

**CELLULAR INTERACTIONS BETWEEN  
LYMPHOCYTES AND RETINAL PIGMENT EPITHELIAL <sup>u.m</sup> CELLS ~~IN VITRO~~**

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This thesis is submitted for the degree  
of Doctor of Philosophy at the University of London  
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**ABSTRACT**

Posterior uveitis is an ocular inflammatory condition which can cause extensive retinal damage. Evidence indicates that this is a T cell mediated disorder and it is likely that induction of the disease is brought about by the migration of lymphocytes from the circulation into the neuroretina. For this to occur lymphocytes must first cross the blood-retinal barrier (BRB) which consists of the retinal vascular endothelium in the anterior portion of the retina and the retinal pigment epithelium (RPE) at the posterior aspect. Lymphocyte migration into the retina can therefore occur via these two separate routes; a direct route by crossing the vascular endothelium and an indirect route via the RPE. For migration to occur by this latter path lymphocytes must first be captured from the circulation by the choroidal endothelium. From here they must then migrate into the extracellular space and penetrate Bruch's membrane, before interacting with adhesion molecules expressed on the RPE which are likely to control migration into the retina.

The role of the vascular endothelium in controlling lymphocyte migration has been extensively studied but the role of other cellular barriers in modulating lymphocyte passage is poorly understood. This project was therefore undertaken to investigate the factors involved in lymphocyte adhesion to and migration across RPE monolayers *in vitro*. PVG rat RPE cells were first isolated and characterised by immunostaining techniques for the expression of cytokeratins and the rat RPE specific monoclonal antibody PE2. In addition, these cells were shown to constitutively express ICAM-1 and MHC class I which was upregulated upon activation with IFN- $\gamma$ . MHC class II expression, however, was only found on IFN- $\gamma$  activated cells. Due to the difficulty associated with primary cell culture a rat RPE cell line was developed by immortalising primary cultures of rat RPE using SV40 large T. These cells were characterised and shown to express similar levels of RET-PE2 and cytokeratins as primary cultures.

Rat lymphocytes used in this study were characterised using flow cytometric analysis to determine the phenotype and state and mode of activation of each group studied. The ability of peripheral lymph node (PLN) cells (activated and non-activated) and antigen specific CD4<sup>+</sup> T cell line lymphocytes to adhere to RPE cells *in vitro* was evaluated. Results demonstrate that the state and mode of lymphocyte activation is crucial in determining their ability to adhere to and migrate across the monolayers. Antigen specific T cell lines were more adhesive and migratory than untreated or activated PLN



cells. The effect of activating the RPE with the cytokines interferon- $\gamma$  (IFN- $\gamma$ ) and interleukin-1 (IL-1) on adhesion and migration were also studied. It was found that adhesion was not increased by cytokine activation whereas migration was increased by activation of cells with IFN- $\gamma$  but not IL-1.

The adhesion molecules involved in the migration of antigen specific T cell lines across RPE monolayers was also studied. Migration was found to be predominantly ICAM-1/LFA-1 mediated, with VCAM-1/VLA-4 interactions playing a role only when monolayers had been activated for 72 hours with IFN- $\gamma$ . In addition, the ability of the immortalised RPE cells to support lymphocyte migration was determined. The migration of PLN cells was similar to that observed with primary cultures, although the migration of antigen specific T cell line lymphocytes was found to be lower.

To compare results from RPE-lymphocyte interactions with that of retinal endothelial cell-lymphocyte interactions, it was necessary to study lymphocyte migration across PVG rat derived retinal endothelial cells as retinal endothelial studies in our department had employed endothelium isolated from Lewis rats. During the course of this study it was noted that lymphocyte migration was significantly lower across PVG retinal endothelium compared with Lewis rat derived retinal endothelium. Lewis rats are more susceptible to the animal model for uveitis, therefore this may be one of the factors responsible for this. To study this further, ICAM-1 and VCAM-1 expression were compared on retinal endothelium isolated from the two rat strains and it was found that PVG rats expressed lower constitutive levels of ICAM-1.

## ABBREVIATIONS

|               |  |
|---------------|--|
| APC           | antigen presenting cell                                  |
| BBB           | blood-brain barrier                                      |
| BRB           | blood-retinal barrier                                    |
| BSA           | bovine serum albumin                                     |
| CNS           | central nervous system                                   |
| Con A         | concanavalin A   |
| EAE           | experimental allergic encephalomyelitis                  |
| EAU           | experimental autoimmune uveoretinitis                    |
| EC            | endothelial cell   |
| ECGS          | endothelial growth supplement                            |
| ELISA         | enzyme-linked immunosorbent assay                        |
| FCS           | foetal calf serum  |
| GM-CSF        | granulocyte macrophage colony stimulating factor         |
| HBSS          | Hanks' balanced salt solution                            |
| HEV           | high endothelial venule                                  |
| HUVEC         | human umbilical vein endothelium                         |
| ICAM          | intracellular adhesion molecule                          |
| IFN- $\gamma$ | interferon gamma   |
| IL            | interleukin  |
| IRBP          | interphotoreceptor binding protein                       |
| LFA           | lymphocyte function-associated antigen                   |
| mAb           | monoclonal antibody                                      |
| MBP           | myelin basic protein                                     |
| MCP           | monocyte chemoattractant protein                         |
| MHC           | major histocompatibility complex                         |
| MIP           | macrophage inflammatory protein                          |
| NRS           | normal rat serum   |
| PDS           | plasma derived serum                                     |
| PLN           | peripheral lymph node                                    |
| PMA           | phorbol myristate acetate                                |
| RANTES        | regulated on activation, normal T expressed and secreted |
| RPE           | retinal pigment epithelium                               |
| S-Ag          | retinal soluble antigen                                  |
| TGF $\beta$   | transforming growth factor beta                          |
| TNF           | tumour necrosis factor                                   |
| VCAM          | vascular cell adhesion molecule                          |
| VLA           | very late antigen  |

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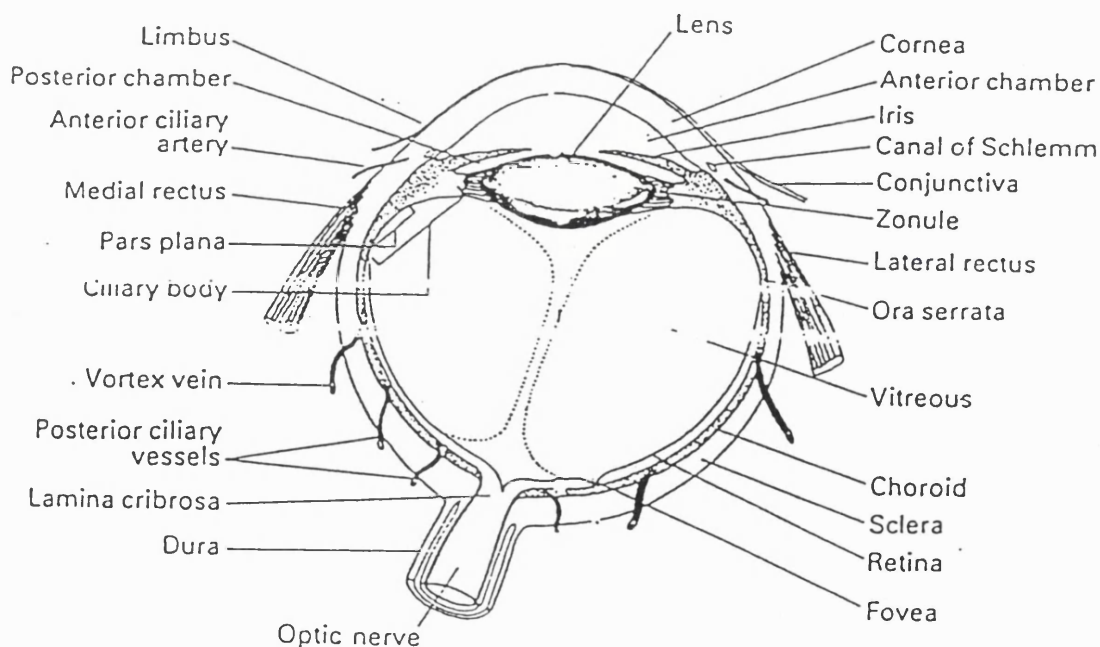
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## INTRODUCTION

### 1.1 OCULAR INFLAMMATORY CONDITIONS.

Immunological conditions of the eye can be divided into two groups in that the eye may be the sole target of attack or it may be one of many tissues involved in a systemic process. There are a number of different inflammatory conditions of the eye and they are named depending on which part of the eye is inflamed ie. conjunctivitis, keratitis, scleritis and, of relevance to this thesis, uveitis. The structure of the eye is shown below (Figure 1.1)



**Fig 1.1** Diagrammatic horizontal section of the eye.

### 1.1.1 Uveitis

Uveitis is a relatively common clinical disorder occurring at a yearly rate of 20/100,000 (Forrester et al., 1990). Many clinical conditions are encompassed in the term uveitis which refers to an inflammation of the uveal tract. Depending on the anatomical location of the inflammation this condition can be classified into anterior or posterior uveitis.

Anterior uveitis involves an inflammation of the iris and ciliary body and often occurs as a self-limiting disorder in which there is a close linkage with Class I MHC, in particular with HLA-B27. Although infective agents have been implicated in the pathogenesis of acute anterior uveitis, in most cases the aetiology remains obscure.

Posterior uveitis is classified by inflammation of the choroid and retina in which there is often an association with Class II MHC molecules (Lightman and Chan, 1990). This condition may occur in association with systemic disorders, such as sarcoidosis and Behçet's disease, or as an organ specific autoimmune condition as in sympathetic ophthalmia. Most forms of the disease have several features in common including vitreous inflammatory cells, retinal vasculitis and macular oedema. This condition usually runs a chronic relapsing course that may be difficult to manage clinically and can result in blindness.

It is now generally accepted that T cells play a dominant role in mediating posterior uveitis (Lightman and Chan, 1990; Lightman and Towler, 1992). Evidence for this comes from histological and immunohistochemical examination of eyes from patients with sympathetic ophthalmia, Vogt-Koyanagi (VKH) disease and birdshot chorioretinitis, where significant numbers of T cells were observed (Chan et al., 1988; Jakobiec et al., 1983). The majority of infiltrating cells found in the enucleated eyes from patients with sympathetic ophthalmia were CD4<sup>+</sup> T cells in the early stage of the disease, with increased numbers of CD8<sup>+</sup> cells found during the chronic phase of disease (Chan et al., 1988). It does not appear that antibodies or complement play a role in this inflammatory process as very few B cells or immunoglobulin have been detected in the retina suggesting that there is no evidence of immune complex deposition (Lightman and Chan, 1990). The beneficial effects of cyclosporin in the clinical management of patients with uveitis is further evidence for the role of T cells in this inflammation.

The aetiology of posterior uveitis in most cases is unknown, with known infective

agents being the cause in only a few cases. Although the initiating antigen responsible for activation of the T cells remains undefined there are a number of candidate retinal antigens which may induce this inflammatory response. For example, retinal soluble antigen (S-Ag) and interphotoreceptor binding protein (IRBP) have been shown to induce uveoretinitis in animal models of the disease (Caspi, 1989). In addition some patients with uveitis have been shown to have a significant frequency of CD4<sup>+</sup> T cells in their peripheral blood which respond to S-antigen (Opremcak et al., 1991; de Smet et al., 1990) and IRBP (de Smet et al., 1990).

### 1.1.2 Experimental Autoimmune Uveoretinitis

In order to try to elucidate some of the immunopathogenic mechanisms involved in uveitic conditions in humans, it has been necessary to employ experimental animal models of uveitis. One of the most extensively studied of these models is experimental autoimmune uveoretinitis (EAU) which has a number of similarities to the experimental model for multiple sclerosis (MS), experimental allergic encephalomyelitis (EAE) (Calder and Lightman, 1992). A number of rodent models of EAU have been developed in the rat, guinea pig, rabbit and mouse. In all cases, susceptible strains of the animal are injected with one of several antigens extracted from the retina as an emulsion in complete freund's adjuvant (CFA) at a site distant from the target organ. EAU was first induced by injection of retinal extracts in emulsion with CFA (Wacker and Lipton, 1968). Since then at least three different uveitogenic proteins isolated from this extract have been identified: they are retinal soluble antigen (S-Ag), interphotoreceptor binding protein (IRBP) and rhodopsin (reviewed by Caspi, 1989). EAU is characterised by destruction of the photoreceptors and a loss in integrity of the retinal layers.

Evidence indicates that susceptibility to autoimmune diseases is largely genetically determined (reviewed by Theofilopoulos, 1995) with major histocompatibility genes being a major factor. Different strains of rats display different susceptibilities to induction of animal models such as EAU or the animal model of multiple sclerosis (MS), experimental allergic encephalomyelitis (EAE). Lewis and PVG rats are both sensitive to bovine S-Ag (de Kozak et al., 1981), whereas Brown Norway rats are not (Mirshahi et al., 1990). However, the disease induced in PVG rats is less severe and more chronic in nature than that observed in Lewis (de Kozak et al., 1981). Similar results are observed with EAE,



in that Lewis rats are the most susceptible, with PVG rats showing an intermediate susceptibility while Brown Norway rats are resistant to disease (Male and Pryce, 1989; Happ et al., 1988).

Several lines of evidence indicate that, as with posterior uveitis, T cells play a major role in EAU. Athymic nude rats fail to develop EAU following immunisation with S-Ag (Salinas-Carmona et al., 1982), although spontaneous EAU has been reported in nude mice reconstituted with embryonic rat thymus (Ichikawa et al., 1991). In addition the development of EAU has been shown to be inhibited by treatment with the immunosuppressive agent cyclosporin (Nussenblatt et al., 1981). However, the most direct evidence that T cells are involved in mediating EAU comes from adoptive transfer studies in which S-antigen specific T cell line lymphocytes injected into naive rats are capable of inducing EAU (Caspi et al., 1986).

The increase in leucocyte recruitment into the retina in EAU is associated with breakdown of the blood-retinal barrier (BRB) (Lightman and Greenwood, 1992) and oedema formation (de Kozak et al., 1981), both of which are involved in the pathogenesis of the disease.

## **1.2 THE BLOOD RETINAL BARRIER**

### **1.2.1 Structure and Function**

The retina lines the whole inner surface of the eye behind the ora serrata and is considered to be an extension of the central nervous system (CNS). One of the most notable morphological difference between the CNS and other tissues is the existence of a selective cellular barrier which separates the CNS tissue from the blood and which, thereby, preserves the CNS as a protected environment. In the retina this barrier is formed by the retinal vascular endothelium and the retinal pigment epithelium (RPE) known collectively as the blood-retinal barrier (BRB)(Figure 1.2).

The anterior retinal barrier is formed by the vascular endothelium and nutrients are supplied via the retinal vasculature using special transport mechanisms. The endothelial cells which form this aspect of the BRB are functionally and structurally identical to those of the brain in that they form tight junctions with extremely high electrical resistance (Towler et al., 1994) which limit paracellular diffusion. In addition

to the specialised junctions, the endothelial cells also express low levels of pinocytosis, have no fenestrae or transendothelial pores.

The RPE which forms the posterior BRB separates the outer retina from its blood supply formed by the choriocapillaris of the choroid. Choroidal endothelium are comparable to that of the choroid plexus of the brain in that they are permeable and fenestrated. The tight apical junctions of the RPE are therefore responsible for controlling the passage of substances into and out of the outer retina via the choroidal circulation.

Together these cellular barriers are believed to be responsible for the limited traffic of leucocytes into the retina during normal immune surveillance.

### 1.2.2 Inflammation and the Blood-Retinal Barrier.

The BRB plays an important role in the pathogenesis of inflammatory eye conditions. One of the main problems associated with these diseases is a dramatic increase in the permeability of the BRB which, although not being the central cause, does lead to the development of oedema and subsequent functional impairment of vision. There are a number of possible causes of this increase in permeability. Breakdown of the BRB in EAU occurs in association with lymphocytic infiltration in the retina and does not occur prior to this event (Lightman and Greenwood, 1992). This suggests that it is the migration of leucocytes that causes physical disruption of the BRB, although it is more likely that increased permeability is a result of locally released vasoactive inflammatory mediators resulting from the leucocytic infiltration.

In areas of the greatest leucocyte infiltration the endothelia have been reported to display a number of morphological features characteristic of high endothelial venules. Furthermore, they have increased amounts of cytosol and an upregulation of cytosolic organelles such as rough endoplasmic reticulum and ribosomes (Greenwood et al., 1994). These changes are thought to be the consequence of increased metabolic activity as the endothelial cell will be induced to synthesise immunologically important molecules such as adhesion molecules and cytokines.

In EAU the RPE remains well preserved throughout the disease process with only small changes in permeability (Greenwood et al., 1994). It retains its monolayer configuration even in advanced EAU (Greenwood et al., 1994; Dua et al., 1991), the only notable difference being that cell processes may become stunted or lost

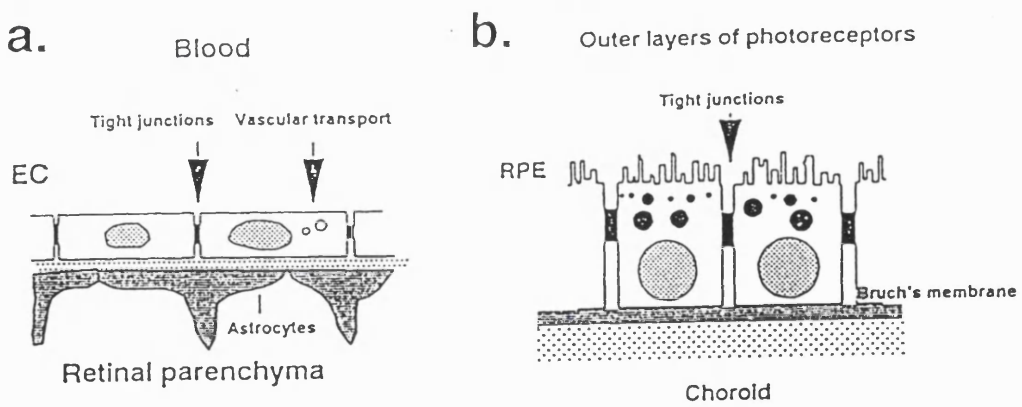
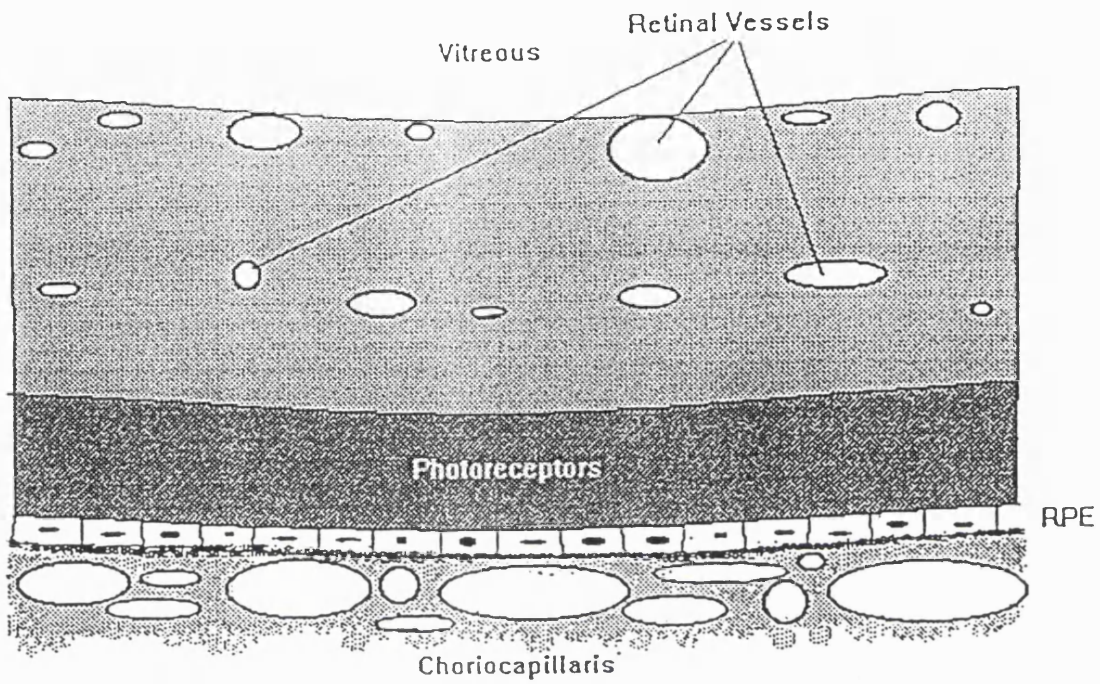


Fig 1.2 Schematic drawing of the retinal layers and the cells of the BRB ie. (a) retinal vascular endothelium (b) the RPE.

(Dua et al., 1991).

The route of leucocyte entry into the retina remains uncertain. Due to the endothelia of the retinal vasculature being in direct contact with circulating cells, leucocytes are more likely to cross the BRB at this site. Inflammatory cells crossing the BRB at the RPE must first be captured by the choroidal endothelia from where they must extravasate and penetrate Bruch's membrane before crossing the RPE. Despite this being a more tortuous route, there is ultrastructural evidence that leucocytes can cross the posterior barrier, but mainly during the latter stages of the disease (Greenwood et al., 1994; Lin et al., 1991; Dua et al., 1991). Moreover, in the guinea pig model of EAU the inflammatory cells are likely to enter the retina via the RPE as in this animal the retina is avascular (Caspi, 1989). In all models of EAU there is a significant recruitment of leucocytes by the choroidal vasculature leading to the choroid becoming packed with inflammatory cells. This may result in the separation of Bruch's membrane into its outer collagenous layer at the choriocapillaris and the inner cuticular layer at the RPE (Dua et al., 1991). From Bruch's membrane leucocytes can pass through the inner cuticular layer, migrate beneath and between the RPE cells eventually reaching the photoreceptors (Greenwood et al., 1994; Dua et al., 1991). There is also evidence that inflammatory cells have been seen apparently within RPE cells (Greenwood et al., 1994) but the direction of leucocyte migration in this case could not be determined.

### 1.3 LEUCOCYTE TRAFFICKING

Lymphocytes continually patrol the body for foreign antigen by recirculating from blood, through tissue, into lymph and back to blood. This trafficking is very important in maintaining immunological surveillance throughout the body and in launching an inflammatory response. The migration pattern of leucocytes, including lymphocytes, through a tissue dramatically alters during inflammatory conditions. The acute inflammatory response is characterised by an early influx of neutrophils, with lymphocytes and mononuclear phagocytes entering the lesion at a later stage where they may then become the predominant cell type. The arrival of different immune cells at the inflammatory site is controlled by the action of various cytokines that regulate expression of adhesion molecules on vascular endothelium. These adhesion molecules interact with

receptors on leucocytes and this determines which leucocytes leave the vasculature and enter the inflammatory site and the extent of the recruitment. However, receptors for extracellular matrix and tissue cells on the leucocytes determine if the leucocytes move through the tissues or are retained.

Chemotactic factors are also important in controlling cell migration, some of which can induce the expression of adhesion molecules.

### 1.3.1 Adhesion Molecules

As already mentioned, a dramatic increase in the expression of adhesion molecules to support the entry of leucocytes to the inflammatory site is central to the process of inflammation. The adhesion molecules expressed by cells is dependent on their state of activation, the cytokine profile of the local environment and their maturation. Different adhesion molecules control the traffic of different cells, however some are common to a wide variety of leucocytes. Three families of adhesion receptors mediate these interactions: the immunoglobulin superfamily; the integrins and the selectins.

#### *Immunoglobulin Gene Superfamily*

The complex array of structures found within this family are thought to have evolved from a single ancestral unit, the immunoglobulin fold, which has undergone divergence and duplication. A number of members of this family are involved in cellular interactions, for example the CD4 and CD8 molecules interact with MHC class II and I antigens respectively which is important in antigen recognition and T cell activation. Of particular importance in leucocyte trafficking, however, are the adhesion molecules intercellular adhesion molecule-1 and -2 (ICAM-1, ICAM-2) and vascular cell adhesion molecule-1 (VCAM-1).

**ICAM-1** :- In the absence of an inflammatory response, ICAM-1 is basally expressed on only a few cell types, including monocytes and endothelial cells. However during an inflammatory response this adhesion molecule can be induced or upregulated on a wide variety of cells, including B and T lymphocytes, thymocytes, dendritic cells, endothelial cells, fibroblasts, keratinocytes, chondrocytes and epithelial cells (Dustin et al., 1986; Dustin and Springer, 1988; Dustin et al., 1988; Haskard et al., 1987; Mentzer et al.,

1988; te Velde et al., 1987; Garner et al., 1994). This adhesion molecule has been shown to be important in mediating immune and inflammatory responses. It contributes to the adhesion between interacting lymphocytes, and between lymphocytes and antigen-presenting cells or target cells (Springer, 1990; Koopman et al., 1991). In addition the expression of ICAM-1 on endothelial cells and its induction on a wide variety of tissue cells controls the migration of leucocytes into inflammatory sites. The expression of ICAM-1 *in-vitro* is induced or upregulated by the cytokines IFN- $\gamma$ , IL-1 $\beta$ , TNF- $\alpha$  or lipopolysaccharide (LPS) (Dustin et al., 1986) reaching maximal levels after 24hr and increasing lymphocyte adhesion and migration (Issekutz, 1990). The ligand for ICAM-1 is LFA-1 which is expressed on leucocytes.

**ICAM-2** :- Unlike ICAM-1, this adhesion molecule is expressed constitutively at high levels on endothelial cells and its expression is not inducible or upregulated by cytokines (Staunton et al., 1989). ICAM-2 is also found on T cells, monocytes and dendritic cells. It is an alternative ligand for the leucocyte receptor LFA-1 and this interaction is thought to be responsible for the normal traffic of lymphocytes.

**ICAM-3** :- Present on all leucocytes, but not on EC and does not appear to be involved in migration but is important in generation of the immune response (de Fougères and Springer, 1992).

**VCAM-1** :- Expression of VCAM-1 in normal tissues and untreated cultured endothelial cells is minimal (Osborn et al., 1989; Carlos et al., 1990). However VCAM-1 has been found in a wide variety of inflammatory lesions (Rice et al., 1991) as well as on activated cultured endothelial cells (Osborn et al., 1989; Carlos et al., 1990; Schwartz et al., 1990; Rice and Bevilacqua, 1989; Rice and Bevilacqua, 1989). This adhesion molecule has also been found on tissue macrophages, dendritic cells and can be induced on some epithelial cells (Rice et al., 1991; Garner et al., 1994; Nakajima et al., 1995, Engelhardt et al., 1995). VCAM-1 is particularly important in the recruitment of leucocytes to sites of inflammation as it mediates the adhesion of lymphocytes (Rice et al., 1990; Nakajima et al., 1994), monocytes (Carlos et al., 1991) and eosinophils (Nakajima et al., 1994) to activated endothelium. An additional function of importance in lymphocyte migration, is

that it is required for the induction of a gelatinase produced by T cells which may facilitate migration (Romanic and Madri, 1994). VCAM-1 may also play a role in normal immune function as it is involved in the interaction of lymphocytes and dendritic cells and the binding of VCAM-1 to T and B lymphocytes can induce antigen-receptor dependent activation (Springer, 1990; Koopman et al., 1991). In addition, immobilised VCAM-1 has been demonstrated to induce proliferation of CD4<sup>+</sup> T cells when co-stimulated with the TcR/CD3 complex (Damle and Aruffo, 1991). There are two possible ligands for VCAM-1, the integrins  $\alpha_4\beta_1$  (VLA-4) or  $\alpha_4\beta_7$ . The expression of VCAM-1 can be induced by the cytokines IL-1 $\beta$ , IL-4, TNF- $\alpha$ , and IFN- $\gamma$  (Masinovsky et al., 1990; Dinarello, 1988; Old, 1985; Beutler and Cerami, 1986) and, on endothelial cells, has been reported to reach maximal levels after 4 hours stimulation and to remain at this level for 48-72 hours (Carlos et al., 1990; Osborn et al., 1989; Rice et al., 1991).

### *Integrins*

Each integrin molecule comprises of an  $\alpha$ - and  $\beta$ -sub-unit and three sub-families of integrins can be distinguished by their  $\beta$ -sub-units; these are known as  $\beta_1$  (CD29),  $\beta_2$  (CD18) and  $\beta_3$  (CD61) integrins (Springer, 1990).

$\beta_1$  integrins are also known as VLA proteins (very late activation) and include receptors that bind to the extracellular matrix components fibronectin, laminin and collagen. The expression of VLA-4 on resting lymphocytes has been shown to be involved in lymphocyte recirculation.

**VLA-4 :-** VLA-4 is found on various leucocytes including thymocytes, peripheral blood lymphocytes, monocytes, NK cells, and eosinophils, but is absent from most tissue cells (Hemler et al., 1990). It was first described as an  $\alpha_4\beta_1$  heterodimer, appearing as the predominant VLA protein complex on multiple T lymphoblastoid cell lines (Hemler et al., 1987). It can function as either a matrix receptor, binding to CS1 domain of fibronectin (Wayner et al., 1989), or as a cell receptor binding to VCAM-1 expressed on activated endothelium (Elices et al., 1990). VLA-4/VCAM-1 interactions play a major role in mediating leucocyte migration during inflammation.

The  $\beta_2$  integrins, also known as leucocyte integrins, consists of the molecules

LFA-1 (lymphocyte function activation antigen), Mac-1 and p150,95. Of particular interest in the trafficking of lymphocytes is LFA-1.

**LFA-1 :-** Lymphocyte function associated molecule-1 (LFA-1) is expressed, at different levels, on most leucocytes (Krensky et al., 1983) with the exception of some murine macrophages (Kurzinger et al., 1982). During the acute inflammatory response LFA-1 is involved in a number of antigen-independent processes such as interaction of leucocytes with endothelium, epithelial cells, synovial cells, keratinocytes and fibroblasts through binding to ICAM-1 (Dustin and Springer, 1988; Dustin et al., 1986; Haskard et al., 1987; Mentzer et al., 1988; te Velde et al., 1987; Haskard et al., 1986). It is also involved in most immune phenomena involving T lymphocytes such as adhesion of cytotoxic T cells to their target cells, mixed lymphocyte reactions, antigen-specific and Con A induced T cell proliferation and T cell dependent antibody response (Martz, 1987). It is mainly constitutively expressed, with an increase in avidity for its ligand ICAM-1 upon activation, rather than a quantitative increase in cell surface expression. Activation can be triggered through the T cell receptor complex or by treatment with phorbol ester (Dustin and Springer, 1989).

The  $\beta_3$  integrins consist of the vitronectin receptor and Gp IIb/IIIa found on platelets.

### *Selectins*

There are three known members of this family: L-selectin, P-selectin and E-selectin. They have a number of structural similarities including a lectin-like domain and all help regulate leucocyte binding to endothelium at inflammatory sites.

**L-Selectin :-** Expressed constitutively on all circulating leucocytes except a subpopulation of memory lymphocytes (Bevilacqua, 1993). This molecule binds to its ligand on high endothelial venules of peripheral lymph nodes and hence is also known as the peripheral lymph node homing receptor. In addition to this function, it mediates PMN, lymphocyte and monocyte binding to endothelium at inflammatory sites (Spertini et al., 1991; Lewinsohn et al., 1987). L-selectin has been shown to be involved in leucocyte rolling



on the vessel wall which precedes firm attachment and extravasation during inflammation (von Andrian et al., 1991; Ley et al., 1991; Lawrence et al., 1995). After the initial adhesion, L-selectin is shed from the surface of lymphocytes and neutrophils which may allow leucocytes to detach (Jung et al., 1988; Kishimoto et al., 1989; Berg and James, 1990; Tedder, 1991). The ligand for L-selectin incorporates sialic acid, eg. MECA-79 (peripheral lymph node addressin) (Streeter et al., 1988; Spertini et al., 1991).

**P-Selectin** :- P selectin is expressed on activated platelets and endothelial cells. This adhesion molecule mediates the adhesion of neutrophils and monocytes to activated platelets (Larsen et al., 1989; Hamburger and McEver, 1990; de Bruijne-Admiraal et al., 1992) and endothelial cells (Etingin et al., 1991; Geng et al., 1990). It has also been reported that P-selectin can mediate the initial adhesion of CD4<sup>+</sup> lymphocytes to TNF- $\alpha$  activated endothelium (Luscinskas et al., 1995). The expression of P-selectin occurs within minutes of stimulation with thrombin, histamine, PMA or peroxides as it is translocated to the cell surface from  $\alpha$ -granules of platelets or Weibel-Palade bodies of endothelial cells (Bevilacqua, 1993; McEver et al., 1989; Bonfanti et al., 1989). P-selectin interacts with sialylated, fucosylated lactosaminoglycans including sialyl Lewis<sup>x</sup> found on neutrophils (Polley et al., 1991).

**E-selectin** :- This selectin has only been found on activated endothelial cells and is important in the regulation of inflammatory and immunological events at the vessel wall. E-selectin mediates the adhesion of neutrophils (Bevilacqua et al., 1989; Hession et al., 1990), monocytes (Carlos et al., 1991), and some CD4<sup>+</sup> memory cells (Picker et al., 1991; Shimizu et al., 1991). *In vitro* activation of endothelium by cytokines such as IL-1 $\beta$  and TNF- $\alpha$  results in a rapid though transient upregulation of E-selectin reaching a peak at 4-6 hours and declining to baseline levels by 24-48 hours (Bevilacqua et al., 1989; Bevilacqua et al., 1987). The ligands for E-selectin are thought to be sialyl Lewis<sup>x</sup>, sialyl Lewis<sup>a</sup> and related fucosylated N-acetyl-lactosamines found on leucocyte glycolipids and glycoproteins (Polley et al., 1991; Berg et al., 1991).

### 1.3.2 Adhesion Molecules and Leucocyte Trafficking

The adhesion molecules constitutively expressed by endothelial cells lining the post capillary venules and microcirculation interact with receptors on circulating leucocytes during normal immune surveillance. However during inflammation, in response to a wide variety of inflammatory mediators, there is an alteration in the expression of adhesion molecules on both endothelium and leucocytes. Adhesion molecule expression may be altered quantitatively through induction, upregulation or by qualitative changes in their affinity for receptors. These modifications result in increased recruitment and an accumulation of leucocytes at the inflammatory site.

A general model has now been proposed in which leucocyte-endothelial cell recognition is viewed as an active process requiring at least three sequential events (Butcher, 1991; Springer, 1994).

The first stage in the process is mediated by selectins. *In vitro* studies suggest that L-selectin mediates leucocyte rolling which slows their transit through inflamed venules, allowing further activation or more stable adhesion to occur (Springer, 1994; Butcher, 1991; Lawrence et al., 1995). This initial adhesion is transient and reversible and may bring leucocytes into closer proximity to chemoattractants which are displayed on or released from the endothelial lining of the vessel wall. The activation of the leucocyte by specific chemoattractants or cell contact-mediated signals capable of triggering activation dependent secondary adhesion molecules is the second step in the process. Chemoattractants are important in activation of integrin adhesiveness and therefore in directing the migration of leucocytes. This is because chemoattractant receptors are coupled to G proteins which transduce signals that results in an increase in integrin adhesiveness. The chemokines produced by the endothelium selectively attracts different leucocytes to the inflammatory site. Of particular importance to the recruitment of lymphocytes are the chemokines MIP-1 $\beta$  (macrophage inflammatory protein 1 $\beta$ ) which enhances the adhesion of naive CD8<sup>+</sup> T lymphocytes (Tanaka et al., 1993); RANTES (Regulated on Activation, Normal T Expressed and Secreted) which selectively attracts a subset of memory T lymphocytes (Schall et al., 1990; Murphy, 1994) and MCP-1 (monocyte chemoattractant protein-1) (Carr et al., 1991) which is abundantly expressed at sites of antigenic challenge and autoimmune disease (Miller and Krangel, 1992). The neutrophil chemotactic protein IL-8 has also been shown to be chemotactic for

lymphocytes (Larsen et al., 1989).

The increase in adhesiveness in integrins (ICAM-1/VCAM-1) allows them to bind to their ligands on the endothelium (ie. LFA-1/VLA-4), which is the third step of the process resulting in firm adhesion and migration.

Lymphocytes which accumulate in tissues in autoimmune disease are almost always memory cells (Mackay, 1992). Memory lymphocytes are imprinted so that they are more likely to return to the type of tissue, such as skin or mucosa, where they first encountered antigen, although there is no evidence for this in the CNS or elsewhere. The phenotype of the cells at sites of inflammation is quite similar to that of lymphocytes which traffic through these sites under basal conditions, suggesting that the same molecular mechanisms that mediate basal trafficking may be upregulated in inflammation (Mackay, 1992). Memory lymphocytes have been demonstrated to be more efficient than naive cells in inducing the expression of E-selectin and VCAM-1 (Damle et al., 1991) which could explain their preferential migration into inflammatory sites. It has also been reported that memory lymphocytes have an increased expression of adhesion molecules known to be involved in migration, in comparison with naive T cells (Sanders et al., 1988; Damle et al., 1991).

#### **1.4 LYMPHOCYTE MIGRATION INTO THE CNS**

The central nervous system was, until recently, considered an "immune privileged site" as it was thought that leucocytes could not cross the blood-CNS barrier under normal circumstances. However it has since been shown that there is limited access of activated lymphocytes and other leucocytes into the CNS during normal immune surveillance (Hickey et al., 1991; Lassmann et al., 1993). In fact it appears that activated T lymphocytes can enter the CNS in a random manner, irrespective of antigen-specificity or MHC compatibility of the lymphocytes with the host nervous system (Hickey et al., 1991). The control of migration of immune cells has been attributed to the highly specialised nature of cerebral endothelium (Male and Pryce, 1989; Male et al., 1992). *In vitro* studies have shown that lymphocyte adhesion to endothelium derived from CNS tissue is much lower (Hughes et al., 1988; Wang et al., 1993; Male et al., 1990) in comparison with microvascular endothelia from other tissues such as the skin (Haskard

et al., 1987). This low level of lymphocyte adhesion to brain and retinal endothelium is believed to be a consequence of the limited level of adhesion molecule expression and not due to the presence of tight junctions between CNS-endothelia.

Multiple sclerosis, posterior uveitis and their animal models, EAE and EAU are believed to be mediated by CD4<sup>+</sup> T cells. *In vitro* studies have shown that the adhesion of CD8<sup>+</sup> T cells to CNS-endothelia is greater than that of CD4<sup>+</sup> T cells (Pryce et al., 1991; Wang et al., 1993). On the other hand CD4<sup>+</sup> T cells can migrate across these monolayers to a greater extent than CD8<sup>+</sup> cells (Pryce et al., 1994; Male et al., 1992). A possible explanation for this comes from experiments carried out on HUVEC where it was demonstrated that CD4<sup>+</sup> T cells adhering to HUVEC are more efficient than CD8<sup>+</sup> T cells in inducing the expression of adhesion molecules on the endothelium (Damle et al., 1991). However, in adoptive transfer studies both CD4<sup>+</sup> and CD8<sup>+</sup> cells are seen within lesions (Hickey et al., 1991). Therefore the evidence as to whether the phenotype of T cells plays a role in their capacity to migrate is confusing although *in vivo* evidence suggests that CD4<sup>+</sup> cells cross the barrier first as CD8<sup>+</sup> T cells are not seen until later in disease states (Jakobiec et al., 1983; Chan et al., 1985; Hickey and Gonatas, 1984).

#### 1.4.1 Adhesion Molecule Expression on cells of the CNS and there involvement in leucocyte trafficking.

As already mentioned, the degree of leucocyte migration into different sites is governed by a variety of factors such as the state of activation of the leucocyte and the local endothelia as well as the repertoire of adhesion molecules which the endothelia are capable of expressing.

There is no evidence, as yet, of CNS specific adhesion molecules. However it is known that the endothelium of the CNS use many of the adhesion molecules involved in the trafficking of lymphocytes across other endothelia. For example, as with non-CNS endothelia, some adhesion molecules such as ICAM-2 are expressed constitutively whereas others like VCAM-1 require induction.

ICAM-1 has been found at low levels on endothelia in normal brain in man and mice. This expression is upregulated in a variety of pathological conditions, including viral encephalomyelitis, brain abscess, active MS, EAE and brain tumours (Lassmann et al., 1991; Sobel et al., 1990; Wilcox et al., 1990; O'Neill et al., 1991). During EAE the

level of ICAM-1 expression is upregulated from basal levels during the active stages of disease (Raine et al., 1990; Cannella et al., 1991; Cannella et al., 1990; Wilcox et al., 1990) and correlates with the induction of addressins during relapse.

These *in vivo* studies have been complemented by *in vitro* studies which have demonstrated that activation of CNS-derived endothelial monolayers with cytokines such as IFN- $\gamma$ , TNF- $\alpha$  and IL-1 increases ICAM-1 expression over 24 hours (Male et al., 1994; Wilcox et al., 1990; Fabry et al., 1992; Wong and Dorovani-Zis, 1992; McCarron et al., 1993; Greenwood et al., 1995). Moreover, this increase in ICAM-1 expression is associated with an increase in lymphocyte adhesion (Hughes et al., 1988; Male et al., 1990; Male et al., 1992; Liversidge et al., 1990; Wang et al., 1993; de Vries et al., 1994; McCarron et al., 1993). Adhesion of lymphocytes to CNS-endothelia appears to be only partly inhibited by pretreatment of the endothelia with antibody to ICAM-1 (McCarron et al., 1993; Greenwood et al., 1995). It is possible, however, that this adhesion is due to an alternative ligand for LFA-1, other than ICAM-1, as it has been reported that antibodies directed against LFA-1, cause a greater inhibition of adhesion (Waldschmidt et al., 1991; Male et al., 1994). On the other hand, lymphocyte migration across CNS-endothelium, does appear to be inhibited by blocking both ICAM-1 on the endothelium or LFA-1 on lymphocytes (Greenwood et al., 1995). Blocking VLA-4 only reduced migration significantly across IL-1 $\beta$  activated endothelia, when VCAM-1 is presumably expressed (Greenwood et al., 1995). However, this apparent decrease in migration may be a consequence of inhibiting the initial adhesion as VLA-4 has been shown to play a role in lymphocyte adhesion to cytokine activated brain endothelium (Male et al., 1994; de Vries et al., 1994). On the whole it appears that, as with non-nervous system endothelia, the LFA-1/ICAM-1 pathway is more dominant in migration than adhesion in comparison with the VLA-4/VCAM-1 pathway (Oppenheimer-Marks et al, 1991). Unlike adhesion, the level of migration across brain (Male et al., 1992) and retinal (Greenwood and Calder, 1993) endothelium is not increased by activation with IFN- $\gamma$  even though ICAM-1 expression is upregulated. However IL-1 $\beta$  stimulation of retinal endothelial cells does increase migration (Greenwood et al., 1995). This may be a characteristic of CNS endothelium as activation with IFN- $\gamma$  does increase migration across other tissue endothelium (Oppenheimer-Marks and Ziff, 1988).

In support of the view that ICAM-1 is involved predominantly in diapedesis and

VCAM-1 in adhesion is the observation that the former is expressed on both the luminal and abluminal membrane of the endothelia whereas VCAM-1 expression is restricted to luminal membrane only. Contrary to this, however, is evidence from *in vivo* studies in which blocking LFA-1 *in vivo* with monoclonal antibodies did not reduce the severity of EAE but instead increased the severity of disease (Welsh et al., 1993; Cannella et al., 1993). In addition, VLA-4 has been demonstrated to be required for T cell entry into brain parenchyma (Baron et al., 1993) and antibodies directed to VLA-4 *in vivo* have been reported to be capable of blocking the development of EAE lesions (Yednock et al., 1992).

#### 1.4.2 Leucocyte migration into the retina.

In the eye, lymphocyte migration is controlled not only by the vascular endothelium but also by the retinal pigment epithelium which overlies the relatively leaky choroidal endothelium. In contrast to migration across the vascular endothelium, relatively little research has been carried out on migration across these cells. *In vitro* studies have demonstrated that RPE also constitutively express ICAM-1, although reports vary as to the levels expressed, with both high (Liversidge et al., 1990) and relatively low levels (Elner et al., 1992) being reported. This discrepancy may be due to differences in culture techniques as both experiments were carried out on cultured cells at different passages. However in both cases the level of expression of ICAM-1 was increased upon activation with the cytokine IFN- $\gamma$ . This adhesion molecule was shown to be functional in the binding of neutrophils (Elner et al., 1992) and lymphocytes to monolayers of RPE (Liversidge et al., 1990; Elner et al., 1992). While ICAM-1 appeared to be the only adhesion molecule involved in neutrophil adhesion, an additional adhesion molecule was thought to be involved in the adhesion of lymphocytes.

Unlike endothelial cells, RPE cells do not capture immune cells from the circulation. This step in the migratory process is a function of the choroidal endothelium. Therefore the adhesion molecules expressed by RPE cells and the role they play in the recruitment of leucocytes may be different from those found at the vascular endothelium. For example, it is unlikely that any of the selectins would be found on RPE, as rather they should be expressed on the choroidal endothelial cells.

In one of the few comparative studies of lymphocyte migration across non-CNS

endothelium and epithelium it was reported that while both cell types were capable of expressing similar levels of the adhesion molecules ICAM-1 and VCAM-1, the cytokines responsible for maximal expression of these molecules differed in that IL-1 produced maximal adhesion molecule expression on endothelial cells whereas IFN- $\gamma$  was responsible for inducing maximal adhesion molecule expression on epithelial cells (Nakajima et al., 1995). Therefore it is possible that the cytokines produced by the immune cells could determine the preferential route of leucocyte entry into the eye, by preferentially upregulating adhesion molecule expression on either the retinal endothelial cells or the retinal pigment epithelium.

