use of such molecules (Oppenheimer-Marks et al., 1991; Kavanaugh et al., 1991). In this study, we have investigated the molecular control of lymphocyte migration across cultured retinal EC monolayers by using S-Ag specific T cells which are more migratory than resting or Con A activated lymphocytes.

The ICAM-1/LFA-1 receptor pairing was found to play an important role in mediating lymphocyte migration across retinal EC monolayers which is similar to previous reports on lymphocyte migration across HUVEC (Oppenheimer-Marks et al., 1991), suggesting that these molecules are equally important in lymphocyte trafficking through CNS and non-CNS vascular EC. This result is, however in contrast to the finding in adhesion where ICAM-1 did not appear to be the important ligand for LFA-1. This difference indicates that there is differential usage of adhesion molecules in the adhesive and migratory processes of lymphocytes. With IL-1 activated retinal EC, lymphocyte migration appeared to be partly independent of ICAM-1, but not LFA-1 (CD11a, Fig.3.12) despite an increase in ICAM-1 expression, further supporting a previous suggestion that LFA-1 interaction with retinal EC can be via an ICAM-1 independent pathway. This ICAM-1 independent pathway is unlikely to be ICAM-2, as this molecule is not inducible, although the possibility that IL-1 activation of EC alters the properties of ICAM-2, such as affinity, exists. In addition the expression of ICAM-2 has been described to be important for lymphocyte trafficking in normal tissues, as in lymphocyte recirculation (Springer 1994).

Although the mAb against both subunits of LFA-1 blocked S-Ag specific T-cell migration, anti-CD11a mAb showed more efficient blocking than anti-CD18 mAb

(Fig.3.12). This result is in contrast to the effect of these two mAb on adhesion in which they functioned equally well. This difference may suggest that CD11a molecules are more important in mediating lymphocyte migration although it is more likely to be due to the mAb binding to a more functional determinant of the molecule.

VLA-4 has been found to mediate lymphocyte migration across IL-1 activated, but not resting, retinal EC. This probably indicates that resting cultured retinal EC do not express VCAM-1, the ligand for VLA-4, and the expression of this ligand can be induced following EC activation. IL-1 has been shown to activate EC by inducing or enhancing a number of adhesion molecules, and we demonstrate in this study that it increases ICAM-1 expression on retinal EC. Although we were unable to identify VCAM-1 on retinal EC, previous studies on HUVEC have demonstrated IL-1 is able to induce VCAM-1 expression (Graber et al., 1990).

Recently it has also been suggested that VLA-4 affinity is important in lymphocyte migration (Hourihan et al., 1993). Cytokines such as IL-1, TNF and IFN-γ can stimulate EC to release chemokines some of which have been shown to be able to increase the lymphocyte integrin activity such as VLA-4 and therefore to enhance adhesion and migration (Tanaka et al., 1993).

The ability of VLA-4 to mediate lymphocyte migration across retinal EC monolayers is in contrast to the findings in lymphocyte migration across HUVEC. In HUVEC, VLA-4 is incapable of mediating normal or activated human peripheral blood lymphocyte migration, regardless of EC activation (Oppenheimer-Marks et al., 1991; Kavanaugh et al., 1991) although this molecule was found to be capable of functioning partly in the migration of LFA-1 deficient lymphocyte clones (Kavanaugh et al. 1991). This different role of VLA-4 in mediating lymphocyte migration across CNS and non-CNS vascular EC suggests genuine differences between vascular EC derived from the CNS and non-CNS vasculature in their interactions with lymphocytes.

The important role of LFA-1, VLA-4 and ICAM-1 in lymphocyte migration has also been demonstrated in *in vivo* studies in which administration of anti-LFA-1, VLA-4 and ICAM-1 mAb can significantly reduce the number of inflammatory cells into the non-CNS tissues (Issekutz 1991; 1992; Iigo et al., 1991). In the CNS, VLA-4

is thought to be an important molecule in brain inflammatory reactions as mAb to the VLA-4 integrin has been used successfully to prevent experimentally induced inflammation of the CNS in rats (Yednock et al., 1992). There is also evidence to indicate that the loss of encephalitogenic potential of myelin basic protein-specific $CD4^+$ T-cell clones may correlate with a decreased level of VLA-4 on the cell surface, and a corresponding reduced capacity of these T cells to migrate into the brain (Baron et al., 1993; Kuchroo et al., 1993). In contrast, the role of LFA-1 and ICAM-1 appeared not to play a significant part in the development of CNS inflammation since mAb to LFA-1 and ICAM-1 did not suppress the entry of inflammatory cells into the CNS (Cannella et al., 1993; Willenborg et al., 1993) although our *in vitro* study showed LFA-1 and ICAM-1 mediated lymphocyte migration across retinal EC monolayers. Whether these conflicting *in vitro* and *in vivo* results are due to differences of animal species or properties of the mAb is unknown. Nevertheless, it indicates that *in vivo* lymphocyte extravasation is a more complex process than in an *in vitro* model.

4.5. EXPRESSION OF ICAM-1 AND MHC MOLECULES ON RETINAL EC

4.5.1. ICAM-1 expression on retinal EC

ICAM-1 is a single chain glycoprotein of 90 KD, expressed on EC and other cell types (Springer 1990). It is thought that resting cultured HUVEC express low levels of ICAM-1, but these low levels can be markedly increased by treatment with IL-1α, IL-1ß, TNF, IFN-γ, LT, and LPS (Pober et al., 1986a, 1988). Although CNS vascular EC differ considerably from extra-cerebral microvessel EC in their range of surface molecules, primary cultures of brain EC have also been found to express ICAM-1, and its expression can also be increased by IFN-γ and TNF (Wong and Dorovini-Zis 1992; McCarron et al., 1993; Male et al., 1994)

Using flow cytometry we have found that a larger proportion (60%) of resting cultured retinal EC constitutively express ICAM-1, which correlates with the finding that lymphocyte migration to resting retinal EC can be mediated by this molecule. In contrast to our finding of a constitutive expression of ICAM-1 on cultured retinal EC,

several other studies have shown that ICAM-1 is not constitutively expressed on resting cultured human retinal EC and on normal retinal tissues. The expression of this molecule on retinal EC has been found only under inflammatory conditions of the retina (Whitcup et al., 1992; Wakefield et al., 1992) or following activation of retinal EC with IFN-γ in the *in vitro* experiments (Liversidge et al., 1990). These differences may result from various factors such as the use of different species, the purity of cultured cells or the culture conditions.

The expression of ICAM-1 on retinal EC can be increased following IFN-γ activation. This increased expression was found within 18 hours and could be maintained up to 5 days in the presence of IFN-γ. It has been suggested that the time-course expression of ICAM-1 following IFN-γ and TNF activation of CNS vascular EC matches the increased ability of the EC to bind lymphocytes (Male et al., 1994). Since ICAM-1 is unable to directly mediate lymphocyte adhesion, it indicates that there may be a concomitant increase in other adhesion molecules on the EC responsible for lymphocyte adhesion. Furthermore, ICAM-1 was demonstrated to be an important molecule in lymphocyte migration across retinal EC monolayers. Therefore, the time-course expression of ICAM-1 would suggest that IFN-γ activated CNS vascular EC recruit lymphocytes rapidly and persistently in the inflammatory processes.

4.5.2. Induction of MHC antigens on retinal EC

In previous studies, it has been found that cultured brain EC can be induced to express MHC class II molecules, and that therefore they may play a role as APC within the CNS and thus in the development of the immune response (Male et al., 1987; Wilcox et al., 1989b; Hart 1990). In this study using the techniques of immunocytochemistry, ELISA and flow cytometry, we have demonstrated that the expression of MHC class II molecules on retinal EC can be induced by IFN-γ whereas resting retinal EC do not express these molecules. Furthermore, IFN-γ also causes a concomitant increase in MHC class I expression.

Retinal EC respond rapidly to low doses of IFN-γ, producing enhanced MHC class I expression within 18 hours of stimulation. In contrast, MHC class II induction

requires higher doses of IFN-γ and develops over a longer time course with detectable levels of class II molecules only occurring 2 days after stimulation (Fig.3.15). This different expression of MHC class II and class I in response to IFN-γ is believed to occur at the level of the MHC gene, as it has been found that the expression of new class I mRNA synthesis starts immediately within 1 hour after EC triggering (Male and Pryce 1988) and MHC class II mRNA increases from undetectable to significant levels by 24-48 hours following IFN-γ treatment (Collins et al., 1984). The significance of this differential expression in the eye is not clear but it may suggest that in *in vivo* situations, immune responses can produce a rapid and widespread enhancement of MHC class I, whereas MHC class II expression is confined to the vicinity of active T-cell infiltration during chronic inflammation of the retina.

In the induction of MHC class II antigens on retinal EC we found that only I-A, but not I-E is inducible by IFN-γ (Fig.3.15, 3.17). This is in contrast to macrophages which express both I-A and I-E molecules. MHC class II I-A and I-E molecules are products coded by the mouse I-A and I-E region of the H-2 complex, whilst in the rat the mAb OX-6 and OX-17 detect the equivalent products. It has been demonstrated that both OX-6 (I-A) and OX-17 (I-E) are expressed on APC such as thymocytes (Fukumoto et al., 1982). The reason for the failure to detect I-E molecule on retinal EC with OX-17 following IFN-γ activation is not clear but is not due to impaired binding efficiency as macrophages are shown to be positive. It is more likely, however, that the switching on of the MHC class II I-A and I-E encoded gene in retinal EC is differentially controlled following IFN-γ activation. This differential induction of class II allelic gene coded products has also been observed in HUVEC in which MHC class II DQ was found to be a major molecule induced by IFN-γ, whereas class II DR and DP were induced to much a lesser degree (Collins et al., 1984).

The immunotherapeutic potential of anti-I-A and I-E mAb for suppressing EAU has been investigated and it was found that anti-I-E antibody failed to prevent the onset of EAU (Rao et al., 1989). Thus, I-E expression is not thought to be important in the pathogenesis of EAU. Furthermore, it has been found that the I-E

molecule is not involved in antigen presentation by brain EC, since brain EC treated with anti-I-E rather than I-A antibodies did not inhibit antigen-specific cytotoxicity of $CD4^+$ T cells (Risau et al., 1990; Sedgwick et al., 1990). In this study we also confirm that MHC class II I-A but not I-E molecules were important in retinal EC stimulation of T-cell proliferation (Tab.3.7).

By comparison of the expression of MHC class II molecules between retinal EC and macrophages, it is clear that there is a difference in IFN-γ responsiveness between these two cell types. Macrophages were shown to be more sensitive to IFN-γ with a rapid enhancement of both class II I-A and I-E within 4 hours with low doses (5 U/ml) of IFN-γ (Fig.3.16). This difference in responsiveness may be due to a heterogenous distribution of the IFN-γ receptor on different cell types where bone marrow derived cells have been shown to express a greater number and higher affinity of IFN-γ receptors than non-haematopoietic cells (Orchansky et al., 1986; Gerrard et al., 1988).