#### 1.4.3 Additional Methods by which Cells of the CNS Influence Lymphocyte Migration.

A consequence of inflammation is the release of a variety of cytokines which, by upregulating certain adhesion molecules or by acting as chemokines or chemotactic agents, influence the cell types which enter the inflammatory site. Cells of the CNS have been found not only to respond to cytokines, but can also be stimulated to produce a variety of cytokines or polypeptides with cytokine-like properties (Chang et al., 1994).

RPE cells have also been reported to produce TNF- $\alpha$  *in vitro*, in response to activation by IFN- $\gamma$  or LPS (Tanihara et al., 1992; de Kozak et al., 1994). This cytokine also induces or upregulates the expression of adhesion molecules. Moreover, the ability of RPE cells and retinal Müller cells from different strains of rats to produce TNF- $\alpha$ , correlates with the susceptibility of these strains to EAU (de Kozak et al., 1994).

The inflammatory cytokine, IL-1, has also been reported to be produced by RPE cells. It is not yet known if CNS endothelium can produce IL-1, although Non-CNS endothelial cells have been shown to produce little basal IL-1, and can be stimulated by LPS, IL-1 or TNF to produce IL-1 $\alpha$  and to a lesser degree IL-1 $\beta$  (Libby et al., 1986; Kurt-Jones et al., 1987). The IL-1 produced by RPE cells does not appear to be secreted but is sequestered within the cell (Planck et al., 1993). RPE cells have been reported to produce IL-1 $\beta$  in response to IL-1 $\alpha$  and vice versa (Planck et al., 1993; Jaffe et al., 1992). The expression of a number of adhesion molecules can be induced or upregulated by this cytokine.

As already mentioned, the production of chemokines by vascular endothelial cells is a critical step in the migratory process. These chemoattractants activate integrins,

thereby increasing their adhesiveness for the relevant ligand. In the eye, in addition to vascular endothelial cells, RPE cells have also been shown to be capable of producing chemokines and chemoattractants. Cultured RPE cells have been shown to express mRNA for, and secrete, IL-8 when stimulated with IL-1 $\beta$  or TNF- $\alpha$  which can act as a chemoattractant for neutrophils (Benson et al., 1992). In addition RPE cultures have been shown to produce monocyte chemoattractant protein (Elner et al., 1990; de Vries et al., 1994) which has been reported to be a chemoattractant for T lymphocytes as well as monocytes (Carr et al., 1991).

Therefore, these studies show that RPE have a number of common features with vascular endothelium which allows them to support leucocyte migration into the retina.

RPE cells co-cultured with lymphocytes have been demonstrated to produce prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). This prostaglandin has been reported to both increase (Renkonen, 1990) and inhibit (Oppenheimer-Marks et al., 1994) lymphocyte migration across endothelial cells. It is widely believed that E-series prostaglandins down-regulate the immune system, and that they do this by increasing cAMP levels (Phipps et al., 1991). However, increased cAMP levels in endothelial cells can increase lymphocyte adhesion mediated by LFA-1 (Haverstick and Gray, 1992) and lymphocyte penetration through these endothelial monolayers (Turunen et al., 1990). Therefore the effect, if any, on lymphocyte migration by RPE PGE<sub>2</sub> production remains to be determined.

These studies suggest that the RPE may influence leucocyte recruitment by production of cytokines, chemokines or prostaglandins. These compounds would then act upon the choroidal endothelium, upregulating adhesion molecule expression resulting in an increase in leucocyte recruitment via the choroidal vasculature. Moreover the production of such agents may act in an autocrine fashion altering the ability of the RPE to support lymphocyte penetration of the barrier.

## 1.5 ANTIGEN PRESENTATION

It is now well established that T cells can only recognise antigen in association with self MHC molecules on the surface of cells known as antigen presenting cells (APCs) and that this involves processed antigen-binding in a groove on the MHC molecule. In most cases, CD4<sup>+</sup> T cells recognise antigen in association with MHC class



II, while CD8<sup>+</sup> T cells require MHC class I molecules. Although most cells of the body express class I MHC molecules and can therefore serve as APCs for class I restricted T cells, the term APC normally refers to a more limited set of cells (known as professional antigen-presenting cells) capable of presenting antigen in association with class II molecules. The major professional antigen presenting cell types are macrophages, dendritic cells, Langerhans' cells and B cells.

Proliferation of most T cells, particularly resting T cells, requires a second signal other than that produced by the interaction of TCR/CD3 with antigen and MHC. This second co-stimulatory signal comes from the antigen presenting cells and may include cytokines such as IL-1 and IL-6, although in most instances it appears that co-stimulatory signals are provided by adhesion molecules on APCs interacting with their ligands on T cells. ICAM-1 has been implicated in playing a role in this process (Van Severter et al., 1990), although the most important is believed to be the interaction of CD28 on T cells with one of its ligands (B7-1 or B7-2) on professional APCs (Nickoloff and Turka, 1994).

Although MHC class II molecules are usually expressed only on professional APCs, following cytokine activation *in vitro*, or during an inflammatory condition *in vivo* MHC class II molecules are induced on a wide variety of epithelial and endothelial cells. The ability of epithelial or other non-haematopoietic, organ resident cells to participate in immune regulation, either by stimulating or inhibiting lymphocyte proliferation has been demonstrated in several *in vitro* models of autoimmunity. The aberrant expression of MHC class II molecules by organ-resident cells may permit the presentation of tissue antigens to random circulating autoreactive T cells. Indirect evidence for such mechanisms has been shown in autoimmune thyroiditis (Bottazzo et al., 1986), and insulin-dependent diabetes (Dean et al., 1985) but definitive proof that such a process occurs *in vivo* is still lacking.

### 1.5.1 MHC Class II Expression on Cells of the CNS.

Expression of MHC class I and class II antigen's in normal CNS is very low (Lassmann et al., 1991). This low expression is probably a major protective mechanism against the induction of auto-immunity to the vast number of tissue-specific antigens. However, it has been shown that expression can be induced under appropriate conditions on a variety of cells. Class II expression can be induced in the CNS in viral encephalitis

or in the experimental models EAE (Matsumoto et al., 1986; Fontana et al., 1987) and EAU (Lightman, 1987) and MHC expression is enhanced in the region of MS plaques (Traugott and Raine, 1985) and in uveitis (Chan et al., 1986).

Potential candidates for tissue-resident antigen presenting cells in inflammatory conditions of the CNS are cells of the blood-CNS barriers. For example, both rat brain and retinal endothelium have been shown to constitutively express MHC class I molecules with class II antigens expressed upon stimulation with IFN- $\gamma$  (Male et al., 1987; Wang et al., 1995). In addition, it is notable that in both cases, expression of I-A was more readily induced than I-E (Male et al., 1987; Wang et al., 1995). At the other site of the blood-retinal barrier, it has been demonstrated that RPE cells also express MHC class I constitutively (Clark and Hall, 1986) and while they do not normally express MHC class II, HLA-DR expression has been detected on RPE cells from patients with uveitis (Chan et al., 1986). Human and rat RPE cells have also been shown to express MHC class II antigens after stimulation with IFN- $\gamma$  (Liversidge et al., 1988; Percopo et al., 1990; Detrick et al., 1985). The expression of MHC class II molecules on cells of the blood-CNS barrier appears to occur prior to clinical signs of disease (McLaren et al., 1993; Chan et al., 1986).

### 1.5.2 Cells of the CNS as Antigen-Presenting Cells.

The inducibility of MHC class II molecules suggests that CNS vascular endothelial cells and RPE may be able to act as antigen-presenting cells during inflammatory reactions. Nevertheless, the evidence as to whether these cells can act as antigen presenting cells is confusing. It has been shown that MHC class II positive HUVEC can present antigen to either allogeneic or syngeneic T cells resulting in T cell activation and proliferation (Pober et al., 1983). However, it has been reported that confluent cultures of brain and retinal endothelium do not induce T cell proliferation to any appreciable extent, (Pryce et al., 1989; Wang et al., 1995) although they can support CD4<sup>+</sup> T cell-mediated cytotoxicity (Sedgwick et al., 1990). In contrast to these results others have shown that brain endothelial cells can present antigen to antigen-specific T cells resulting in a significant degree of T cell proliferation (McCarron et al., 1985; Wilcox et al., 1989). A possible explanation for this discrepancy comes from a study which demonstrated that although confluent CNS endothelial cultures could not induce T cell

proliferation, sub-confluent cultures could (Wang et al., 1995), therefore the ratio of T cells : endothelia may be crucial in determining the ability of these endothelial cells to act as antigen presenting cells *in vitro*.

One of the many functions of the RPE is the phagocytosis of photoreceptor outer segments (Elner et al., 1981; Bok and Young, 1979). This ability of the RPE cell is reminiscent of macrophage phagocytic capabilities. RPE cells manifest all of the classical properties of phagocytic cells, including discrimination between particles of different types, ingestion of particulate matter into vacuoles bounded by a plasma membrane, production of a large variety of hydrolytic enzymes, and the capacity for digestion of engulfed particles. The functional similarity to the macrophage suggests that the RPE cells may possess the potential to process protein as the macrophage does during presentation. However, as with CNS endothelial cells, RPE cells do not induce T cell proliferation. Most reports indicate that RPE suppress T cell proliferation, even in the presence of professional antigen-presenting cells (Percopo et al., 1990; Liversidge and Forrester, 1992; Liversidge et al., 1993; Liversidge et al., 1994). Although T cell proliferation was not observed, it was demonstrated that RPE cells could stimulate sensitised T cells to produce IL-2 when co-cultured in the presence of antigen (Percopo et al., 1990). This effect was inhibited by chloroquine, indicating that processing of antigen is required prior to presentation.

In addition to producing factors which enhance the immune response, it also seems likely that cells of the blood-CNS barriers produce immunosuppressive agents. One possible candidate is eicosanoid production. RPE cells have been shown to produce PGE<sub>2</sub> and T cell proliferation induced by RPE was shown to be improved in the presence of the prostaglandin inhibitor, indomethacin (Liversidge et al., 1993). In another study, using retinal endothelial cells however, no further T cell proliferation was observed in the presence of indomethacin (Wang et al., 1995). This would imply that PGE<sub>2</sub> may be involved in the suppression observed with RPE cells, but not endothelial cells, although, indomethacin did not reverse the inhibition of IL-2 driven proliferation of T cells indicating that other immunosuppressive agents may be involved (Liversidge et al., 1993).

The production of the cytokine TGF- $\beta$  has been reported in both RPE and retinal EC (Tanihara et al., 1993; Wang et al., 1995). This cytokine has a number of

immunosuppressive, as well as pro-inflammatory, properties one of which is the inhibition of T cell proliferation (Wahl, 1992; Wahl et al., 1988). Furthermore, nitric oxide has also been proposed to have an immunoregulatory role on T cell activation within the retina (Liversidge et al., 1994). RPE cells have been shown to express an inducible form of nitric oxide synthase and produce high levels of nitric oxide (Liversidge et al., 1994), although, its production by retinal endothelium was not found despite using a variety of cytokines (Mesri et al., 1994). This report stated that RPE cells could induce T cell proliferation despite production of high levels of nitric oxide (Liversidge et al., 1994).

As already mentioned, expression of peptide/MHC complexes are rarely sufficient to stimulate most T cells, they usually require additional signals before they can be activated. There are a number of other ways in which the cells of the blood-retinal or blood-brain barrier may provide the necessary signals at the site of inflammation.

Several cytokines have now been described that can provide these co-stimulatory signals to T cells. IL-1 $\alpha$  and  $\beta$ , IL-4, 6 and 7 as well as TNF- $\alpha$  and possibly IFN- $\gamma$  are possible candidates for having co-stimulatory roles in the activation of T cells (March et al., 1985; Balkwill and Burke, 1989; Ceuppens et al., 1988). Of these HUVEC, but not CNS endothelial cells (Hughes et al., 1990) and RPE cells (Planck et al., 1992; Planck et al., 1993; Tanihara et al., 1992; Benson et al., 1992) have been found to synthesise IL-1, IL-6 and TNF- $\alpha$ .

The surface expression of ICAM-1 and VCAM-1 may also function as co-stimulatory molecules, since it was found that purified forms of these molecules, acting through their respective ligands LFA-1 and VLA-4, can provide co-stimulatory signals to trigger CD4<sup>+</sup> T-cell proliferation (Van Seventer et al., 1990; Van Seventer et al., 1991).

The inability of some cells which have been induced to express MHC class II to activate T cells, may be because they cannot provide other appropriate co-stimulatory signals. This may lead to the induction of T-cell unresponsiveness rather than activation. There is evidence that cells of the BRB can cause this, as pre-exposure of T cells to a confluent monolayer of retinal endothelium rendered them unresponsive to further antigen-stimulation with professional antigen presenting cells (Wang et al., 1995). There is also evidence that non-professional APC may alter the cytokine profile of T cells, due

to the absence of some co-stimulatory signals (eg, altering a Th1 response to a Th2 response) (Nickoloff and Turka, 1994).

Despite all of the *in vitro* evidence, there is no evidence *in vivo* for the activation and proliferation of T cells in response to these tissue-resident cells. Therefore, the ability of cells of the blood-retinal or blood-brain barrier to act as potential antigen presentation cells is still inconclusive. However, it does appear that the ability of these cells to express MHC class II molecules does correlate with susceptibility of certain strains of animals to both EAE and EAU (Fujikawa et al., 1990; Male and Pryce, 1989; Happ et al., 1988; Jemison et al., 1993; Caspi, 1989).

## 1.6 PURPOSE OF THIS STUDY

The aim of this project was to investigate the cellular and molecular interactions of lymphocytes with rat retinal pigment epithelial cells *in vitro* and to compare this with similar interactions with retinal endothelial cells.

### 1.6.1 Control of lymphocyte adhesion to and migration through RPE monolayers.

Lymphocyte adhesion to RPE monolayers will be quantified using <sup>51</sup>chromium labelled lymphocytes and migration measured using time-lapse videomicroscopy. The factors involved in controlling adhesion and migration will also be evaluated, these include (1) comparison of the effect of lymphocyte activation with mitogen (Con A), CD3 cross-linking and antigen (S-antigen, PPD and ovalbumin). (2) the effect of cytokine activation (IFN- $\gamma$  and IL-1) of RPE monolayers on lymphocyte adhesion and migration.

### 1.6.2 Adhesion molecule and MHC expression on RPE.

Expression of MHC class I and II molecules on RPE and the effect on their expression of activation with IFN- $\gamma$  will be determined by enzyme-linked immunoassay (ELISA), immuno-histochemistry and flow cytometry. The expression of the adhesion molecules ICAM-1 and VCAM-1 will be examined on resting and IFN- $\gamma$  activated RPE monolayers using ELISA and flow cytometry.

### 1.6.3 Adhesion molecules controlling lymphocyte migration across RPE monolayers.

The role of the adhesion molecules ICAM-1 and VCAM-1 in controlling lymphocyte migration across resting and cytokine activated RPE monolayers will be determined. This will be done by adding monoclonal antibodies to block these molecules on the RPE monolayers prior to carrying out lymphocyte migration assays. The ligands for these adhesion molecules (LFA-1 and VLA-4 respectively) on lymphocytes will also be blocked using monoclonal antibodies. The ability of these molecules to control lymphocyte migration across both normal and cytokine activated monolayers will be investigated.

### 1.6.4 Comparison of lymphocyte migration across RPE and REC monolayers.

Lymphocyte migration across RPE monolayers derived from PVG rats will be compared with the migration across both PVG and Lewis derived retinal endothelial monolayers.

### 1.6.5 Immortalisation of primary cultures of rat RPE

Due to the limitations associated with primary culture, primary cultures of rat RPE cells will be immortalised by transfection with SV40 large T antigen. The resulting cell lines will be cloned and characterised for the expression of epithelial markers, MHC and adhesion molecule expression. The ability of these cells to support lymphocyte migration will also be determined.

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## CHAPTER 2

### MATERIALS & METHODS

#### 2.1 ANIMALS

PVG rats, bred in-house (originally from Harlan-Olac, Oxon, UK) were used to isolate retinal pigment epithelial cells; retinal capillary endothelium; lymph nodes and isolating CD4<sup>+</sup> antigen-specific T cell lines. 6-10 week old specific pathogen-free female Lewis rats were also used for isolation of retinal endothelium and for the production of antigen-specific T cell lines (Charles River, Kent, UK).

#### 2.2 REAGENTS

##### 2.2.1 Chemicals

*Sigma, Poole, UK* :- Concanavalin A (Con A); deoxyribonuclease (DNase); heparin; polyethyleneglycol; N $\alpha$ -p-Tosyl-L-lysine chloromethyl ketone (TLCK); vitamin C; Hams F'10; RPMI-1640, Hanks' balanced salt solution (HBSS); Ca<sup>2+</sup> and Mg<sup>2+</sup> free HBSS, non-essential amino acid, 1mM sodium pyruvate; penicillin/streptomycin; L-glutamine; 20mM HEPES; 7.5% sodium bicarbonate; trypsin/EDTA; sodium metrizoate and gentamicin.

*Gibco, Paisley, UK* :- Foetal calf serum (FCS) and G418.

*Boehringer-Mannheim, Sussex, UK* :- Collagenase/Dispase; type 1 collagen and dispase.

*Amersham International plc, Buckinghamshire, UK* :- Sodium chromium-51 (<sup>51</sup>Cr) and <sup>3</sup>H-thymidine.

*Advanced Protein Products, West Midlands, UK* :- Bovine serum albumin; plasma-derived serum and endothelial cell growth supplement (ECGS).

### 2.2.2 Antibodies

A comprehensive list of monoclonal antibodies used is found in table 2.1.

| Code    | Specificity                         | Source              |
|---------|-------------------------------------|---------------------|
| OX-6    | anti-rat class II I-A               | *                   |
| OX-8    | anti-rat CD8                        | *                   |
| OX-17   | anti-rat MHC class II I-E           | *                   |
| OX-18   | anti-rat MHC class I                | Serotec, Oxford, UK |
| OX-19   | anti-rat CD5                        | Serotec             |
| OX-21   | anti-human C3b inactivator          | *                   |
| OX-22   | anti-rat leucocyte common antigen   | Serotec             |
| OX-33   | anti-rat CD45ABC, B-cell restricted | Serotec             |
| OX-39   | anti-IL2R, CD25                     | Serotec             |
| W3/25   | anti-rat CD4                        | Serotec             |
| WT.1    | anti-rat CD11a                      | Serotec             |
| WT.3    | anti-rat CD18                       | Serotec             |
| 1A29    | anti-rat ICAM-1                     | Serotec             |
| TA-2    | anti-rat CD49d                      | AMS biotech, UK.    |
| 5F10    | anti-rat VCAM-1                     | **                  |
| K8.13   | anti-human cytokeratin              | DAKO, Oxon, UK.     |
| HIS52   | anti-rat RECA-1 antigen             | Serotec             |
| RET-PE2 | specific for rat RPE                | ***                 |

\* *These monoclonal antibodies were prepared from hybridoma cell lines (a generous gift from Dr. M. Puklavec, MRC, Oxford, UK). The hybridoma cell lines were cultured for 48hr in 10% FCS RPMI-1640 medium, and the supernatant, containing the antibody, collected.*

\*\* *The anti-rat VCAM-1 (5F10) was a generous gift from Dr Roy Lobb, Biogen, MA, USA.*

\*\*\* *RET-PE2 was a generous gift from Prof. C.J.Barnstable, Yale, CT, USA.*

In addition, the fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse immunoglobulin G F(ab)<sub>2</sub> fragment (RAMIG) was purchased from Serotec and the peroxidase conjugated rabbit anti-mouse IgG from Sigma. Biotin-conjugated sheep anti-mouse antibody, peroxidase conjugated rabbit anti-mouse and avidin labelled peroxidase were obtained from R&D systems.



### 2.2.3 Cytokines

Rat recombinant IFN- $\gamma$  was purchased from GIBCO Life Sciences (Paisley, UK) and murine recombinant IL-1 $\beta$  was obtained from Genzyme (Oxford, UK).

All cytokines were aliquoted and stored at -70°C.

## **2.3 RETINAL SOLUBLE ANTIGEN (S-Ag)**

2.3.1 Buffer:- 0.01M NaCl + Inhibitors (0.2mM of N-ethyl maleimide (Sigma) and 0.2Mm of pheylmethylsulfonyl fluoride (Sigma) pH 7.2)

### 2.3.2 Isolation and Purification

S-Ag was extracted from bovine retinas by gel filtration followed by ion exchange according to the method described by Wacker et al. (1977). All procedures were carried out at 4°C where possible.

Retinas were removed from 50-100 bovine eyes, homogenised in buffer and the protein precipitated with saturated ammonium sulphate. This was then centrifuged at 60,000g (Suprafuge 22, Hereaus Sepatech, Osterode, Germany) and the pellets collected and resuspended in buffer. To remove insoluble proteins the solution was centrifuged again for 1 hour at 41,000g (Centrikon T-2070, Kontron Instruments AG, Zurich, Switzerland).

The dissolved proteins were separated according to their molecular weight by gel filtration chromatography using gel specific for 20,000-350,000 (Ultra-gel AcA 34 IBF Biotechnics, France). The solution was added at a flow rate of 17ml/h, and the protein was eluted with 500ml of buffer at the same flow rate. Protein fractions of 5ml were collected using a superac system (LKB-Pharmacia, Sweden). The fraction containing the S-Ag was determined by ELISA using a monoclonal antibody to S-Ag (gift from Dr. J. Liversidge, Aberdeen, UK) and immunodiffusion on Micro-Ouchterlony plates with hyperimmune serum raised against S-Ag.

The positive fractions were pooled and loaded onto a Sepharose-Q ion-exchange column (FastFlow, Pharmacia Ltd, Milton Keynes, UK) with a continuous gradient buffer of 0.01M NaCl to 0.5M NaCl at a flow rate of 80ml/h. S-Ag was usually eluted out at about 0.3M NaCl.

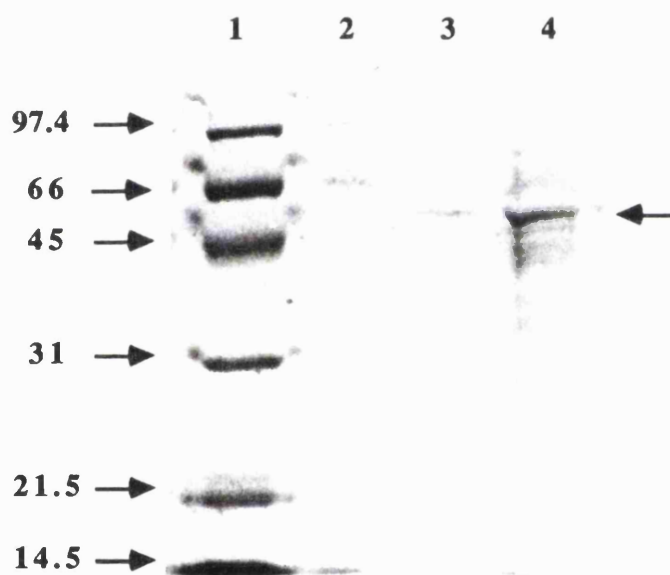
Positive fractions were again detected by ELISA and immunodiffusion as before

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and the protein concentrated by dialysis in 35 % polyethylene glycol (Sigma) using a pore size of 12-14,000 MWt cut off.

### 2.3.3 Protein concentration and purity.

The purity of the protein was determined by SDS-PAGE (Miniprotein II, BIORAD, Richmond, CA, USA). 12 % polyacrylamide gels were used and the molecular weight was compared with low molecular weight standards (Biorad). Separated protein fractions were stained using Coomassie blue, 0.2 % in 40 % methanol and 10 % acetic acid for 1 hour and destained using 40 % methanol in 10 % acetic acid until the bands were clearly seen (Figure 2.1). Protein concentration was evaluated using a Coomassie blue assay (Pierce & Warriner, Chester, UK). The protein was stored in 1mg/ml aliquots at -70 °C.



**Fig 2.1** SDS-PAGE for S-Ag purified by Ultrigel 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 concentrated and a 1:10 dilution of S-Ag solution. The proteins were stained with Coomassie blue.

## 2.4 ISOLATION AND CULTURE OF RAT RPE.

### 2.4.1 Culture Medium

HAMS F-10 medium supplemented with 20% FCS, 20mM HEPES, 7.5% sodium bicarbonate, 2mM glutamine and 100U/ml of penicillin streptomycin was used for RPE cultures.

### 2.4.2 Isolation of RPE

#### *Method 1*

Initial attempts at isolating RPE were carried out using adult Lewis rats (4-6 weeks). At the time of starting this study rat RPE cells had only been cultured using young rats less than 15 days old (Edwards, 1981; Chang et al., 1991; Mayerson et al., 1985). All of these techniques employed soaking the intact globes in enzyme prior to dissection. In this instance eyes were first dissected to remove the anterior section of the eye and retina. The posterior eye cup was then placed onto sterile wax moulds, in which grooves had been made to fit the eye cup. 1mg/ml trypsin and 70U/ml collagenase were then added to the eye cup and incubated for 45 minutes at 37°C. Following this incubation, the enzyme mixture was titrated up and down to dissociate the RPE from the choroid. The RPE was then washed twice in Hank's Balanced Salt Solution (HBSS) and finally resuspended in growth medium and plated out onto tissue culture flasks.

#### *Method 2*

The second method employed was based on that according to Edwards (1981). Eyes were enucleated from 6-8 day old rats and soaked overnight in phosphate buffered saline (PBS) containing penicillin/streptomycin in the dark at 4°C. Eyes were then incubated with 0.5ml trypsin-collagenase per eye (1mg/ml trypsin and 70U/ml collagenase). Immediately following the incubation in the enzyme solution the eyes are immersed in culture medium. The dissection was carried with the aid of a dissection microscope and with the eyes submerged in culture medium. The incision was made just posterior to the ora serrata and extended circumferentially with microdissecting scissors

separating the anterior segment from the posterior eye cup. Before removing the retina, the posterior eye cup had to be soaked in medium for 15 minutes to prevent damage to the RPE microvilli. The posterior eye cup was then gently stretched using two pairs of forceps and agitated, by holding the optic nerve head, to dislodge the cells. The RPE was then removed from the petri dish with a pipette and placed into tissue culture flasks, pre-coated with foetal calf serum.

### *Method 3*

The method used was that described by Chang et al.(1991). Briefly, eyes were enucleated from 6-8 day old PVG rats and the intact globes incubated for 30 minutes in 2% Dispase in HAMS F-10 at 37°C. After rinsing the globes with warm medium twice, the eyes were dissected as described above. The cornea, lens and vitreous were discarded and, with the aid of a dissecting microscope, the retina, with the adherent RPE, carefully lifted from the choroid. The retina was then incubated for a further 15 minutes at 37°C in culture medium. Following this incubation the RPE could be separated from the retina using forceps. The RPE tended to come off in sheets and had to be trypsinised to obtain a single cell suspension. Cells were washed with calcium-magnesium-free Hank's Balanced Salt Solution (HBSS) and treated with 1% trypsin (v/v) until the RPE sheets were partially dissociated into single cells or small clusters of 2-10 cells. Cells were then plated out onto a variety of tissue culture plates.

## **2.5 IMMORTALISATION OF RAT RETINAL PIGMENT EPITHELIAL CELLS**

Primary cultures of rat RPE cells were immortalised by transfection with the SV40 large T antigen.

### **2.5.1 Virus Producing Cells**

The replication deficient SV40 retrovirus was collected from the supernatant of the cell line SVU19.5 in which a packaging defective mouse moloney leukemia provirus was present (a generous gift from Dr. P.S. Jat, Ludwig Institute, London, UK). The retroviral vector encoded a temperature sensitive (tsa58), non SV40-origin binding mutant

of the large T-antigen and a selectable neomycin resistance gene (aminoglycoside phosphotransferase I). Cells were maintained in DMEM medium supplemented with 10% FCS and 100U/ml penicillin/ streptomycin and had to be passaged at least once prior to collecting the virus. When the producer cells were 80% confluent, they were maintained in a minimal amount of media (to ensure that the virus is as concentrated as possible) for no more than 10 hours. The supernatant containing the virus was collected and filtered (0.45 $\mu$ m pore size) to remove any unwanted SVU19.5 producer cells. The virus was then stored in 0.5ml aliquots at -70°C.

### 2.5.2 Transfection of RPE Cells

Primary cultures of RPE cells, isolated using method 3 (section 2.4.2) plated out 2-3 days prior to transfection were used. 200 $\mu$ l of virus in 2ml of media containing 8 $\mu$ g/ml polybrene (Aldrich, Dorset, UK) was added to the RPE cells. This was left for 2 hours at 37°C in a CO<sub>2</sub> incubator, gently shaking the flask at 15 minute intervals. Following this incubation, 5ml of medium (that described for RPE previously) was added to the flask overnight. Cells were then maintained as normal for 2-3 days before adding 300 $\mu$ g/ml G418 (neomycin) to the media to ensure that only cells which had taken up the virus would grow (virus also contained the neomycin resistance gene). Cells were maintained in G418 media for up to 2 weeks, changing the media every 2-3 days. Following selection of transformed cells by this process, G418 was removed from the culture media. Resulting cell line were cloned, by plating cells out into 96 well plates at a concentration of < 1 cell per well.

## **2.6 CHARACTERISATION OF PRIMARY AND TRANSFORMED RPE CULTURES.**

### 2.6.1 Immunohistochemistry

To examine the purity of RPE cultures the cells were investigated for their expression of cytokeratins and a rat RPE specific antigen (RET-PE2)(Neill and Barnstable, 1990). Cells were grown on permanox Lab-Tek culture chamber slides for this purpose (Nunc, Gibco, UK). Cells which were stained for cytokeratins were first

fixed in pre-cooled (-20° C) methanol and acetone before incubating with the anti-cytokeratin antibody 8.13 (clone K8.13 which labels cytokeratins 1,5,6,7,8,10,11 and 18 from Dako, Buckinghamshire, UK) for 45 minutes. The RET-PE2 antibody was added directly to unfixed cells, for 45 minutes at 4°C. The cells were then washed in phosphate buffered saline (PBS) and incubated with either an anti-mouse peroxidase (1:50) conjugate or anti-mouse-FITC conjugate (1:100) for 30 minutes. The peroxidase was developed in AEC, giving a red reaction product.

Transformed RPE cells were also stained for the presence of the large T antigen. Cells were fixed as before with methanol and acetone before adding a 1:4 dilution of the antibody (a gift from P.S. Jat) for 45 minutes at 4°C. Then the anti-mouse-FITC conjugate was added as before.

### 2.6.2 Flow Cytometry

Flow cytometry was performed on a FACScan (Becton-Dickinson, Oxford, UK). Staining for flow cytometry was carried out as above, using the FITC-conjugated anti-mouse secondary antibody. However, in this instance cells were removed from the culture flask and dissociated to obtain single cell suspensions prior to staining. A number of different methods were used to obtain single cell suspensions. While both trypsin and enzyme-free cell dissociation solution (Sigma) removed cells from the plastic tissue culture flask, cells remained in clumps. Best results were achieved using collagenase/dispase. Cells were incubated in this solution at 37°C for 45-60 minutes. Single cells could then be obtained by pipetting up and down a few times.

Gates were set so as to exclude non-viable cells and at least 5000 events were counted within this gate. Cells stained with the secondary antibody alone were used to set the background control. Results are expressed as a percentage of positive cells with background staining of the control antibody having been subtracted.

### 2.6.3 Electron Microscopy

RPE cells and RPE/T-cell line co-cultures were fixed *in situ* by the addition of a mixture of 1% paraformaldehyde and 3% glutaraldehyde in 0.1M sodium cacodylate-HCl (pH 7.4) or 2.5% glutaraldehyde in 0.1M sodium cacodylate buffered to pH 6.9 by the

addition of 0.5% (w/v) tannic acid. Following 3 x 5 minute rinses in sodium cacodylate buffer (pH7.4), cells were secondarily fixed for 2hr in darkness at 4°C using a 1% aqueous solution of osmium tetroxide, dehydrated through ascending alcohols (1 x 10 min 50 - 90, 4 x10 min 100%) and embedded in araldite cured at 60°C for 12hr. Semi-thin (1µm) and ultra-thin (50nm) sections were cut using a Leica Ultracut S microtome, fitted respectively with a glass or diamond knife. Semi-thin sections were stained for light microscopy with 1% toluidine blue in 50% ethanol and ultra-thin sections stained sequentially with 1% lead citrate in 50% ethanol and Reynold's lead citrate and viewed and photographed in a JEOL 1010 TEM operating at 80kV.

#### 2.6.4 Electrical Resistance

RPE cells were grown on falcon cell culture inserts (pore size 0.45µm, Marathon, London, UK) to measure electrical resistance. Primary cultures did not grow well on these inserts despite coating the filters with matrigel or collagen. This may be due to the requirement for a higher seeding density of cells for these filters (Hall and Quon, 1981) and the viability of passaged cells was not sufficient to overcome this problem. However, the transepithelial electrical resistance of immortalised cells was determined. Measurements were taken daily once the cells reached confluence. Transwells were placed in a chamber containing medium and resistance was measured using an EVOM voltohmmeter with Endohm tissue chambers (World Precision Instruments, Hertfordshire, UK).

## **2.7 ISOLATION OF RAT RETINAL MICROVESSEL ENDOTHELIAL CELLS**

### 2.7.1 Buffers

*Buffer:-* 100ml of Ca<sup>2+</sup>& Mg<sup>2+</sup>free HBSS containing 10mM HEPES and 200U/ml penicillin/streptomycin was gassed for 10 min with oxygen enriched gas (95% O<sub>2</sub> plus 5% CO<sub>2</sub>) after which 2.272ml of 22% BSA was added. The pH of the buffer was adjusted with 1M NaOH to approximately 7.2 by eye with the indicator phenol red.

*Digestion Buffer:-* 5ml of collagenase/dispase supplemented with 0.147µg/ml TLCK and



200U/ml DNase was gassed, as above, for a few minutes and the pH restored to 7.2 with 1M NaOH.

*Percoll Gradient:-* Percoll (Sigma) was autoclaved before preparing the gradient mixture. 50 ml of Percoll was mixed with 45ml of HBSS and 5ml of 10x concentrated HBSS to restore the osmolarity. High speed centrifuge tubes (Sorvall Instruments, Du Pont, USA) were sterilised with 75% ethanol, washed and coated for at least 2hr with buffer. To establish a gradient, 7ml of the Percoll mixture was added to the sterile centrifuge tubes and centrifuged for 1hr at 25,000g at 4°C in a high speed centrifuge (Heraeus-Suprafuge 22, Osterode, Germany).

*Culture Medium:-* HAMS F'10 medium was supplemented with 17.5% plasma derived serum (PDS), 7.5µg/ml ECGS, 80µg/ml heparin, 2mM glutamine, 0.5µg/ml of vitamin C and 100U/ml penicillin/streptomycin to culture endothelial cells.

### 2.7.2 Isolation of Retinal Microvessels

Rat retinal microvessels were isolated according to the method described by Greenwood (1992).

For each preparation 6 PVG rats were sacrificed by asphyxiation with CO<sub>2</sub>. The eyes were removed and dissected under sterile conditions. Using a dissecting microscope the cornea, lens and vitreous body were discarded before the retina was carefully removed and transferred into buffer at 4°C.

The retinas were then digested for 1 hour in 5ml of digestion buffer at 37°C. Following this incubation, the digested tissue was centrifuged for 15 minutes at 1000g in 22% BSA solution to remove contaminating lipids. The remaining tissue was incubated for a further 1 hour in 5ml digestion buffer to separate pericytes from the microvessels. At the end of this incubation, the digestion buffer was removed and the tissue pellet resuspended in 1ml of buffer.

The suspension was then loaded onto the pre-formed Percoll-gradient and centrifuged for 15 minutes at 1000g. Microvessels formed a layer about a third of the way up the Percoll gradient which was removed using a sterile pasteur pipette. The

microvessel fragments were washed twice before being resuspended in culture medium.

The cells were then maintained at 37° C in 5% CO<sub>2</sub> and given fresh medium every 3 days until confluent.

### 2.7.3 Characterisation of retinal endothelial cultures

Endothelial cells isolated using this method had previously been extensively characterised (Greenwood, 1992). The cells grew out from isolated capillaries, forming a confluent monolayer of spindle shaped cells within two-three weeks. To determine the purity, endothelial cells were stained with the rat endothelial cells specific monoclonal antibody RECA-1.

## **2.8 ISOLATION OF ANTIGEN SPECIFIC CD4<sup>+</sup> T-CELL LINE LYMPHOCYTES**

### 2.8.1 Culture Media

Dutch modified RPMI 1640 medium was used supplemented with 2mM L-glutamine, 1mM sodium pyruvate, 1% non-essential amino acids, 50µg/ml gentamycin, 5 x 10<sup>-5</sup>M 2-mercaptoethanol (2-ME), and either 1% syngeneic rat serum or 10% heat-inactivated FCS was used as complete medium in all experiments.

### 2.8.2 Antigens

PPD was diluted in RPMI-1640 at a concentration of 10mg/ml and stored at 4° C. For stimulating lymphocytes it was diluted to 15µg/ml.

S-Ag was also used at concentration of 10µg/ml and ovalbumin at 20µg/ml to stimulate lymphocytes.

### 2.8.3 Source of IL-2

The IL-2 used to maintain the T cell lines was obtained from the supernatant of hybridoma cells transformed with mouse IL-2 cDNA (a kind gift from Prof. F. Melchers, Karasuyama and Melchers, 1988).

Hybridoma cells were grown at a concentration of 2 x 10<sup>5</sup> cells/ml and cultured with geneticin (G418) at 1mg/ml in 5% FCS-RPMI without 2-ME. Before collecting the conditioned media, the cells were washed twice to remove the G418 and cultured for a

further 48hr in the absence of G418. All supernatants were filter sterilised and stored at -20° C.

An IL-2 proliferation assay was carried out to determine the optimum concentration of supernatant to use.

#### 2.8.4 Preparation of Antigen Presenting Cells (APC)

Irradiated thymocytes were used as antigen presenting cells. Thymuses were removed from syngeneic rats and a single cell suspension obtained by grinding the tissues through a sterile nylon mesh. The cells were washed twice and resuspended in 5ml of complete medium and exposed to 3000 rads of gamma radiation (Pantak, Windsor, UK).

#### 2.8.5 Metrizoate Ficoll (M-F) Separation of Cells

M-F at a specific density of 1.088 was used to separate live cells from accessory cells. Ficoll 400 was dissolved in distilled water at 14g/100ml and mixed with 32.9% (w/v) sodium metrizoate at a ratio of 10 vols of metrizoate to 24 vols of Ficoll. This was then sterilised by autoclaving and stored at 4°C in the dark.

Before use, the M-F was allowed to warm up to 20°C, then 5ml of cell suspension (up to  $5 \times 10^7$  cells) in complete media was overlaid onto 5ml M-F. The cells were centrifuged at 1000g for 30 minutes at 20°C. Viable cells could be seen at the M-F /media interface while non-viable cells, including dead feeder cells, formed a pellet at the bottom of the universal.

#### 2.8.6 Preparation of T cell Lines.

Lewis or PVG rats were immunised subcutaneously in a hind leg footpad with 50µg of S-Ag or ovalbumin emulsified in complete freund's adjuvant (CFA) and in the case of S-Ag specific T cell lines an intraperitoneal injection of 100µg of killed Bordetella pertussis. In order to establish PPD T cell lines rats received an injection of CFA diluted 1:1 with sterile PBS.

7-10 days after injection of the antigen inguinal and popliteal lymph nodes were removed, prepared as a single cell suspension and resuspended at  $2 \times 10^6$  viable cells/ml in complete media containing 1% (v/v) NRS and antigen (PPD, ovalbumin or S-Ag) at

15, 20 or 10 $\mu$ g/ml respectively. Viable cells, including blast cells, were then isolated 4-5 days later by density centrifugation over M-F (as described previously). Cells from the interface were collected, washed twice and cultured in complete medium containing 10% FCS and 1% IL-2 at 2 x 10<sup>5</sup>/ml for 6-9 days. Following this cells were then restimulated every 10-12 days with antigen and irradiated thymocytes (50x number of T cells) followed by M-F separation and culture in IL-2 containing medium.

### 2.8.7 Phenotyping of Cell Lines

The phenotype of all cell lines used was determined by flow-cytometry. Cells were incubated with the following antibodies which were either FITC or PE conjugated: W3/25 (anti-CD4; Mason et al., 1983); MRC OX-8(anti-CD8; Mason et al., 1983), MRC OX19 (anti-CD5; Dallman et al., 1982) and MRC OX22 (anti-CD45R; Spickett et al., 1983). Isotype matched monoclonal antibodies were used as controls. All antibodies were used at a concentration of 10 $\mu$ l/10<sup>6</sup> cells and incubated for 30min at 4°C. Cells were washed twice with PBS before acquiring as described previously.

### 2.8.8 Proliferation Assay

The antigen specificity of the cell lines used was determined using a proliferation assay (Sedgewick et al., 1989). 2 x 10<sup>4</sup> lymphocytes were added to each well of a 96 well plate. In addition 10<sup>6</sup> thymocytes (APC, 3000 rads) and a range of concentrations of the specific antigen (at 1,5,10,15 and 20 $\mu$ g/ml) were added to the wells. The proliferative response to an irrelevant antigen (BSA at 1 and 10 $\mu$ g/ml) and Con A (5 $\mu$ g/ml) were tested for comparison. Cells were then cultured for 72hr at 37°C in a 5% CO<sub>2</sub> incubator and pulsed with 1 $\mu$ Ci of <sup>3</sup>H-thymidine 8hr prior to termination of the assay. Cultures were then harvested using a cell harvester (Dynatech, U.K.)and thymidine incorporation was measured with a  $\beta$ -scintillation counter (Tricarb, Canberra Packard).

## 2.9 ISOLATION OF PERIPHERAL LYMPH NODE (PLN) LYMPHOCYTES

### 2.9.1 Resting PLN Lymphocytes

PLN lymphocytes were obtained from cervical and mesenteric peripheral lymph nodes. Single cell suspensions were obtained by grinding the lymph nodes through a sterile nylon mesh. The cells were washed twice, resuspended in 10% FCS RPMI medium and incubated overnight at 37°C in a 5% CO<sub>2</sub> incubator.

### 2.9.2 Activation of PLN Lymphocytes

The effect of different modes of activation on the ability of lymphocytes to adhere to or migrate across RPE monolayers were investigated.

*Con A* :- Lymphocytes were treated 24hr prior to the experiment with the optimal concentration (5µg/ml) of the mitogen Con A. For lymphocyte migration studies, lymphocytes were also activated with Con A for 48 and 72hr.

*Con A + IL-2* :- Con A activated PLN (24hr and 72hr) cells were further activated with 1% exogenous IL-2 for 24, 48 and 72hr.

*Con A + PMA* :- Con A activated PLN (24hr) were further stimulated with 10ng/ml PMA for 10 minutes.

*CD3*:- 25cm<sup>2</sup> flasks were coated overnight at 4° C with 5µg/ml of an anti-CD3 monoclonal antibody. The flask was washed twice with sterile PBS before adding lymphocytes isolated from peripheral lymph nodes. Lymphocytes were stimulated for up to 72 hours in the presence of 1% exogenous IL-2.

The phenotype of PLN cells was determined as for T cell line lymphocytes using flow cytometry.

## **2.10 ASSAY OF LYMPHOCYTE ADHESION TO CULTURES OF RPE.**

### **2.10.1 RPE**

RPE cells were grown to confluence in 96-well plates for use in adhesion assays. Cells were screened prior to use to ensure that only confluent wells, with no contaminating cells, were used.

The effect on lymphocyte adhesion of activating the RPE with cytokines was determined. Selected wells were activated with a range of concentrations of IFN- $\gamma$  (1-200U/ml) and IL-1 $\beta$  (0.05-100U/ml). The RPE monolayers were washed three times prior to addition of lymphocytes.

### **2.10.2 Lymphocytes**

PLN lymphocytes were prepared as previously described. Either resting or Con A activated (with and without further activation with exogenous IL-2) were used for the adhesion assay.

PPD, ovalbumin and S-Ag specific T-cell lines were used for migration and adhesion assays 3-4 days after Ag stimulation and culture in IL-2 conditioned medium.

### **2.10.3 $^{51}\text{Cr}$ Labelling of Lymphocytes**

Lymphocytes were washed twice with HBSS before labelling. Cells were spun down and the cell pellet was incubated for 90 minutes with 3 $\mu\text{Ci}$  of  $^{51}\text{Cr}$  per  $10^6$  cells at 37°C, after which the cells were washed three times to remove any non-incorporated radiation.  $^{51}\text{Cr}$  labelled cells were then resuspended in 10% FCS-RPMI medium at a concentration of  $1 \times 10^6$  cells/ml.

### **2.10.4 Adhesion Assay**

The extent of lymphocyte adhesion to RPE cells was determined according to a previously described method (Haskard et al., 1987; Male et al., 1990).  $2 \times 10^5$   $^{51}\text{Cr}$  labelled lymphocytes were added per well of confluent RPE. Lymphocytes were also added to blank wells as a background control. The cells were incubated for 2hr in a 5% CO<sub>2</sub> incubator at 37°C. Non-adherent cells were washed off with pre-warmed HBSS four

times (using a multichannel pipette) from the four poles of the wells. The adherent cells were lysed with 200 $\mu$ l of 2% SDS and the  $\gamma$ -emissions of the lysate counted on a gamma emission counter (Dynatech, UK). The percentage of adherent cells was calculated using the formula:

$$\% \text{ adhesion} = \frac{\text{cpm of lysate} - \text{cpm of background}}{\text{cpm of cells added}}$$

## 2.11 LYMPHOCYTE MIGRATION ASSAY

The lymphocyte migration assay was carried out using time-lapse videomicroscopy by the method described by Greenwood & Calder (1993).

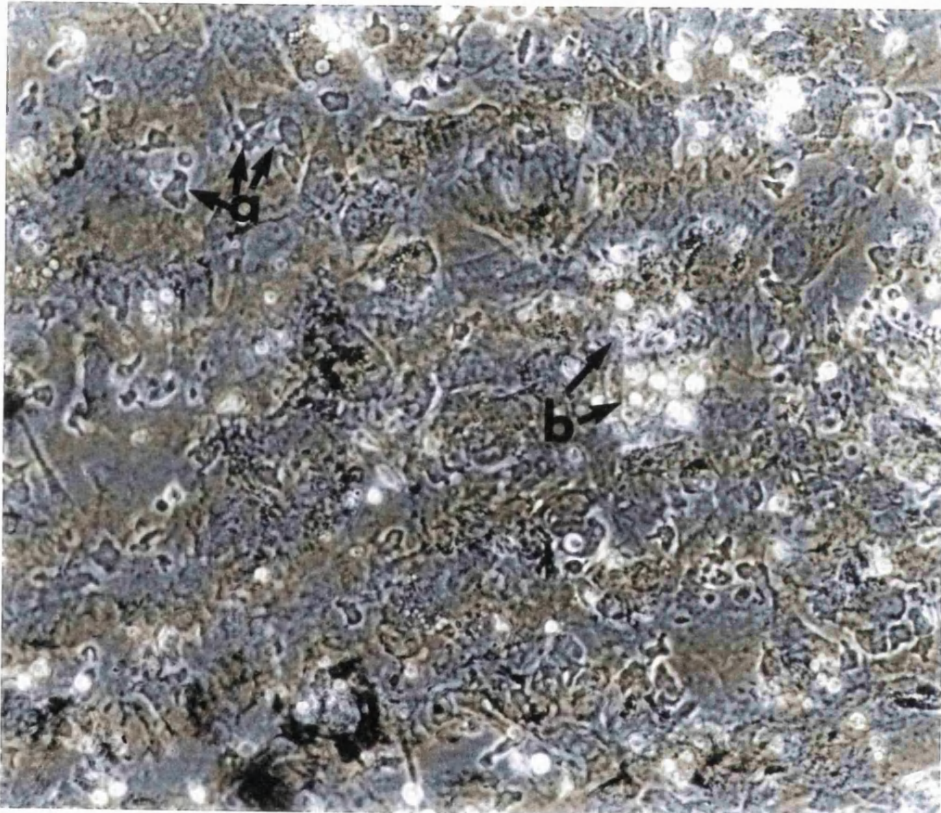
**2.11.1 Groups Studied:-** Non-activated PLN; Con A activated PLN (24, 48 and 72hr); Con A activation (24hr) + exogenous IL-2 (up to 3 days); Con A (24hr) + PMA (10min); CD3 activated PLN in the presence of exogenous IL-2 (48hr); PPD, ovalbumin and S-Ag specific CD4<sup>+</sup> T cell lines.

### 2.11.2 Migration Assay

2 x 10<sup>5</sup> lymphocytes in 1ml of medium were added to RPE or REC monolayers grown in 24 well plates. The culture was then placed onto the stage of an inverse-phase contrast microscope which was temperature (37°C) and CO<sub>2</sub> (5%) controlled. A field of 200 $\mu$ m<sup>2</sup> was selected and, using time-lapse videomicroscopy, the interactions of the cells were recorded over a period of 4 hours. The percentage of cells which had migrated through the monolayer was calculated at 30 minute time-points by replaying the tape 160x normal speed. Lymphocytes could be seen moving above and below the monolayer and could easily be distinguished by their morphology and phase contrast appearance (Figure 2.2).

Having established the kinetics of lymphocyte migration across the RPE, lymphocytes were added to the wells at 10min intervals and recorded for 10 minutes (5 minutes either side of the 4 hour time-point) in future experiments.

**CO-CULTURE OF T CELL LINE LYMPHOCYTES WITH RPE  
CELLS.**



**Fig 2.2** *Phase contrast image of co-culture of RPE with T cell line lymphocytes. Lymphocytes below the monolayer (examples marked a) appear phase dark while those above the monolayer are phase bright (examples marked b). Magnification = x240.*



### 2.11.3 Effect of Cytokines

RPE cells were activated with 100U/ml IFN- $\gamma$  or 5U/ml IL-1 for 24 hours prior to the migration assay. A minimum of four wells were used per cytokine and lymphocytes were also added to untreated RPE as a control for each experiment. The effect of cytokine treatment on the migration of untreated PLN, Con A activated PLN, CD3 activated PLN (up to 72hr) and the antigen specific T cell line lymphocytes was investigated.

### 2.11.4 Antibody Blockade Experiments

The role of adhesion molecules expressed on both lymphocytes and the RPE on lymphocyte migration was investigated. Antibodies against rat adhesion molecules were added to the RPE or lymphocytes for 1h at 4°C before the migration assay. 100 $\mu$ l/well of anti-ICAM-1 supernatant or 10 $\mu$ g/ml of anti-VCAM-1 was added to RPE; 100 $\mu$ l supernatant per 5 $\times$ 10<sup>6</sup> cells of anti-LFA-1 (CD11a;WT.1 and CD18; WT.3) antibody or 10 $\mu$ g per 1 $\times$ 10<sup>6</sup> cells of anti-VLA-4 (anti-CD49) monoclonal antibody were added to the lymphocytes. In control experiments, lymphocytes were incubated with an irrelevant antibody OX-21 (anti-human C3b inactivator mAb) as a control or in the absence of mAb. The adhesion molecules involved in lymphocytes migration across resting and IFN- $\gamma$  (100u/ml, 24h and 72h) activated RPE monolayers were investigated.

## **2.12 EXPRESSION OF MHC CLASS I AND II & ADHESION MOLECULES ON RPE**

### 2.12.1 ELISA

The expression of MHC molecules (class I and II) and the adhesion molecules ICAM-1 and VCAM-1 on RPE monolayers were determined using this technique. The kinetics of expression of these molecules was measured by treating the cells for up to 5 days with 100U/ml IFN- $\gamma$ . As a positive control for MHC class II, peritoneal macrophages were also cultured in 96 well plates and activated with IFN- $\gamma$ . RPE cells were also treated for 5 days with a range of concentrations of IFN- $\gamma$  (0-1000U/ml) to

establish the optimum concentration requires to induce class II expression.

Cells were first fixed for 15 minutes in 0.1% glutaraldehyde in PBS before blocking for 20 minutes with 0.05M Tris-HCl (pH 7.4). The cells were then incubated for 1h with the primary antibodies: OX18 (1:12 dilution, anti-MHC class I mAb); OX6 (1:5 dilution, anti-class II I-A mAb); OX-17 (1:5 dilution, anti-class II I-E mAb); 1A-29 (anti-CD54/ICAM-1 mAb) and 5F10 (anti-VCAM-1). After washing three times with 0.3% BSA-PBS cells were incubated for 1hr incubations with biotinylated sheep anti-mouse IgG (1:10 000 dilution) and streptavidin-peroxidase (1:5000). Again, well was washed three times with 0.3% BSA-PBS between each step.

The plates were developed using 100 $\mu$ g/ml tetramethylbenzidine in 97mM sodium acetate/3mM citric acid, containing 0.5 $\mu$ l/ml H<sub>2</sub>O<sub>2</sub> for approximately 15 minutes. The reaction was stopped with 1M H<sub>2</sub>SO<sub>4</sub> and the optical density measured at 450nm using an ELISA reader (Titertek, Multiskan, Flow, Oxon, UK).

### 2.12.2 Flow Cytometry

Confluent RPE and REC cells (unstimulated and cytokine activated) cultured in 25cm<sup>2</sup> flasks were dissociated as described previously and incubated with 1A-29 (anti-ICAM-1) and 5F2 (anti-VCAM-1) antibody for 45 minutes followed by FITC-RAMIG. The fluorescence labelled cells were analysed by flow cytometry.

## **2.13 STATISTICS**

All experiments were carried out a minimum of three times and results expressed as the mean  $\pm$  S.D. (standard deviation). In general, experiments in which n=9 or greater, results are expressed as mean  $\pm$  S.E.M (standard error of mean).

Statistical analysis of migration kinetics was compared at each time point using chi-squared analysis. This method allowed the comparison of the total number of cells above and below the monolayer, as opposed to just the percentage of migrated cells. All other statistical comparisons were made using the students t-test. The decision to use the chi-squared test for migration results was taken upon consulting Dr Darwin Minnasian for statistical advice.

## CHAPTER 3

### RPE:- ISOLATION AND CHARACTERISATION

#### 3.1 INTRODUCTION

The retinal pigment epithelium (RPE) is a single layer of cuboidal epithelial cells located between the choriocapillaris and photoreceptor cells. It has many complex functions such as absorption of light, storage and conversion of vitamin A esters, and phagocytosis of rod and cone outer segments. Apical processes of RPE and rod outer segments (ROS) of photoreceptor cells interdigitate with each other and this close contact appears to be essential for photoreceptor cell development and function (Wang et al., 1993). The strong attachment of rat RPE to Bruch's membrane makes it impossible to "brush" off the RPE (as is done in bovine and frog) without damaging the cells. One of the main problems in isolating viable rat RPE is to separate the retina from the RPE without tearing the apical microvilli of the RPE as the strong interdigitation of RPE microvilli with ROS makes the separation of the two structures difficult in adult rodents (Wang et al., 1993). By using young animals (less than 15 days old), ROS have not fully developed and RPE cells can be isolated without tearing away their apical microvilli (Edwards, 1981; Mayerson et al., 1985).

Rat RPE cells, like other epithelia have abundant cytokeratin intermediate filaments (Owaribe, 1988), therefore, in the absence of a RPE specific monoclonal antibody, is commonly used to prove the purity of RPE cultures. However, the cytokeratins expressed by each cell varies within the culture, depending on the maturity of the cell (Robey, 1994).

Although there is no commercially available monoclonal antibody specific for RPE, a few have been developed against human (Hooks et al., 1989) and rat (Neill and Barnstable, 1990) RPE. RET-PE2, the monoclonal which recognises rat RPE, labels a 50kDa cell surface protein exposed on the cell surface of RPE of adult rats and RPE in culture (Neill and Barnstable, 1990).

## 3.2 RESULTS

### 3.2.1 Morphology

RPE cells grown on plastic assume a flattened morphology, often projecting long pseudopod like extensions to contact adjacent cells. Isolated RPE settled as small clusters of hexagonal, pigmented cells. As cells grew out from this central plaque they became increasingly less pigmented and a number of multi-nucleated cells were seen. The degree of cell spreading and therefore also cell size was variable, with some "giant" cells, several times the size of most cells, observed. During the initial phase of culture (1-3 days) purity was easily assessed as only pigmented cells were present, any cultures containing non-pigmented cells were discarded. Confluent cultures of RPE cells were obtained within 1-2 weeks and cultures used within this time period. (Figure 3.1)

### 3.2.2 Immunohistochemistry

RPE cultures were examined as to their expression of cytokeratins using immunohistochemistry. Cells were stained positive using the wide-spectrum antibody K8.13 which labels cytokeratins 1,5,6,7,8,10,11 and 18 (Figure 3.2a).

Cultures of RPE were also positive when stained with RET-PE2 (Figure 3.2b).

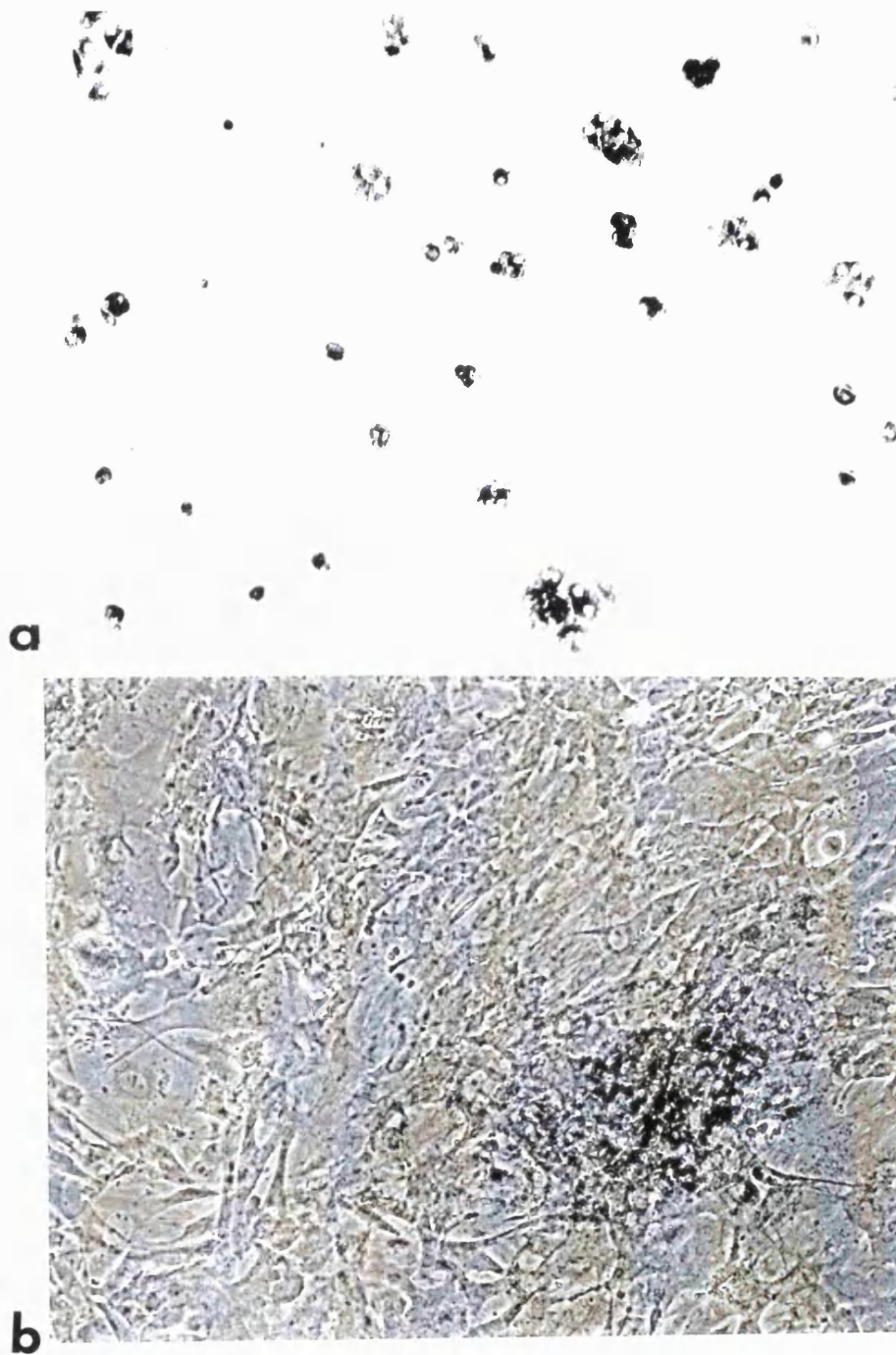
### 3.2.3 Flow cytometry

The expression of cytokeratins and RET-PE2 was also examined using flow-cytometry, with the majority of the cells staining positive with RET-PE2, but much lower levels of cytokeratin expression (Figure 3.3).

### 3.2.4 Electron Microscopy

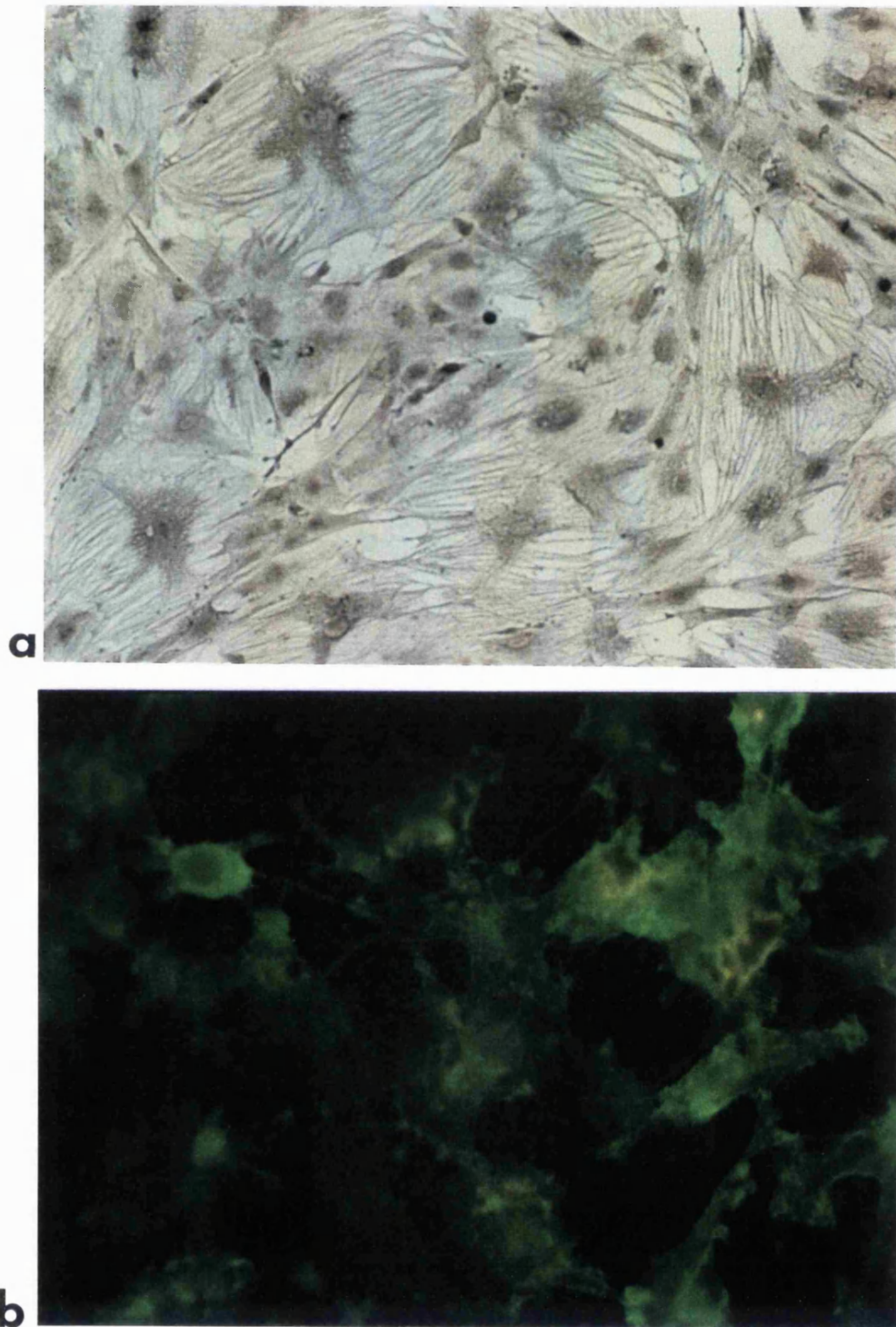
Transmission electron micrographs showed an abundance of smooth and rough endoplasmic reticulum, and mitochondria, suggesting that these cells were metabolically active as well as coated pits and long attenuated strands, possibly microvilli. Melanosomes, at different stages of development, could be seen in some cells (Figure 3.4).

## CULTURES OF RAT RPE

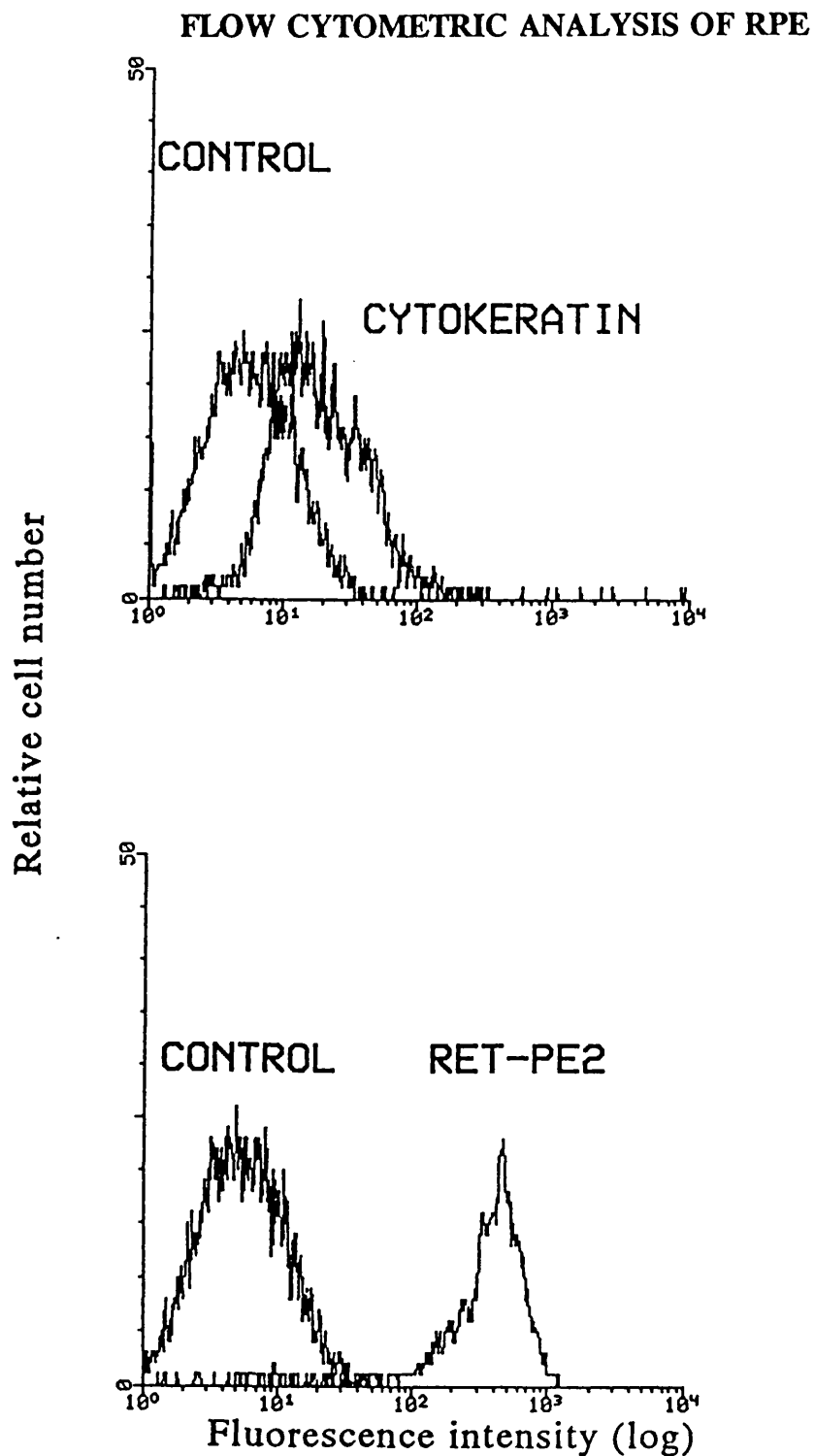


**Fig 3.1** RPE isolated from PVG rats. (a) RPE 1 days after isolation; (b) RPE after 3 days in culture; (c) Confluent RPE cultures after 1 week in culture (x 240 magnification).

## IMMUNOHISTOCHEMICAL ANALYSIS OF RPE

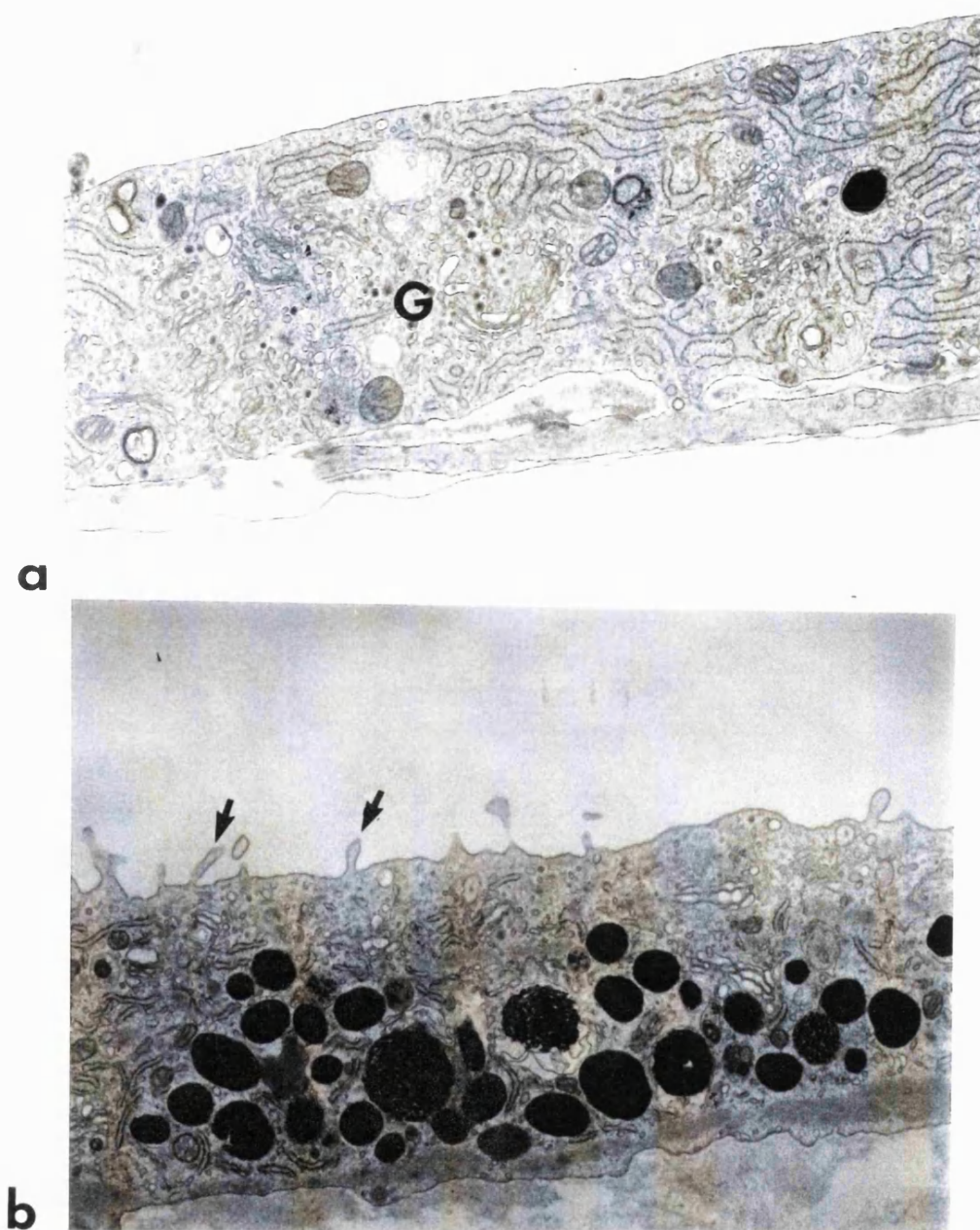


**Fig 3.2** (a) *Immunoperoxidase staining on RPE for cytokeratins and (b) Immunofluorescence staining with the rat RPE specific monoclonal RET-PE2 (x500 magnification).*



**Fig 3.3** Single cell suspensions of normal cultured RPE were stained with anti-cytokeratin and RET-PE2 monoclonal antibody. Control histogram is background staining of FITC-labelled RAMIG. 40% of cells stained positive for cytokeratin, while 100% of cells were positive when stained with the rat RPE specific monoclonal antibody RET-PE2.

## ULTRASTRUCTURE OF RPE



**Fig 3.4** Transmission electron micrographs of vertically sectioned primary rat RPE showing (a) well developed perinuclear Golgi system (G) and (b) melanosomes at varying degrees of maturity. Note apical microvilli (arrows) and basal membrane associated actin stress fibre in (b). (Scale bars  $a=0.5\mu\text{m}$  and  $b=1\mu\text{m}$ . Magnification (a)  $\times 10,000$  and (b)  $\times 8000$ ).



### 3.3. DISCUSSION

The isolation of rat RPE, as opposed to human, bovine, chick etc. is extremely difficult due to the strong adhesion between the RPE and both Bruch's membrane and the choroid. A number of methods were employed at the start of this project to ensure that the cell cultures were pure and that maximal numbers of viable cells were isolated from each preparation.

Initial attempts were carried out on adult Lewis rats (Method 1, Chapter 2). At this time RPE had only been isolated from rats less than 15 days old. The enzyme cocktail used was the same as had been used for young rats (Edwards, 1981), but the eyes were dissected prior to incubation with the enzyme, to allow the enzyme to act directly on the RPE. While RPE cells were dissociated using this technique, they did not grow in culture. This may be due to the cells being damaged from the enzyme treatment or through trauma. Alternatively, the enzyme treatment may cleave the surface molecules required for adhesion to the tissue culture plate resulting in cell death. A method for isolating RPE from adult rats has since been developed (Wang et al., 1993), although the cells were not subsequently cultured, but used for biochemical analysis.

The second method used was that developed by Edwards (1981). However the problem with this technique is that the retina is removed first, leaving the RPE attached to the choroid. Since both RPE and choroid cells are pigmented, separation of the RPE from the choroid following removal of the retina can be ambiguous, resulting in contamination of the cultures with choroidal cells. As many of these cells (fibroblast. dendritic cells etc.) grew at a faster rate than the RPE, which were relatively slow growing, the cultures soon became overgrown with other cell types and thus could not be used.

The third method used proved to be the best and was used for the majority of RPE cultures described in this thesis. This method used Dispase which allows the RPE to remain attached to the retina, but not the choroid, during the isolation procedure. The length of time required for the enzyme digestion was also shorter, which decreased the possibility of injury due to prolonged enzyme treatment. RPE cells were also isolated from 7 day old Lewis pups, however, due to these animals being non-pigmented it was impossible to distinguish between RPE and other cells during the physical isolation step and hence the purity of cultures was variable. Therefore pigmented PVG rats were used for subsequent cultures.

Twenty four hours after seeding, 80 -100% of cells attached, forming a confluent monolayer within 7 days. The morphology of RPE cultures was consistent with other descriptions of rat RPE cultures. Cells covered the plate both by spreading and by cell division resulting in a monolayer of cells of different sizes with the loss of pigment in actively dividing cells a characteristic of these cultures (Albert and Buyukmihci, 1979). The "giant" cells observed are cells which do not readily divide in culture, but spread to become several times larger than most other RPE cells. These have also been described previously.

Morphological descriptions of rat RPE cells in culture vary because cell shape, degree of pigmentation and growth rate of these cells is dependent on culture conditions, such as serum concentration and the substrate on which the cells grow. For example, it has been shown that when these cells were grown on plastic in RPMI in high serum (20%) the cells assumed a fibroblastic shape and appeared lightly pigmented. However, if they were maintained in DMEM with low serum (3%) over several weeks they gradually assumed a more epithelioid shape and exhibited pigmentation in the perinuclear region, consistent with a more differentiated phenotype (McLaren et al. 1993). Due to their irregular shape, the ability to assess accurately the purity of these cultures is extremely important. Immunohistochemical examination of cytokeratin expression was employed to prove the purity of RPE cultures as possible contaminants (Muller glia, astrocytes, endothelium and fibroblasts) do not express this epithelial marker (Robey et al., 1992). However, cytokeratin expression may change with differentiation (Robey, 1994) and flow cytometric analysis implied lower levels of expression than observed immunohistochemically. The cell surface marker RET-PE2 was therefore used for this purpose and as a positive control in future flow-cytometric analysis. Unlike cytokeratins, this monoclonal antibody is specific for a cell surface molecule. Flow cytometric analysis revealed that 100% of cells were positive for this marker, indicating that the cultures were extremely pure, something which had not previously been shown for primary rat RPE cultures. Previously, purity of these cultures was only estimated by morphology, pigmentation and expression of the epithelial markers, cytokeratins.

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## CHAPTER 4

### EFFECT OF LYMPHOCYTE ACTIVATION ON ADHESION TO AND MIGRATION THROUGH NON-ACTIVATED RPE MONOLAYERS

#### 4.1 INTRODUCTION

Activation of T lymphocytes requires a second signal other than that produced by the interaction of TCR/CD3 with antigen and MHC. *In vitro* activation of T cells can also be induced by the mitogens Con A, and PMA, or by triggering of the CD3 complex with immobilised anti-CD3 plus exogenous IL-2 (this overrides the requirement for accessory cells) (Byrne et al., 1988; Geppert and Lipsky, 1987). This activation of T cells results in both quantitative and qualitative changes in adhesion molecule expression leading to an increased adhesion for endothelium. The mode of lymphocyte activation has been shown to be crucial in determining the level of lymphocyte adhesion to (Wang et al., 1993) and migration across (Greenwood and Calder, 1993) rat retinal endothelial monolayers. The adhesion and migration of CD4<sup>+</sup> T cell lines (ie. activated with antigen) was found to be much greater than both non-activated and Con A-stimulated PLN cells. On the other hand, activation of PLN cells with Con A only increased adhesion to, not migration through endothelial monolayers (Greenwood and Calder, 1993), despite an increase in both LFA-1 expression and cell motility (Greenwood et al., 1995).

Similar experiments were carried out with RPE monolayers to determine if the mode of lymphocyte activation also controlled the level of adhesion to or migration through these cell monolayers.

#### 4.2 RESULTS

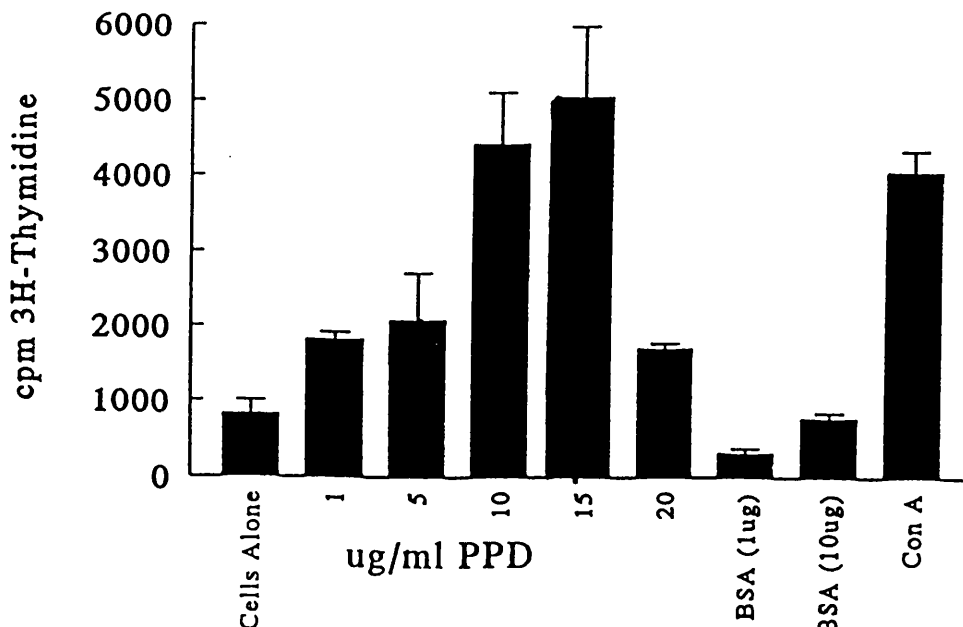
##### 4.2.1 Phenotype of lymphocyte populations studied.

The phenotypes of T cell line lymphocytes and PLN cells (non-activated and activated with Con A or anti-CD3) were assessed using flow cytometry (Table 4.1). Activation with either Con A or anti-CD3 increased the percentage of OX-19 (CD5, pan T cell) positive cells, in particular the percentage of CD4<sup>+</sup> (W3/25) positive cells. As expected the percentage of activated cells (OX-39/IL-2 receptor positive) also increased upon activation. OX-22 expression is much lower in T cell line lymphocytes, indicating

that, as expected, these cells were memory T cells. Antigen specificity was determined by a proliferation assay (Figure 4.1).

| Ab    | Resting PLN | Con A (24hr) | CD3 (24hr) | PPD T-cell line | S-Ag T-cell line |
|-------|-------------|--------------|------------|-----------------|------------------|
| W3/25 | 40.4 ± 4.7  | 67 ± 8.6     | 57 ± 7.7   | 98.3 ± 1.7      | 98 ± 0.9         |
| OX-8  | 34 ± 3.4    | 39 ± 4.9     | 41 ± 4.2   | 4.9 ± 7.8       | 2.5 ± 1.2        |
| OX-19 | 73.4 ± 2.9  | 93 ± 4.7     | 98.5 ± 5.8 | 73 ± 6.3        | 95.3 ± 4.1       |
| OX-39 | 40 ± 11.8   | 78.1 ± 13    | 61.4 ± 3.7 | 55 ± 36.4       | 88.5 ± 5.8       |
| OX-22 | 69.2 ± 3.6  | 85 ± 9.1     | 64 ± 9     | 0.2 ± 0.07      | 1.5 ± 0.9        |

**Table 4.1.** Summary of phenotype of lymphocytes studied. Percentage of lymphocytes stained positive (mean ± S.D., n=3). Cells were stained for the expression of CD4 (W3/25); CD8 (OX-8); CD5 (pan T cell, OX-19); CD25 (IL-2 receptor, OX-39) and leucocyte common antigen (naive cell marker, OX-22).



**Fig 4.1** Proliferation assay demonstrating antigen specificity of PPD T cell line. PPD T cell line lymphocytes stimulated with 1-20µg/ml PPD, 1 or 10µg/ml BSA or the mitogen Con A. Error bars represent S.E.M. (n=9) and results are expressed as counts per minute of incorporated <sup>3</sup>H-thymidine.

#### 4.2.2. Effect of PLN lymphocyte activation with Con A on adhesion to and migration across RPE monolayers.

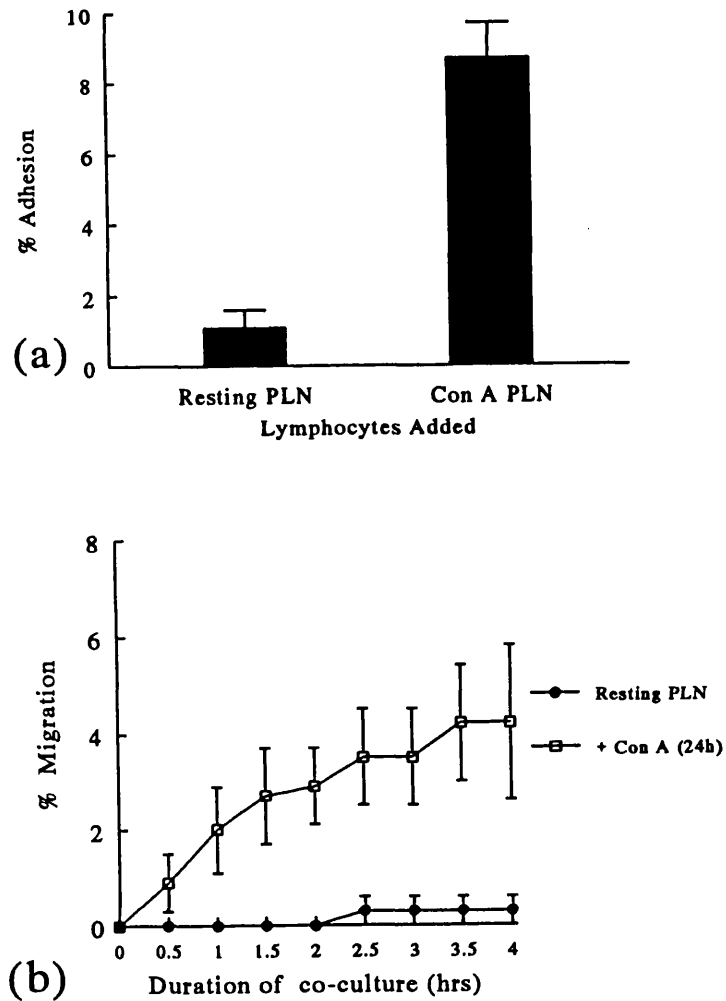
The level of adhesion to and migration across untreated RPE monolayers of untreated PLN cells was very low with  $1 \pm 0.5\%$  adhesion and  $0.3 \pm 0.1\%$  migration. Activation of PLN lymphocytes with Con A for 24hr significantly increased the level of adhesion and migration to  $8.7 \pm 1\%$  and  $4 \pm 1.6\%$  respectively (figure 4.2,  $p < 0.01$ , chi-squared analysis). Activation of lymphocytes with Con A for 48 or 72hrs did not further augment this increase in migration (figure 4.3).

Expression of the adhesion molecules LFA-1 and VLA-4 was examined using flow cytometry to establish if the increase in adhesion or migration was associated with an increase in expression of a particular adhesion molecule. Activation of PLN cells with Con A increased the expression of both VLA-4, and to a greater extent LFA-1 as shown by flow cytometric analysis using antibodies against both CD11a and CD18 (WT-1 and WT-3 for LFA-1) and an antibody against CD49d for VLA-4 (TA-2). No difference was found between the expression of CD11a or CD18. Prolonged activation of PLN cells with Con A also had no effect on the percentage of cells expressing LFA-1 (Table 4.2) or the level of expression, but did increase the percentage of cells expressing VLA-4 (Figure 4.4).

|               | CD11a           | CD18            | CD49d           |
|---------------|-----------------|-----------------|-----------------|
| Untreated PLN | $76 \pm 15.2$   | $75.1 \pm 12.1$ | $24 \pm 4.2$    |
| 24 hours      | $97.3 \pm 2.5$  | $97.3 \pm 7.8$  | $38 \pm 10.6$   |
| 48 hours      | $96.5 \pm 11.2$ | $99.2 \pm 6.4$  | $53.3 \pm 29.7$ |
| 72 hours      | $98.5 \pm 1.4$  | $98.4 \pm 0.6$  | $71.3 \pm 8.2$  |

**Table 4.2.** Percentage of PLN lymphocytes positive for expression of the adhesion molecules LFA-1 (WT-1/WT-3) and VLA-4 (TA-2) (Mean  $\pm$  S.D.,  $n=4$ ). PLN lymphocytes, either not activated and cultured for 24 hours or activated with Con A for 24, 48 or 72 hours.

**PLN LYMPHOCYTE ADHESION TO AND MIGRATION THROUGH RPE  
MONOLAYERS.**



**Fig 4.2** Comparison of (a) adhesion to and (b) migration through untreated RPE monolayers of PLN lymphocytes either untreated or activated with Con A for 24hr. Results from adhesion assays are a mean of 3 experiments with each experiment carried out in triplicate ( $n=9$ ). Migration assay results are a mean of 8 experiments. Results from both are expressed as mean  $\pm$  S.E.M.

MIGRATION THROUGH RPE MONOLAYERS OF CON A ACTIVATED PLN  
LYMPHOCYTES.