The inducibility of MHC class II molecules suggests that retinal EC could potentially play a role as APC in antigen specific T-cell activation. In addition, the capacity for MHC class II molecule expression on CNS vascular EC appears to correlate with the susceptibility of a particular species or strain to experimental autoimmune disease (Male and Pryce 1989; Jemison et al., 1993, Welsh et al., 1993). For example, strains of rat (Lewis) and mouse (SJL/J) whose cerebral EC possess the greatest capacity for MHC class II induction by IFN-γ are also those in which EAE is most readily induced. It is not clear, however, whether this is due to a difference in the MHC regulator genes, to other MHC-linked genes or to a differential ability to respond to cytokine stimulation.

4.5.3. ICAM-1 and MHC expression on brain EC

Recently, there have been a number of reports describing the differential expression of surface antigens by different EC populations. Of these, capillary but not large vessel EC have been shown to express MHC class II (Page et al., 1992). In another study, cultured microvascular EC from skin are reported to be unresponsive to IL-1 with respect to VCAM-1 expression and to display a shorter expression of

VCAM-1 after TNF-α treatment when compared with HUVEC (Swerlick et al., 1992). Furthermore, a differential induction of VCAM-1 on human iliac venous and arterial EC has also been reported (Hauser et al., 1993). These differences in expression and induction of surface antigens on EC populations imply that various EC may operate differently during the development of an inflammatory lesion.

The EC that form the vascular beds of the brain and retina, however, are believed to be identical in that they both form vascular blood-tissue barriers. During the development of inflammation within the brain and the retina, there are also many similarities in the morphological and pathophysiological changes of brain and retinal vascular EC (Greenwood 1992b) and it is therefore assumed that they may play a similar role in control of the immune response within these organs.

In this comparative study we found that CNS vascular EC derived from brain and the retina are similar in respect of their expression of ICAM-1, MHC class I and class II antigens. Despite a lower level of MHC class II I-A molecules induced by IFN-γ, brain EC also exhibit a similar cytokine-responsiveness to retinal EC in the expression of these molecules (Fig.3.17, 3.18; Tab.3.5, 3.6). This study suggests that brain and retinal EC are likely to function similarly in controlling lymphocyte traffic into the CNS. They may also play a similar role in antigen presentation since we demonstrated that both brain and retinal EC stimulate T-cell proliferation in an MHC class II I-A restricted manner. The observation that there was lower level of MHC class II I-A induction by IFN-γ on brain EC than that of retinal EC suggests that there may be some difference in cytokine-responsiveness although the reason for this difference remains to be further elucidated. Their equal ability to present antigen however indicates that the level of MHC class II I-A antigen expression on brain and retinal EC is not critical to their functioning as APC.

4.5.4. Pathogenic expression of ICAM-1 and MHC class II antigens during inflammatory diseases

In normal tissues EC usually express very low levels of ICAM-1, but not MHC class II molecules. The expression of MHC class II molecules on EC, or other cells which do not normally express them, is thought be to an important factor in the

pathogenesis of inflammatory diseases. Moreover, the enhanced or induced expression of adhesion molecules on EC under pathogenic conditions may also correlate with inflammatory cell infiltration.

Aberrant expression of MHC class II molecules has been found on a variety of cell types including astrocytes, microglia cells and brain EC in multiple sclerosis (MS) and EAE (Fontana et al., 1987), thyroid epithelial cells in autoimmune thyroiditis (Matsunaga et al., 1986), renal tubular cells and epidermal keratinocytes in graft versus host disease (Lampert et al., 1981), bile duct epithelial cells in primary biliary cirrhosis (Bottazo et al., 1983), salivary gland epithelial cells in Sjogren's syndrome and Langerhan's islet cells (Dalavanga et al., 1987). In inflammatory disorders of the retina, such as uveitis or the disease model EAU, retinal vascular cells, RPE and Müller cells can express MHC class II molecules (Lightman 1988, Chan et al., 1986a, 1986b, 1988b).

In addition to this aberrant MHC class II expression, an enhanced or induced expression of adhesion molecules has also been found under these similar pathogenic conditions (Koch et al., 1991). Increased ICAM-1 expression has been observed on EC in various forms of hepatitis (Volpes et al., 1990; Adams et al., 1989; Burra et al., 1992), on keratinocytes in various inflammatory dermatoses (Nickoloff et al., 1990) and on renal tubular epithelium during renal allograft rejection (Faull et al., 1989). Within the CNS expression of this molecule has been markedly increased in active MS plaque edges, viral encephalitis lesions, infarcts (Sobel et al., 1990) and EAE animals (Cannella et al., 1990; Wilcox et al., 1990) although normal brain tissues express a low level of adhesion molecules (Lassmann et al., 1991). In the eye ICAM-1 has been found to be expressed on the retinal EC, choroidal blood vessels and the retinal pigment epithelium in all uveitic, but not normal eyes (Whitcup et al., 1992; Wakefiedet et al., 1992).

4.5.5. The role of IFN-γ in the induction of MHC molecules and ICAM-1 on CNS vascular EC

IFN-γ, which is produced by activated T cells and NK cells, has multiple actions in regulating the immune response. In addition to its role in MHC class II

induction and cellular differentiation and activation, its action on EC has been investigated in recent years and it is believed that IFN-γ plays an important role in EC activation and the development of tissue inflammation (Pober 1988). IFN-γ induces a pattern of EC activation characterized by increased class I and ICAM-1 expression and *de novo* induction of class II molecule expression (Pober and Cotran 1991). IFN-γ also synergizes with TNF to produce greater class I and E-selectin expression (Doukas and Pober 1990) and to enhance EC adhesiveness for lymphoytes (Thornhill et al., 1990c, 1991).