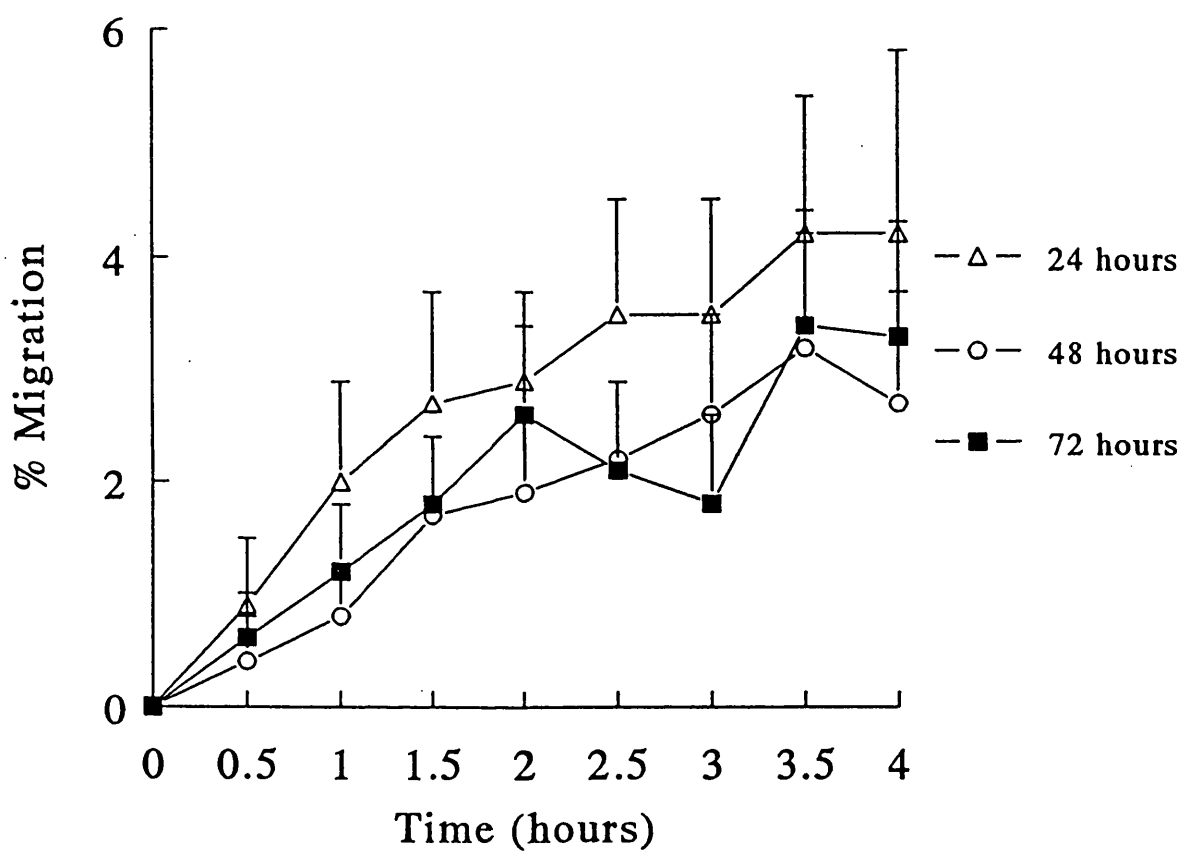
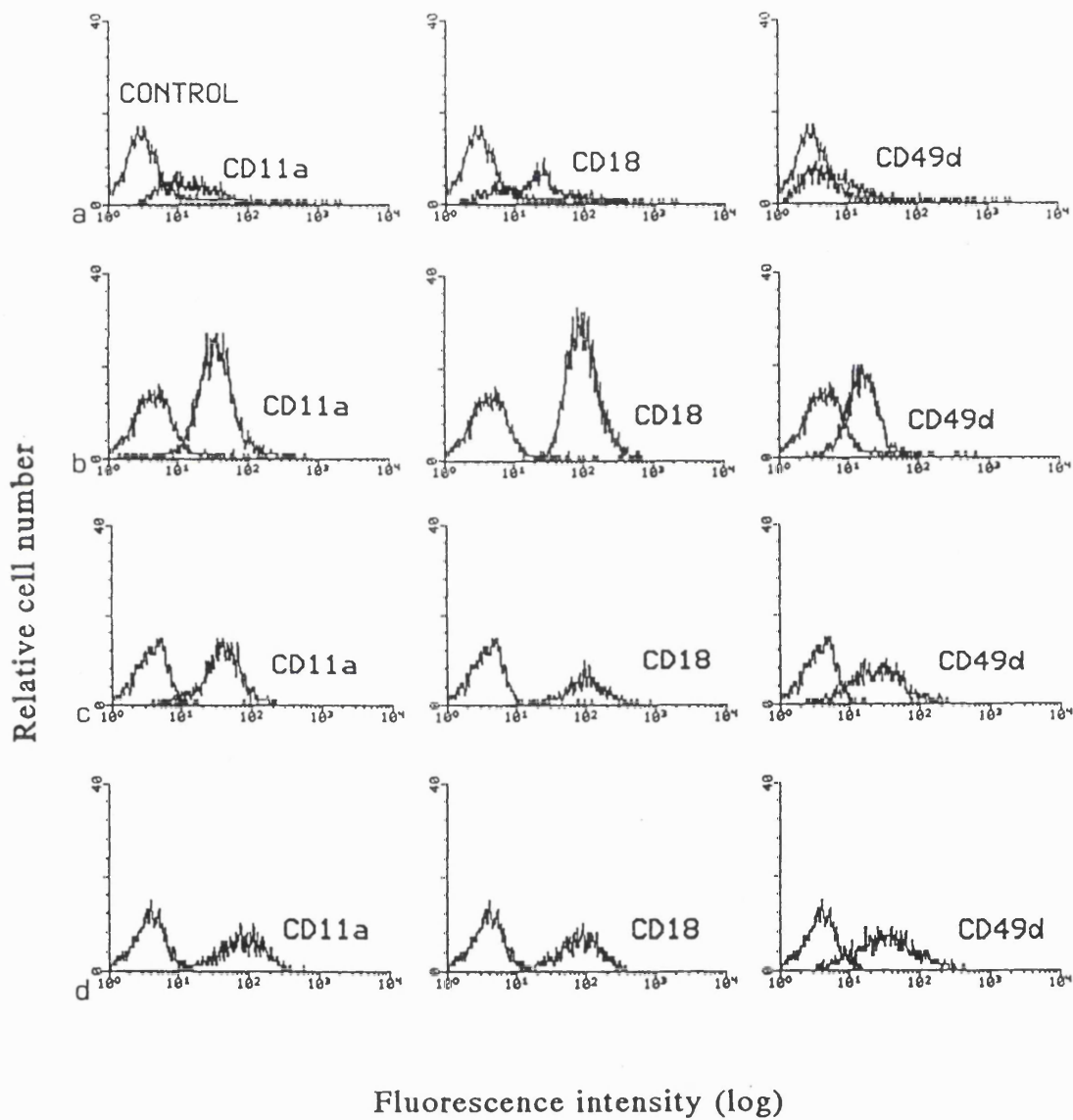


Fig 4.3 Migration of PLN lymphocytes activated with Con A for 24, 48 and 72hrs. Results are expressed as mean migration  $\pm$  S.E.M. ( $n=9$ ). No significant difference was found in migration of any of the groups studied.

**FLOW CYTOMETRIC ANALYSIS OF ADHESION MOLECULE EXPRESSION  
ON PLN LYMPHOCYTES.**



**Fig 4.4** Flow cytometric analysis of LFA-1 (CD11a and CD18) and VLA-4 (CD49d) expressed on (a) untreated and PLN cells activated with Con A for (b)24hr, (c)48hr and (d)72hr. Activation of PLN cells with Con A for 24hr increased the intensity of expression of LFA-1 and VLA-4.



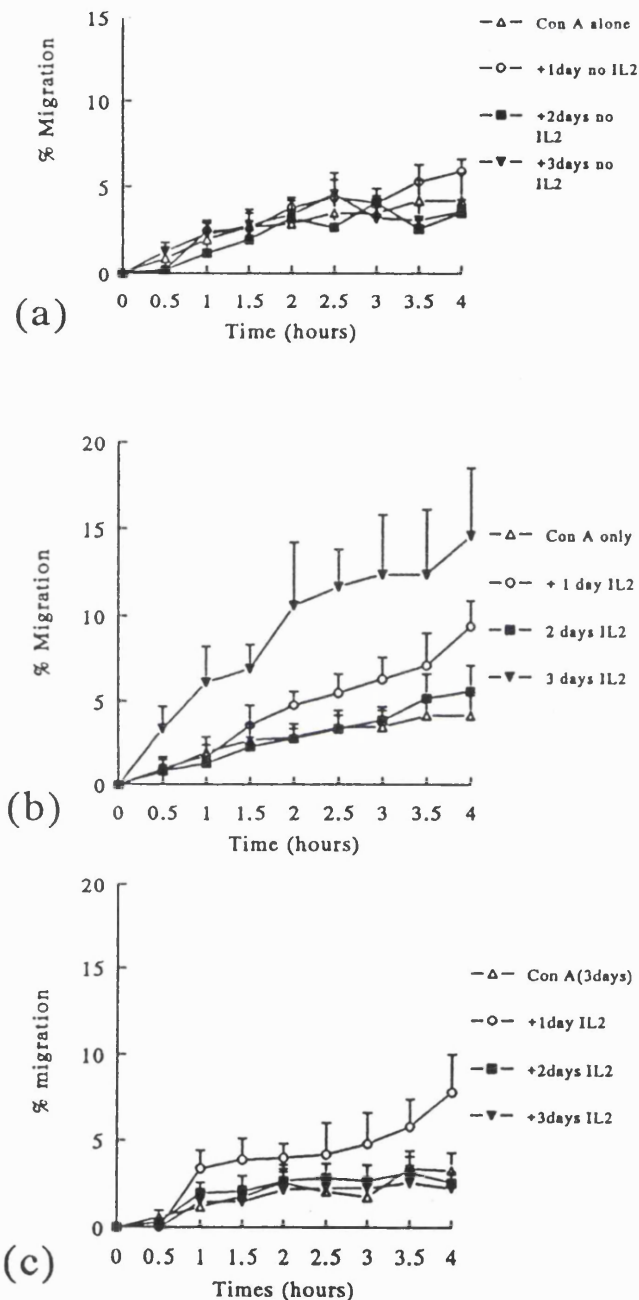
The effect of adding exogenous IL-2 to the culture medium post-stimulation with Con A was also examined and, as a control, cells were cultured in the absence of exogenous IL-2 for a similar time period (Figure 4.5). In addition, exogenous IL-2 was added to cultures activated with Con A for 3 days, as this is comparable with the activation protocol used to maintain the T cell lines.

Adding exogenous IL-2 to PLN cells activated with Con A for 24 hours did enhance their ability to migrate when compared with cells cultured in the absence of IL-2 after 24 hours Con A activation, ( $p < 0.05$ ). However, this increase in migration was only significantly greater than 24hr activation with Con A alone after 3 days post-IL-2 stimulation ( $p < 0.01$ , Figure 4.5b). Addition of exogenous IL-2 to Con A activated PLN cells (24hr) prolonged rather than enhanced, the expression of VLA-4 but did not increase the percentage of cells expressing this molecule (Table 4.3). IL-2 also had no effect on the percentage of cells expressing LFA-1 or the intensity of expression. There was no difference in the level of adhesion molecule expression after 24hr Con A activation followed by 3 days IL-2 stimulation which could account for the difference in migration found at this time point (figure 4.6).

|                      | CD11a (WT-1) | CD18 (WT-3) | CD49d (TA-2) |
|----------------------|--------------|-------------|--------------|
| <b>1 day IL-2</b>    | 99.8 ± 4.3   | 99 ± 1.6    | 64.7 ± 16.2  |
| <b>1 day no IL-2</b> | 89.8 ± 7.3   | 98.8 ± 1.9  | 74 ± 17.1    |
| <b>2 day IL-2</b>    | 89.8 ± 12    | 99 ± 2      | 45.2 ± 0.6   |
| <b>2 day no IL-2</b> | 99.6 ± 0.3   | 99.7 ± 0.1  | 61 ± 15      |
| <b>3 day IL-2</b>    | 93.8 ± 8.9   | 89 ± 5.5    | 33.6 ± 2     |
| <b>3 day no IL-2</b> | 97.7 ± 2.8   | 97.2 ± 3.5  | 54.3 ± 23    |

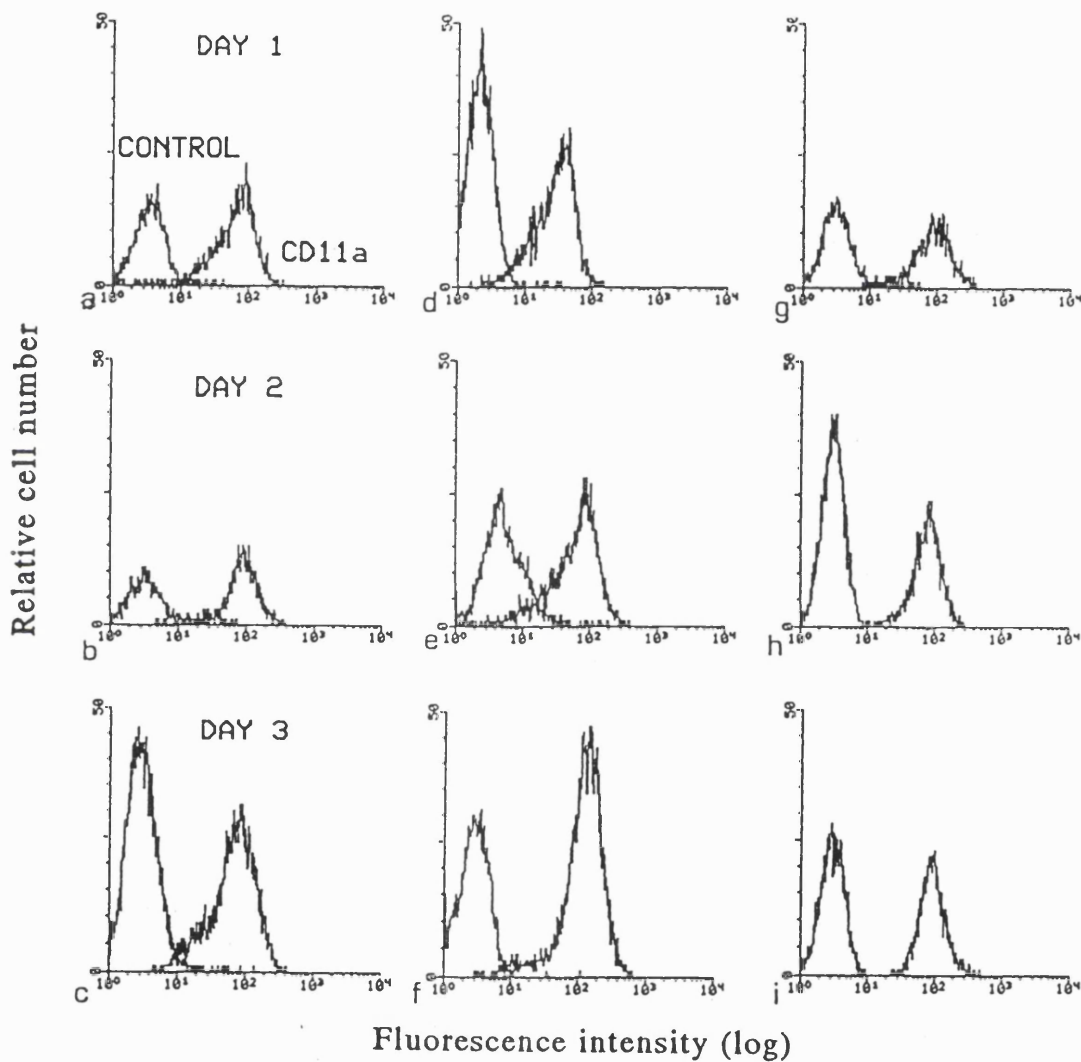
**Table 4.3.** Percentage of PLN lymphocytes positive for the adhesion molecules LFA-1 (CD11a/CD18) and VLA-4 (CD49d). Lymphocytes were activated for 24hr with Con A prior to culture in normal media for 3 days with and without IL-2 (Mean ± S.D., n=4).

### EFFECT OF IL-2 ON LYMPHOCYTE MIGRATION THROUGH RPE MONOLAYERS.



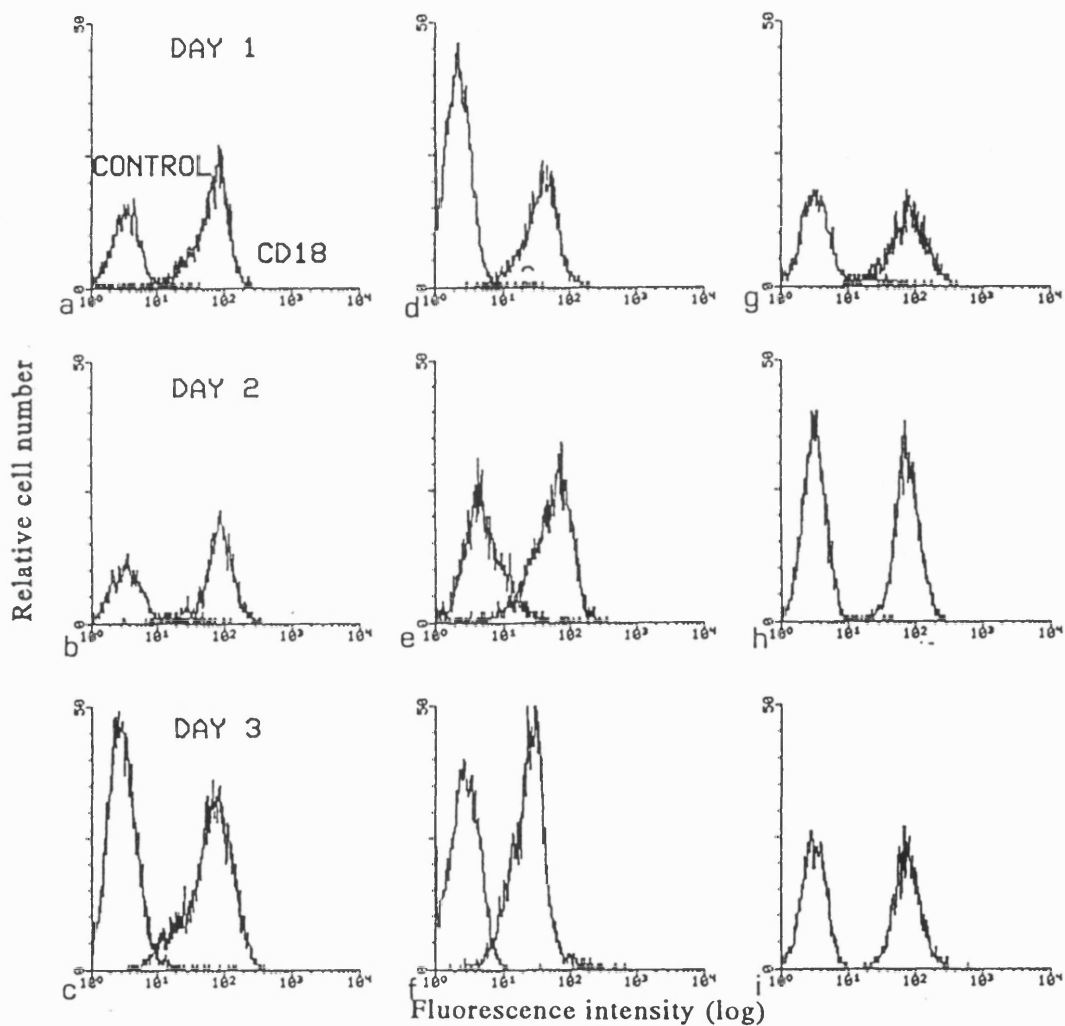
**Fig 4.5** Migration across untreated RPE monolayers of PLN cells activated with Con A for 24hr prior to culture (a) without IL-2 or (b) with IL-2 or (c) activated with Con A for 72hr prior to culture with IL-2. Results are expressed as mean percentage migration  $\pm$  S.E.M. ( $n=10$ ). Only significant difference in migration compared with the 24hr Con A activation alone was PLN cells cultured with IL-2 for 3 days after 24hr Con A activation ( $p < 0.01$ ).

**EFFECT OF IL-2 ON CD11a EXPRESSION ON CON A ACTIVATED PLN LYMPHOCYTES.**



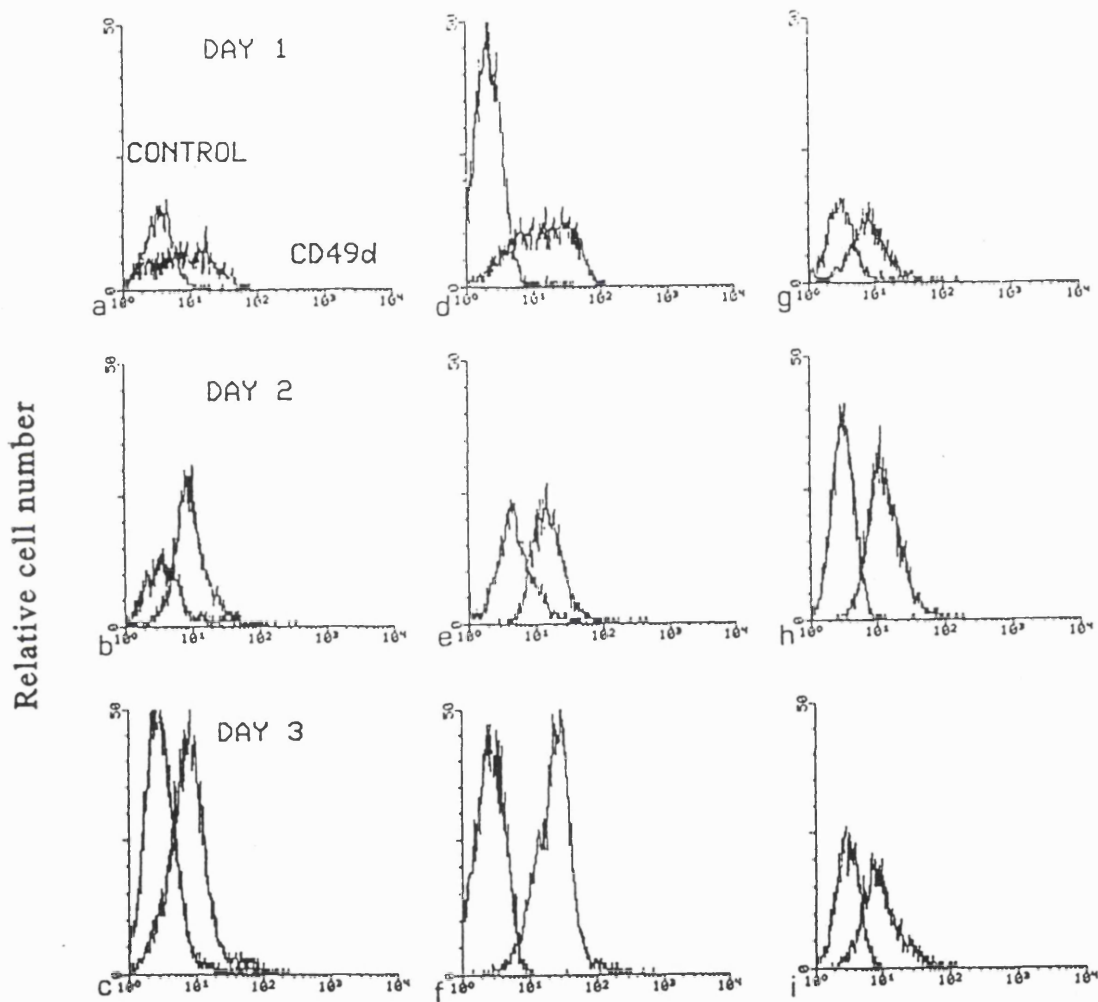
**Fig 4.6** Flow cytometric analysis of CD11a expressed on PLN cells (a-c) cultured in normal media for 3 days without IL-2 after 24hr Con A activation; (d-f) cultured in normal media with IL-2 for 3 days after 24hr Con A activation (g-i) Con A activation for 72hr prior to culture with IL-2 for an additional 3 days.

**EFFECT OF IL-2 ON CD18 EXPRESSION ON CON A ACTIVATED PLN  
LYMPHOCYTES.**



**Fig 4.7** Flow cytometric analysis of CD18 expressed on PLN cells (a-c) cultured in normal media for 3 days without IL-2 after 24hr Con A activation; (d-f) cultured in normal media with IL-2 for 3 days after 24hr Con A activation (g-i) Con A activation for 72hr prior to culture with IL-2 for an additional 3 days.

**EFFECT OF IL-2 ON VLA-4 (CD49d) EXPRESSION ON CON A ACTIVATED  
PLN LYMPHOCYTES.**



**Fig 4.8** Flow cytometric analysis of CD49d expressed on PLN cells (a-c) cultured in normal media for 3 days without IL-2 after 24hr Con A activation; (d-f) cultured in normal media with IL-2 for 3 days after 24hr Con A activation (g-i) Con A activation for 72hr prior to culture with IL-2 for an additional 3 days.

The migration of PLN cells activated for 3 days with Con A prior to adding exogenous IL-2 was also not significantly greater than without IL-2 (figure 4.5c), although the migration of these cells 1 day post IL-2 was significantly greater than that of Con A-activated PLN cells for 72 hours. Activation of PLN cells for 3 days prior to stimulation with IL-2 had no significant effect on the percentage of cells expressing adhesion molecules (Table 4.4) or the intensity of expression (Figure 4.6-4.8).

|            | CD11a (WT-1) | CD18 (WT-3) | CD49d (TA-2) |
|------------|--------------|-------------|--------------|
| 1 day IL-2 | 98.5 ± 2.1   | 98.6 ± 1.9  | 69.4 ± 15    |
| 2 day IL-2 | 100 ± 0      | 100 ± 0.04  | 79.8 ± 18    |
| 3 day IL-2 | 93.3 ± 14    | 99.9 ± 0.1  | 71.9 ± 28    |

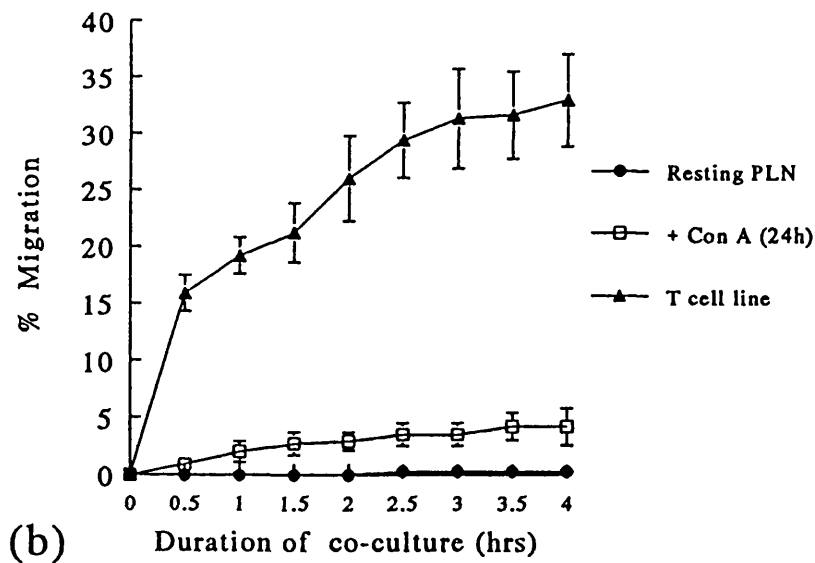
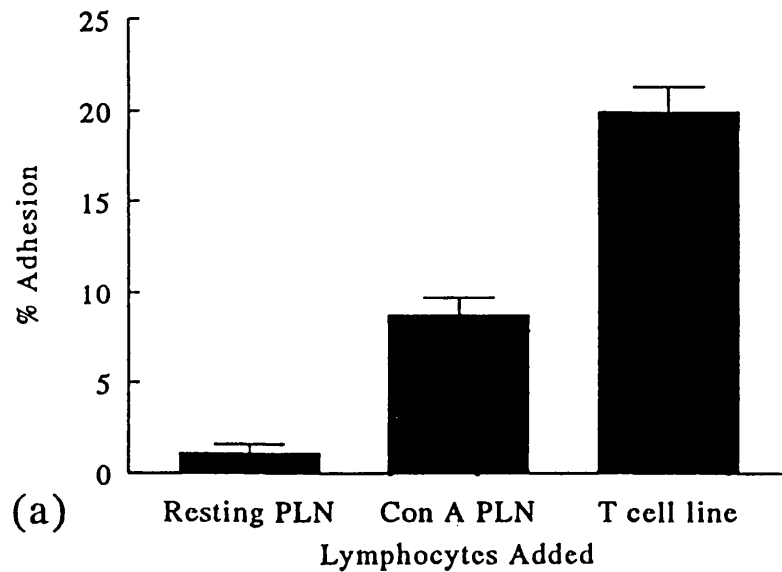
**Table 4.4.** Adhesion molecule expression, as determined by flow cytometry, of PLN cells activated with Con A for 72 hours prior to IL-2 stimulation for up to 3 days (Mean ± S.D., n=4).

#### 4.2.3 Adhesion and migration of antigen-specific T cell lines

Initial comparisons of the adhesion and migration of PPD specific T cell lines compared with non-activated or Con A activated PLN cells revealed that, as with endothelium, both the levels of adhesion and migration were greater with antigen-specific T cell lines (PPD) compared with Con A activated PLN cells. 19.9 ± 1.4% of T cell line lymphocytes adhered to RPE, compared with 8.7 ± 1% of Con A activated PLN cells (figure 4.9). The level of migration for the PPD specific lymphocytes was 32.9 ± 4.2% compared with 4.2 ± 1.6% for Con A activated lymphocytes (Figure 4.9, p < 0.001, chi-squared test).

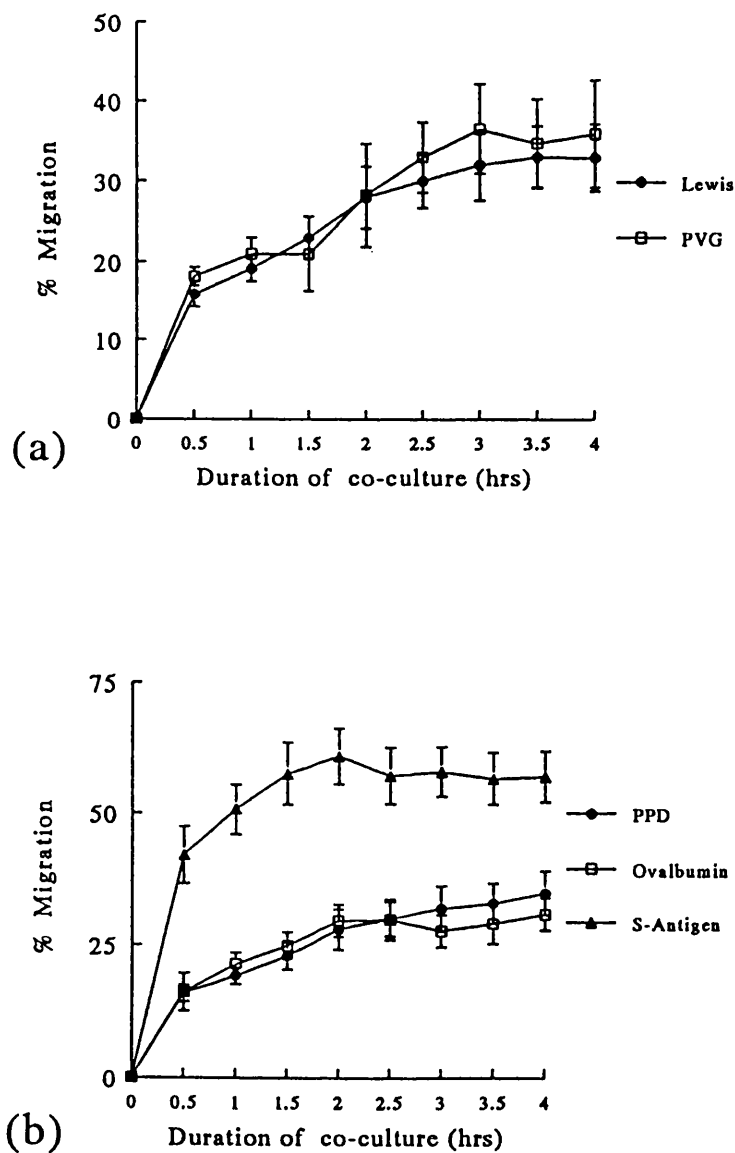
There was no significant difference in the migration of PPD T cell lines derived from Lewis or PVG rats. Therefore Lewis rat derived T cell lines were used in all future experiments as PPD T cell lines proved difficult to maintain. The migration of three different antigen-specific T cell lines were also compared, one specific for the retinal

**COMPARISON OF PLN AND T CELL LINE LYMPHOCYTE MIGRATION  
THROUGH RPE MONOLAYERS.**



**Fig.4.9** Comparison of (a) adhesion and (b) migration of Con A activated PLN cells (24hr) and PPD-specific T cell line lymphocytes. All results are expressed as mean  $\pm$  S.E.M. ( $n=9$ ). Adhesion and migration of PLN cells was significantly increased by Con A activation ( $p < 0.01$ , chi-squared test for migration and  $t$ -test for adhesion). However this was still significantly less than that of T cell line lymphocytes ( $p < 0.001$ ).

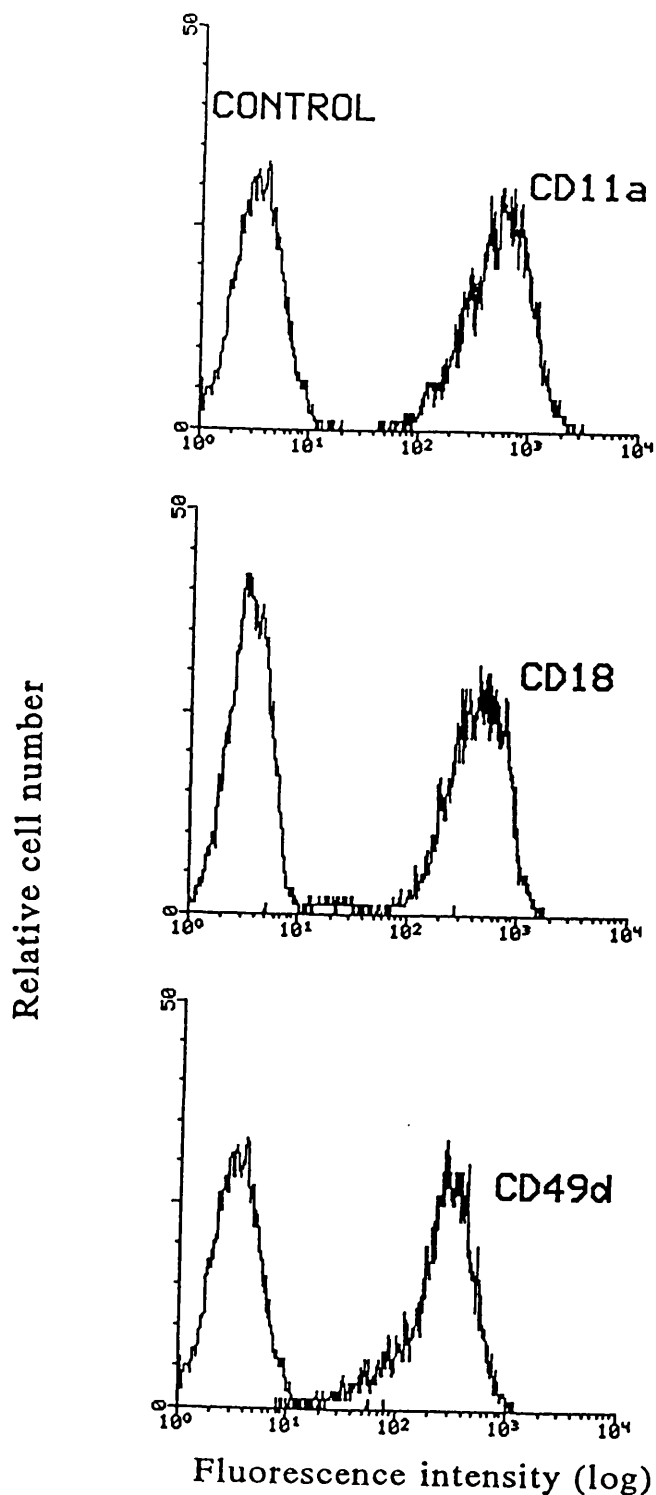
### T CELL LINE MIGRATION ACROSS RPE MONOLAYERS.



**Fig 4.10** Comparison of the migration of (a) PPD T cell lines derived from Lewis or PVG rats and (b) three antigen specific T cell lines; one specific for the ocular antigen S-Ag and two raised against the irrelevant antigens PPD and ovalbumin. Results expressed as mean  $\pm$  S.E.M. (a)  $n=3$  and (b)  $n=10$ . No significant difference was found between the migration of Lewis or PVG derived PPD T cell line lymphocytes. However, Lewis S-Ag specific lymphocytes were significantly more migratory than either PPD or OA T cell lines ( $p < 0.01$ , chi squared).



## ADHESION MOLECULE EXPRESSION ON T CELL LINE LYMPHOCYTES



**Fig 4.11** Expression of LFA-1 (CD11a and CD18) and VLA-4 (CD49d) on T cell line lymphocytes. In each case the histogram on the left is a negative control of cells stained with secondary antibody alone.

antigen S-antigen and two raised against the irrelevant antigens ovalbumin (OA) and purified protein derivative (PPD). In comparison to lymphocyte migration across retinal endothelial monolayers (Greenwood and Calder, 1993), a difference in the migration of the antigen specific T cell line to cross RPE monolayers was observed (figure 4.10b). The migration of S-antigen specific lymphocytes was significantly greater than either PPD or OA-specific lymphocytes ( $57 \pm 4.9\%$  compared with  $32.9 \pm 4.2$  and  $30.9 \pm 3.1\%$  respectively,  $p < 0.01$  chi squared test). This migration was also significantly greater than that of Con A activated PLN cells cultured with exogenous IL-2. The T cell line lymphocytes were all 100% positive for CD11a, CD18 and CD49d, with only the level of VLA-4 expression differing significantly from that of activated PLN lymphocytes (Figure 4.11, compared with figures 4.6-4.8).

#### 4.2.4 Adhesion and migration of CD3-activated PLN cells

To try and elucidate the factors responsible for the higher levels of adhesion and migration of antigen-stimulated T cells compared with Con A stimulated PLN cells, PLN cells were activated via the CD3-complex (part of the T cell receptor) as this is involved in the signalling during antigen-stimulation. PLN cells were added to 25cm<sup>2</sup> flasks coated with anti-CD3 monoclonal antibody for 24, 48 and 72 hours in IL-2 containing medium before adhesion or migration assays. There was no significant difference in the level of adhesion, between PLN cells activated with CD3 for 24 or 48 hours ( $11.8 \pm 2.3\%$  and  $13.5 \pm 2\%$  respectively). Adhesion, however, was significantly reduced following activation of the PLN cells for 72 hours ( $5.2 \pm 0.6\%$ ) ( $p < 0.05$ , figure 4.12a). Migration was measured after 4 hours of co-culture to determine the optimum incubation time with anti-CD3 for maximum migration (Figure 4.12b). The migration of these cells was greatest following 48 hours ( $p < 0.05$ ), with no significant difference found with PLN cells activated for 24 and 72 hours.

Figure 4.13 compares the kinetics of PLN cell migration and adhesion when the lymphocytes were activated with Con A for 24 hours or CD3 for 48 hours. The adhesion and migration of CD3 activated PLN cells was significantly greater than untreated PLN cells ( $p < 0.01$ ), but was not significantly different from 24hr activation with Con A (Figure 4.13).

Activation of PLN cells with anti-CD3 increased the expression of LFA-1 and

### EFFECT OF CD3 ACTIVATION ON LYMPHOCYTE ADHESION AND MIGRATION.

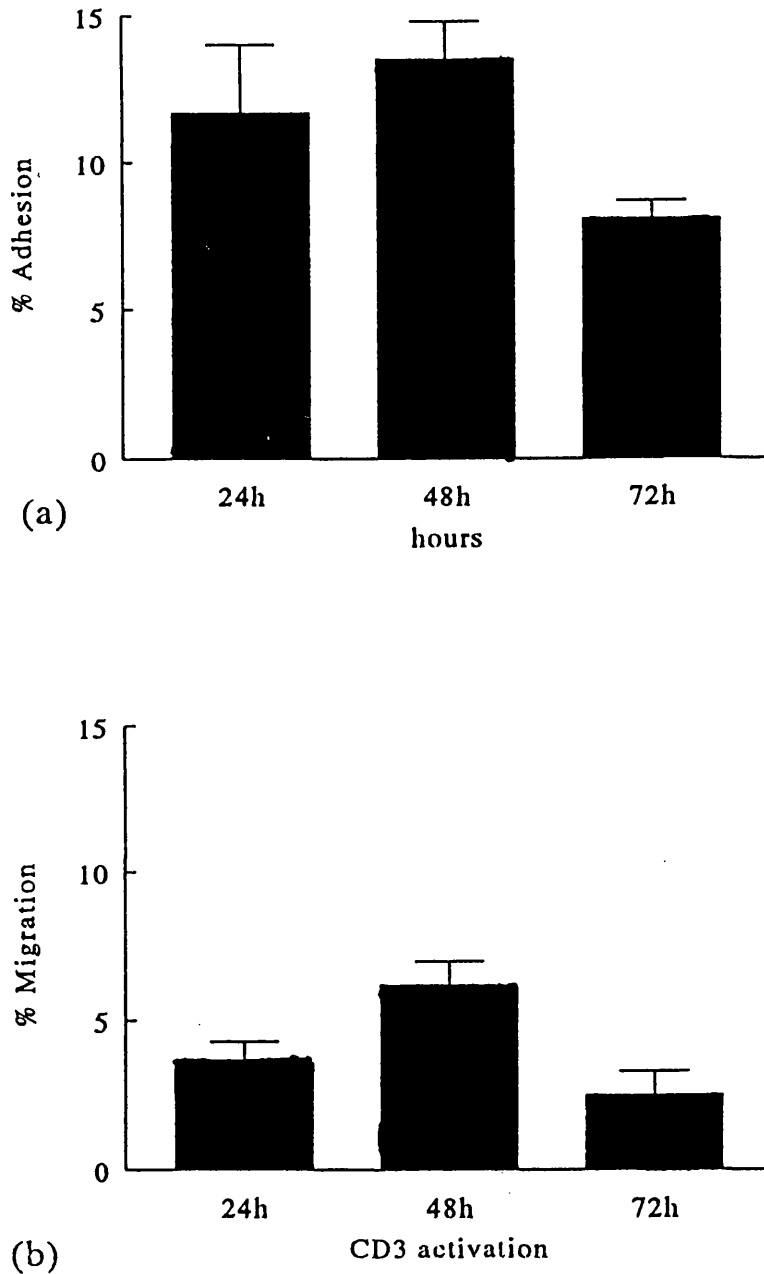
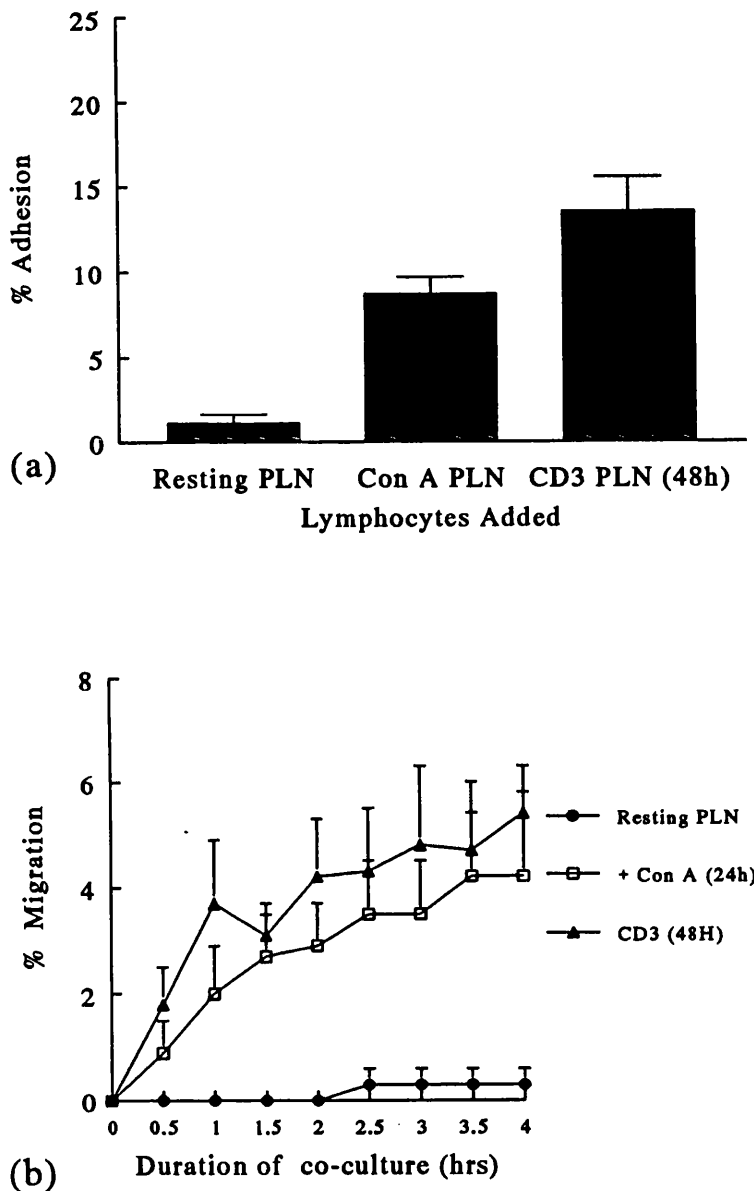


Fig 4.12 Time course of (a) adhesion and (b) migration of PLN activated with immobilised anti-CD3 and IL-2. Results are expressed as mean  $\pm$  S.E.M. ( $n=9$ ). Adhesion was significantly less when PLN cells activated for 72hr ( $p < 0.05$ ,  $t$ -test) but no difference was observed between 24 and 48hr activation. Migration was significantly greater when PLN cells were activated for 48hr ( $p < 0.05$ ).

**COMPARISON OF CON A AND CD3 ACTIVATION ON LYMPHOCYTE  
ADHESION AND MIGRATION.**



**Fig 4.13** Comparison of different modes of lymphocyte activation on (a) adhesion and (b) migration of PLN lymphocytes to and across untreated RPE monolayers. Results are expressed as mean  $\pm$  S.E.M. (adhesion  $n=9$ ). No significant difference was found between Con A (24hr) and CD3 (48hr) activation (ie. optimum activation times), however adhesion and migration of both were significantly greater than untreated PLN cells ( $p < 0.01$ , chi-squared for migration and t-test for adhesion assays).

VLA-4 after 24 hours, both of which remained at a similar intensity after activation for 48 or 72 hours (Figure 4.14, Table 4.5). There did not appear to be any difference in adhesion molecule expression between activation with Con A or anti-CD3.

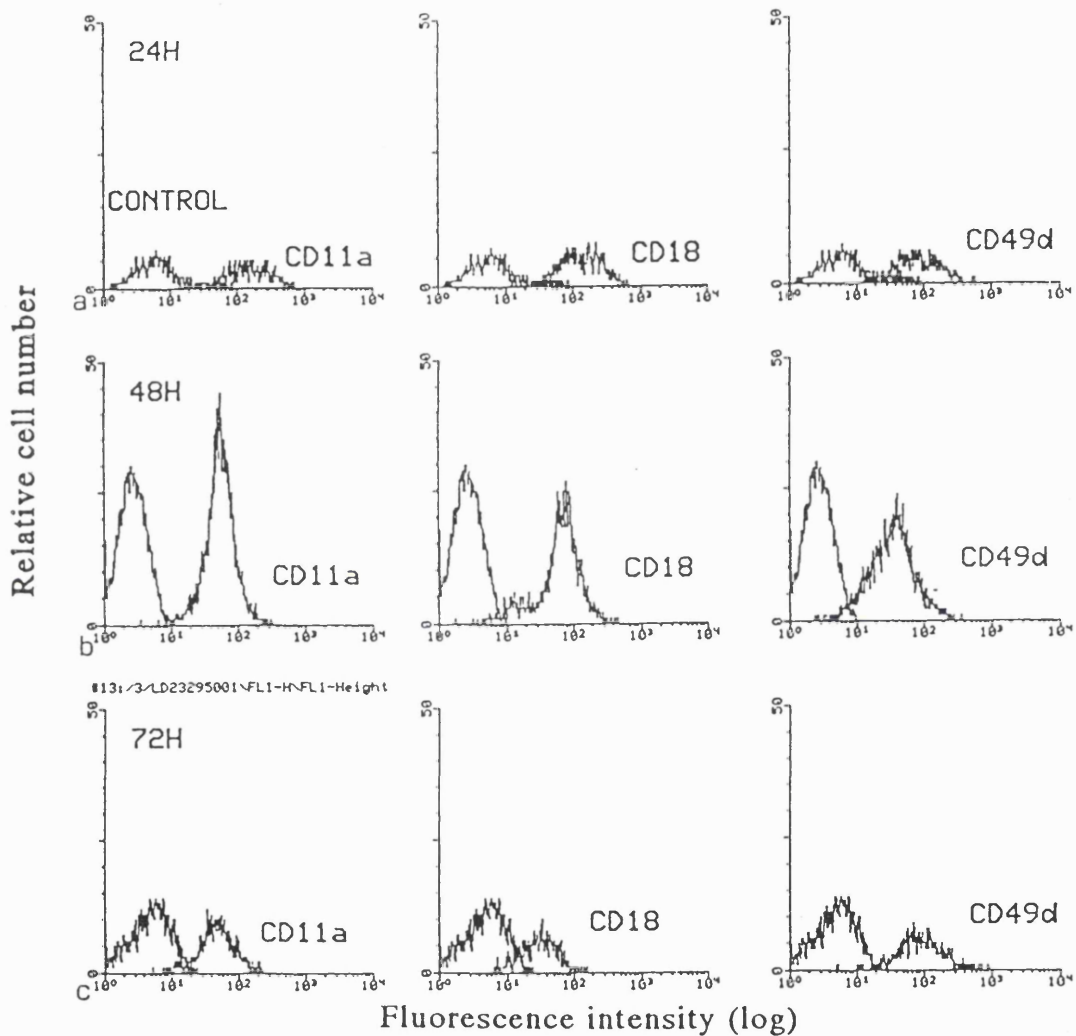
|          | CD11a (WT-1) | CD18 (WT-3) | CD49d (TA-2) |
|----------|--------------|-------------|--------------|
| 24 hours | 89.9 ± 8.7   | 83 ± 9.6    | 55.7 ± 5.9   |
| 48 hours | 96.7 ± 0.6   | 90.1 ± 4    | 84.5 ± 2.6   |
| 72 hours | 81.4 ± 9.8   | 88.7 ± 5.8  | 78.3 ± 4.6   |

**Table 4.5.** Adhesion molecule expression, as determined by flow cytometry, of PLN cells for 24, 48 and 72 hours with immobilised anti-CD3 and IL-2. Results are expressed as mean ± S.D. (n=3).

### 4.3 DISCUSSION

These results demonstrate that the state and mode of lymphocyte activation is crucial in determining the ability of lymphocytes to adhere to, or migrate through RPE monolayers. The level of adhesion and migration of untreated PLN cells was very low, both being increased upon activation with Con A (24hr). Con A has previously been shown to enhance lymphocyte adhesion to rat RPE *in vitro*, although to a much higher level than reported in this study (ie. 52 to 83%) (Mesri et al., 1994). This discrepancy may be due to differences in the technique employed. For example, in the study by Mesri et al., splenocytes were used which may be more adherent than PLN lymphocytes used in this study. Moreover, in this study PLN cells were activated with Con A for 24 hours as this had previously been shown to induce adhesion to both rat brain and retinal endothelium *in vitro* (Male et al., 1990; Wang et al., 1993) whereas in the study of Mesri et al. (1994) high levels of adhesion were reported after 20 minutes Con A activation of the splenocytes. A potential problem is that Con A bound to the surface of the lymphocyte is also binding to mannose residues on the RPE cell surface and producing higher levels of apparent adhesion. This issue has previously been addressed in adhesion

## ADHESION MOLECULE EXPRESSION ON CD3-ACTIVATED PLN CELLS.



**Fig 4.14** Flow cytometric analysis of CD11a, CD18 and CD49d expressed on PLN lymphocytes activated with anti-CD3 for (a)24hr, (b)48hr or (c)72hr. Adhesion molecule expression was enhanced after 24hr activation (compare with Fig 4.4). However this expression was not increased further after longer activation with CD3.

and migration studies with retinal endothelia (Greenwood and Calder, 1993; Wang et al., 1993). Pretreatment of Con A activated PLN cells with 0.1M  $\alpha$ -mannose to block lectin binding sites did not have any effect on the level of lymphocyte adhesion or migration. The mechanisms responsible for the upregulation of the affinity of adhesion molecules by Con A activation are not fully understood. The rapid increase in adhesion to retinal endothelium following Con A activation for 10 minutes of activation suggests that early upregulation of adhesion is unlikely to be due to *de novo* synthesis of adhesion molecules (Wang et al., 1993). As adhesion continues to increase for up to 24 hours activation, and  $\alpha$ -amanitin (which blocks protein synthesis) inhibits the later Con A-induced increase in adhesion of PLN cells to brain endothelium (Male et al., 1990), it is likely that more than one mechanism is operating. The early changes being due to rapid qualitative changes in adhesion molecules and the later quantitative changes requiring protein synthesis. Treatment of lymphocytes with the protein kinase C activator PMA, has previously been shown to increase the affinity of adhesion molecules, possibly by a phosphorylation mechanism, leading to an increase in lymphocyte binding (Haskard et al., 1986; Buyon et al., 1990). Whether Con A initiates a similar signalling system in lymphocytes remains to be determined.

The adhesion assay used does not allow the discrimination between cells which have already migrated and those which are just adherent. Adhesion assays were terminated after 2hr, to be comparable with similar experiments carried out on retinal and brain endothelium (Male et al., 1990; Wang et al., 1993). Comparison of lymphocyte adhesion and migration results with RPE monolayers at this time-point indicated that in most cases, and particularly in the case of the antigen-specific T cell lines, the majority of apparent adhesion measured was, in fact, migration. However it should be noted that the adhesion of both Con A and anti-CD3 activated PLN cells was approximately 50% greater than lymphocyte migration, which is probably due to the lower activation state of these cells. While these results indicate that adhesive forces do exist between the two cell types it should be noted that, unlike endothelial cells, RPE are not involved in capturing cells from the circulation. The adhesion molecules expressed are therefore more likely to be involved in providing points of attachment for cell migration thereby facilitating traffic into the retina once the leucocytes have reached the RPE. For this reason this discussion

will mainly concentrate on the factors controlling lymphocyte migration across RPE monolayers.

Con A activation of PLN led to an increase in the expression of both LFA-1 and VLA-4, although it is likely to be the increase in LFA-1 expression and affinity which is responsible for the increase in migration. Con A activation for up to 3 days resulted in a continuous increase in intensity of VLA-4 expression. The percentage of LFA-1 positive cells and the intensity of expression did not change with prolonged activation by Con A.

The migration of PPD T cell lines derived from either PVG or Lewis rats was not significantly different which confirmed that the difference in the strain of rats from which the RPE or the lymphocytes were derived did not have an effect on lymphocyte migration. Therefore it was concluded that for the purpose of these studies there was no difficulty in using Lewis rat T cell lines co-cultured with PVG derived RPE.

IL-2 activation of T cells has been shown to increase their adhesion to and migration across non-CNS endothelium in a time and dose-dependent manner (Damle et al., 1987; Pankonin et al., 1992). That this also occurred with CNS-endothelium has been confirmed by work carried out in our laboratory in which the level of migration of antigen specific T cell lines across brain-endothelium *in vitro* correlated with the expression of the IL-2 receptor. While the migration of Con A activated PLN cells additionally stimulated with IL-2 was greater than without IL-2, it only reached significance after 3 days in the presence of exogenous IL-2. Expression of either LFA-1 or VLA-4 was not increased during this time-period therefore, it is most likely that additional mechanisms are involved. It is possible that cytokines released by the PLN cells may vary, or additional activation markers may be expressed after prolonged stimulation. For example, CD26 (Dipeptidyl peptidase IV) expression is increased within two days of activation and is further increased after culture in IL-2. This cell surface molecule is expressed on a variety of cells, but only on activated, not resting T cells. On other cells it functions as a proteinase, but is also known to be involved in T cell activation and to bind to components of the extracellular matrix (Fleischer, 1994). Therefore it is possible that further activation of the lymphocytes via this pathway could lead to an increase in migration.



None of these modes of lymphocyte activation achieved levels of migration similar to the high levels observed with antigen-specific T cell lines. In comparison with PLN cells, the T cell lines used were all OX-22<sup>low</sup>, (rat leucocyte common antigen) which is believed to be indicative of the rat memory cell phenotype, whereas PLN cells contained approximately 65% OX-22 positive cells (ie. OX22<sup>high</sup>, naive). Memory cells have been shown to adhere more efficiently than naive cells (Shimizu et al., 1991) and exhibit a greater ability to migrate through endothelial monolayers (Greenwood and Calder, 1993; Pietschmann et al., 1992). Human memory cells have been shown to express higher levels of adhesion molecules than naive cells (Sanders et al., 1988; Shimizu et al., 1990) and an increased capability to bind their relevant ligands (Shimizu et al., 1990). In this study the expression of LFA-1 on T cell line cells was substantially increased. These quantitative changes in LFA-1 on T cell line lymphocytes 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. It has also been shown that OX-22<sup>low</sup> rat T cells express higher levels of VLA-4 than naive cells (Issekutz and Wykretowicz, 1991). In addition to the increased expression of adhesion molecules, memory T cells can also induce endothelial cells to express the adhesion molecules E-selectin and VCAM-1 thereby leading to an increase in adhesion and migration (Damle et al., 1991). This effect was shown to be dependent on direct cellular contact and was not due to cytokines released from the lymphocytes. It remains to be seen if a similar effect could occur with epithelial cells, although it seems unlikely as migration across RPE monolayers appears to occur relatively quickly compared with retinal endothelium. The actual cell-contact time prior to migration across RPE may not therefore be sufficient for this to occur.

The cytokines secreted by lymphocytes may still play a part in determining the level of migration by inducing or upregulating adhesion molecule expression. This may help explain why different modes of lymphocyte activation are crucial in determining the level of migration since it has been shown that the cytokines produced by lymphocytes vary depending on the mode of activation (Zhao et al., 1994). Activation of S-Ag T cell lines with S-antigen was shown to induce secretion of cytokines with a Th1-like profile (ie. IL-2 and IFN- $\gamma$ ) (Zhao et al., 1994). On the other hand, activation of the same T cell line with Con A, induced secretion of IL-4 as well as IL-2 and IFN- $\gamma$  which is a Th2-like

cytokine (cells which produce both Th1 and Th2 cytokines are termed Th0 and are thought to be precursors of either Th1 or Th2 cells). IL-2 and IFN- $\gamma$  are both inflammatory cytokines which have been found in the eye during uveitis (Hooks et al., 1988; Charteris and Lightman, 1992) and therefore have more relevance to the *in vivo* situation.

The antigen-specificity of the T cell has been shown to be irrelevant to their ability to cross CNS endothelium both *in vivo* (Hickey et al., 1991) and *in vitro* (Greenwood and Calder, 1993). Therefore, it was surprising that this was not the case with migration across RPE. This effect was first observed with PPD T cell lines compared with S-antigen, therefore the migration of an ovalbumin T cell line (established in our laboratory by Gareth Pryce) was also examined with similar results. There is no satisfactory explanation to suggest that this is a consequence of the ocular specificity of the antigen involved. It is more likely that this discrepancy is a consequence of a difference in the antigenicity of the antigens involved, resulting in the S-antigen T cell line being more activated and hence migratory than those with which it was compared. Alternatively the cytokine profiles of the T cell lines may differ. In a study comparing the cytokine profiles of PPD and S-antigen T cell lines it was demonstrated that S-antigen T cell lines predominantly expressed cytokines with a Th1 profile, while the cytokines produced by PPD T cell lines were either Th0 or Th2-like. Of particular relevance, may be the level of IFN- $\gamma$ , which was found to be much greater in the supernatant of the activated S-Ag T cell than in the PPD T cell line (personal communication, V. Calder). The reason why a differential ability to migrate is not observed with the endothelial monolayers is that the rate limiting factor may be due to functional aspects of the endothelium, which limits the degree of migration. On the RPE these factors might not be rate limiting thus allowing for differences in the migratory ability of the T cell lines to be observed. For example, retinal endothelial cells express lower levels of ICAM-1 than RPE and as this molecule is central to the process of migration (Greenwood et al., 1995) it may be one of the factors involved.

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**CHAPTER 5**  
**CYTOKINE ACTIVATION OF RPE: EFFECT ON ADHESION MOLECULE**  
**EXPRESSION AND LYMPHOCYTE ADHESION & MIGRATION ACROSS**  
**RPE MONOLAYERS**

### **5.1 INTRODUCTION**

The state and mode of lymphocyte activation is critical in determining the degree of lymphocyte adhesion to and migration through cellular monolayers of the blood-CNS barrier. These processes, however, can also be modulated by the activation state of the endothelium or the RPE. Endothelial cells can be activated by a variety of inflammatory cytokines released by inflammatory cells during an immune response which leads to an upregulation in the adhesion and migration of lymphocytes. Two such cytokines are IFN- $\gamma$  and IL-1 $\beta$ .

IFN- $\gamma$  is an important mediator within the immune system, having a profound effect on immunoregulation and inflammation. It is secreted by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and has also been shown to be produced by NK cells. As well as enhancing MHC class I and inducing class II expression on a variety of cells, it has also been shown to induce or upregulate the expression of a number of adhesion molecules, including ICAM-1 and VCAM-1, which are important in lymphocyte adhesion and migration (Dustin et al., 1986; Issekutz, 1990).

IL-1 is produced by many cell types, most notably by mononuclear phagocytes (Durum et al., 1985) and has been shown to be an important co-stimulatory factor in T cell activation (Weaver and Unanue, 1990). It has a wide range of biological activities some of which account for its important inflammatory role, for example by enhancing or inducing the expression of several adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin (Dustin et al., 1986; Dinarello, 1988; Masinovsky et al., 1990).

IFN- $\gamma$  and IL-1 have both been shown to increase lymphocyte adhesion to and/or migration through non-CNS endothelial monolayers (Cavender et al., 1986; Yu et al., 1985; Bevilacqua et al., 1985; Turunen et al., 1990; Issekutz, 1990). The response of CNS-endothelium to these cytokines is similar in that adhesion molecule expression can be induced or upregulated (McCarron et al., 1993; Greenwood et al., 1995) leading to

an increase in lymphocyte adhesion and/or migration (Hughes et al., 1988; Male et al., 1992; Wang et al., 1993; Greenwood and Calder, 1993; McCarron et al., 1993).

Extensive research has been carried out investigating the role of the adhesion molecules ICAM-1 and VCAM-1 in transendothelial leucocyte migration. However, very little is known of how these molecules are involved in extravascular trafficking during an inflammatory response. ICAM-1 has previously been shown to control the adhesion and migration of neutrophils (Elner et al., 1992) across human RPE cells in culture. Moreover, ICAM-1/LFA-1 interactions have also been reported to play a role in the adhesion of lymphocytes to cultured RPE, although the extent of the inhibition following the blockade of ICAM-1 was variable. In one study adhesion was inhibited by 30% (Elner et al., 1992), whereas in a separate report 85-94% of lymphocyte adhesion was blocked with an anti-ICAM-1 monoclonal antibody (Liversidge et al., 1990). The reasons for the discrepancy is unclear but may be due to a difference in the monoclonal antibodies used.

ICAM-1/LFA-1 interactions have been demonstrated to control the migration, but not adhesion, to untreated retinal vascular endothelium (Greenwood et al., 1995). The VCAM-1/VLA-4 pathway of lymphocyte migration, however, was only involved in migration across activated endothelium, as VCAM-1 expression was not found on untreated endothelium.

Experiments were carried out to determine if activation of RPE monolayers with the cytokines IFN- $\gamma$  and IL-1 $\beta$  enhanced lymphocyte adhesion and/or migration. Moreover, the effect of these cytokines on expression of the adhesion molecules ICAM-1 and VCAM-1, as well as the role of these molecules in lymphocyte migration were studied.

## **5.2 RESULTS**

### **5.2.1 Lymphocyte adhesion to cytokine activated RPE monolayers.**

RPE cells were activated with a range of concentrations of IFN- $\gamma$  (1-200U/ml) or IL-1 $\beta$  (0.05-100U/ml) for 24 hours prior to the adhesion assay. The effect of cytokine activation on the adhesion of untreated, Con A activated and anti-CD3 activated PLN cells as well as PPD T cell lines were investigated (Figure 5.1-5.3).

IFN- $\gamma$  and IL-1 $\beta$  significantly increased the adhesion of untreated PLN cells ( $p < 0.01$ , t-test). This increase occurred at very low concentrations of either cytokine, although adhesion was greatest with 100-200U/ml IFN- $\gamma$  or 0.5U/ml IL-1 $\beta$ . Nevertheless, this increased adhesion, was still significantly lower than the adhesion of Con A activated PLN to untreated RPE (Figure 5.1). The adhesion of all other lymphocytes investigated was not significantly increased by activation of the RPE with either IFN- $\gamma$  or IL-1 $\beta$ .

### 5.2.2 Lymphocyte migration across cytokine activated RPE monolayers.

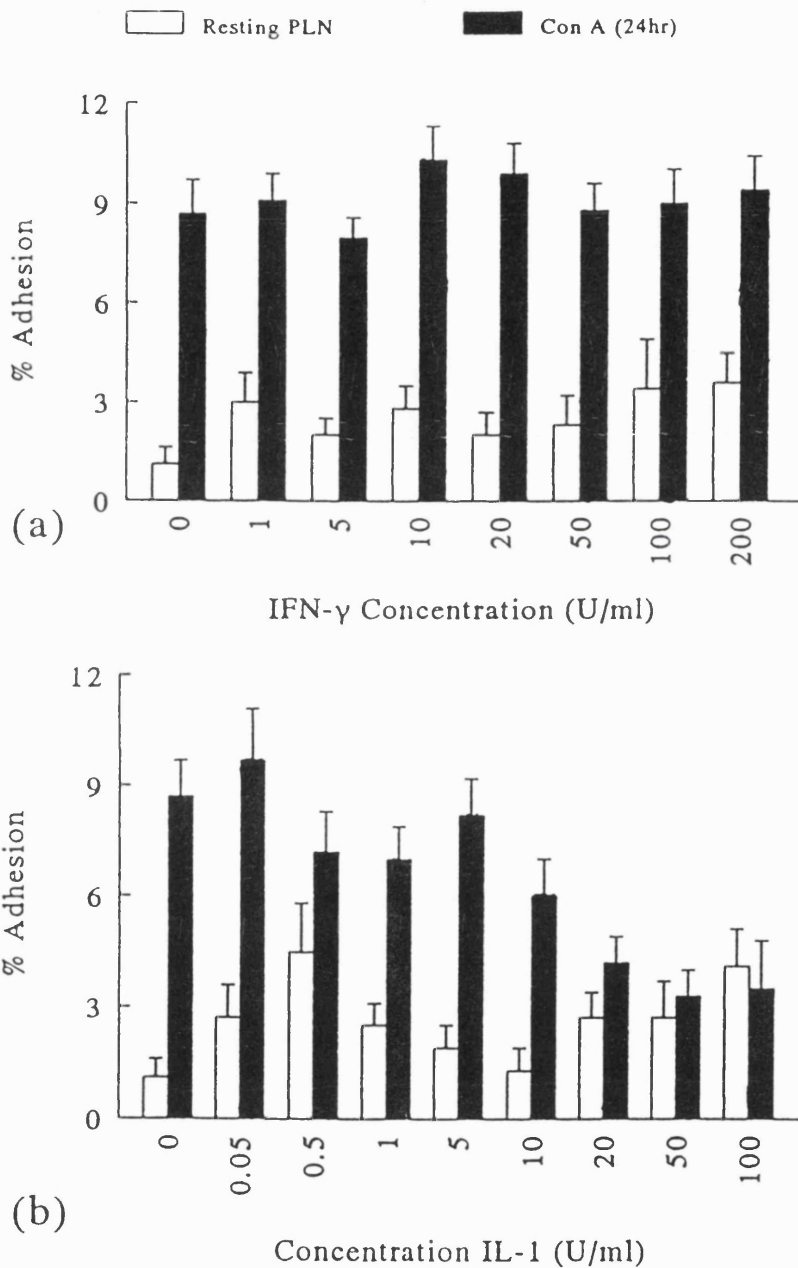
To investigate the effects of these cytokines on lymphocyte migration, RPE monolayers were again activated for 24 hours with either 100U/ml IFN- $\gamma$  or 5U/ml IL-1 $\beta$  (Figure 5.4, Table 5.1). These concentrations were chosen, so as to be comparable with similar experiments carried out with Lewis retinal endothelium (Wang et al., 1993; Greenwood and Calder, 1993; Greenwood et al., 1995).

In all cases IFN- $\gamma$  significantly increased the migration, whereas IL-1 $\beta$  only increased the migration of untreated PLN cells and PLN cells activated via the CD3 complex (this was true for all time-points examined).

|                  | Control        | IFN- $\gamma$  | IL-1 $\beta$   |
|------------------|----------------|----------------|----------------|
| Untreated PLN    | 0 $\pm$ 0      | 1.6 $\pm$ 0.6  | 1.7 $\pm$ 0.5  |
| Con A PLN (24hr) | 4.1 $\pm$ 0.3  | 10.6 $\pm$ 1.5 | 4.3 $\pm$ 0.5  |
| PPD T cell line  | 14.8 $\pm$ 1.5 | 22 $\pm$ 1.6   | 15.2 $\pm$ 1.2 |
| CD3 (24hr)       | 3.6 $\pm$ 0.7  | 5.6 $\pm$ 0.8  | 8.4 $\pm$ 1.6  |
| CD3 (48hr)       | 7 $\pm$ 0.8    | 16.8 $\pm$ 1.5 | 11.9 $\pm$ 1.5 |
| CD3 (72hr)       | 2.5 $\pm$ 0.8  | 4.7 $\pm$ 0.3  | 8.3 $\pm$ 0.9  |

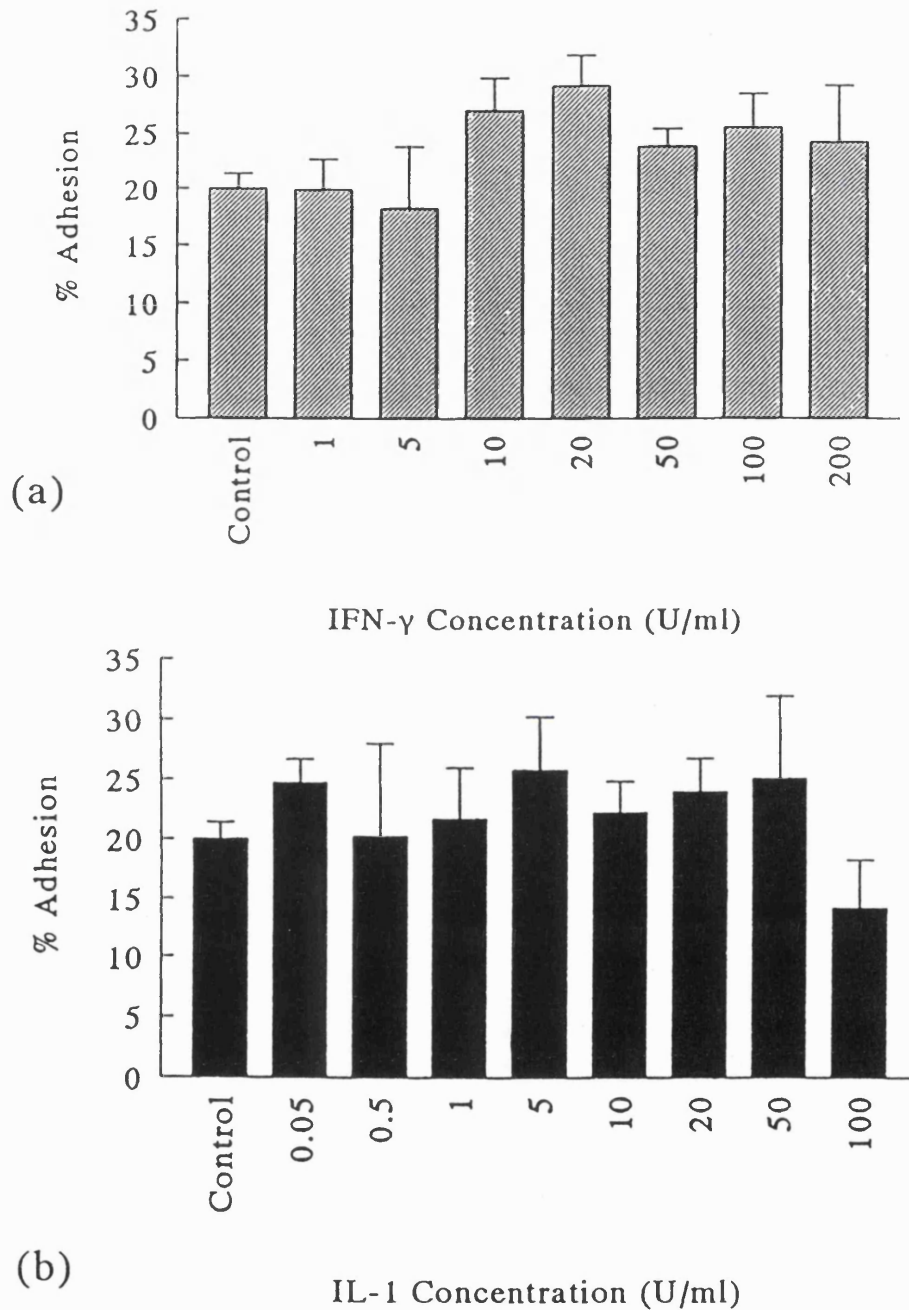
**Table 5.1** Summary of migration results from RPE cells activated with the cytokines IFN- $\gamma$  or IL-1 $\beta$ . Values are mean % migration  $\pm$  S.E.M. ( $n=12$ ).

### EFFECT OF CYTOKINE ACTIVATION ON ADHESION OF PLN LYMPHOCYTES.



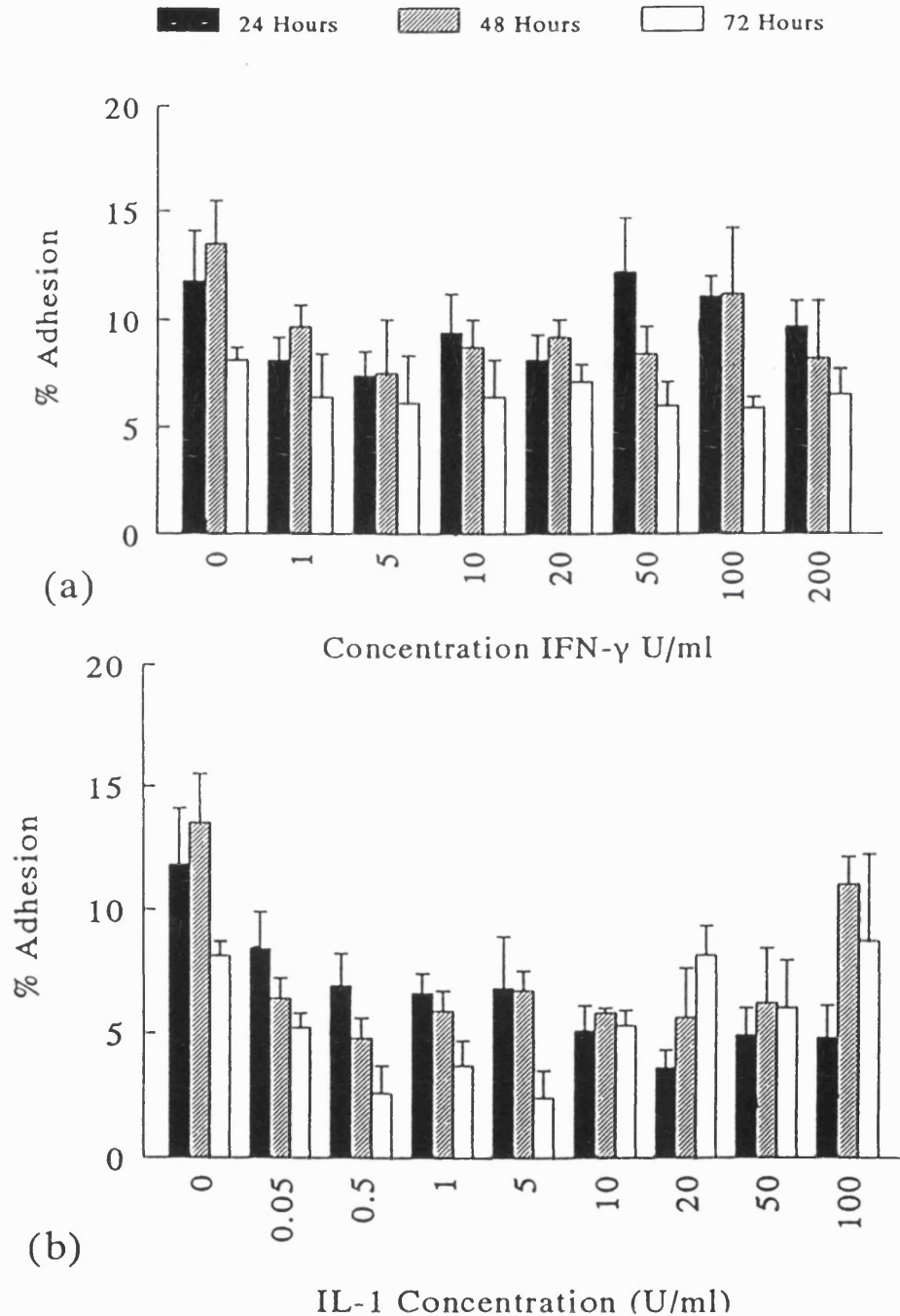
**Fig 5.1** Adhesion of resting or 24hr Con A activated PLN cells to RPE activated with (a) IFN- $\gamma$  or (b) IL-1 $\beta$  for 24 hours. Results are expressed as mean  $\pm$  S.E.M. ( $n=12$ ). Cytokine activation only significantly increased the adhesion of PLN cells ( $p < 0.01$ ,  $t$ -test).

EFFECT OF CYTOKINE ACTIVATION ON ADHESION OF T CELL LINE LYMPHOCYTES.



**Fig 5.2** Adhesion of PPD T cell line lymphocytes to RPE activated with (a) IFN- $\gamma$  or (b) IL-1 $\beta$  for 24 hours. Results are expressed as mean  $\pm$  S.E.M. ( $n=12$ ). No significant increase in adhesion of PPD T cell line lymphocytes was found when RPE cells were activated with either cytokine.

EFFECT OF CYTOKINE ACTIVATION ON ADHESION OF CD3 ACTIVATED  
PLN LYMPHOCYTES.



**Fig 5.3** Adhesion of CD3 activated PLN cells (24-72 hours) to RPE activated with (a) IFN- $\gamma$  or (b) IL-1 $\beta$ . Results are expressed as mean  $\pm$  S.E.M. ( $n=12$ ). No significant increase in adhesion of CD3 activated PLN lymphocytes was found when RPE cells were activated with either cytokine.



### 5.2.3 Adhesion molecule expression on cultured RPE

The expression of the adhesion molecules, ICAM-1 and VCAM-1 on cultured RPE was determined by flow cytometry and ELISA. ELISA results demonstrated that ICAM-1 was constitutively expressed on RPE cells and that expression continued to increase in intensity up to at least five days after activation with IFN- $\gamma$ . VCAM-1, on the other hand, was not constitutively expressed but reached maximal expression 3 days after activation (Figure 5.5).

Flow cytometric analysis confirmed that rat RPE cells in culture constitutively expressed ICAM-1 and could be upregulated upon activation with IFN- $\gamma$  for 24 hours. As the ELISA data indicated that VCAM-1 was not fully expressed until 3 days post IFN- $\gamma$  activation, flow cytometric analysis was only evaluated at this time-point (Figure 5.6).

### 5.2.4 Role of adhesion molecules in lymphocyte migration across untreated RPE monolayers.

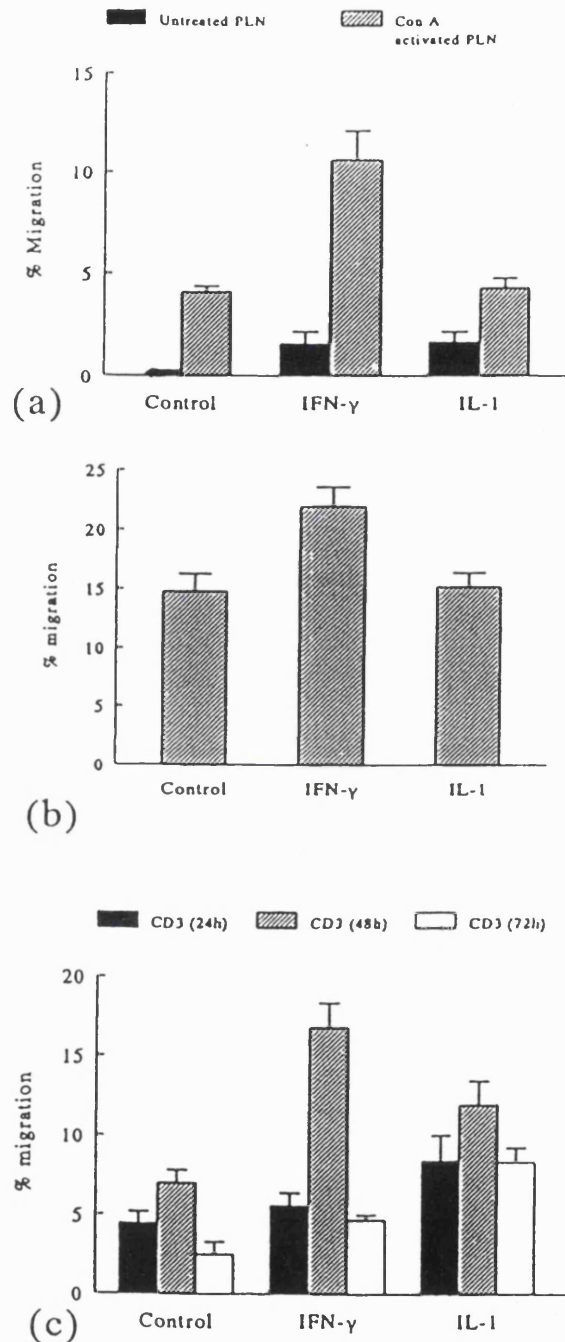
The migration of antigen-specific T cell line lymphocytes across untreated RPE was, in part, mediated by ICAM-1/LFA-1 interactions. Migration was reduced by  $48.6 \pm 3.5\%$  when RPE cell ICAM-1 was blocked with the monoclonal antibody 1A29. This inhibition was not significantly greater than that achieved by blocking the ICAM-1 receptor LFA-1 on lymphocytes. Migration was decreased by  $61 \pm 5.2\%$  and  $63.2 \pm 4.7\%$  with antibodies against LFA-1 $\alpha$  (CD11a) and LFA-1 $\beta$  (CD18) respectively. Blocking with anti-VLA-4 antibody or with the control antibody OX-21 had no significant effect on migration (Figure 5.7a).

### 5.2.5 Role of adhesion molecules in lymphocyte migration across RPE monolayers activated for 24 or 72 hours with IFN- $\gamma$ .

Activation of the RPE with IFN- $\gamma$  significantly increased the percentage of migration which was inhibited by anti-ICAM-1 to  $69.1 \pm 3.5\%$ , when compared to non-activated RPE ( $p < 0.01$ ). As with untreated RPE, VLA-4 was not involved in lymphocyte migration across retinal pigment epithelium activated for 24 hours with IFN- $\gamma$  (Figure 5.7b).

The reduction in migration as a result of blocking ICAM-1 was significantly less

## EFFECT OF CYTOKINE ACTIVATION ON LYMPHOCYTE MIGRATION.



**Fig 5.4** Migration of (a) untreated and Con A (24hr) activated PLN cells; (b) PPD T cell line and (c) CD3 activated (24, 48 and 72hr) PLN cells across RPE activated with either IFN- $\gamma$  or IL-1 $\beta$ . Results are expressed as mean  $\pm$  S.E.M. ( $n=12$ ). IFN- $\gamma$  significantly increased migration ( $p < 0.01$ ,  $t$ -test) in all cases, whereas IL-1 only increased the migration of untreated and CD3 activated PLN cells ( $p < 0.05$ ,  $t$ -test).

TIME COURSE OF ADHESION MOLECULE EXPRESSION ON IFN- $\gamma$   
ACTIVATED RPE.

Time course of adhesion molecule expression

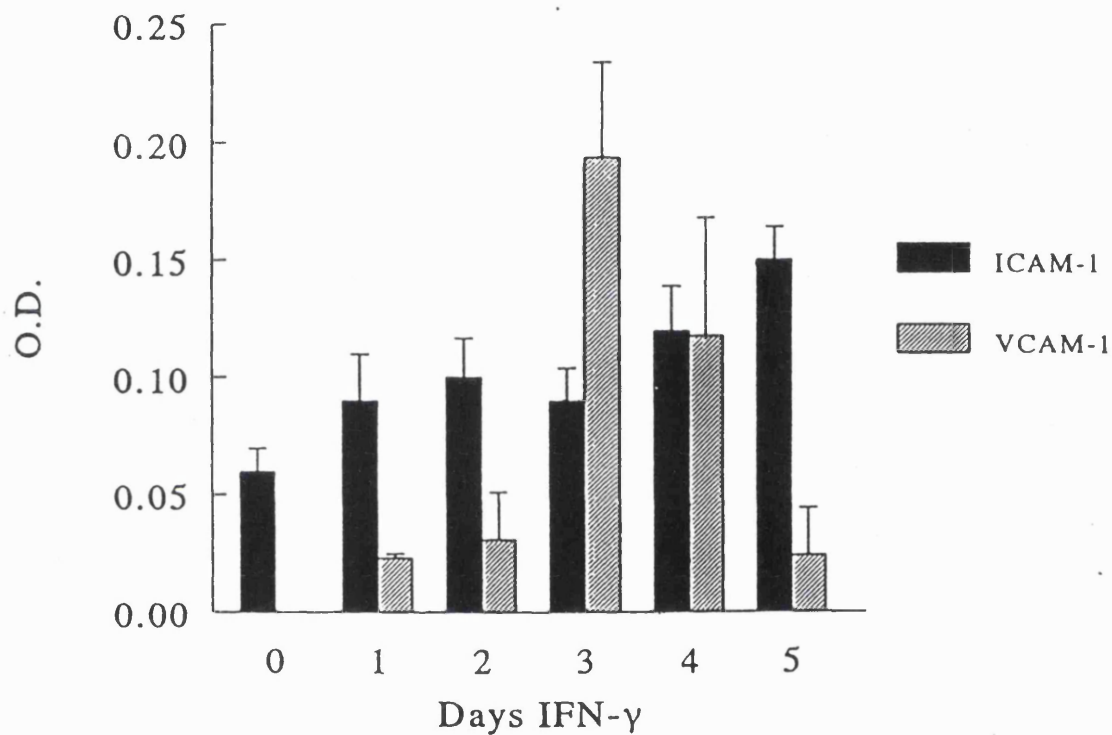
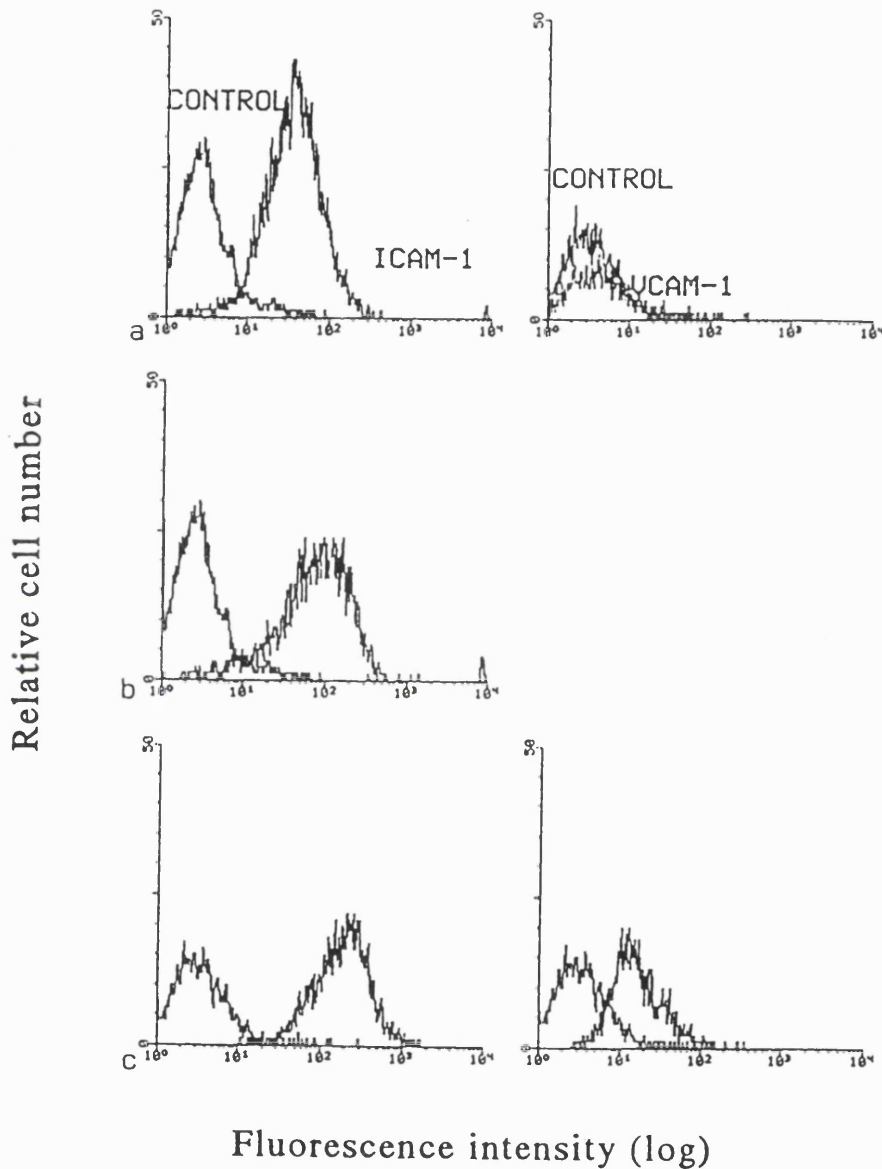


Fig 5.5. Time course of ICAM-1 and VCAM-1 expression on IFN- $\gamma$  activated RPE as determined by ELISA. Optical density values are mean ( $\pm$ S.E.M.,  $n=9$ ) with background absorbance subtracted.

**FLOW CYTOMETRIC ANALYSIS OF ADHESION MOLECULE EXPRESSION  
ON RPE MONOLAYERS.**



**Fig 5.6** Expression of ICAM-1 and VCAM-1 on (a) normal, untreated RPE, (b) RPE activated with IFN- $\gamma$  for 24hr or (c) RPE activated with IFN- $\gamma$  for 72hr. In each case, histogram on the left hand is cells stained with secondary antibody alone. ICAM-1 expression is upregulated upon activation, whereas VCAM-1 is not constitutively expressed but is induced upon activation.

( $P < 0.05$ ) when the RPE was activated for 72 hours than with activation for 24 hours ( $47.7 \pm 4.2\%$  compared with  $69.1 \pm 3.5\%$  respectively). Blocking both VLA-4 and VCAM-1 at 72hr also reduced migration by  $32.3 \pm 6.2$  and  $21.6 \pm 8.4\%$  respectively. The percentage inhibition when both ICAM-1 and VCAM-1 were blocked was significantly greater than either alone (Figure 5.7c,  $70.9 \pm 2.3\%$ ,  $p < 0.01$ ).

## DISCUSSION

The cytokines IFN- $\gamma$ , IL-1, and TNF- $\alpha$  have all been shown to enhance lymphocyte adhesion to non-CNS endothelium (Yu et al., 1985; Bevilacqua et al., 1985; Cavender et al., 1986; Cavender et al., 1987). Furthermore, activation of both brain (Hughes et al., 1988) and retinal endothelium (Wang et al., 1993) with IFN- $\gamma$  has been shown to enhance lymphocyte adhesion, whereas IL-1 has been reported to increase the adhesion to retinal (Wang et al., 1993), but not brain endothelium (Hughes et al., 1988), although this discrepancy may be a result of different sources of IL-1. Conversely, studies investigating adhesion of lymphocytes to cytokine-activated RPE have provided conflicting results. In studies with human RPE, IFN- $\gamma$  activation has been reported to increase lymphocyte adhesion (Liversidge et al., 1990; Elner et al., 1992), whereas in the rat IFN- $\gamma$  had no effect (Mesri et al., 1994). This may be a consequence of a difference between human and rat cultures. The results shown here demonstrate that activation of RPE with either IFN- $\gamma$  or IL-1 only increased the adhesion of untreated PLN cells, but that this adhesion was still extremely low.

In general, activation of the RPE with IFN- $\gamma$ , and not IL-1, increased lymphocyte migration. An increase in migration by IL-1 activation was only seen with untreated PLN cells and PLN cells which had been activated via the CD3 complex. In the case of migration of untreated PLN cells across untreated RPE, the migration was found to be negligible suggesting that an upregulation in adhesion molecule expression was sufficient to result in a small, but significant increase in migration. The lack of an increase in migration of activated T cells (Con A and T cell line) may be due to migration being maximal under these conditions and insensitive to further induction of RPE adhesion molecules by IL-1, but not IFN- $\gamma$ . The reason for the ambiguity in the migration of CD3 activated PLN cells, where IL-1 did increase migration remains unclear, but may be due

to a difference in the signalling involved.