In this study we have demonstrated that IFN-γ can induce the expression of MHC class II molecules and enhance the expression of MHC class I molecules and ICAM-1 on the similar populations of retinal and brain EC although the time-course of induction of these molecules differs. ICAM-1 expression is rapidly increased within 18 hours after treatment suggesting that IFN-γ initially upregulates the ability of retinal and brain EC to recruit lymphocytes from the circulation. In contrast, IFN-γ induced MHC class II expression on the EC which may allow them to function as APC for T cells, is likely to occur later, as it requires higher doses of IFN-γ and a longer induction period. This finding is also consistent with *in vivo* studies in which MHC class II expression is observed 3-5 days following lymphocyte infiltration into the retina in EAU (Chan et al., 1988).

It has also been suggested that IFN-γ is an important cytokine in the induction *in vivo* of both MHC class II expression and adhesion molecules on retinal EC during an inflammatory process. Systemic administration or intraocular injection into the vitreous cavity of rat eyes of recombinant IFN-γ has been demonstrated to induce MHC class II expression on ocular resident cells in the uveal tract, cornea, extraocular conjunctival epithelial cells and retinal vessels (Kusuda et al., 1989). The subretinal injection of recombinant IFN-γ also produces class II expression on RPE cells and retinal EC (Hamel et al., 1986).

In addition, studies have shown that IFN-γ exists in the inflammatory tissues of the retina. By using *in situ* hybridization techniques, it was found that IFN-γ mRNA is expressed simultaneously with the onset of EAU and that this increased as the inflammatory disease progressed (Charteris and Lightman 1992). Activated S-Ag

specific T-cell lines have also been shown to express IFN-γ mRNA and secrete IFN-γ (Calder et al., unpublished data). Therefore, production of IFN-γ *in situ* during the pathogenic process provides a mechanism whereby enhanced expression of ICAM-1 and MHC class II molecules on retinal EC, or other resident cells, can occur.

4.6. ANTIGEN PRESENTATION BY RETINAL EC

4.6.1. Stimulation of T-cell proliferation by retinal EC

Whether CNS vascular EC are able to present antigen resulting in T-cell proliferation remain a contentious issue. Despite their ideal location at the blood-tissue interface several *in vitro* studies have failed to elicit any significant T-cell proliferation in response to EC antigen presentation (Pryce et al., 1989; Risau et al., 1990). In those studies in which a significant response was induced (McCarron et al., 1985; Wilcox et al., 1989a), it has been argued that this may be due to contamination of the assay with other APC.

In this study we have demonstrated that cultured retinal and brain EC under subconfluent conditions are able to present S-Ag to S-Ag-specific $CD4^+$ T-cells *in vitro* resulting in T-cell proliferation. Thymidine incorporation into dividing EC under these conditions is not likely to influence the results as we have found that retinal EC do not take up thymidine when cultured in serum-free medium. The ability of CNS vascular EC to present antigen is similar to that reported for microglia where it was found that T-cell proliferation depended on the ratio of T cell to microglia. The response was reported to be maximal at a ratio of 10:1, declining with increased numbers of microglia (Matsumoto et al., 1992), although no explanation for this observation was given. Poor T-cell proliferation induced by confluent monolayers of retinal and brain EC corroborates previous studies in which only confluent monolayers of EC were employed (Risau et al., 1990; Pryce et al., 1989).

The reason that confluent retinal EC are poor stimulators of T-cell proliferation is not clear but may be due to inhibitory factors produced by non-dividing confluent cells or to the switching off of stimulatory factors, which could lead to T-cell anergy. Synthesis of eicosanoid products by EC is also thought to be

a factor in the inhibition of T-cell proliferation stimulated by EC, and this has been shown in antigen presentation by brain EC (Pryce et al., 1989). However, inclusion of indomethacin in the coculture system of EC and T-cells could only marginally increase T-cell proliferation when confluent EC were used (Fig.3.19), suggesting that eicosanoid production by either retinal or brain EC is not the only factor that leads to poor antigen presentation.

The ability of retinal EC to stimulate T-cell proliferation was found to be independent of pretreatment with IFN-γ, despite T-cell proliferation being MHC class II I-A antigen restricted. In earlier studies this was thought to be due to contamination of the EC by MHC class II bearing cells (Nunez et al., 1983). This however is unlikely to be a case in this study as we have demonstrated that resting EC in culture do not express class II molecules. Furthermore, it was also demonstrated that the culture does not contain any macrophages (detected with ED2 mAb) or dendritic cells (detected with ED7).

A possible explanation for this experimental phenomenon is that S-Ag-specific T-cell lines may induce the retinal EC to express MHC class II molecules. S-Ag-specific T-cell lines used in this study have been shown to produce a pattern of cytokines including IFN-γ, when they are activated with S-Ag or mitogens in the presence of APC (Calder et al., unpublished data). Although these cells were maintained for 10 days in IL-2 conditioned medium prior to the antigen presentation assay and were expected to be in a resting state, they may continue to release low levels of IFN-γ (Sedgwick et al., 1990) sufficient to induce MHC class II antigen expression. Therefore, during the 5 day proliferation assay there was adequate time for MHC class II molecule induction to occur followed by antigen presentation and T-cells proliferation.

4.6.2. Induction of T cell IL-2 production by confluent and subconfluent retinal EC

Activation of resting $CD4^+$ T cells depends upon two processes: (a) acquisition of responsiveness to low levels of IL-2, mediated by *de novo* synthesis and expression of the p33 subunit of the IL-2 receptor; and (b) biosynthesis and secretion of IL-2, the principal autocrine cell cycle progression factor for T cells (Smith 1988). T cells

require at least two signals, both specific (antigen-MHC complex) and non-specific (costimulator), for optimal IL-2 synthesis. Professional APC are able to produce both of these signals and to stimulate T-cell proliferation.