The effect on migration of activation of retinal endothelial cells with these cytokines also appears to be dependent on the cytokine, for although adhesion was enhanced by both cytokines (Wang et al., 1993), only IL-1 $\beta$  (Greenwood et al., 1995; McCarron et al., 1993), but not IFN- $\gamma$  (Greenwood and Calder, 1993), significantly increased migration. However, in a study on lymphocyte migration across brain-derived endothelium, the activation state of the endothelium was reported to be irrelevant in determining the level of migration observed (Male et al., 1992). This may be due to the fact that in this assay the migratory period was 24hr which was considerably greater than the 4hr migration assay used in the present study.

The increased lymphocyte migration upon activation of the RPE with IFN- $\gamma$  is possibly a consequence of an upregulation of the adhesion molecule ICAM-1, as no significant VCAM-1 is present until 3 days post-activation. RPE cells have previously been shown to constitutively express ICAM-1, although the reported levels expressed varied (Liversidge et al., 1990; Elner et al., 1992). From these results it can be concluded that ICAM-1 is constitutively expressed on cultured rat RPE cells and this expression is upregulated after 24hr activation with IFN- $\gamma$  and continues to increase up until at least 5 days post-activation. This is different from brain endothelium, where expression has been reported to peak at 24-72 hours (Male et al., 1992).

VCAM-1 expression has not previously been found on RPE cells. In a study examining the expression of VCAM-1 on cultured RPE cells, only the cytokines IL-1 and TNF- $\alpha$  were assessed and cells were only activated for 24-48 hours. In this study VCAM-1 was found on RPE activated with IFN- $\gamma$  for 72 hours. Therefore, it is possible that only IFN- $\gamma$ , but not IL-1 or TNF- $\alpha$  can induce VCAM-1 on RPE. Alternatively, the time-course of activation may not have been long enough to be able to detect expression. The time course of VCAM-1 expression on RPE is different from that reported on non-CNS endothelium. VCAM-1 expression on HUVEC is maximal 4 hours after activation with IL-1 or TNF- $\alpha$  and this expression is sustained for 24-48 hours (Osborn et al., 1989; Carlos et al., 1990). Although IFN- $\gamma$  did not induce VCAM-1 expression on HUVEC, its expression was increased by this cytokine on high endothelial cells derived from rat peripheral lymph nodes (May et al., 1993). The difference in the time-course

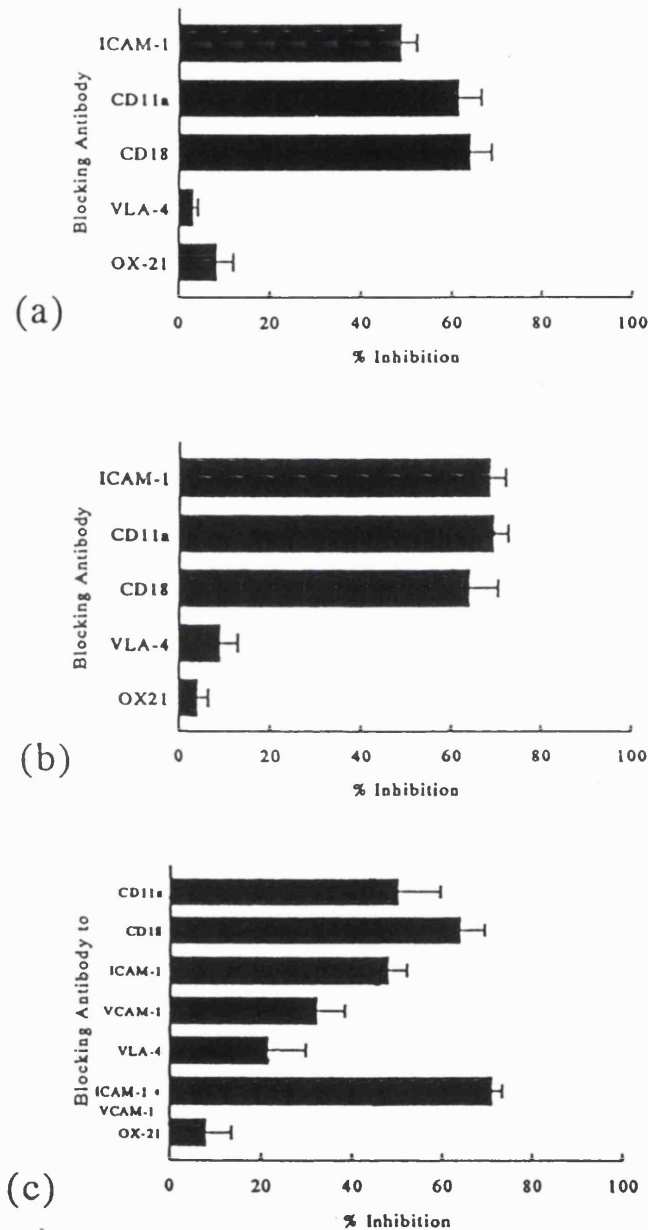
of expression, and the cytokines responsible for inducing VCAM-1 expression, may reflect differences between endothelia from different vascular beds as well as differences between endothelial and epithelial cells.

RPE, unlike endothelial cells, do not capture cells from the circulation as this occurs at the choroidal endothelium. This may explain why cytokine activation did not increase adhesion but did increase lymphocyte migration. Therefore during an inflammatory response it is likely that lymphocyte adhesion to and migration through the choroidal endothelium is enhanced due to an upregulation in adhesion molecule expression which increases the number of inflammatory cells which can cross the RPE.

Lymphocyte migration across cultured RPE was partly mediated by ICAM-1/LFA-1 interactions, with other possible mechanisms also being involved. When the same experiment was carried out with untreated retinal vascular endothelial cells, migration was reduced by approximately 80% (Greenwood et al., 1995) compared to 50% on non-activated RPE. It is unlikely, therefore, that the lack of total inhibition was related to the ability of the antibody to cause sufficient functional blockade of the adhesion molecules although, as RPE cells have been reported to express greater amounts of constitutive ICAM-1 than retinal endothelial cells, this is not impossible. Nevertheless, similar results have been observed with lymphocyte adhesion to human RPE cells, in that the level of apparent adhesion was reduced by 30% when ICAM-1 was blocked. This was in contrast to neutrophil adhesion, which was almost completely blocked by anti-ICAM-1 antibodies (Elner et al., 1992). Lymphocyte adhesion to RPE, however, has also been reported to be reduced by approximately 90% with anti-ICAM-1 antibodies (Liversidge et al., 1990) although results from these studies do not differentiate between lymphocyte adhesion and migration. The former study also indicates that alternative adhesive or migratory pathways may exist in lymphocyte trafficking through RPE monolayers.

The results obtained in this study with the anti-VLA-4 antibody implied, as expected, that under conditions where VCAM-1 was not expressed, the VLA-4/VCAM-1 pathway was not involved in transepithelial migration. Nevertheless, when VCAM-1 was expressed (72 hours post IFN- $\gamma$  activation), the VLA-4/VCAM-1 pathway could operate. Lymphocyte adhesion to RPE cells activated with 500U/ml IFN- $\gamma$  have been shown to employ an ICAM-1 independent adhesion (Liversidge et al., 1990). Under these

**ADHESION MOLECULES INVOLVED IN LYMPHOCYTE MIGRATION  
THROUGH RPE MONOLAYERS.**



**Fig 5.7** Percentage inhibition of lymphocyte migration upon blocking adhesion molecules with specific monoclonal antibodies on (a) untreated RPE; (b) RPE activated with IFN- $\gamma$  for 24hr or (c) RPE activated with IFN- $\gamma$  for 72hr. Results are expressed as mean  $\pm$  S.E.M. ( $n=12$ ). Antibodies against ICAM-1, VCAM-1 and the negative control OX-21 were added to RPE and antibodies against LFA-1 (CD11a, CD18) and VLA-4 were added to lymphocytes.



conditions, the results shown here indicate that VCAM-1 may be the alternative ligand in use. Blocking both ICAM-1 and VCAM-1, so that both possible migratory pathways were inhibited, reduced migration to a greater extent than either alone. Therefore, during inflammatory conditions, when the RPE would be activated, it is possible that lymphocyte migration across the posterior BRB could occur using either ICAM-1/LFA-1 or VCAM-1/VLA-4 interactions.

Despite some differences, these results were similar to those observed with lymphocyte migration through retinal endothelial monolayers (Greenwood et al., 1995). The migration of lymphocytes across normal retinal endothelium is also predominantly ICAM-1/LFA-1 dependent. Cytokine activation of the endothelium for 24 hours was sufficient to allow migration to occur via the VCAM-1/VLA-4 pathway, however for this pathway to operate at the RPE a longer activation period was required. This may help explain why leucocyte migration in EAU occurs primarily via the retinal vascular endothelium (Greenwood et al., 1994), in that a longer period of activation may also be required *in vivo* before the RPE are equipped to allow extensive leucocyte trafficking.

It remains to be elucidated if the same adhesion molecules are involved in the migration of Con A activated PLN cells. Adhesion of Con A activated T cells to RPE (Mesri et al., 1994) and retinal endothelium (Greenwood et al., 1995) have been reported to be ICAM-1 independent. This could explain why IFN- $\gamma$  activation did not increase lymphocyte adhesion, despite an increase in the intensity of ICAM-1 expression, but was able to increase migration. The increase in migration of Con A activated PLN cells as well as T cell line lymphocytes could be due to the increase in ICAM-1 expression as well as to qualitative changes.

Under no circumstance was lymphocyte migration completely abolished, which may indicate that, unlike transendothelial migration, other adhesion molecules are involved in migration across RPE monolayers. As trafficking across the RPE is actually migration within the tissue, it is possible that extracellular matrix components are also involved. For example, fibronectin and laminin can interact with VLA integrins on lymphocytes (VLA-4 and VLA-5 bind to receptors on fibronectin and VLA-6 can bind to laminin) (Shimizu et al., 1990; Li and Cheung, 1992; Hauzenberger et al., 1994). RPE cells *in vitro* release fibronectin constitutively (Osusky et al., 1994) which may be

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involved in attracting lymphocytes across the monolayer. However, as the migration was not blocked by anti-VLA-4, it is possible that an alternative ligand is involved or that the monoclonal antibody used does not block the fibronectin binding site. This may also explain the enhanced migration of the memory T cell line lymphocytes, as memory lymphocytes express three to four fold more VLA-4, VLA-5 and VLA-6 than do naive cells, and bind more efficiently through them to fibronectin and laminin (Shimizu et al., 1990).

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## CHAPTER 6

### MHC CLASS I AND II EXPRESSION ON CULTURED RPE CELLS

#### 6.1 INTRODUCTION

The cytokines produced during an inflammatory response not only induce or upregulate adhesion molecule expression on non-immune cells at the site of inflammation, but can also alter the expression of major histocompatibility molecules (both class I and II). Of particular importance in modulating MHC expression is the cytokine IFN- $\gamma$ . Most cells of the body express MHC class I, but class II molecules are normally restricted to cells of the immune system. However, expression of MHC class II molecules on non-immune cells during an inflammatory response is thought to allow these cells to act as organ resident antigen presenting cells which would enhance the inflammatory response (Bottazzo et al., 1986; Dean et al., 1985).

Most evidence indicates that posterior uveitis is a class II restricted, CD4<sup>+</sup>-mediated condition and thus MHC class II expression on cells of the blood-retinal barrier may initiate or enhance the inflammatory response. Under normal physiological conditions RPE and retinal vascular endothelium do not express MHC class II antigens. However, expression can be induced on both cells types prior to clinical and histological signs of EAU (Fujikawa et al., 1987; Chan et al., 1986; Fujikawa et al., 1990). Moreover, expression has also been found on these cells in enucleated eyes from patients with uveitis (Chan et al., 1986). *In vitro* activation of cultured RPE or retinal endothelial cells with IFN- $\gamma$  can also induce expression of MHC class II molecules (Liversidge et al., 1988; Liversidge et al., 1988; Fujikawa et al., 1989; Percopo et al., 1990; Wang et al., 1995) as well as upregulating MHC class I expression (Clark and Hall, 1986; Wang et al., 1995).

The level of expression of MHC class I and MHC class II molecules (both I-A and I-E) were determined on untreated and IFN- $\gamma$  activated RPE cultures using ELISA, flow cytometry and immunohistochemistry.

## 6.2 RESULTS.

### 6.2.1 Constitutive and inducible MHC class I expression on cultured RPE.

Class I MHC antigens were constitutively expressed on primary cultures of RPE isolated from PVG rats. Activation of RPE with IFN- $\gamma$  significantly increased expression after 2 days (from  $0.04 \pm 0.01$  to  $0.09 \pm 0.02$ ,  $p < 0.05$ , t-test) and remained stable at this level for at least five days after the initial stimulation (Figure 6.1a).

Flow cytometric analysis of cells demonstrated that  $47 \pm 16.7\%$  of cells constitutively expressed class I MHC, which was increased to  $73 \pm 7.6\%$  upon stimulation with IFN- $\gamma$  for 5 days (results expressed as mean  $\pm$  S.E.M.,  $p < 0.05$ , Figure 6.2).

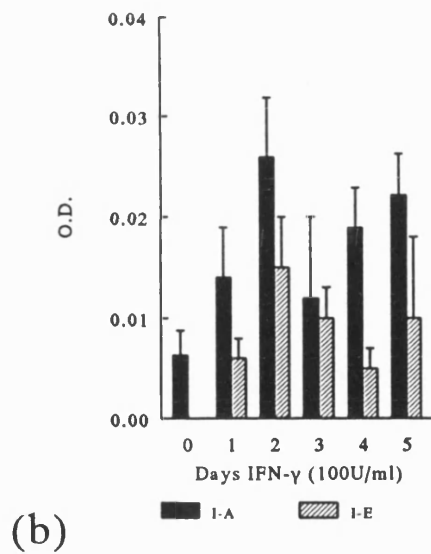
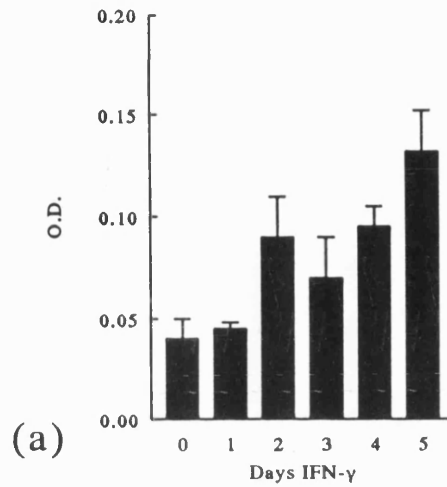
### 6.2.2 Constitutive and inducible MHC class II expression on cultured RPE.

The constitutive expression of MHC class II molecules was negligible, but was induced upon activation with IFN- $\gamma$ , although the level of expression was still low compared with that of MHC class I. Initial experiments were carried out using 100 U/ml of IFN- $\gamma$  for up to 5 days as this had previously been shown to induce class II expression on  $> 85\%$  of RPE cells (Percopo et al., 1990). The level of class II (both I-A and I-E) induced with this concentration (as determined by the optical density) was extremely low, although after 2 days stimulation, it was significantly greater than unstimulated RPE (Figure 6.1b,  $p < 0.05$ , t-test). Flow cytometric analysis of MHC class II expression was variable, but on average  $55.5 \pm 18.7\%$  of activated RPE cells were MHC class II I-A positive and  $45.6 \pm 20\%$  were positive for MHC class II I-E after 4-5 days (Figure 6.3). Untreated RPE cells did not express MHC class II.

Due to the low levels of class II obtained with the ELISA, a range of concentrations of IFN- $\gamma$  (0-1000U/ml) were tested, and the MHC expression assessed after 5 days. These results demonstrated that 500U/ml appeared to be the optimal concentration required to induce class II expression, although all concentrations above 50U/ml gave significantly higher results than untreated RPE (Figure, 6.4a,  $p < 0.01$  for 500U/ml all others  $p < 0.05$ ).

To ensure that this low level of Class II expression was not due to poor antibody binding, the expression of both I-A and I-E class II MHC molecules was examined on

## EXPRESSION OF MHC MOLECULES ON CULTURED RAT RPE.



**Fig 6.1** Expression of (a) MHC class I and (b) MHC class II (I-A and I-E) on RPE activated with 100U/ml of IFN- $\gamma$  for up to 5 days (mean  $\pm$  S.E.M.,  $n=9$ ). MHC class I expression was significantly upregulated after two days activation with IFN- $\gamma$  ( $p < 0.05$ ,  $t$ -test). Low level MHC class II expression also induced ( $p < 0.05$ ).

FLOW CYTOMETRIC ANALYSIS OF MHC CLASS I EXPRESSION ON  
RPE CELLS IN CULTURE.

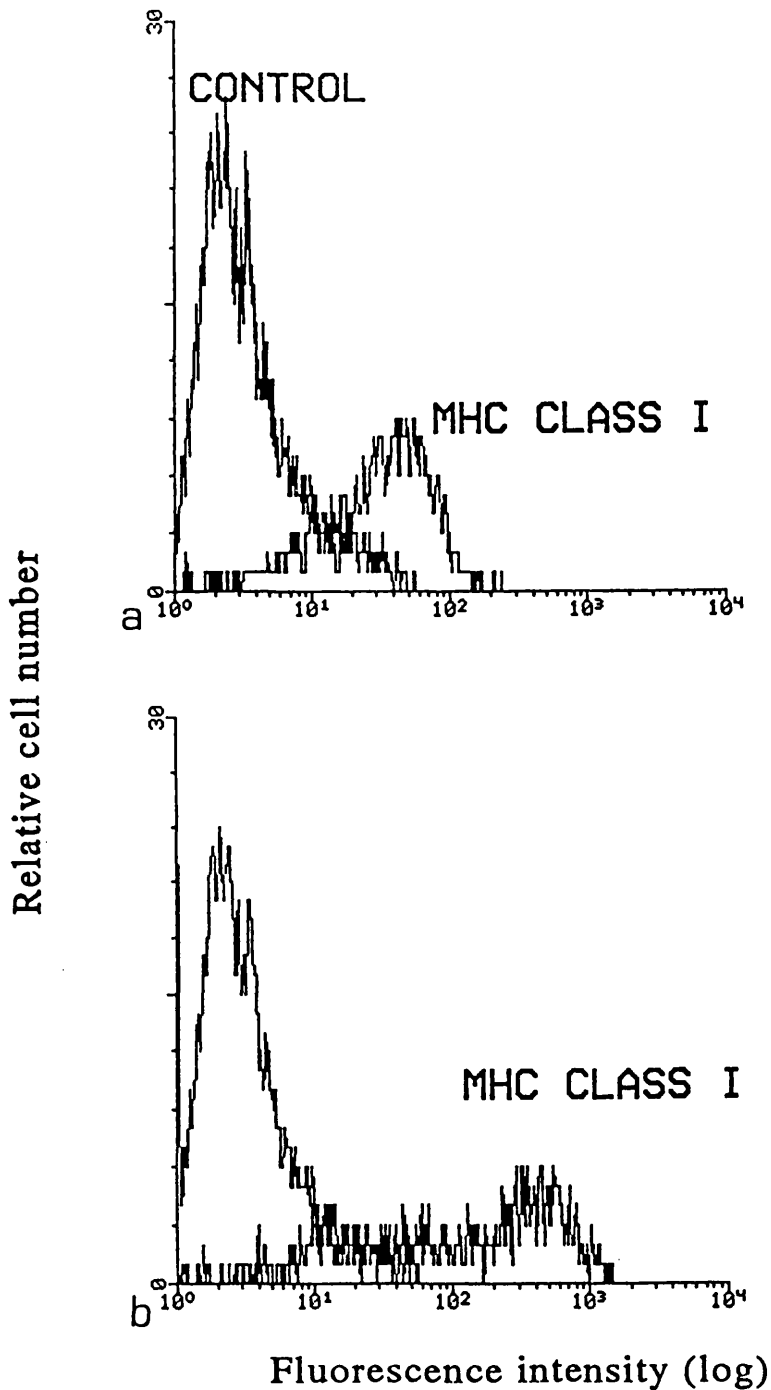
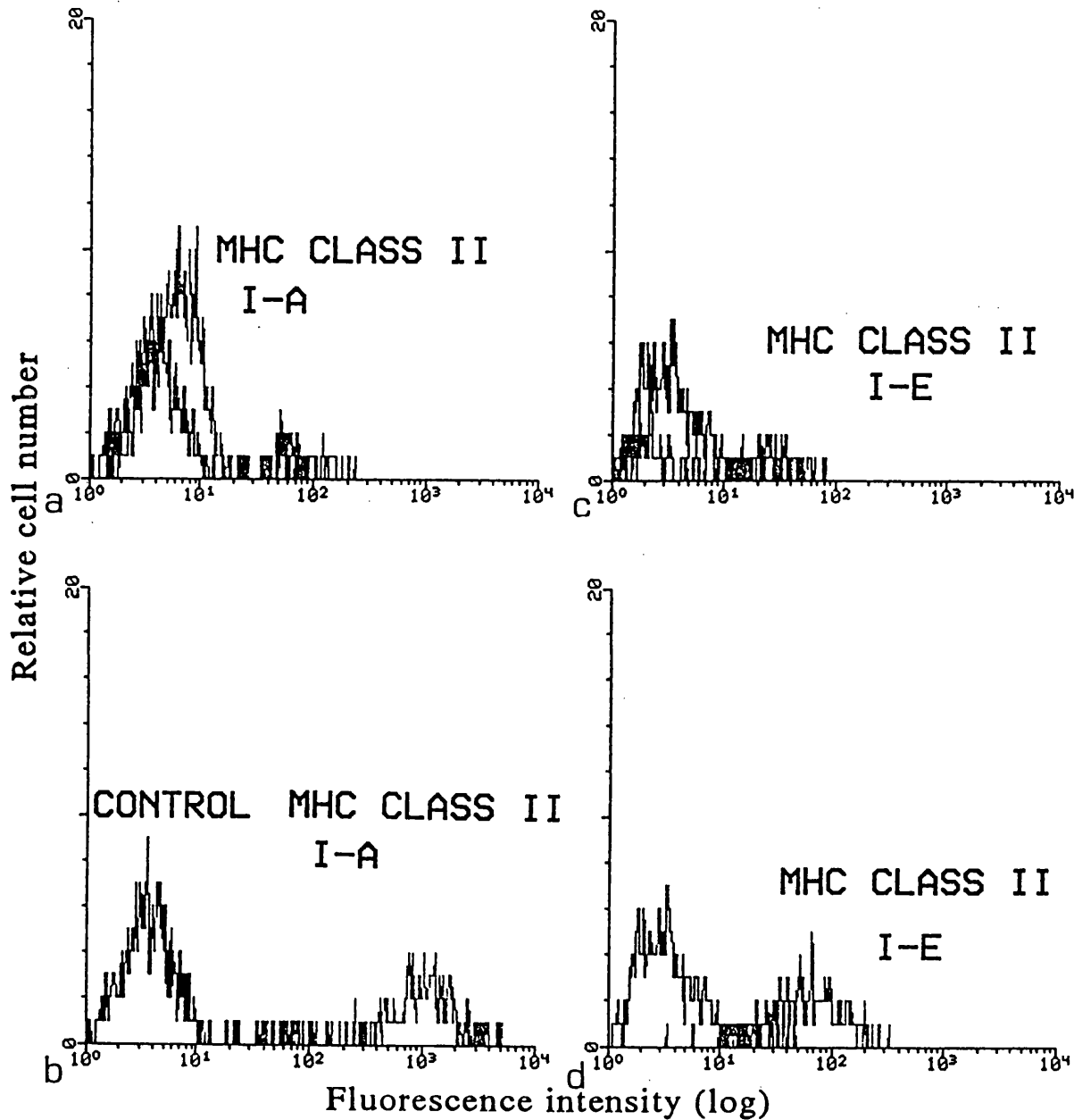


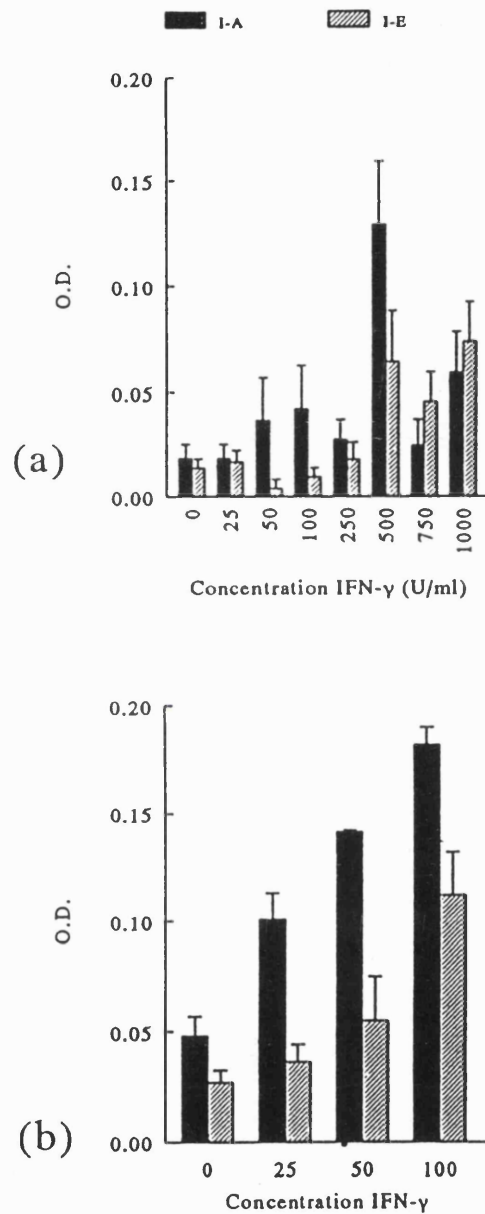
Fig 6.2 Expression of MHC class I on (a) normal and (b) IFN- $\gamma$  activated (5 days 100U/ml) RPE. In each case, histogram on left is negative control of cells stained with secondary antibody alone. Cells constitutively expressed MHC class I, and this expression was upregulated upon activation with IFN- $\gamma$ .

**FLOW CYTOMETRIC ANALYSIS OF MHC CLASS II EXPRESSION ON RPE  
CELLS IN CULTURE.**



**Fig 6.3** Expression of MHC class II (I-A and I-E) on (a and c) normal and (b and d) IFN- $\gamma$  activated (5 days 100U/ml) RPE. Control histogram of cells stained with secondary antibody alone on left in each diagram. Cells did not constitutively express MHC class II, however both I-A and I-E expression was induced upon activation with IFN- $\gamma$ .

### DOSE RESPONSE OF IFN- $\gamma$ ACTIVATION ON MHC CLASS II EXPRESSION.



**Fig 6.4** Expression of MHC class II on (a) RPE and (b) intra-peritoneal macrophages activated with various concentrations of IFN- $\gamma$ . RPE stimulated with 0-1000U/ml and macrophages stimulated with 0-100U/ml IFN- $\gamma$ . Results are expressed as mean  $\pm$  S.E.M. ( $n=12$  for RPE and  $n=9$  for macrophages). MHC class II significantly increased by IFN- $\gamma$  ( $p < 0.01$  for 500U/ml activation of RPE,  $p < 0.05$  for all other concentrations tested,  $t$ -test).



intraperitoneal macrophages isolated from Lewis rats. Unstimulated cells were I-A and I-E positive and this expression was enhanced after stimulation with IFN- $\gamma$  (Figure 6.4b).

### 6.3 DISCUSSION

Human RPE cells have been previously shown to express MHC class I antigens *in vitro* the expression of which was upregulated by the cytokines IFN- $\alpha$ , IFN- $\gamma$  and TNF- $\alpha$  (Clark and Hall, 1986). This study has shown that rat RPE also constitutively expressed class I MHC which could be upregulated upon activation with IFN- $\gamma$ , as has been shown with both brain and retinal endothelium (Wang et al., 1995). The relevance of an increase in class I expression during ocular inflammatory conditions, however, is unclear although may be related to the increase in CD8<sup>+</sup> cells seen during the later stages of disease in EAU (Chan et al., 1985).

The expression of class II MHC molecules on cultured RPE was much lower than that of the professional antigen presenting cells examined (ie. intraperitoneal macrophages). RPE cells did not constitutively express MHC class II molecules and required activation with relatively high concentrations of IFN- $\gamma$  (500U/ml) to induce levels of expression similar to those observed with macrophages stimulated with much lower levels of IFN- $\gamma$ . This result may be due to the culture conditions as MHC class II was not expressed on either unstimulated or IFN- $\gamma$  stimulated rat or guinea pig RPE when the cells were grown in foetal calf serum (Liversidge et al., 1988), but when grown in homologous serum expression was detected on activated cells. The explanation given for this was that alpha-fetoprotein, found in foetal calf serum, could apparently inhibit Ia expression on macrophages. This explanation seems unlikely as in this study macrophages used as a positive control were also cultured in foetal calf serum. Macrophages were isolated from Lewis rats, not PVG, as a positive control for antibody staining. The strain difference was not considered a problem for this purpose as MHC inducibility is tissue specific, and does not apply to macrophages (Massa et al., 1987). Further experiments are required to determine if there is a strain difference in class II expression on RPE cells.

A more plausible explanation for the low level expression of MHC class II on RPE relates to strain differences in MHC inducibility which have been found with brain and retinal endothelium as well as astrocytes (Male and Pryce, 1989; Fujikawa et al., 1989; Linke and Male, 1994). The ability of these cells to express MHC class II, and the

level expressed after stimulation with IFN- $\gamma$ , relates to susceptibility of these animals to the animal models EAU, or EAE (Massa et al., 1987; Caspi, 1989). Previous studies investigating MHC class II expression on rat RPE cultures have used RPE derived from either Lewis rats, which are highly susceptible to EAU (Caspi, 1989), or Lewis/PVG F<sub>1</sub> crosses (Liversidge et al., 1988; Percopo et al., 1990). Brain endothelium derived from Lewis rats (also highly susceptible to EAE), express relatively high levels of class II in comparison with EAE-resistant strains (including PVG rats) upon IFN- $\gamma$  stimulation (Linke and Male, 1994). Susceptibility to EAE is inherited as a dominant trait which has been shown to be associated with a permissive MHC (Gasser et al., 1973). It is therefore likely that RPE derived from a Lewis/PVG cross would express higher levels of class II than RPE isolated from PVG rats.

Interestingly, although I-A class II expression was consistently higher than I-E, both were expressed on RPE. This is in contrast to brain and retinal endothelium from Lewis rats, in which only I-A class II molecules were observed upon stimulation with IFN- $\gamma$  (Male et al., 1987; Wang et al., 1995). This may also be a variation between the different rat strains and not between the different cell types studied.

IFN- $\gamma$  is the most potent modulator of MHC class II expression on most cells, although other cytokines, including TNF- $\alpha$ , IL-1 and IFN- $\beta$  can act additively or synergistically with IFN- $\gamma$  to enhance this expression (Calder et al., 1988). Therefore, it is possible that using combinations of different cytokines the low class II expression on PVG derived RPE cells may be enhanced.

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## CHAPTER 7

### COMPARISON OF LYMPHOCYTE MIGRATION ACROSS PVG AND LEWIS DERIVED RETINAL VASCULAR ENDOTHELIUM.

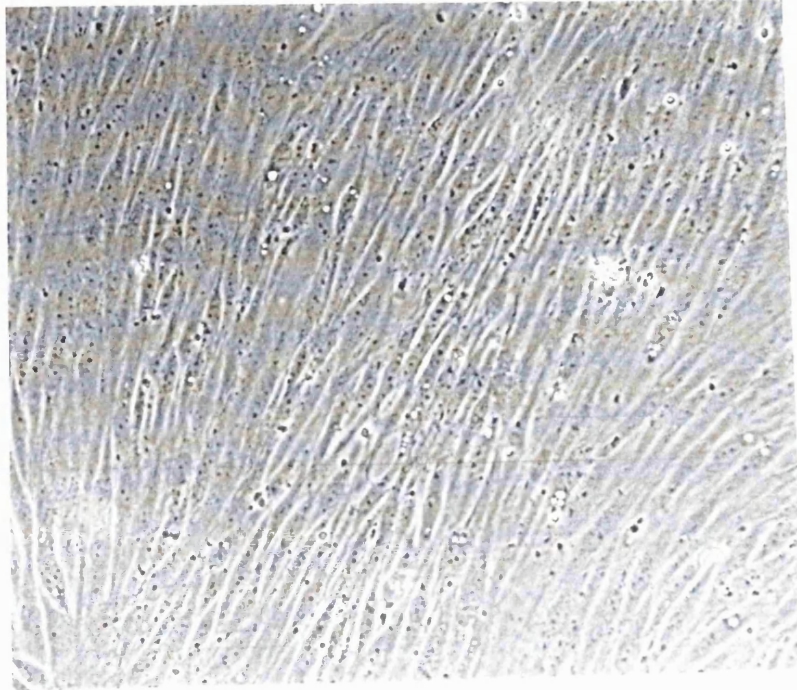
#### 7.1 INTRODUCTION

Studies investigating the interactions of lymphocytes with cells of the blood brain and blood retinal barriers in vitro are normally undertaken with cells derived from Lewis rats as this strain which is most susceptible to induction of EAU (Caspi, 1989) and EAE (Happ et al., 1988). However, as these rats are non-pigmented the isolation of pure cultures of RPE cells is rather difficult, which is why for this project PVG rats were used. PVG rats were chosen as they have been demonstrated to be susceptible to EAU, although the disease is less severe and more chronic in nature (de Kozac et al., 1981). By using PVG animals a direct comparison could not then be made with similar studies carried out in the laboratory as these involved retinal endothelial cells derived from Lewis rats. Therefore, experiments were carried out to determine if lymphocyte interactions with PVG derived retinal endothelium were similar to that of Lewis retinal endothelial cultures. Other studies have concentrated on differences in class II inducibility (as described in the previous chapter). However, other factors are also involved in determining susceptibility to experimental models of autoimmune conditions as congenic Brown Norway rats (EAE-resistant) which have a permissive Lewis MHC superimposed, remain resistant to EAU (Happ et al., 1988). Therefore, it was necessary to determine if this was the only difference between lymphocyte interactions between Lewis and PVG-derived endothelium.

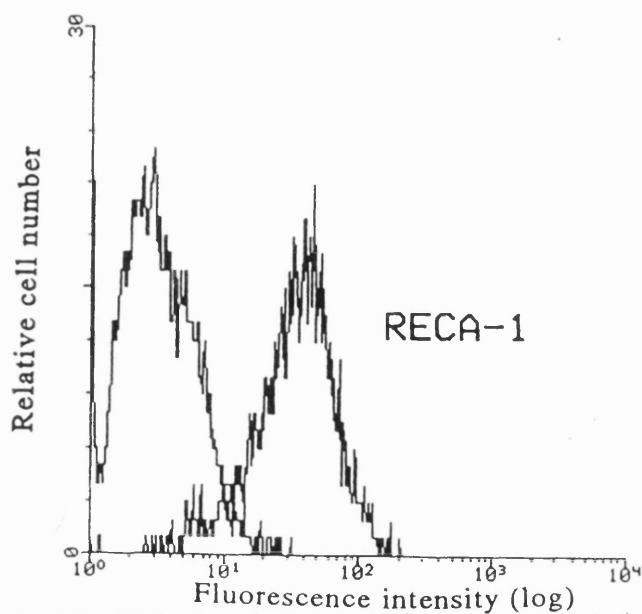
#### 7.2 RESULTS

##### 7.2.1 Characterisation of retinal endothelial monolayers.

Endothelial cells isolated using the method described previously have been extensively characterised (Greenwood, 1992) and, as has been described, grew out from capillaries forming a confluent monolayer of contact-inhibited, spindle shaped cells after approximately two weeks (Figure 7.1). For the purpose of this work, the purity of the endothelial cells was determined by using the anti-rat endothelial cell antibody RECA-1. Flow cytometric analysis revealed that cells were >95% pure (Figure 7.2).

**CHARACTERISATION OF RETINAL ENDOTHELIUM.**

**Fig 7.1** Phase contrast image of confluent PVG retinal endothelial cell culture (x240 magnification).



**Fig 7.2** RECA-1 staining of PVG retinal endothelium as determined by flow cytometry. Histogram on the left is negative control of cells stained with secondary antibody alone. Retinal endothelial cells were >95% positive for the rat endothelial cell marker RECA-1.

### 7.2.2 Migration of lymphocytes across PVG derived endothelium.

Untreated, resting PLN cells did not migrate across retinal endothelium derived from PVG animals which was only marginally, but not significantly, increased when the PLN cells were activated with Con A ( $0.6 \pm 0.4\%$ )(Figure 7.3a).

The migration of the three antigen-specific T cell lines described previously were also investigated using time-lapse videomicroscopy. As before, the cells above and below the monolayer could be distinguished by their phase contrast appearance (Figure 7.4) allowing for the enumeration of migration. No significant difference was found in the migration of PPD ( $22.6 \pm 4.9$ ), ovalbumin ( $29.1 \pm 2.8\%$ ) or S-antigen ( $22.2 \pm 3.1\%$ ) specific lymphocytes (Figure 7.3b).

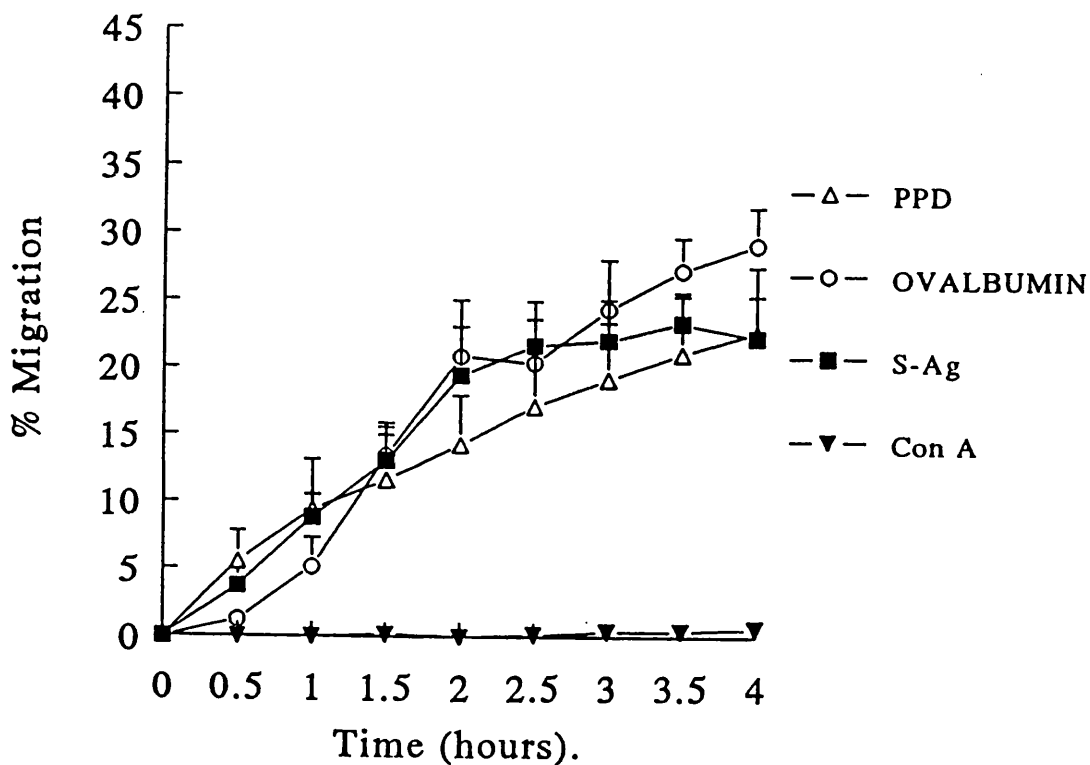
### 7.2.3 Migration of lymphocytes across Lewis derived endothelium.

The level of migration of PLN cells (untreated and activated), as well as T cell line lymphocytes, across PVG-derived endothelial cells appeared to be lower than had been reported with Lewis derived endothelium (Greenwood and Calder, 1993). However, as different T cell lines were used in this study, T cell line lymphocyte migration across Lewis retinal endothelium was repeated using the same T cell lines as had been added to PVG retinal endothelium. As with the PVG endothelium there was no significant difference between the migration of any of the T cell lines. However, the percentage of cells which had migrated after four hours was significantly higher than that of the same T cell line lymphocytes across PVG derived endothelium ( $p < 0.01$ , chi-squared). The average migration across Lewis derived endothelium was  $44 \pm 2.8\%$  for PPD,  $38.6 \pm 5.6\%$  for ovalbumin and  $40.3 \pm 4.4\%$  for S-antigen specific lymphocytes (Figure 7.5).

### 7.2.4 ICAM-1 expression on untreated and activated REC derived from PVG and Lewis derived endothelium.

The level of constitutive ICAM-1 expression on PVG and Lewis retinal endothelial cells was compared using flow cytometry (Figure 7.6). PVG retinal endothelial cells were found to express lower constitutive ICAM-1 expression than Lewis retinal endothelium (Figure 7.6a). Activation of endothelium with IL-1 $\beta$  from both strains resulted in an increase in intensity of ICAM-1 expression, but the ICAM-1 expression was still lower on PVG than that of Lewis derived retinal endothelium. Moreover, the response to cytokine activation, as measured by the overall increase in intensity, was greater with

**LYMPHOCYTE MIGRATION ACROSS PVG-DERIVED RETINAL  
ENDOTHELIUM.**



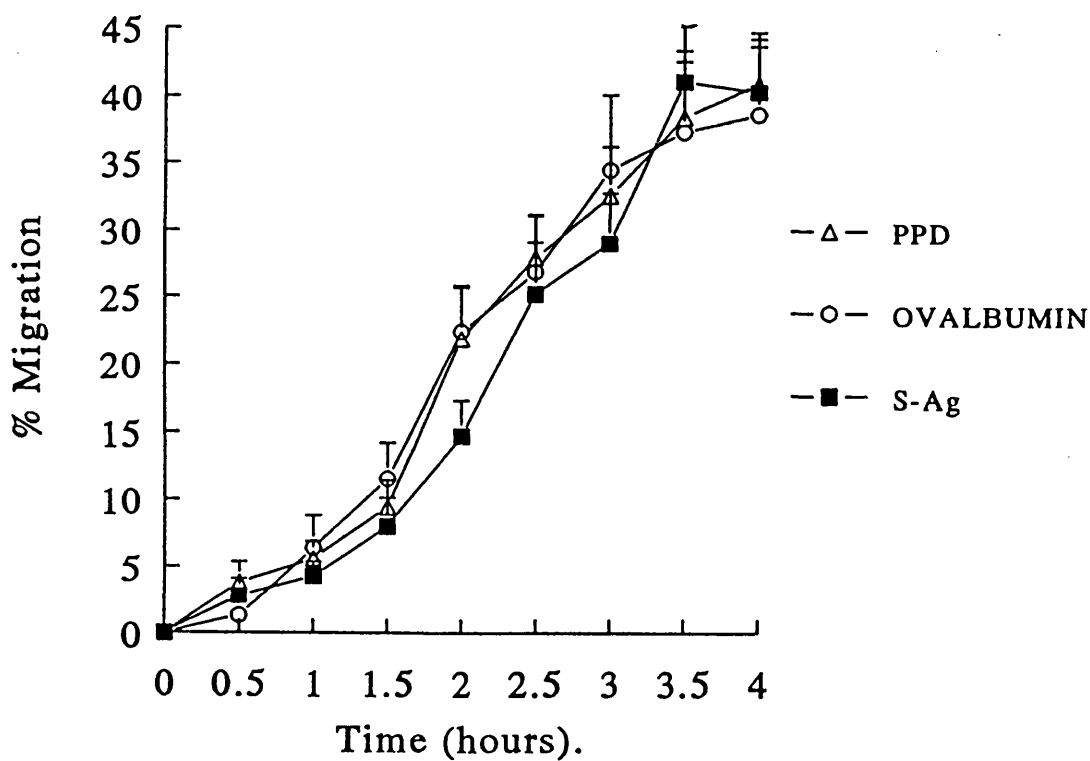
**Fig 7.3** Migration across untreated PVG derived retinal endothelium of Con A activated PLN cells (24hr and three antigen-specific T cell lines, specific for PPD, OA, S-Ag (mean  $\pm$  S.E.M.,  $n=9$  for T cell lines and  $n=6$  for Con A activated PLN). No significant difference was found in the migration of T cell line lymphocytes, however, the migration of Con A activated PLN was significantly lower than any of the T cell line studied ( $p < 0.01$ , chi-squared).