Previous studies have demonstrated that HUVEC are able to play a role in providing accessory factors for T-cell production of IL-2 (Hughes et al., 1990), and are therefore able to induce T-cell proliferation. The ability of EC to increase T-cell IL-2 production has also been suggested to be an important feature in EC acting as APC (Geppert et al., 1990; Hughes et al., 1990). In this study it was found that retinal EC were able to increase PHA- and antigen-stimulated $CD4^+$ T-cell IL-2 production. This ability, however, was dependent on the concentration of EC as subconfluent retinal EC caused significantly higher IL-2 production than confluent EC. Corresponding with this raised IL-2 production, subconfluent EC were also able to stimulate a significantly higher level of T-cell proliferation. However, since exogenous IL-2 was present in the last 2 days of the assay, the increase in T-cell IL-2 production is unlikely to be the reason for increased levels of T-cell proliferation stimulated by subconfluent EC. Instead, it suggests that other factors exist in confluent EC that can impair either T-cell proliferation or IL-2 production.

4.6.3. The role of TGF-ß in T-cell proliferation and IL-2 production

TGF-ß is the member of a family of structurally related polypeptides referred to as the TGF superfamily. Three TGF-ß isoforms, termed TGF-ß1, 2 and 3, have been identified in mammals. TGF-ß1 elicits a diverse range of cellular responses depending on cell type, state of differentiation and culture conditions. In the past few years, TGF-ß has been shown to be an immune regulator, playing an important role as an immunosuppressive agent (Shull et al., 1992). It has been shown to suppress B-cell proliferation and immunoglobulin (IgG and IgM) secretion, inhibit T-cell proliferation and functions, suppress natural and lymphokine-activated killing by large granular lymphocytes, and inhibit the generation of cytotoxic T lymphocytes (Wahl et al., 1989).

Since it was found that confluent retinal EC did not stimulate T-cell proliferation and IL-2 production, it was considered that TGF-ß, which we have

shown to be constitutively produced by retinal EC (Fig.3.24) and which may accumulate in the media, may be a factor inhibiting antigen presentation by retinal EC. However, we found that neutralizing TGF-ß with a specific mAb did not overcome the inability of confluent retinal EC to support T-cell proliferation and IL-2 production. In contrast, its deactivation led to a significant inhibition of S-Ag and PHA stimulated T-cell proliferation and IL-2 production by subconfluent retinal EC. This finding implies that TGF-ß is not the inhibitor of antigen presentation by confluent retinal EC, but rather, it appears that it may be a contributing accessory factor. This effect of TGF-ß seemed to be optimal, as the addition of excess TGF-ß neither improved T-cell proliferation and IL-2 production with confluent retinal EC nor further increased these parameters with subconfluent retinal EC. These findings suggest that sufficient inhibitory factors are produced by confluent EC which are thus able to overcome the stimulatory effect of TGF-ß.

Although it is generally thought that TGF-ß is an immune suppressive factor, it has recently been reported that TGF-ß induces the ''memory'' phenotype and potentiates Ag-dependent encephalitogenic and DTH-inducing functions (Weinberg et al., 1992), which suggests that TGF-ß could promote the development of helper ''memory'' cells. Despite having an inhibitive effect on IL-4 and IL-5 synthesis TGF-ß does not have any significant effect on IFN-γ, lymphotoxin or IL-2 production by polyclonally activated human T cells, suggesting a modulatory effect of TGF-ß on the Th1/Th2 balance of immune responses (Fargeas et al., 1992). Furthermore, TGF-ß added to $CD4^+$ precursors, suppresses the development of IL-4/IL-5 secreting effectors and results instead in the development of cells secreting IL-2 and IFN-γ (Swain et al., 1991). All these studies indicate that TGF-ß differentially regulates the functions of T-cell subsets, ie. it enhances Th1 and inhibits Th2. S-Ag specific T-cell line lymphocytes are ''memory'' cells and secrete a pattern of Th1-like cytokines (including IL-2, IFN-γ and low levels of IL-4; Calder et al., unpublished data). Thus, this T-cell line may require TGF-ß as one of the costimulatory signals in effecting T-cell proliferation.

4.6.4. Comparison of retinal and brain EC with professional APC

In this study it was found that the ability of brain and retinal EC to present antigen to S-Ag specific T cells and stimulate T-cell proliferation are comparable despite brain EC expressing slightly lower levels of MHC class II I-A molecules. In comparison with professional APC, retinal and brain EC are not as effective as thymocytes (which contain dendritic cells), but are comparable to splenic adherent cells (which are rich in macrophages; Fig.3.22).

The mechanisms of these different antigen presenting abilities are not fully understood although it has been shown that optimal T-cell proliferation is dependent on both the source of the APC and the T-cell subsets (Th1 or Th2) (Gajewski et al., 1991). There are several possible explanations for these differential T cell and APC interactions: 1) the APC might inhibit proliferation at the same time as activating the T cell, 2) the APC might not express the appropriate MHC-restricting element for a given T-cell subset, 3) the APC might lack one or more important accessory molecules for optimal cell-cell interaction for a given T-cell subset, 4) different APC may be differentially able to process Ag into immunogenic peptides recognized by a given T-cell subset, and 5) Different APC might be able to provide cofactors necessary for optimal T-cell proliferation of one subset but not another.

The limited capability of brain and retinal EC to present antigen may be due to secreted factors that inhibit proliferation despite activating the T cell. Alternatively, retinal and brain EC may lack one or more of the important accessory molecules required for optimal cell-cell interactions. Furthermore, it has been reported that murine brain EC, in contrast to professional APC which stimulate both Th1 and Th2 cells, preferentially activate Th2 $CD4^+$ T cells (Fabry et al., 1993). The cells used in this study are Th1-like and the reduced level of T-cell proliferation by CNS EC may be due to the absence of the full complement of accessory factors required for inducing Th1-like T-cell proliferation.

4.6.5. The potential role of antigen presentation by CNS vascular EC *in vivo* in inflammatory diseases

Although it is difficult to determine how antigen presentation occurs *in vivo*

within the CNS, a reported study using a chimeric rat model has demonstrated that there is indeed an antigen specific, MHC class II restricted $CD4^+$ encephalogenic T-cell mediated immune response within the brain (Hinrichs et al., 1987). The antigen presenting cells in this study were found to be bone marrow derived cells, such as microglia cells and infiltrated monocytes. However, in a separate study using a similar model it was demonstrated that in addition to bone-marrow derived cells, CNS resident cells, such as astrocytes and vascular EC, are also important antigen presenting cells in CNS inflammatory diseases (Myers et al., 1993). We have been able to confirm that both retinal and brain vascular barrier cells can be induced to express MHC class II molecules and present antigen to antigen-specific T cells resulting in T-cell proliferation. This implies that CNS EC possess the potential to play a role as APC in the pathogenesis of certain diseases of the CNS. Furthermore, the antigenic activation of T cells by these vascular barrier cells may provide a mechanism for T-cell migration across the BBB and BRB, since it has been demonstrated that only activated T cells are capable of crossing the barriers and that the migratory ability is independent of their antigen specificity (Hickey 1991; Greenwood and Calder 1993).

Although we have demonstrated *in vitro* that subconfluent monolayers of CNS vascular EC are able to present antigen, it remains to be seen whether such *in vitro* manipulations have any bearing on the *in vivo* condition. It may be argued that confluent monolayers equate more to the *in vivo* state than subconfluent monolayers. CNS EC however are not entirely quiescent but do show signs of continual slow turnover. Furthermore, if the most important component in determining antigen presentation is the presence of an inhibitory factor that suppress T-cell proliferation, then a confluent monolayer would be less representative as build up of inhibitory factors *in vivo* would be rapidly removed by the circulation unless specifically trapped by the glycocalyx. It is likely therefore, that although TGF-ß may play an important part in the signalling responsible for antigen presentation, other inhibitory factors are also influential.

4.6.6. Cytotoxicity of retinal EC mediated by S-Ag specific $CD4^+$ T cells

T-cell mediated cytotoxicity can be demonstrated in both $CD4^+$ and $CD8^+$ T-cell subsets. $CD8^+$ cytotoxic T lymphocytes (CTL) specific for or restricted to MHC class I antigen are considered to be important in the recovery from viral infection (Zinkernagel and Welsh 1976). $CD4^+$ CTL restricted to MHC class II antigen were described later (Swain et al., 1981; Tite and Janeway 1984; Lukacher et al., 1985) and their potential protective benefit is not known but there is evidence that such cells are important in the aetiology of EAE (Sun and Wekerle 1986). In recent studies on the pathogenesis of EAE, cytotoxicity of brain EC by encephalogenic $CD4^+$ T cells has also been demonstrated (Sedgwick et al., 1990; Risau et al 1990; McCarron et al., 1991). This effect has been shown to be both antigen specific and MHC class II restricted and the cytotoxic damage to brain EC could potentially lead to a significant disruption of the integrity of the BBB in CNS inflammatory diseases.

In this study we have demonstrated that S-Ag specific $CD4^+$ T cells can mediate cytotoxic damage to cultured retinal EC monolayers which supports the previous findings with brain EC. It should be noted however that the concentrations of T cells required to elicit a cytotoxic response in both this and previous studies (Sedgwick et al., 1990; McCarron et al., 1989) are much greater than those used in the T-cell proliferation assay, and it is therefore unlikely that cytotoxicity occurs in the proliferation assay.

$CD4^+$ T-cell mediated cytotoxicity in response to S-Ag further demonstrates the antigen presenting ability of retinal EC. In contrast to T-cell proliferation, the cytotoxicity has been demostrated on confluent EC monolayers which are unable to stimulate T-cell proliferation (Risau et al., 1990; Pryce et al., 1989), indicating that the mechanisms of cytotoxic damage mediated by $CD4^+$ T cells are independent of T-cell proliferation.

Although the factors that mediated damage to the EC monolayers are not completely clear, our findings indicate that T-cell activation is crucial to cytotoxicity. Previous studies have shown that activated $CD4^+$ T cells produce cytokines such as TNF which have been shown to be important factors for $CD4^+$ T-cell mediated cytotoxicity (Tite 1990). Whether the cytokines produced by activated S-Ag specific

$CD4^+$ T-cell lines are responsible for the retinal EC monolayer disruption is not clear since TNF containing supernatant from MBP specific $CD4^+$ T-cell lines does not damage brain EC monolayers (Sedgwick et al., 1990). An alternative explanation for the damage mediated by activated S-Ag specific T-cell lines is that lymphocytes produce an heparan sulfate endoglycosidase which severely disrupts the integrity of the EC monolayers by degrading the matrix substance (Naparstek et al.,1984). This enzyme has been shown to be produced only by activated, but not resting $CD4^+$ T cells. Therefore, both Con A and antigen activated S-Ag specific T cells may be able to produce this substance and thereby result in the damage to the monolayers.

In addition to soluble factors, it has also been suggested that strong cellular adhesion plays an important role in $CD4^+$ T-cells mediated cytotoxicity (Sedgwick et al., 1990). In our study we have shown that S-Ag specific $CD4^+$ T cells adhered to retinal EC with a high efficiency and have the ability to cross EC monolayers. Such efficient cellular interactions may assist cytotoxicity of $CD4^+$ T cells if the cytotoxic effect is cellular contact dependent. To support this proposal, it has been shown that blocking adhesion molecules with mAb, such as ICAM-1/LFA-1 can prevent T-cell mediated cytotoxicity (Figdor et al., 1990).