**CO-CULTURES OF LYMPHOCYTES WITH RETINAL ENDOTHELIAL  
CELLS.**



**Fig 7.4** *Phase contrast image of lymphocyte/retinal endothelial co-culture (x240 magnification). Lymphocytes underneath monolayer are phase dark (a) and on top of monolayer are phase bright (b).*

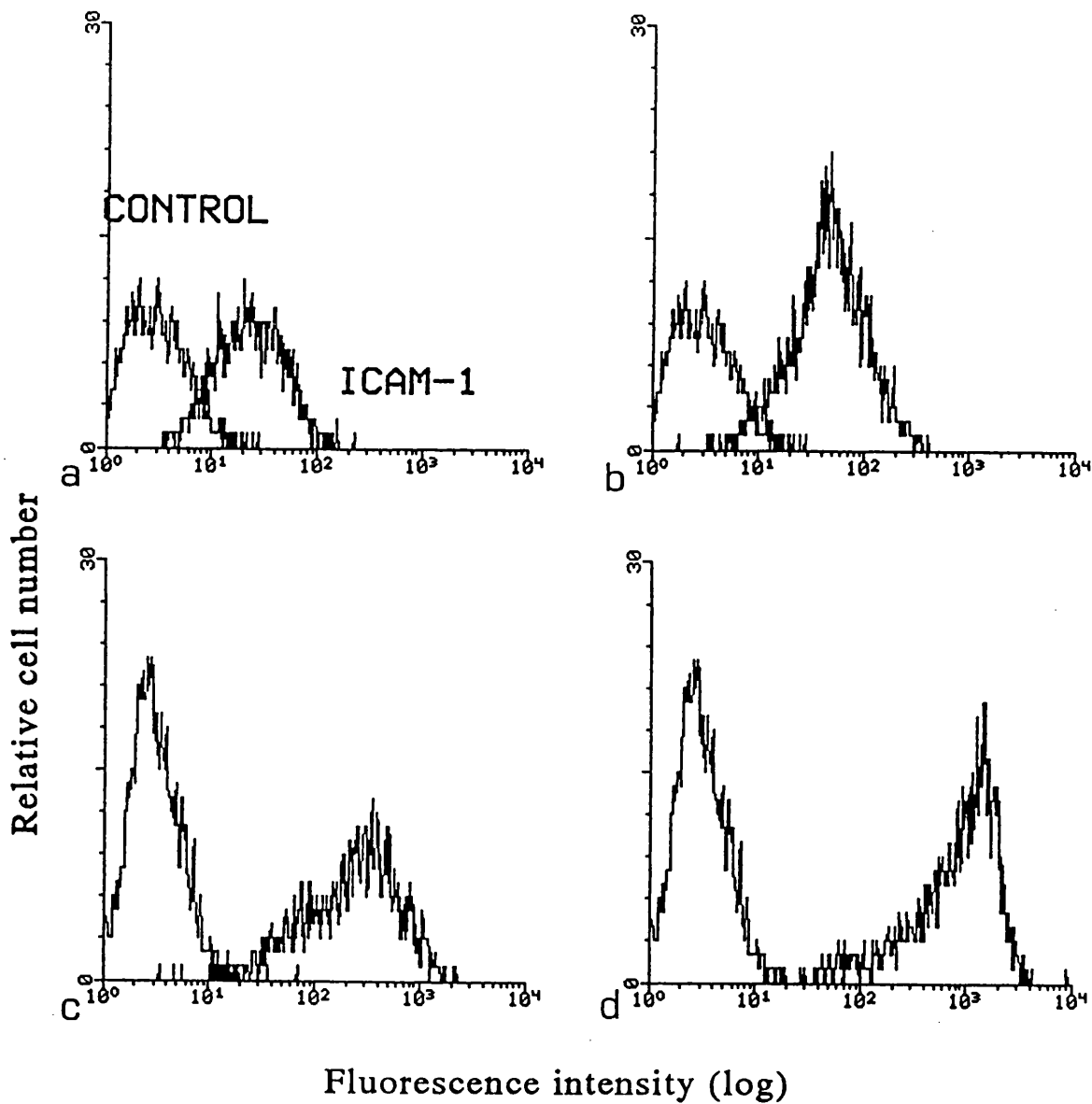
**LYMPHOCYTE MIGRATION ACROSS LEWIS-DERIVED RETINAL  
ENDOTHELIUM.**



**Fig 7.5** *T cell line migration across Lewis derived retinal endothelial cells. Average migration of PPD, OA and S-Ag specific T cell line lymphocytes across untreated Lewis retinal endothelium ( $\pm$ S.E.M., n=9). No significant difference was found in migration of T cell lines studied.*

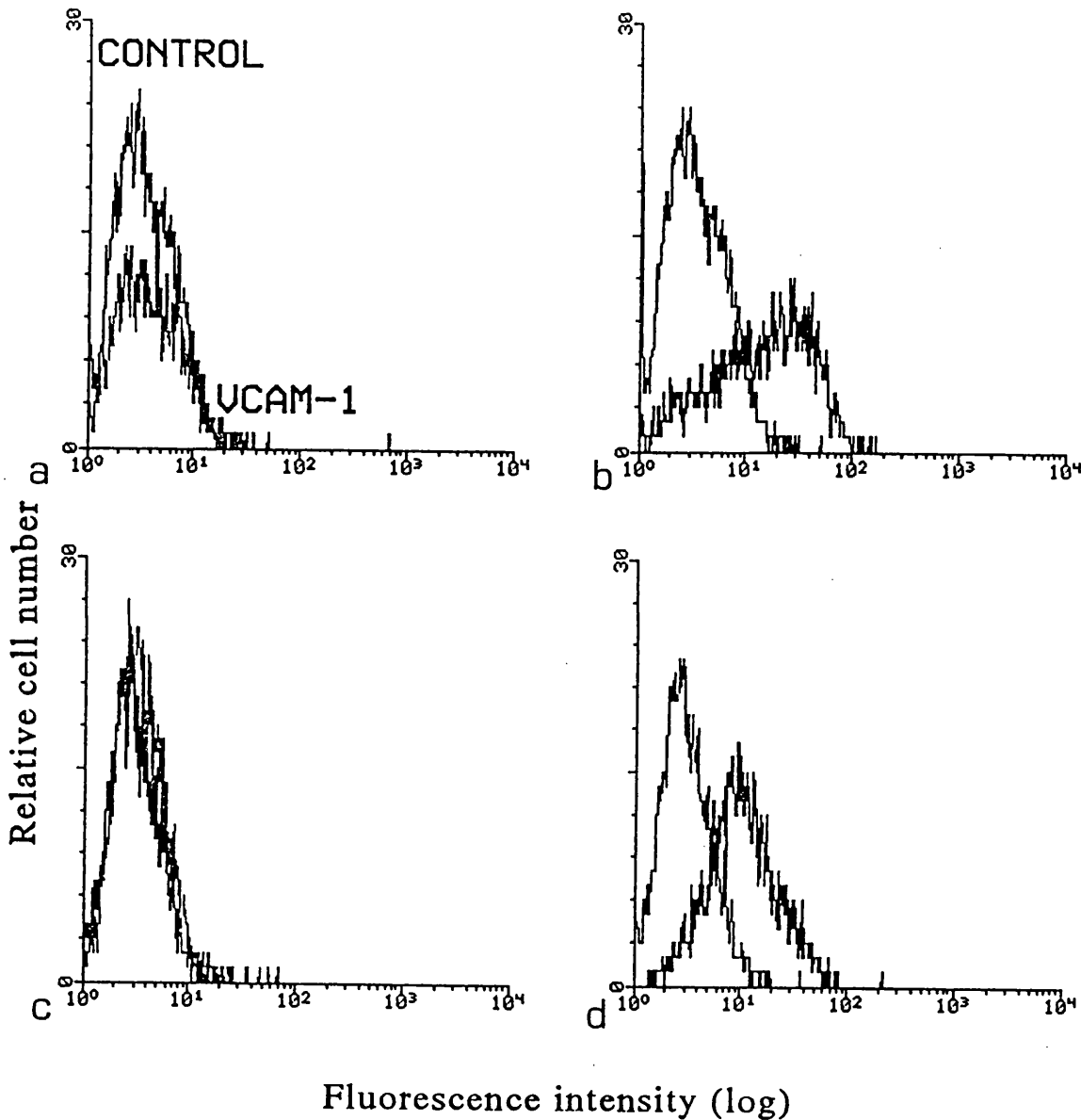


COMAPRISON OF ICAM-1 EXPRESSION ON LEWIS AND PVG DERIVED  
RETINAL ENDOTHELIAL CELLS.



**Fig 7.6a** Comparison of ICAM-1 expression on PVG (a&b) and Lewis (c&d) derived retinal endothelium. Expression was measured on (a&c) normal, untreated endothelium or (b&d) IL-1 $\beta$  activated endothelium (5U/ml IL-1 $\beta$  for 24hr). The level of ICAM-1 expression was found to be greater on Lewis derived retinal endothelium.

**COMAPRISON OF VCAM-1 EXPRESSION ON LEWIS AND PVG DERIVED  
RETINAL ENDOTHELIAL CELLS.**



**Fig 7.6b** Comparison of VCAM-1 expression on PVG (a&b) and Lewis (c&d) derived retinal endothelium. Expression was measured on (a&c) normal, untreated endothelium or (b&d) IL-16 activated endothelium (5U/ml IL-1 $\beta$  for 24h). VCAM-1 was not constitutively expressed but was induced upon IL-16 activation. No difference in VCAM-1 expression was noted between endothelium from the two rat strains.

Lewis endothelium. VCAM-1 was not expressed on non-activated retinal endothelial cells isolated from either rat strain nor was there any difference in VCAM-1 expression following IL-16 activation.

### 7.3 DISCUSSION

The retinal vascular endothelium, compared with the RPE, is most likely to be the primary route of entry into the eye due to its close association with circulating inflammatory cells. As has been shown previously, antigen specificity of the T cell line was irrelevant in determining the level of migration observed across retinal endothelium (Greenwood and Calder, 1993).

The results described here demonstrate for the first time, that the source of the endothelium, ie. which strain of rats the endothelium was derived from determines the level of lymphocyte migration. Migration of lymphocytes across endothelium derived from PVG rats was significantly lower than that across Lewis retinal endothelium which may be related to the lower level of ICAM-1 expressed on the former. ICAM-1 expression on brain endothelial cells has also been shown to vary depending on the strain of rat (Linke and Male, 1994). Activated brain endothelial cells isolated from rats which were susceptible to EAE expressed more ICAM-1 than endothelial cells from EAE-resistant strains (although the difference in constitutive ICAM-1 was not significant in this instance). Therefore, it is feasible that lower ICAM-1 expression on these cells results in lower lymphocyte trafficking which contributes to a reduced susceptibility to disease.

It has been suggested that the differences found in inducible MHC class II and ICAM-1 expression on endothelial cells from different rat strains is due to a common feature affecting the IFN- $\gamma$  signalling pathway involved in the induction of both class II and ICAM-1, but not class I (Linke and Male, 1994). There is, however, no direct evidence for this, but if this hypothesis were true, it would be expected that this effect would be seen in all cells and not just endothelium.

The data presented here indicates that a lot more work is required to determine the full extent of differences between retinal endothelial cells from different strains. Due to time constraints only differences in lymphocyte migration across untreated endothelium were studied. It can, however, be speculated that as ICAM-1 expression remains lower on IL-16 activated PVG retinal endothelium, lymphocyte migration would also be less compared with Lewis derived endothelium. Further experiments are required to confirm

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or disprove this hypothesis. It would also be interesting to determine if lymphocyte adhesion is also less on PVG derived compared with Lewis derived retinal endothelium.

In addition, further work is required to determine if this effect is unique to the endothelium, or if there is a similar difference in RPE cells from both strains. It has been shown that RPE isolated from EAU-susceptible and resistant strains produce different amounts of the inflammatory cytokine TNF- $\alpha$ , with cells from susceptible strains producing significantly greater amounts (de Kozak et al., 1994). Therefore, strain differences do appear to play a role in the immunological properties of RPE. It is likely that a number of factors determine the susceptibility or resistance of certain rat strains to animal models of disease, including MHC class II and ICAM-1 inducibility and the cytokines produced in response to certain inflammatory stimuli.

## CHAPTER 8

### IMMORTALISATION OF PRIMARY CULTURED RAT RPE

#### 8.1 INTRODUCTION

The isolation of rat RPE cells is extremely time-consuming as well as expensive. Primary rat RPE cultures do not passage very well, therefore establishing cell lines that retain their characteristics was not feasible. Moreover, obtaining sufficient rat litters and a constant supply proved to be a problem throughout this study. Thus, transformed cell lines were developed using a temperature sensitive form of the SV40 large T antigen which had previously been shown to immortalise rat cells.

#### 8.2 RESULTS

##### 8.2.1 Characterisation of immortalised cultures of RPE.

The morphology of transformed cultures (parent line designated IO.LD7 and clones IO.LD7:1 to IO.LD7:6) were similar to that of primary cultures, although they were not pigmented. Like primary cultures, the cells settled in clusters from which they grew out, but as they grew much faster, the cells were much more regular in shape and did not exhibit the same degree of spreading (Figure 8.1).

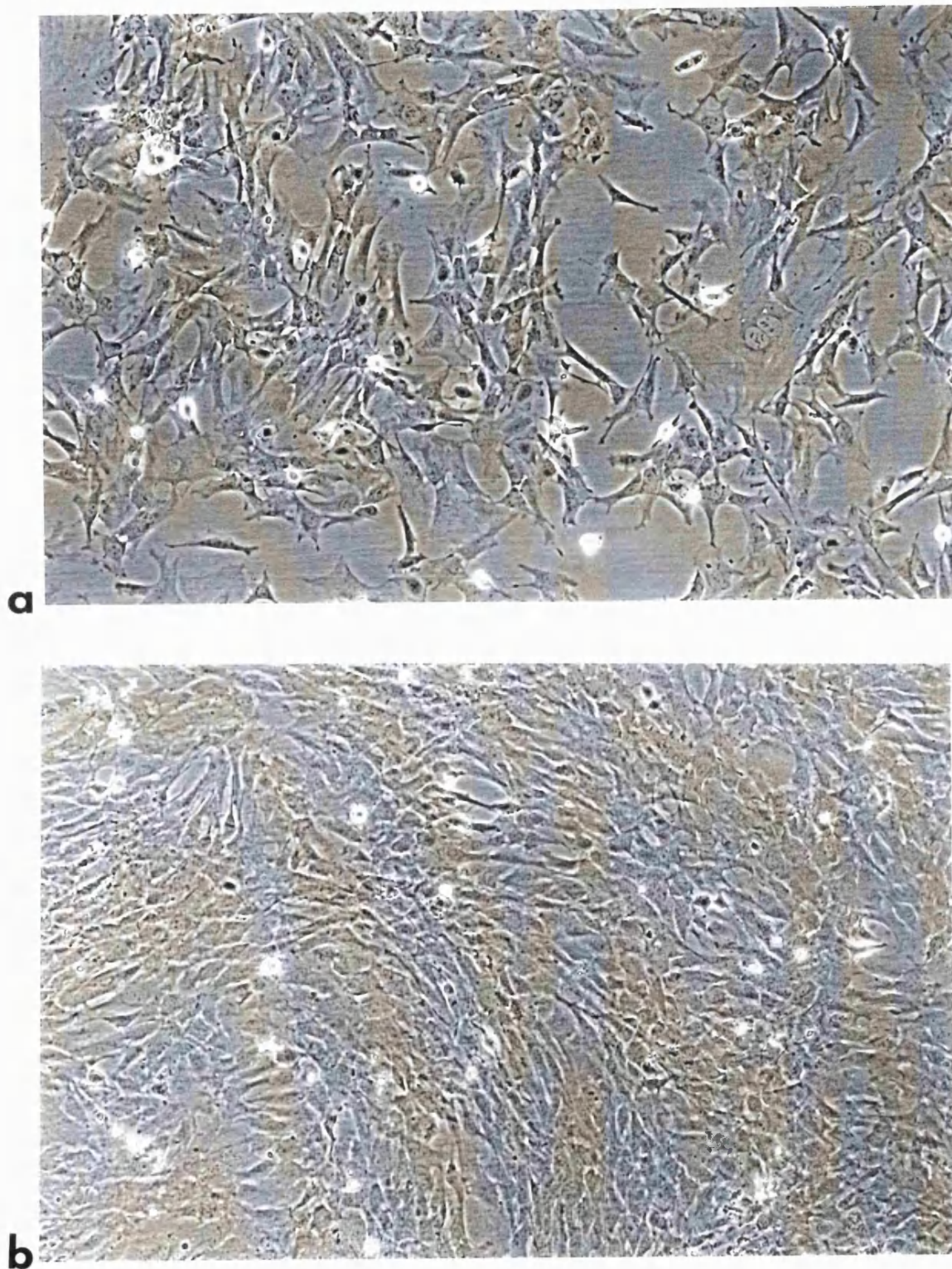
The expression of cytokeratins and the RPE specific marker RET-PE2, as determined by flow cytometry, were similar to that observed with primary cultures (Figure 8.2) in that RET-PE2 proved to be a more useful marker than cytokeratins. Cells were 100% positive when stained with RET-PE2, indicating the purity of the cultures. In general these cells were much more amenable to flow cytometric analysis than primary cultures perhaps due to their more uniform shape. Cells were also positive for Large T antigen used to immortalise them (Figure 8.3).

Transmission electron microscopy also revealed similar ultrastructure to primary cultures, with dense bodies, possibly premelanosomes observed (Figure 8.4).

##### 8.2.2 MHC and adhesion molecule expression on immortalised RPE.

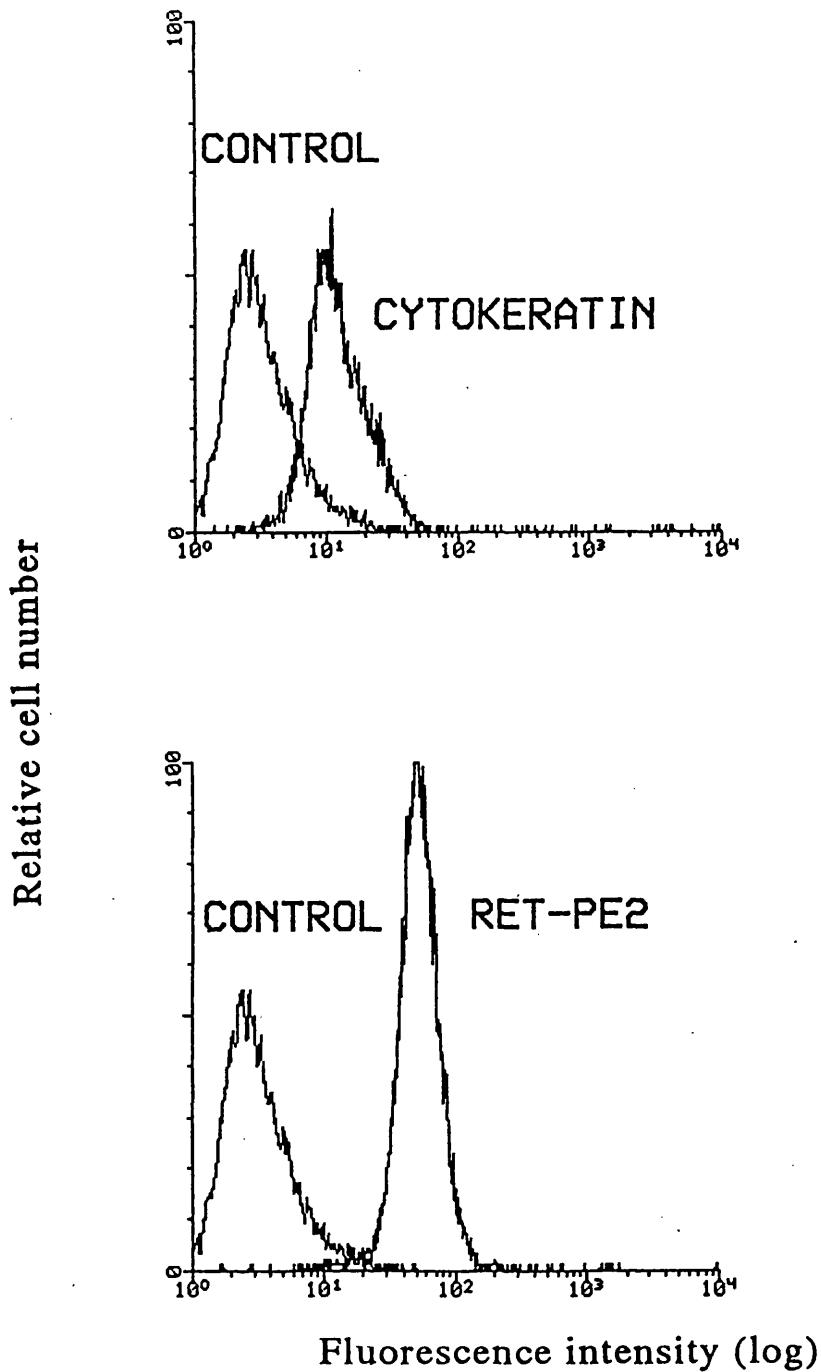
The expression of MHC class I and II and the adhesion molecules ICAM-1 and VCAM-1 were determined by ELISA and flow cytometry.

IO.LD7 cells constitutively expressed MHC class I which was significantly increased upon activation, from an optical density value of 0.134 to 0.258 (figure 8.5a)

**IMMORTALISED RPE CULTURES (IO.LD7).**

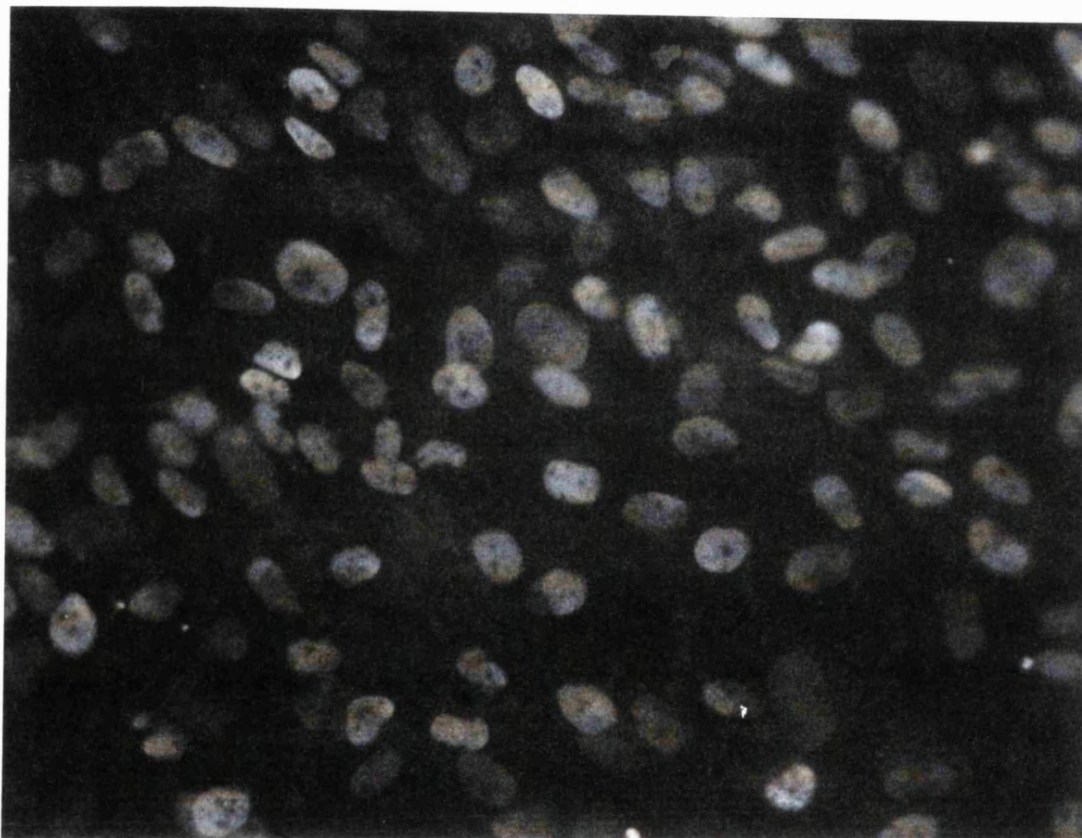
**Fig 8.1** Phase contrast image (x240 magnification) of IO.LD7 cells (a) sub-confluent and (b) confluent. Cells reached confluence within 2-3 days of plating.

## CHARACTERISATION OF IO.LD7 CELLS.



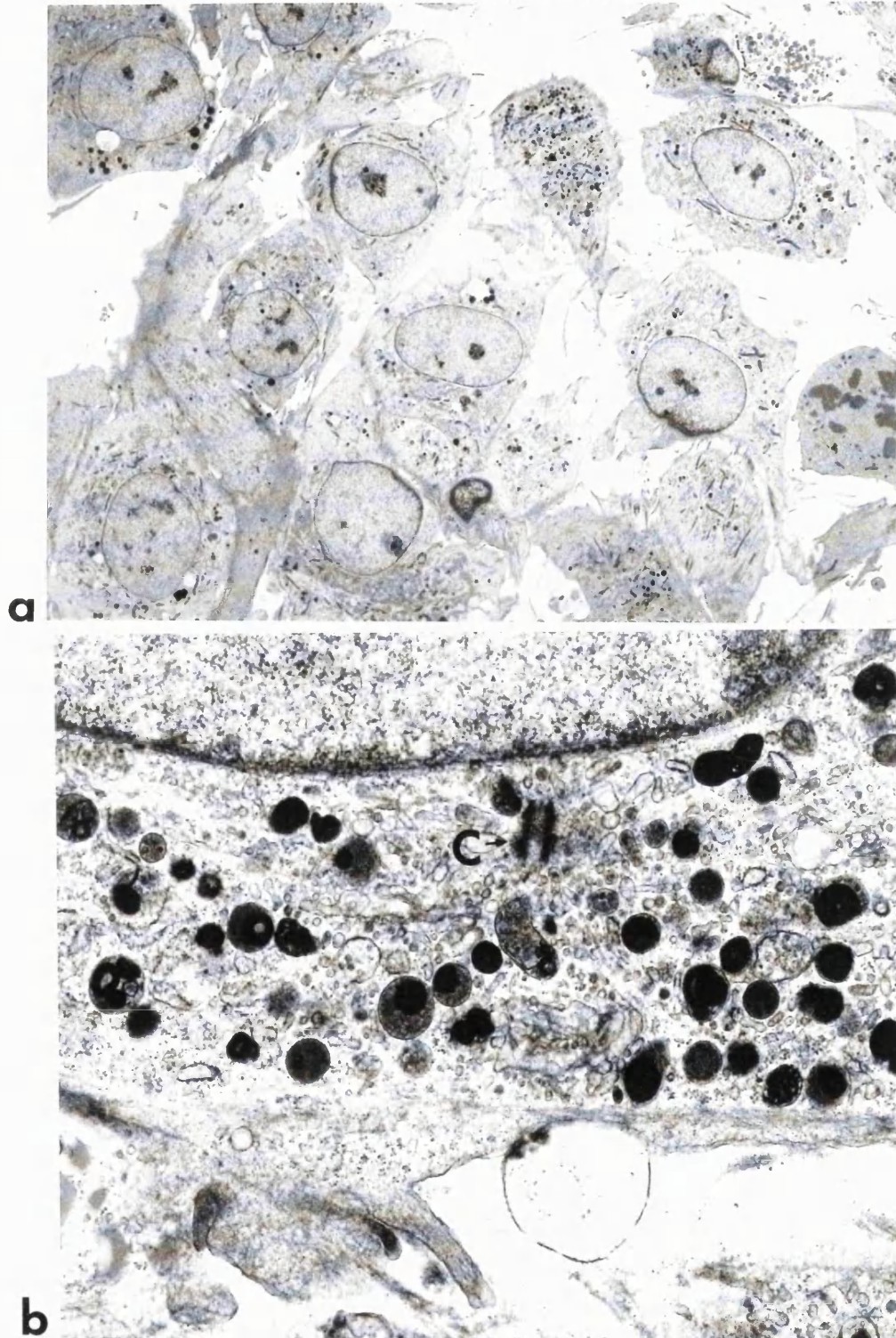
**Fig 8.2** Flow cytometric analysis of IO.LD7 cells stained for cytokeratins and RET-PE2. 100% of cells stained positive with RET-PE2 antibody, demonstrating that these cells had retained the marker for rat RPE.

**SV40 LARGE T ANTIGEN EXPRESSION IN  
TRANSFORMED RPE CELLS.**



**Fig 8.3** *Immunofluorescent staining of large T antigen in nucleus of transformed RPE. SV40 large T antigen was used to immortalise primary RPE. (Magnification x480).*



**ULTRASTRUCTURE OF IMMORTALISED RPE CELLS.**

**Fig 8.4** Transmission electron micrographs of horizontally sectioned IO.LD7:4 clones showing (a) details of nuclear morphology and perinuclear organelle clustering and (b) selective enlargement showing melanolipofuscin-like bodies and a centriole (C). (Scale bars:  $a=5\mu\text{m}$  and  $b=0.5\mu\text{m}$ . Magnification (a)  $\times 1000$  and (b)  $\times 15,000$ .)

$p < 0.01$ ). MHC class II molecules (figure 8.5b), however were not found on untreated transformed RPE cultures, but low levels of expression were induced upon activation with 100U/ml IFN- $\gamma$  (maximal optical density values of 0.0814 for I-A and 0.078 for I-E, 5 days after activation). Flow cytometric analysis of MHC class I expression indicated that 10% of untreated cells were positive which was increased to 100% after 24 hours activation with IFN- $\gamma$  (Figure 8.6).

ICAM-1 was constitutively expressed on IO.LD7 cells, the expression of which was increased when the cells were activated with IFN- $\gamma$ , although ELISA results indicated that this was only significant after 4 days activation ( $p < 0.05$ , Figure 8.7). The percentage of cells which were positive for ICAM-1, as determined by flow cytometry, however, was approximately 73%. Activation with IFN- $\gamma$  for 24 hours increased the percentage of cells which were positive to 100% (figure 8.8).

These cells did not constitutively express the adhesion molecule VCAM-1, although expression could be induced with IFN- $\gamma$ . Expression was greatest at two days ( $p < 0.05$ ) but was expressed at levels significantly greater than control after activation for 1 day (Figure 8.7,  $p < 0.01$ ).

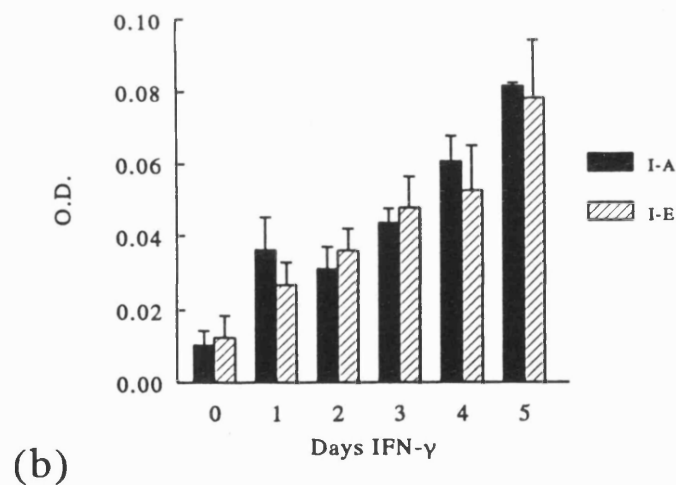
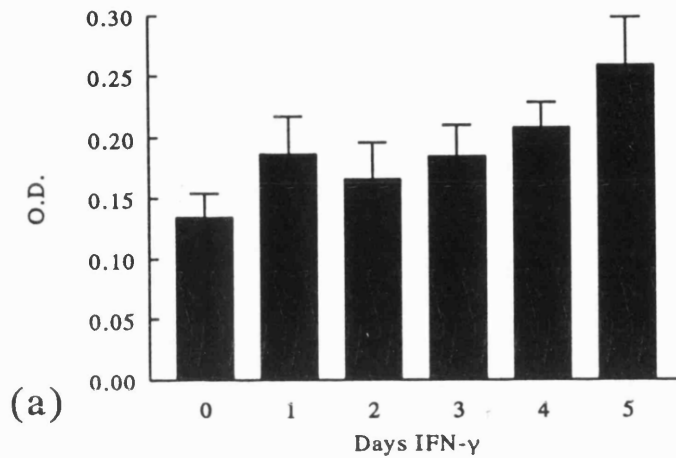
### 8.2.3 Lymphocyte migration across immortalised RPE.

Similar to migration across primary RPE cultures, the migration of untreated PLN cells was very low ( $2.5 \pm 0.2\%$ ) and was significantly increased ( $p < 0.05$ ) upon activation with Con A or immobilised anti-CD3 ( $4.2 \pm 0.7\%$  and  $5.7 \pm 0.7\%$  respectively, figure 8.9). Antigen specific T cell line lymphocytes were more migratory with an average migration of  $16.5 \pm 1.6\%$ , although this was significantly less than migration of the same T cell line lymphocytes across primary RPE (ie.  $27.6 \pm 2.5$ ,  $p < 0.05$ ).

### 8.2.4 Electrical resistance measurements.

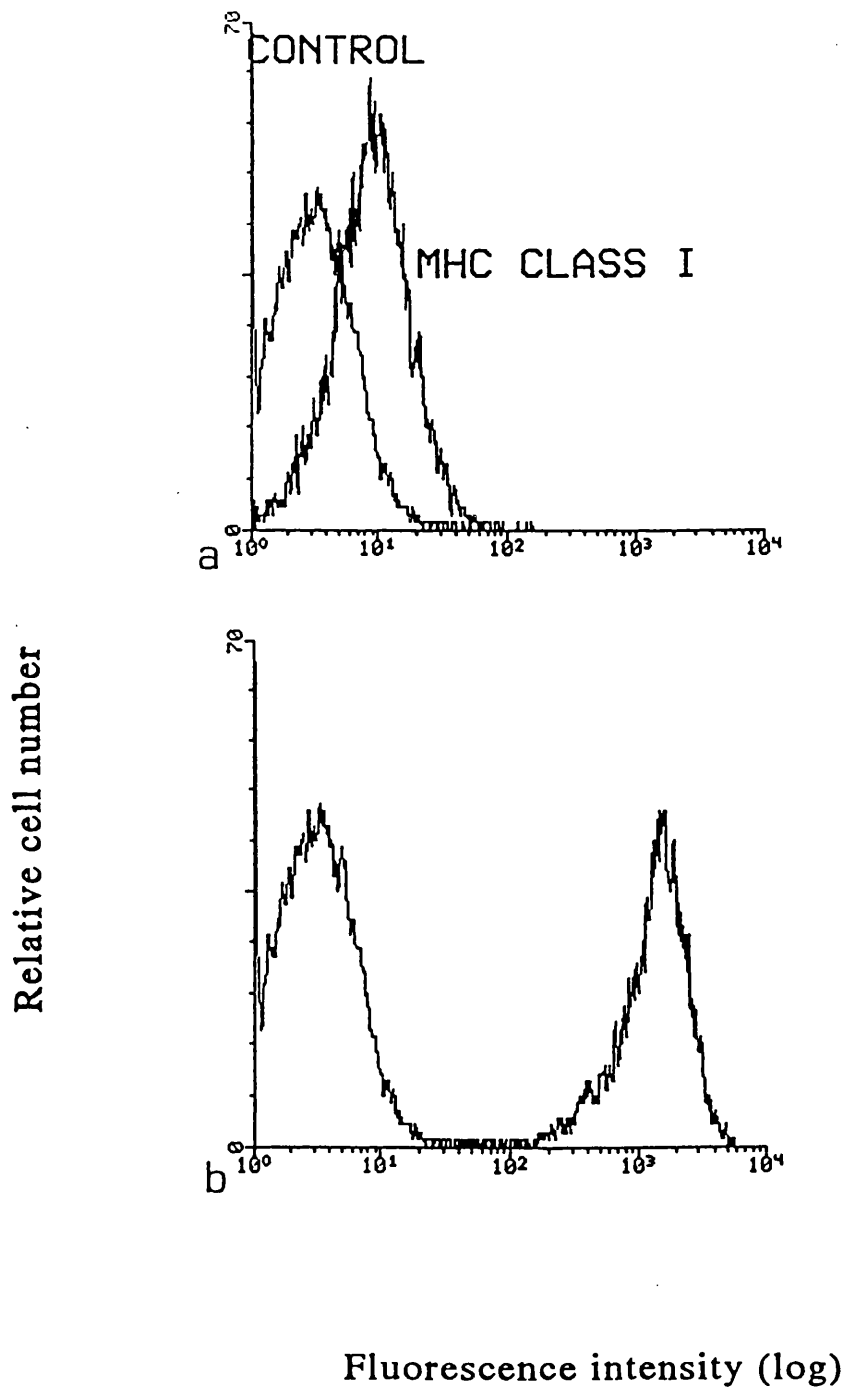
The transepithelial electrical resistance of transformed RPE cells grown to confluence on filters was measured (Figure 8.10). Electrical resistance peaked after 3 days after cells reached confluence as determined by phase contrast microscopy ( $564\Omega \cdot \text{cm}^2 \pm 397$ ), but declined rapidly, as cells began to overgrow and lift off the filter. The high error bar associated with the maximal resistance measurement was due to a reading of over 4000 ohms.cm<sup>2</sup>, which had increased from 900 ohms.cm<sup>2</sup> recorded 24h previously.

## MHC EXPRESSION ON IO.LD7 CELLS.



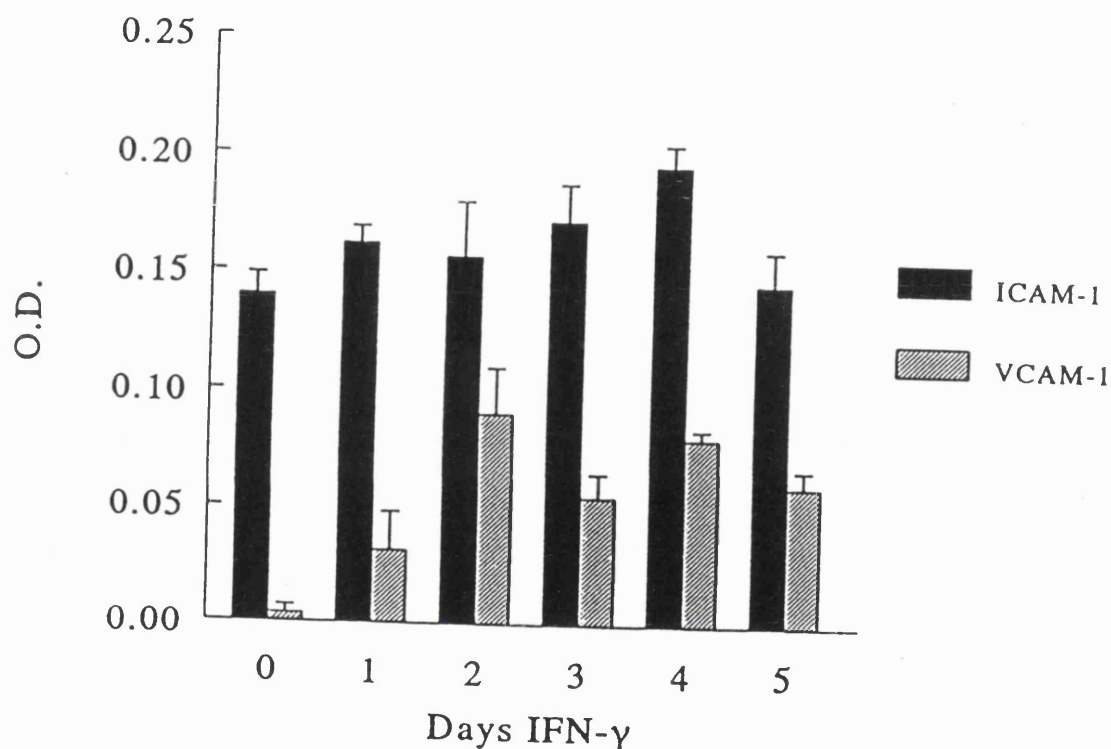
**Fig 8.5** (a) MHC class I and (b) MHC class II expression (I-A and I-E) on IO.LD7 cells as determined by ELISA. Optical density (O.D.) values shown have the background reading (secondary antibody and substrate added to cells) subtracted. Results are expressed as mean  $\pm$  S.E.M. ( $n=9$ ). MHC class I and class II expression were significantly increased upon activation ( $p < 0.01$  for MHC class I and  $p < 0.05$  for MHC class II).

## FLOW CYTOMETRIC ANALYSIS OF MHC CLASS I EXPRESSION.



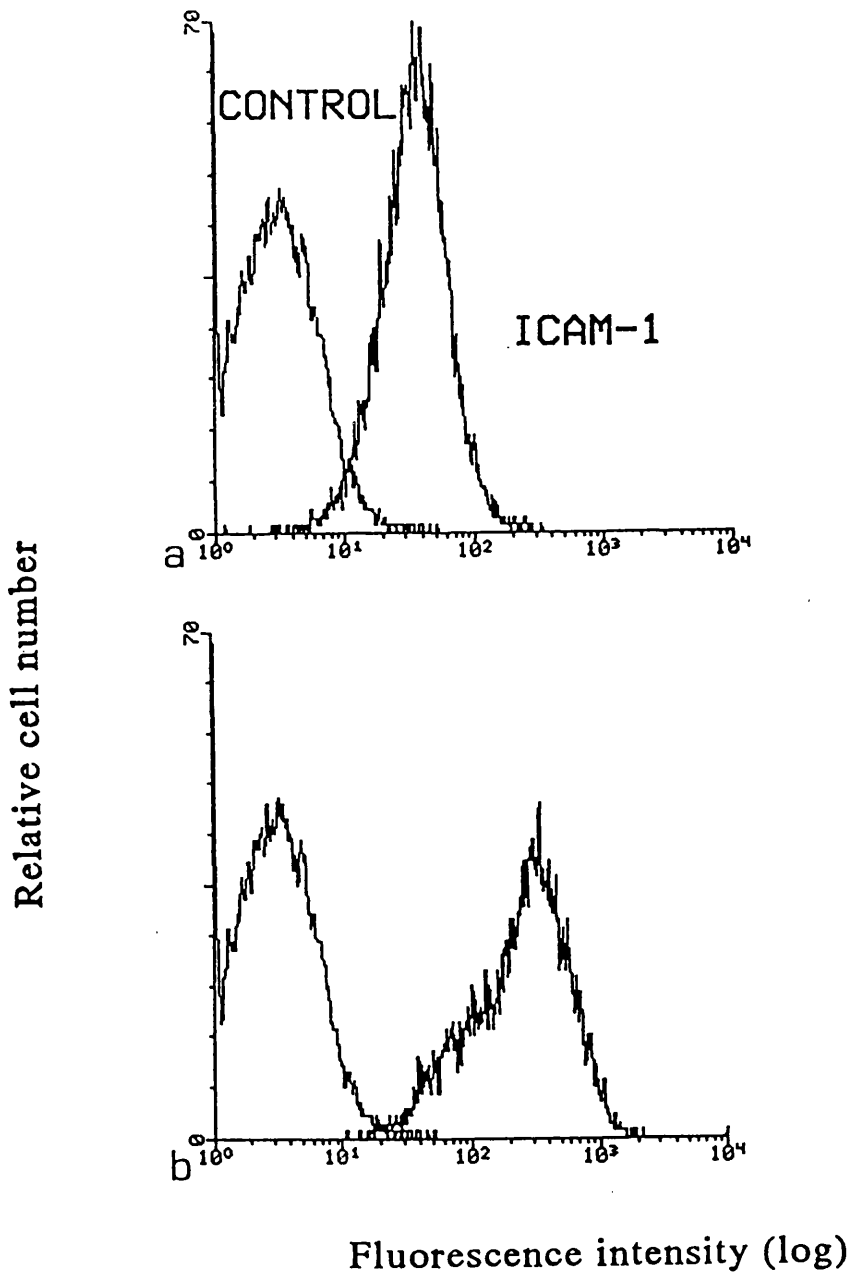
**Fig 8.6** MHC class I expression on (a) untreated and (b) IFN- $\gamma$  activated (100U/ml 5 days) IO.LD7 cells as determined by flow cytometry. Control histogram on the left hand side in each case, is cells stained with secondary (FITC) antibody alone. MHC class I expression is shown to be upregulated upon activation.

EXPRESSION OF THE ADHESION MOLECULES ICAM-1 AND  
VCAM-1 ON IO.LD7 CELLS.



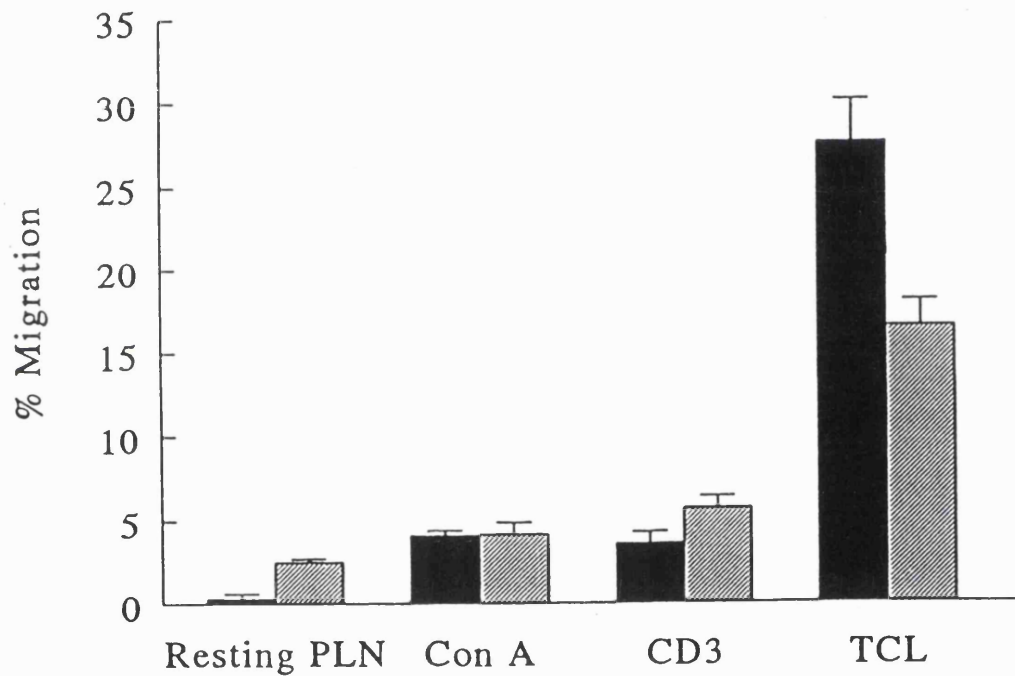
**Fig 8.7** Expression of ICAM-1 and VCAM-1 on IO.LD7 cells as determined by ELISA. O.D. values are mean  $\pm$  S.E.M. ( $n=9$ ) with background values subtracted. ICAM-1 expression was significantly upregulated after 4 days IFN- $\gamma$  activation ( $p < 0.05$ ). VCAM-1 expression was significantly upregulated after 1 day IFN- $\gamma$  activation ( $p < 0.01$ ) and expression was maximal after 2 days activation ( $p < 0.05$ ).

FLOW CYTOMETRIC ANALYSIS OF ICAM-1 EXPRESSION  
ON IO.LD7 CELLS.