Finally, one should consider whether damage to retinal vascular EC by $CD4^+$ T cells is likely to be important *in vivo* and if so what the possible mechanisms involved are. The increased permeability of BRB in EAU (Lightman and Greenwood 1992) would suggest that there are indeed substantial changes in the retinal vasculature under inflammatory conditions. Whether these effects are mediated directly by $CD4^+$ T cells is difficult to determine, but the demonstration in a mouse model of acute graft-vs-host disease of vascular leakage syndrome, mediated by passively transferred $CD4^+$ T cells (Lehmann et al., 1990), further supports the concept that $CD4^+$ T cells may be capable of mediating pathologically important effects *in vivo* that are targeted toward the vasculature (Sun and Wekerle 1986).

CHAPTER 5 CONCLUSIONS

At the boundary between blood and the retinal and brain parenchyma lies the vascular EC barrier. As a result of this unique anatomical position and their specialized characteristics, retinal and brain EC play a key role in the regulation of a variety of pathophysiological processes of the CNS. The data presented in this thesis indicates that retinal EC are important cells contributing to the development of T-cell mediated immune reactions. They play a role in both antigen independent T-cell recruitment and the potential for antigen dependent T-cell activation.

Lymphocyte adhesion and migration is a key step in lymphocyte entry into the retina, and this process is largely dependent on cellular activation of both lymphocytes and retinal EC in a manner similar to that described for lymphocyte interaction with EC derived from other vascular beds. Adhesion molecules, such as LFA-1, ICAM-1 and VLA-4 play an important role in both lymphocyte adhesion and migration, and the process of the adhesion and migration depends on the different use of these molecules. Although retinal and brain EC do not constitutively express MHC class II molecules, they may be induced to do so. The capacity of retinal and brain EC to present antigen raises the possibility that they could be antigen presenting cells to circulating ''memory'' T cells.

It is clear that these two processes of lymphocyte interactions with retinal and brain EC are controlled by different, but related, molecular mechanisms and the molecules expressed on the surface of vascular EC play an important part. In response to the inflammatory cytokine IFN-γ, the expression of MHC class II molecules on retinal and brain EC can be induced and the expression of adhesion molecule ICAM-1 is also enhanced. Since the expression of class II and ICAM-1 differentially responds to IFN-γ in a time-dependent manner, the processes associated with these molecules may occur at different stages during the cellular interactions between CNS vascular EC and lymphocytes.

Finally, it should be pointed out that mechanisms that control cellular interactions between lymphocytes and vascular EC have not been rigorously studied

in vivo. Particularly, the antigen presenting ability of vascular EC *in vivo* is largely unknown. In this *in vitro* study we have found that the ability of retinal and brain EC to present antigen is less than that of professional antigen presenting cells, such as thymocytes. This would suggest that antigen specific T-cell activation is mainly dependent on T cells interacting with professional APC, and that antigen presentation by retinal and brain EC may play a partial role in the development or perpetuation of CNS inflammatory diseases. Nevertheless, the *in vivo* implications of this *in vitro* study remain to be elucidated although some immunocytochemical studies of experimental and pathological tissues of the retina and brain have provided support for this proposal. In the future, investigations should be further focused on the molecular basis of these cellular interactions both *in vitro* and *in vivo*.

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APPENDIX

I. SDS-PAGE

Reagents

A: Stock solutions:

30% Acrylamide/Bis (Biorad, USA), TEMED (Biorad), 1.5 M Tris-HCL, pH 8.8, 0.5 M Tris-HCL, pH 6.8, 10% SDS.

B: Sample buffer (x4):

Distilled water	4.0 ml
0.5 M Tris-HCL (pH 6.8)	1.0 ml
Glycerol (Biorad)	0.8 ml
10% SDS	1.6 ml
2-ß mercaptoethanol	0.4 ml
0.05% Bromophenol blue	0.2 ml

C: 5X electrode (running) buffer, pH 8.3

Tris base	9.0 g
Glycine	43.2 g
SDS	3.0 g
H_2O	600.0 ml

D: 12% separating gel preparation

H_2O	3.35 ml
1.5 M Tris-HCL (pH 8.8)	2.50 ml
10% SDS	100 µl
30% Acrylamide/Bis	4.00 ml
TEMED	5 µl

After degassing for 15 min, 50 µl of 10% fresh ammonium persulfate were added.

C: 4.0% stacking gel preparation

H_2O	6.1 ml
0.5 M Tris-HCL (pH 6.8)	2.5 ml
10% SDS	100 µl
30% Acrylamide/Bis	1.3 ml
TEMED	10 µl
10% ammonium persulfate	50 µl

Procedures

Separating gel was poured into a sandwich chamber of the Mini-protean II Dual slab cell (Biorad). After polymerization, stacking gel was added. The protein sample (S-Ag) was incubated for 5 min with sample buffer (1:4) at 90°C, and then loaded onto stacking gel at concentrations of 5-20 μg/ml. After running for 45 min at 200 volts in the running buffer, the gel was stained for 30 min with Commmassie blue R-250 (Biorad).

II. PREPARATION OF PLASMA DERIVED SERUM (PDS):

Chemicals: 0.38% Citric Acid, trisodium salt (Sodium citrate, Sigma); 10 mM HEPES buffer (Sigma); 1 M $CaCL_2$ (Sigma).

CM-Sephadex (Sigma) was swollen in 0.9% saline buffer containing 10 mM HEPES and washed three times with the saline buffer. The beads were resuspended in the buffer and stored at 4°C.

Method: Two litres of fresh cow blood were collected, placed into the plastic container and mixed with 200 ml of 0.38% sodium citrate. The blood cells were removed by centrifuged at 1200g for 20 min at 4°C in plastic centrifuge tubes, and supernatant was carried out for a further centrifugation at 24500g for 20 min at 4°C to remove platelets. Plasma was collected and mixed with 1 M $CaCL_2$ to give a final concentration of 20 mM of $CaCL_2$, and incubated at 50°C for 30 min, then placed in 4°C for overnight. The clotted plasma was centrifuged for 30 min at 25,000 g, and fibrin was removed. The supernatant (ie. PDS) was dialysed 24 hours in 3500 MW dialysed membrane (Millipore) against 20 times of volume of 10 mM HEPES buffer at 4°C. At the end of dialysis, remaining fibrin was removed by further centrifugation.

PDS was mixed with prepared CM-Sephadex beads in the ratio of 10:1, and incubated for 5-10 min at room temperature to remove platelet derived growth factor (PDGF). Following centrifugation for 15 min at 1000g, the supernatant was collected, heat-inactivated for 30 min at 56°C and sterilized by filtering through 0.22 μm filter.

III. TITRATION OF CON A AND PHA CONCENTRATIONS

Con A and PHA were dissolved in HBSS at a concentration of 1 mg/ml. The chemicals were sterilized by filtration with 0.22 μm filters and stored at -20°C.

Lymphocytes obtained from rat peripheral lymph nodes were placed to 96-well plates at a concentration of $5x10^4$ cells/well in Nutridoma-SP conditioned RPMI medium. A range of concentrations of Con A (0.1-10 μg/ml) and PHA (1-20 μg/ml) was added. The cells were then incubated for 3 days at 37 °C, in a 5% CO_2 incubator. At last 8 hours 1 μCi/well of ^{3}H-thymidine was pulsed. Cells were harvested onto nitrocellulose paper and ^{3}H-thymidine incorporation was counted on a ß-scintillation counter. The results are shown in the figures.

IV. TEST OF α-METHYL-ß-MANNOSIDE

The concentrations of α-methyl-ß-mannoside that are able to effectively neutralize Con A were tested by inhibition of Con A stimulated lymphocyte proliferation.

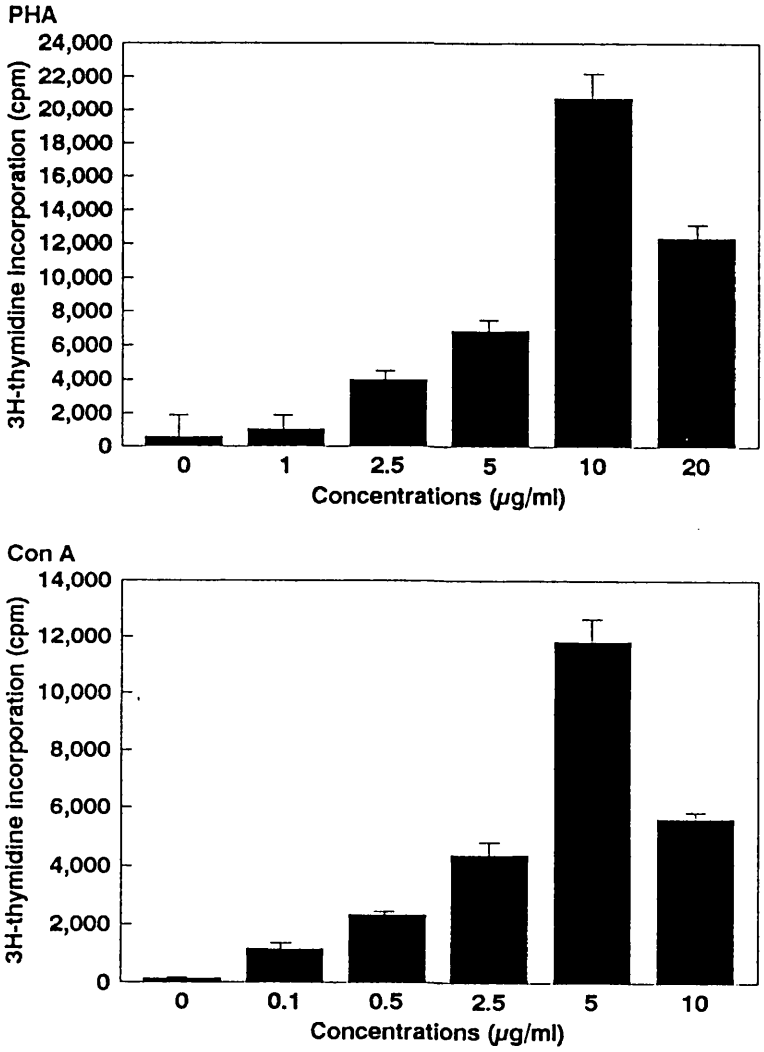
Con A stimulated lymphocyte proliferation was carried out by the same method described above. The wells were included with 5 μg/ml Con A and different concentrations of α-methyl-ß-mannoside. After 3 days cultures, lymphocyte proliferation was assayed by ^{3}H-thymidine incorporation. The results are shown below.

Inactivation of Con A by α-methyl-ß-mannoside

Conc.of α-methyl-ß-mannoside	cpm
Control	3099±892
0	24801±6463
0.05	16160±2733
0.1	2083±821

Data given as means±SD

Lymphocyte proliferation stimulated by mitogens



$5x10^4$ cells/well were incubated for 3 days with a range of concentrations of the mitogen PHA and Con A, and proliferation was measured by ^{3}H-thymidine incoporation.

PUBLICATIONS

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