**Fig 8.8** Expression of ICAM-1 on (a) untreated and (b) IFN- $\gamma$  activated (100U/ml, 24h) IO.LD7 cells. Left hand histogram in each case is a negative control of cells stained with secondary (RAMIG-FITC) alone. ICAM-1 expression shown to be upregulated upon IFN- $\gamma$  activation.

COMPARISON OF LYMPHOCYTE MIGRATION ACROSS PRIMARY  
AND IMMORTALISED RPE CELLS.



**Fig 8.9** Comparison of lymphocyte migration across primary (solid bars) and immortalised RPE monolayers (shaded bars). Results are expressed as mean  $\pm$  S.E.M. ( $n=9$ ). Only significant difference between migration across primary and immortalised RPE was in the migration of T cell line lymphocytes, which was lower across immortalised RPE monolayers ( $p < 0.05$ ).

### 8.3 DISCUSSION

The immortalised cell lines obtained from the infection with SV40 large T, of primary rat RPE cultures retained the majority of characteristics associated with primary cultures. The main difference between primary and transformed cultures was the loss of pigment, however this has been reported previously for RPE immortalised using SV40 large T antigen (McLaren et al., 1993; Albert and Buyukmihci, 1979), and pigmentation decreases in primary cultures as cells grow out from the heavily pigmented central plaque. The transformed cells are more uniform in shape, and do not exhibit the same degree of spreading as primary cultures, which is probably due to the increased growth rate associated with transformed cells. Although no pigment could be seen at the light microscopy level, dark bodies which could possibly be premelanosomes could be seen ultrastructurally.

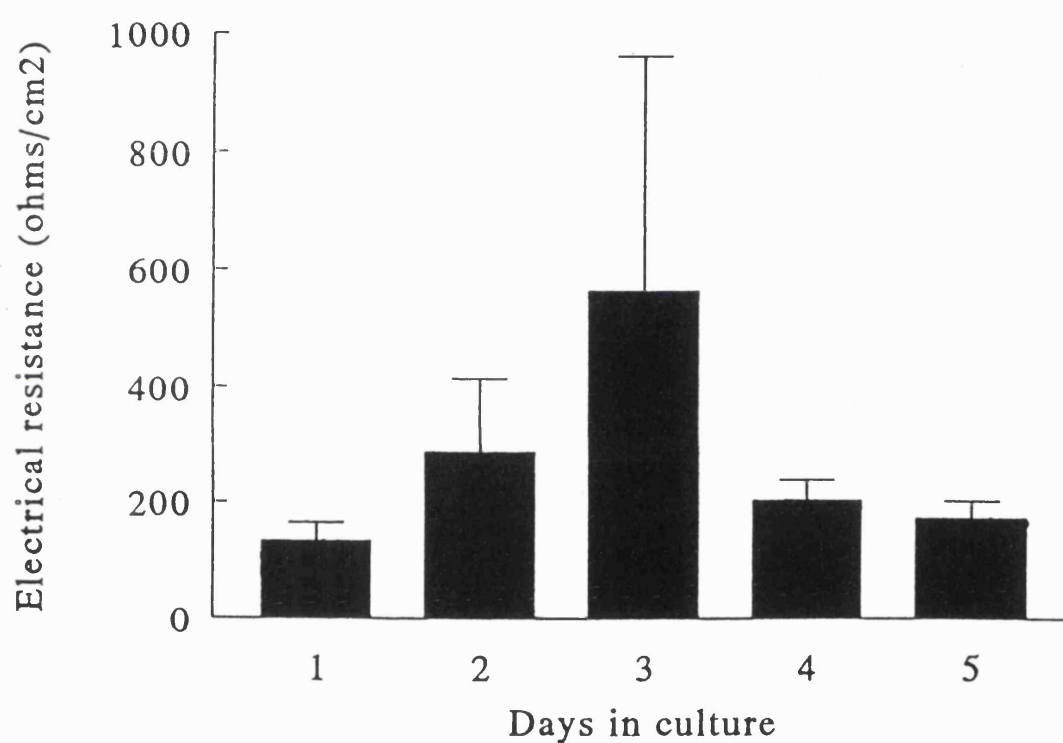
Expression of both MHC class I and II was the same as had already been demonstrated for primary cultures, in that only MHC class I was constitutively expressed, while relatively low levels of MHC class II molecules were found only on IFN- $\gamma$  activated IO.LD7 cells. As with primary cultures, expression of both I-A and I-E class II MHC molecules were induced.

Adhesion molecule expression of the transformed cells was also shown to be similar to that of primary cultures. ICAM-1 molecules were found constitutively, and could be significantly upregulated upon activation for four days with IFN- $\gamma$  ( $p < 0.05$ ) as determined by ELISA. Flow cytometric analysis suggested a much more pronounced increase than ELISA, however this may be due to differences in the sensitivity of these methods. VCAM-1 was not found on untreated IO.LD7 cells, but could be induced by IFN- $\gamma$ . The time-course of expression was slightly different in that expression peaked two days post-activation, whereas this did not occur until 3 days with primary cultures. Moreover the level of VCAM-1 expressed by the transformed cells appeared to be less than that of primary cultures.

Immortalised RPE cell lines were also able to support lymphocyte migration, although the level of migration of antigen specific lymphocytes was less across the immortalised RPE than had been observed across primary cultures. This may be due to less ICAM-1 being expressed on the transformed cells, however the results shown here do not demonstrate that this is the case. Flow cytometric analysis suggests that the



## ELECTRICAL RESISTANCE MEASUREMENTS OF IO.LD7 CELLS.



**Fig 8.10** *Electrical resistance measurements of confluent cultures of IO.LD7 cells taken over a 5 day time period. Results are expressed as mean  $\pm$  S.E.M. ( $n=8$ ). Resistance was greatest at 3 days post confluence ( $p < 0.05$ ).*

intensity of ICAM-1 is the same on both primary and transformed cultures. An alternative possibility is that the junctions between IO.LD7 cells were tighter. Transepithelial resistance measurements were not obtained with primary cultures in this study due to difficulties with growing these cells on filters, however one study has reported resistance measurements of between 85-190 ohms cm<sup>2</sup> for primary rat RPE cultures (Chang et al., 1991). Significantly higher resistance measurements were found with the transformed cells at 2-4 days post confluence, however the transepithelial resistance at the time of the migration assays could not be determined as the cells were not grown on filters for this purpose.

Immortalised cells may not retain all of the characteristics of primary cultures and therefore results must be interpreted with care. It is unlikely that they will completely replace the need for primary cultures. However the availability of these cells, particularly when large numbers are needed, for example in biochemical studies, should prove invaluable for future research. Due to the number of functions associated with the RPE, could facilitate research not just associated with uveitis, but in other conditions such as age related macular degeneration. For example, these cells have been injected subretinally and shown to improve the symptoms in the animal model of this sight-threatening condition (R.Lund, personal communication).

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## CHAPTER 9

### GENERAL DISCUSSION & FUTURE WORK

One of the first steps in the development of an inflammatory response is the migration of leucocytes to the site of inflammation. During ocular inflammatory conditions, such as posterior uveitis, a large number of inflammatory cells enter the retina via the blood-retinal barrier. Of particular importance in this condition are CD4<sup>+</sup> T lymphocytes, which can cross either the anterior barrier, formed by the retinal vascular endothelium, or the posterior aspect of the BRB formed by the RPE. The majority of leucocyte infiltration appears to occur via the vascular endothelium, which is probably due to the endothelium being more readily accessible to the inflammatory cells in the circulation, whereas, in order to reach the RPE, inflammatory cells must first cross the choroidal endothelium and penetrate Bruchs' membrane. Nevertheless, migration across the RPE does occur, and can in some cases be the primary route of infiltration.

Comparison of lymphocyte migration across both aspects of the BRB *in vitro* did not reveal any significant difference in the overall level of migration after four hours of co-culture. Moreover the factors controlling this migration were similar, in that the state and mode of lymphocyte activation was crucial in determining the level of migration observed. The migration of antigen-activated long term cell lines was much greater across both retinal endothelium and RPE than PLN activated with mitogen or immobilised anti-CD3 monoclonal antibody.

Some differences were found in lymphocyte migration across RPE compared with retinal endothelial monolayers. Comparison of results with that of Greenwood et al. (1993,1995) demonstrated that although cytokine activation of the retinal endothelium or the RPE enhanced migration, the cytokines responsible for the increase differed. IL-1 $\beta$  but not IFN- $\gamma$  significantly increased migration across the endothelium, whereas with RPE cells IFN- $\gamma$ , not IL-1 $\beta$ , significantly increased migration. The rate at which lymphocyte migration occurred, also differed. Migration across RPE monolayers occurred quickly, with lymphocytes migrating minutes after contact with the RPE, with the majority of migration occurring within the first 30 minutes and reaching a plateau after approximately two hours. Migration across the retinal endothelium, on the other hand, was a much slower process, with fewer cells migrating in the first 90 minutes of co-culture, increasing more rapidly during the latter period of the co-culture. A possible

explanation for this may be that the endothelial cells require activation, by cytokines produced by the lymphocytes, which may upregulate adhesion molecule expression (eg ICAM-1 expression). The RPE, however, has been shown to express higher levels of ICAM-1 (Liversidge et al., 1990) than retinal endothelium, which may be sufficient to support the initial migration. As RPE cells are not as accessible to circulating inflammatory cells the faster rate of migration across RPE cells *in vitro* may not have relevance to the *in vivo* situation because, as described below, lymphocyte migration via the RPE is a more tortuous route. An additional difference that was noted was that with T cell line migration across RPE monolayers, but not across retinal endothelium, antigen specificity did appear to have an effect on the level of migration observed. S-antigen T cell line lymphocytes were the most migratory. The most likely explanation for this is that the higher ICAM-1 expression on RPE cells, allows T cell lines to achieve maximal migration, whereas with endothelial monolayers, it is the ICAM-1 expression which is the limiting factor. It would be interesting to investigate the migration of other ocular specific T cell lines across RPE monolayer, to determine if this higher level of migration is still observed.

A major difference between the two cell types is that, unlike retinal endothelium, the RPE is not directly responsible for recruiting cells into the retina from the circulation. This may explain why cytokine activation of the RPE did not enhance lymphocyte adhesion as has been reported for retinal endothelium (Wang et al., 1993). However, the RPE can influence lymphocyte migration across the choroidal endothelium by producing a variety of cytokines which upregulates adhesion molecules on the endothelium of the choroidal vasculature (Tanihara et al., 1992; Planck et al., 1993; Planck et al., 1992; de Kozak et al., 1994). Alternatively, RPE cells can produce chemokines attracting leucocytes towards the RPE (Elner et al., 1990; Elner et al., 1991).

This study has shown, using antibody blockade studies, some of the adhesion molecules involved in lymphocyte migration across RPE monolayers. While ICAM-1/LFA-1 interactions were demonstrated to play a role in migration across RPE monolayers, results indicated that an alternative route may also be involved as anti-ICAM-1 on untreated monolayers caused only 50% inhibition of migration. The VCAM-1/VLA-4 migratory pathway however, was only involved when monolayers had been activated with IFN- $\gamma$  for 72 hours. Possible candidates for this ICAM-1 independent migratory pathway are members of the  $\beta$ -1 integrin family, such as extracellular matrix

components, which have previously been shown to be involved in controlling lymphocyte migration. Using this technique, it would be interesting when antibodies become available, for future work to determine if these adhesion molecules are involved. In addition, the role of chemokines in this migratory process should be investigated. RPE cells have been shown to produce the chemokines IL-8 (Benson et al., 1992) and monocyte chemoattractant protein (Elner et al., 1990; de Vries et al., 1994), however it is possible that these cells could produce others such as RANTES or MIP-1 $\beta$ . Therefore it would be interesting to determine the range of chemokines produced by the RPE constitutively and under inflammatory conditions, and the role these chemokines play in controlling lymphocyte migration.

Lymphocytes crossing the posterior barrier to enter the retina *in vivo* would migrate across the basal surface of the RPE first, whereas in this study RPE cells were grown directly on plastic resulting in lymphocytes interacting with the apical surface of the RPE. Adhesion molecule expression on the apical and basal surface may differ, therefore it would be of interest for future work to concentrate on investigating this aspect of migration. It was hoped that this would have been done during this project, but due to time constraints and technical difficulties it was not possible. As the immortalised cell lines grow better on membranes than primary culture the use of these cells may facilitate this work. Nevertheless, the expression of adhesion molecules demonstrated here, and their capacity to mediate lymphocyte migration is indicative of their contribution to the migratory process. RPE cells do express ICAM-1 on their apical surface *in vivo* and it has been shown that human RPE, *in situ*, express more ICAM-1 on the apical rather than the basal surface (Elner et al. 1992). The function of this may be to act as a haptotactic gradient, thereby attracting leucocytes across the posterior barrier (Springer review?). ICAM-1 expressed on the apical membrane may also be acting as a co-stimulatory molecule involved in activation of lymphocytes. However, there is no evidence that ICAM-1 expressed on RPE is involved in activating T cells, therefore further work is required to assess this possibility.

Genetic factors have been shown to be an important factor in the susceptibility of an individual to autoimmune conditions (Theofilopoulos, 1995) and of particular importance are differences in MHC class II expression. This study has shown that retinal endothelial cells isolated from different strains of rats, which have different susceptibilities to EAU, also express different constitutive levels of the adhesion molecule

ICAM-1. Moreover, the lower level of ICAM-1 found on endothelium derived from the less susceptible strain (ie PVG) could be responsible for the lower level of lymphocyte migration observed across these endothelial cells. Preliminary results from a separate study carried out within our department, indicate that this effect is not seen with RPE, as no significant difference was found in lymphocyte migration across RPE isolated from PVG and PVG/Lewis hybrids. It is intended to investigate the expression of ICAM-1 and the migration of T cells across retinal EC and RPE from Brown Norway rats which are not susceptible to EAU and to clarify these results. The number of factors thought to be involved in controlling susceptibilities to autoimmune conditions is continually increasing. The difference in level of constitutive and inducible ICAM-1 expression on endothelium from Lewis and PVG rats, may also be observed with other adhesion molecules, although no difference was apparent in VCAM-1 expression. Moreover, the less susceptible strains of rat are less immunoreactive in general, as demonstrated by the failure to successfully maintaining long term T cell lines from PVG rats. Therefore in Lewis rats it is likely that a number of predisposing factors, which are genetically linked, result in an overall increase in disease susceptibility.

The adhesion molecules involved in controlling lymphocyte migration across both sites of the blood-retinal barrier are similar. ICAM-1/LFA-1 interactions are the main known ligands involved in lymphocyte migration across normal retinal vascular endothelium (Greenwood et al., 1995) or, as has been shown in this study, the RPE. This is the first study to demonstrate VCAM-1 expression on RPE cells and that lymphocyte migration can be mediated via the VCAM-1/VLA-4 pathway. The difference in VCAM-1/VLA-4 mediated lymphocyte migration across RPE as opposed to migration across the retinal endothelial cells is that VCAM-1 expression on endothelial cells is sufficient after 24 hours (Greenwood et. al 1995) cytokine activation, whereas RPE cells have to be activated for 72 hours *in vitro*. The difference in time required for induction of VCAM-1 expression may be an explanation as to why leucocyte migration via the posterior barrier does not occur until the latter stages of the disease process.

The route the lymphocytes take through the barrier formed by the RPE, or the retinal vascular endothelium, is a matter of some contention despite several ultrastructural studies. There are two possible pathways for the lymphocyte, either through the junctions between the cells or through the cells of the blood-retinal barrier themselves. However, there is now increasing evidence for lymphocyte migration through endothelial cells

(Raine et al., 1990; Greenwood et al., 1994). There is also some *in vivo* evidence that this is the route of lymphocyte transepithelial migration through the RPE (Greenwood et al., 1994). Transmission electron micrographs of lymphocyte/RPE co-cultures, did not reveal any lymphocytes in the process of migrating, however, lymphocytes could be seen on the surface and below the monolayer, as well as within the RPE cells themselves (Figure 9.1). As lymphocytes were found apparently within the RPE cells suggests that passage across the posterior BRB may occur through RPE cells and not via the tight junctions. How lymphocytes enter the RPE is still unknown but may occur by phagocytic mechanisms which is one of the many functions of this cell.

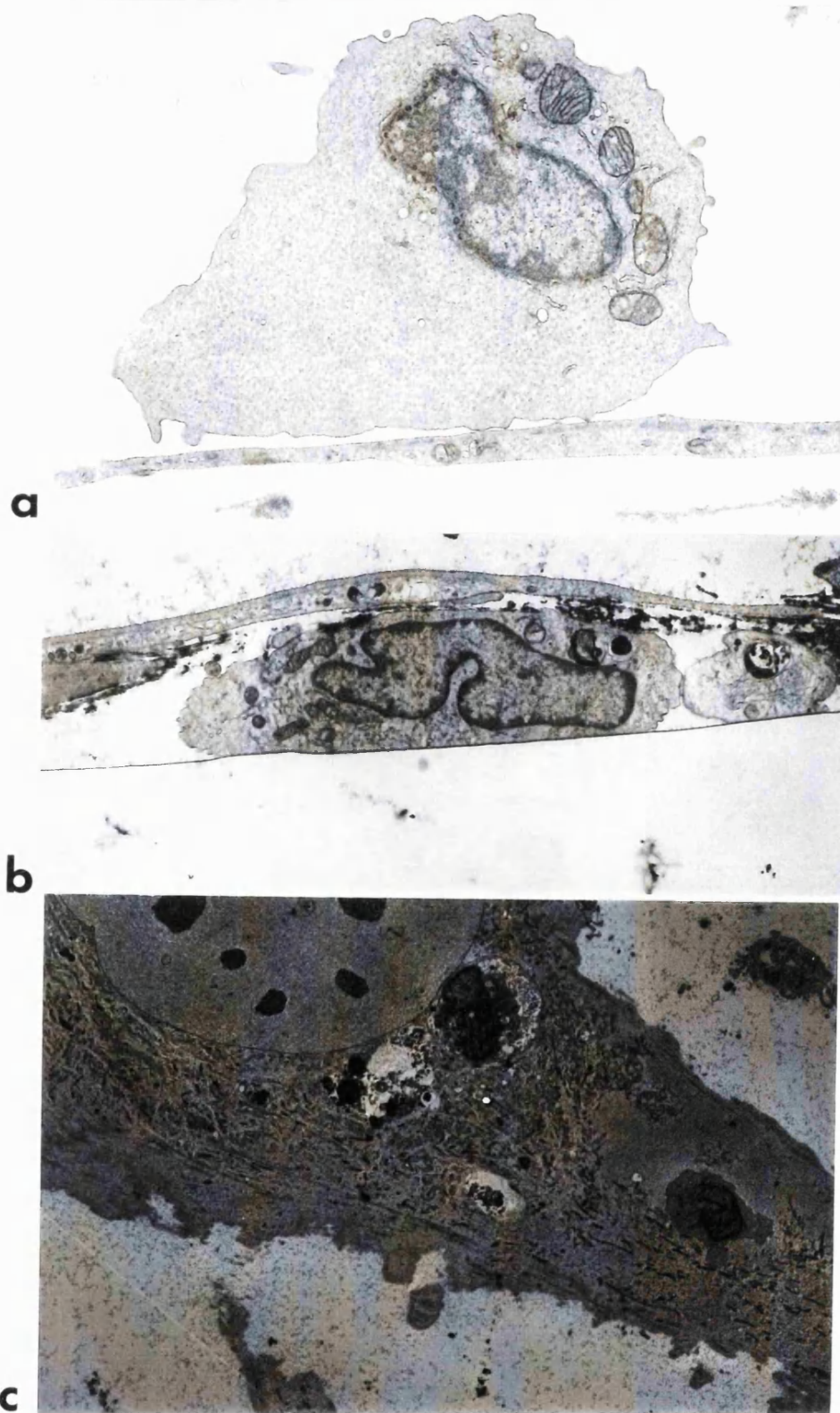
One important aspect of RPE function that was not investigated during the course of this study, was the ability of RPE cells to act as antigen presenting cells. The factors responsible for initiating the inflammatory response still remain unknown. Aberrant MHC class II expression on the cells of the BRB could activate CD4<sup>+</sup> T cells which may initiate, or potentiate the inflammatory response. Moreover, this is another possible explanation as to why the susceptibility of different strains of rat to EAU or EAE correlates with the level of MHC class II which is expressed on the endothelium following activation. However, as discussed previously, although these cells can readily be induced to express MHC class II, the evidence for their ability to act as antigen presenting cells is inconclusive. This is thought to be due the absence of secondary signals from the endothelium. The RPE may be better equipped to act as antigen presenting cells as they not only can express MHC class II, but also phagocytose rod outer segments which contain S-antigen and other potentially uveitogenic molecules as well as the necessary enzymes to process the antigens. There is one study which suggests that antigen presentation is the main function of the RPE during the development of EAU as destruction of the RPE resulted in a lower incidence of EAU and the disease was less severe (Konda et al., 1994). The ability of the RPE cells in this study to present antigen was not examined, primarily due to the low level of MHC class II found. However, further studies are being carried out using RPE cells isolated from PVG/Lewis hybrids. It would also be interesting to study the expression of B7 on RPE cells as this is the ligand for the co-receptor CD28 which has been shown to be important in T cell activation (Nickoloff and Turka, 1994). An additional molecule which may be expressed on RPE cells is CD95, or fas-ligand which is important in inducing apoptosis, or programmed cell death. This molecule has been shown to prevent graft rejection in the

testis and is thought to be important in establishing immune privileged sites (Bellgrau et al, 1995).

Isolation from pure cultures of RPE cells from rat eyes is an extremely difficult procedure and very few cells could be isolated from one eye, which therefore required the use of large litters to obtain sufficient cells. To facilitate research involving these cells, rat RPE cells were immortalised using SV40 large T. The resulting clones were characterised and found to express cytokeratins and RET-PE2, markers for epithelial and rat RPE cells respectively. The only notable difference between immortalised and primary culture RPE was that T cell line migration was lower across the immortalised cell monolayers. The reason for this is unclear, as the level of ICAM-1 was similar to that of primary cultures. However, the uniformity and closer packing of the immortalised cells may provide a greater physical barrier for the lymphocytes to migrate across. In support of this theory is the high level of transmonolayer electrical resistance observed with these cells. An other possible explanation, may be that although ICAM-1 mediated migration can occur, the ICAM-1 independent pathway thought to occur, does not.

One of the most exciting areas of research just now is gene therapy and immortalised cells are considered to be an important tool for this process. In relation to this, a separate study using the immortalised cells described has shown that the cells can integrate into the rat eye and form functional RPE monolayers when injected subretinally. This approach is being used as a potential therapy for the animal model of age related macular oedema, a condition which in humans is a major cause of blindness. The availability of these cells in the future should facilitate rat RPE research.





**Figure 10.1** Transmission electron micrographs of RPE /T cell line co-culture showing lymphocytes (a) above (x8000 magnification) and (b) below the monolayer (x4000 magnification) as well as (c) lymphocytes apparently within the RPE cell itself (x1000 magnification).

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**PUBLICATIONS ARISING FROM THIS WORK**

Devine L., Lightman S. and Greenwood J. (1996) Control of lymphocyte migration across the anterior and posterior blood-retinal barrier in vitro. *Cell. Immunol.* 168, 267-275.

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## Lymphocyte Migration across the Anterior and Posterior Blood-Retinal Barrier *in Vitro*

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The migration of lymphocytes through monolayers of rat retinal pigment epithelium (RPE) and retinal vascular endothelium, which form the posterior and anterior blood-retinal barrier (BRB) respectively, was investigated *in vitro*. After a 4-hr assay the migration of untreated peripheral lymph node (PLN) cells through RPE monolayers was negligible (<1%) with only a small increase found after activation of the PLN cells with concanavalin A or by cross-linking CD3. Activation of the RPE with IFN- $\gamma$  augmented migration with maximal PLN cell migration being achieved with a combination of CD3 cross-linking and IFN- $\gamma$  activation (17% migration). The highest level of lymphocyte migration was observed with three CD4<sup>+</sup> antigen-specific T cell lines specific for purified protein derivative (PPD; 33% migration), ovalbumin (OA; 31%), and S-antigen (S-Ag; 57%). Migration of both untreated and Con A-activated PLN cells through retinal endothelial cells (EC) from PVG rats was negligible, whereas the migration of the antigen-specific T cell lines was 23, 29, and 23% for PPD, OA, and S-Ag lines, respectively. Migration of these cell lines through retinal endothelium derived from Lewis rats was significantly greater (44% for PPD, 39% for OA, and 39% for S-Ag) which corresponded with a greater expression of ICAM-1 on the EC. © 1996 Academic Press, Inc.

### INTRODUCTION

The blood-retinal barrier (BRB) is located at two separate sites and is comprised of the retinal pigment epithelium (RPE) and retinal vascular endothelium which forms the posterior and anterior barrier, respectively. Under normal conditions, this barrier restricts the entry of molecules and cells into the neuroretina but during ocular inflammatory conditions, such as posterior uveitis, lymphocytes cross the BRB and enter the retina in large numbers (1, 2). In conjunction with this cellular infiltration, there is also frequent disruption

of the BRB (3) leading to retinal edema and associated loss of vision.

At present, the differential role played by the two barrier sites in lymphocyte infiltration into the neuroretina during inflammatory diseases remains largely unresolved. In general, lymphocyte migration into sites of inflammation depends on the interactions between molecules expressed on the surface of the vascular endothelium and the leukocyte. Within the retina, the vascular endothelial cell (EC) is in direct contact with circulating lymphocytes and interactions between these cells can directly control lymphocyte extravasation. The posterior barrier formed by the RPE, however, lies beyond the vasculature and within the cellular architecture of the eye. For lymphocytes to cross this cellular barrier they must first be recruited by the highly permeable choroidal (non-CNS) endothelium, enter the extracellular space, and penetrate Bruch's membrane. At the posterior barrier, therefore, lymphocyte recruitment is initially regulated by the choroidal endothelium which is known to be under the direct influence of the RPE through the production of cytokines such as interleukin-8 (4), monocyte chemotactic factor (5), and interleukin-6 (6, 7). These inflammatory mediators may induce adhesion molecules on the choroidal endothelia which will lead to an increase in leukocyte infiltration. Alternatively, they may assist in recruitment by binding to the luminal surface of the endothelia (8) or act as chemotactic gradients for leukocyte migration. Whatever additional role the RPE has in assisting in the recruitment of leukocytes from the choroidal circulation, to enter the neuroretina leukocytes must penetrate the RPE monolayer with their tight intercellular apical junctions. The degree of control afforded by the RPE in lymphocyte entry into the neuroretina, however, remains unclear. It is possible that the RPE utilize some of the cellular adhesion molecules which regulate lymphocyte migration through the retinal vascular endothelial barrier. If this is so, their expression and function is likely to be subject to modulation which would provide a degree of control over lymphocyte migration at this site.

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Previous studies have shown that the adhesion (9) and migration (10) of both resting and concanavalin A (Con A)-activated peripheral lymph node (PLN) cells across retinal EC monolayers isolated from Lewis rats is significantly less than that of IL-2-dependent antigen-specific T cell lines. Moreover, evidence indicates that the ability to migrate across an endothelial barrier both *in vitro* (10) and *in vivo* (11) is independent of antigen specificity. Antibody blockade studies suggest that the transmigration (diapedesis) of antigen-specific T cells through both retinal (12) and brain endothelia (manuscript in preparation) *in vitro* is controlled primarily by the leukocyte function-associated antigen-1 (LFA-1)/intercellular adhesion molecule-1 (ICAM-1) receptor pairing with the very late activation antigen-4 (VLA-4)/vascular cell adhesion molecule-1 (VCAM-1) pairing playing a role on activated EC. These studies have also indicated that with these antigen-specific IL-2-dependent cells, migration requires IL-2 stimulation (unpublished data).

Lymphocytes can adhere to cultures of rat (13) and human RPE cells (14, 15), but although this demonstrates that adhesive forces are able to operate between these cells *in vitro* the relevance of these interactions in lymphocyte migration across the barrier *in vivo* is unclear. In this study we have investigated the migration of lymphocytes through RPE monolayers and compared this with lymphocyte migration across primary cultures of retinal vascular endothelium.

## MATERIALS AND METHODS

**Reagents.** Rat recombinant IFN- $\gamma$  was obtained from Holland Biotechnology (Leiden, The Netherlands) and recombinant mouse IL-1 $\beta$  was obtained from R&D Systems (Oxford, UK). Monoclonal antibodies specific for rat CD54 (ICAM-1, 1A-29) (16), CD3 (IF4), rat endothelial cells (RECA-1) (17), and anti-mouse FITC antibodies were obtained from Serotec (Oxford, UK). Chromium-51 was purchased from Amersham International (Buckinghamshire, UK). The rat specific RPE antibody RET-PE2 was a generous gift from Professor C. J. Barnstable, Yale University (New Haven, CT) (18). All other reagents were obtained from Sigma (Dorset, UK).

**Retinal pigment epithelium.** Specific pathogen-free PVG rats (6–8 days old bred in-house) were used for the isolation of RPE cells according to a previously described method (19). Briefly, eyes were enucleated and the intact globes were digested with 2% dispase for 30 min. Following this incubation the eyes were then dissected with the aid of a dissection microscope and the cornea, lens, and vitreous were removed and discarded. The retina was then carefully isolated and incubated in culture medium for an additional 15 min, after which sheets of pigmented RPE cells could be sepa-

rated from the neuroretina. The culture medium consisted of Hams' F10 (Sigma), 20% fetal calf serum, (Gibco, Paisley, UK), 20 mM Hepes, 7.5% sodium bicarbonate, 2 mM glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (all from Sigma). The sheet of RPE cells was then trypsinized to obtain a suspension of single or small clumps of cells which were then plated out and grown to confluence in 24-well plates for migration assays, 96-well plates for adhesion assays, and flasks for flow cytometry. Primary cultures were used throughout.

The effect of activating the RPE monolayers with IFN- $\gamma$  and IL-1 $\beta$  on lymphocyte adhesion and migration was investigated as follows. In the adhesion assay, RPE cells were stimulated for 24 hr prior to the assay with a range of concentrations of either IFN- $\gamma$  (1–200 U/ml) or IL-1 $\beta$  (0.05–100 U/ml). The effect on migration was investigated by pretreating the RPE for 24 hr with either IFN- $\gamma$  (100 U/ml) or IL-1 $\beta$  (5 U/ml).

**Endothelial cells.** Retinal microvessels were isolated from both specific pathogen-free PVG (bred in-house) and Lewis rats (Charles River, Kent, UK) using a previously described method (20). Briefly, retinas were removed and digested in collagenase/dispase for 1 hr. Microvessel fragments were isolated from contaminating single cells by density centrifugation, further enzymatic digestion and separation on a preformed 50% Percoll gradient. The isolated microvessels were then plated out and grown to confluence on collagen coated flasks for flow cytometric studies or 24-well culture plates for use in the migration assay. The culture medium comprised of Hams' F10 medium supplemented with 7.5  $\mu$ g/ml endothelial cell growth supplement, 80  $\mu$ g/ml heparin, 2 mM glutamine, 0.5  $\mu$ g/ml vitamin C, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin and 17.5% plasma-derived serum (Advanced Protein Products, West Midlands, UK). Primary cultures were used throughout.

**Lymphocytes.** Terminally differentiated, interleukin-2 (IL-2)-dependent CD4<sup>+</sup> T cell lines specifically recognizing the nonocular irrelevant antigens, purified protein derivative (PPD), and ovalbumin (OA) or the ocular antigen, retinal soluble antigen (S-Ag), were prepared using a previously described protocol (21). T cell lines were obtained from Lewis rats because PVG rat cell lines proved difficult to maintain. However, for purpose of comparison enough PVG T cell lines were produced to allow for a few migration assays to be carried out to ensure that the MHC differences did not affect the migration. The use of nonsyngeneic T cells was not considered to have a bearing on the outcome of the short adhesion and migration assays used. Antigen specific T cell lines were grown at  $2 \times 10^5$ /ml in the presence of IL-2 and restimulated weekly with antigen (PPD, OA, or S-Ag at 10  $\mu$ g/ml), using 50-fold irradiated autologous thymocytes as feeder cells. Three days after

antigen restimulation, dead feeder cells were removed by density centrifugation over metrizoate-Ficoll followed by culture in the presence of purified recombinant IL-2. The cell lines were used between passages and no earlier than the third passage, with no functional differences being observed between them. The cells were found to be CD4<sup>+</sup>, CD45Rc<sup>low</sup>, and CD25<sup>+</sup> (unpublished observations). These T cell line lymphocytes are highly migratory (10) exhibiting maximal migration between 3 and 5 days post-Ag stimulation (unpublished observations).

Peripheral lymph node (PLN) cells from PVG rats were isolated from cervical and mesenteric lymph nodes as described previously (22) and were suspended in RPMI containing 10% FCS. The PLN cells were used either as untreated cells or were activated by (i) 5 µg/ml Con A for 1 to 4 days, (ii) Con A for 1 day followed by 3 days in the presence of exogenous IL-2, (iii) Con A for 3 days and IL-2 for an additional 3 days, (iv) Con A for 24 hr followed by phorbol myristate ester (PMA) (10 ng/ml) for 10 min, and (v) CD3 cross-linking by coating a tissue culture flask with CD3 monoclonal antibody at 5 µg/ml (for up to 72 hr). PLN cells were washed thoroughly to remove any residual mitogen before being added to RPE cells.

**Flow cytometry.** To examine the purity of RPE cultures the cells were investigated for their expression of the rat RPE-specific cell-surface antigen, RET-PE2, (generous gift from C. J. Barnstable, Yale University) (18). The RET-PE2 antibody was added directly to unfixed cells, which were obtained as a single cell suspension using collagenase/dispase, for 45 min over ice. The cells were then washed in phosphate-buffered saline (PBS) and incubated with an anti-mouse-FITC conjugate (1:100) for 30 min. Positive staining was determined using flow cytometry (FACScan, Becton-Dickinson, Oxford, UK).

The purity of retinal EC cultures was also assessed by flow cytometry, as described above, using the RECA-1 antibody, which is specific for rat EC (17).

Using flow cytometry, the expression of the adhesion molecule ICAM-1 on both untreated and activated RPE and Lewis or PVG-derived endothelial cultures was also determined using the rat anti-ICAM-1 antibody 1A-29. RPE cells were activated with 100 U/ml IFN-γ and retinal endothelial cells were activated with 5 U/ml IL-1β for 24 hr.

**Adhesion assay.** The adhesion assay was carried out using a well-characterized method (9, 22, 23). Lymphocytes were washed three times before labeling with Hanks' balanced salt solution (HBSS) before labeling with 3 µCi chromium-51 (<sup>51</sup>Cr) per 10<sup>6</sup> cells for 90 min at 37°C. Following this incubation cells were washed twice to remove excess <sup>51</sup>Cr and then resuspended in RPMI containing 10% FCS. RPE monolayers were washed four times and 2 × 10<sup>5</sup> lymphocytes were added

to each well and incubated for 2 hr at 37°C. At the end of the incubation time, nonadherent cells were washed off from the four poles of the wells with HBSS warmed to 37°C. Adherent cells were lysed with 2% SDS and the γ emissions of the lysate counted. Each adhesion assay was repeated three times and the data are expressed as the mean (± standard error of mean).

**Migration assay.** PLN cells or antigen-specific T cells 3–5 days post-antigen stimulation (2 × 10<sup>5</sup> cells/well in 1 ml) were suspended in culture medium and added to primary cultures of RPE or retinal EC monolayers. The coculture was then placed onto the stage of an inverted phase-contrast microscope in a temperature- and CO<sub>2</sub>-controlled environment (37°C and 5% CO<sub>2</sub>). To investigate the kinetics of migration the interactions were recorded using time-lapse videomicroscopy over a period of 4 hr. The percentage of cells which had migrated through the monolayer was then calculated at 30-min time points by replaying the tape 160× normal speed. The cells above and below the monolayer could easily be distinguished by their morphology and phase-contrast appearance (10). Migration assays were carried out a minimum of six times and the data are expressed as the mean (± standard error of mean).

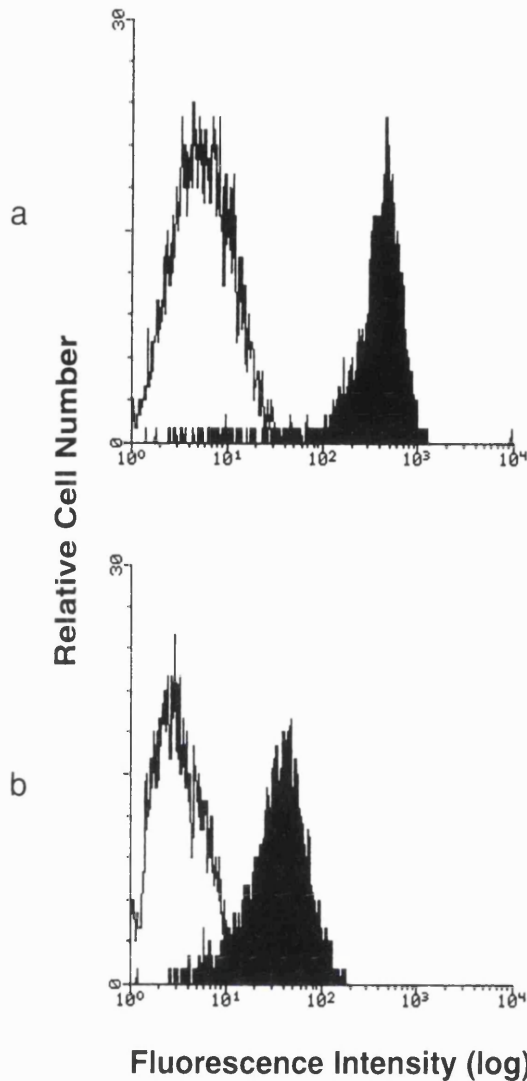
## RESULTS

**Cell cultures.** The purity of the primary cultures of RPE and retinal endothelial cells was determined by both morphological and flow cytometric examination. RPE cells were roughly hexagonal, pigmented, and were positive for the rat specific RPE antibody RET-PE2 as determined by flow cytometry (Fig. 1a).

The retinal endothelial cells were spindle-shaped and contact-inhibited. These cells which have previously been thoroughly characterized (20) stained positive with the rat endothelial cell-specific antibody RECA-1 (Fig. 1b).

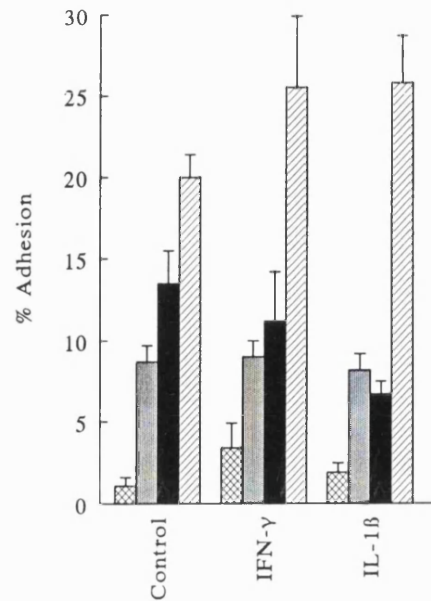
**Lymphocyte adhesion to RPE monolayers.** The level of adhesion of resting PLN cells to untreated RPE was very low with only 1.1 ± 0.5% of cells adhering (Fig. 2). Activation of PLN cells with Con A or with anti-CD3 antibody significantly increased this adhesion to 8.7 ± 1 and 13.2 ± 0.8%, respectively (*P* < 0.01, *t* test). There was no significant difference in the adhesion of PLN cells activated with either Con A or anti-CD3. The adhesion of the PPD-specific T cell line lymphocytes derived from Lewis rats, however, was much greater with 20 ± 1.4% of cells adhering. Activation of the RPE cells with the cytokines IFN-γ (1–200 U/ml) or IL-1 β (0.05–100 U/ml) did not significantly increase lymphocyte adhesion at any of the concentrations used (results shown for 100 and 5 U/ml, respectively).

**Lymphocyte migration across RPE monolayers.** As with lymphocyte adhesion, the level of migration of



**FIG. 1.** Flow cytometric analysis of (a) RPE cells stained with the rat RPE-specific monoclonal antibody RET-PE2 and (b) retinal endothelial cells stained with the rat endothelial-specific monoclonal antibody RECA-1. The controls (left-hand open histograms) were set using FITC-labeled RAMIG.

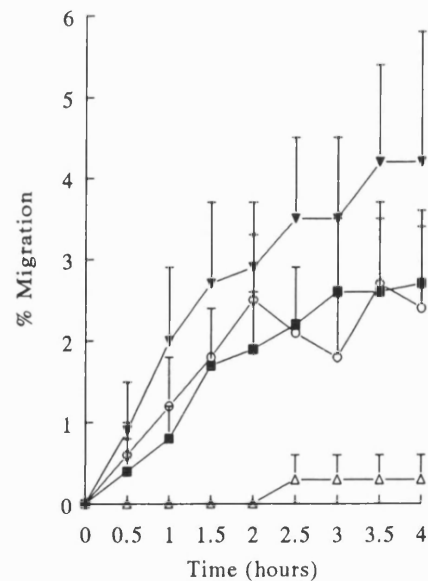
resting PLN cells through RPE monolayers was minimal with only  $0.8 \pm 0.3\%$  of cells crossing the monolayer by 4 hr. Activation of PLN cells with Con A for 24 hr caused a small, but significant, increase in migration to  $4.2 \pm 1.6\%$  ( $P < 0.01$ ,  $\chi^2$  analysis), although increasing the duration of activation to 48 or 72 hr did not further increase migration (Fig. 3). However, when antigen-specific T cell lines from Lewis rats were added to RPE monolayers, the percentage of cells which migrated was much greater with  $32.9 \pm 4.2\%$  of PPD-specific T cells,  $30.9 \pm 3.1\%$  of OA-specific T cells, and  $57 \pm 4.9\%$  of S-Ag-specific T cells crossing the RPE by 4 hr (Fig. 4). The migration of S-Ag-specific T cells was significantly greater than that of either PPD- or OA-specific T cells ( $P < 0.01$ ,  $\chi^2$ ). There was no significant difference in the migration across PVG RPE mono-



**FIG. 2.** The percentage adhesion of untreated PLN cells (cross-hatched bars), Con A-activated PLN cells (shaded bars), CD3-activated PLN cells (solid bars), and PPD-specific T cell line lymphocytes (hatched bars) to untreated, IFN- $\gamma$ -activated (100 U/ml) and IL-1 $\beta$ -activated (5 U/ml) RPE monolayers.

layers of PPD T cell line lymphocytes derived from either PVG or Lewis rats ( $35.9 \pm 6.7\%$  compared with  $32.9 \pm 4.2\%$ , respectively).

The presence of exogenous IL-2 after 24-hr Con A activation of PLN only caused a significant increase in migration after 3 days ( $P < 0.01$ ) (Fig. 5a) compared



**FIG. 3.** Percentage migration over a 4-hr period of untreated PLN cells (open triangles) and PLN cells activated with Con A for 24 hr (solid triangle), 48 hr (solid square) and 72 hr (open circles) across untreated RPE monolayers.

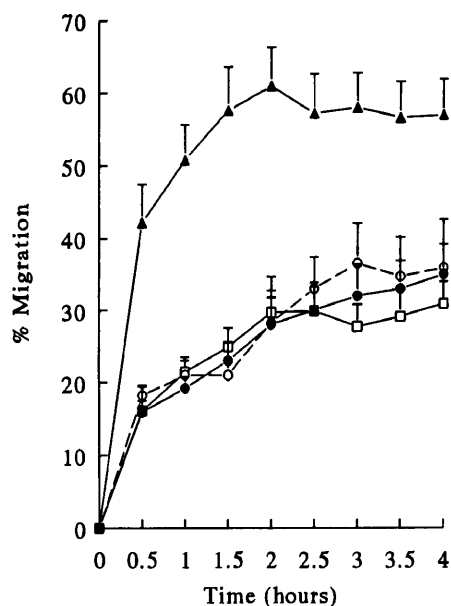


FIG. 4. The percentage migration over a 4-hr period of antigen-specific T cell line lymphocytes across untreated PVG-derived RPE monolayers. The PPD-specific T cell line (solid circles), OA-specific T cell line (open squares), and S-Ag T cell line (solid triangle) were all derived from Lewis rats. The PPD-specific T cell line (open circles with broken line) were derived from PVG rats.

with an identical time course in the absence of IL-2 (Fig. 5b). In an attempt to mimic antigen stimulation, cells were activated with Con A for 72 hr and then maintained in IL-2-containing medium for an additional 72 hr. However, this also failed to significantly increase the level of migration (Fig. 5c), although the

migration of PLN cells stimulated with IL-2 for 1 day after Con A activation for 72 hr was significantly greater than Con A activation for 72 hr alone ( $P < 0.05$ ). Moreover, stimulation of Con A-activated PLN cells with PMA for 10 min did not increase migration (data not shown).

The possibility that activation of PLN cells via the CD3 complex can increase migration was also investigated as this forms part of the T cell receptor complex. However, under the described conditions the level of migration observed after 4 hr was not significantly greater than that with activation by Con A (Fig. 6).

Activation of RPE monolayers with IFN- $\gamma$  (100 U/ml) increased lymphocyte migration significantly in all cases ( $P < 0.01$ ), whereas activation with IL-1 $\beta$  (5 U/ml) only increased the migration of untreated PLN cells ( $P < 0.01$ ) and CD3-activated PLN cells ( $P < 0.05$ ) over 4 hr (Fig. 6).

*Lymphocyte migration across retinal endothelial monolayers.* Both untreated and Con A-activated PLN cells displayed negligible migration through untreated PVG retinal EC monolayers. Conversely, the antigen-specific T cell lines were all highly migratory with  $22.6 \pm 4.9\%$  of PPD-specific lymphocytes,  $29.1 \pm 2.8\%$  of OA-specific lymphocytes, and  $22.5 \pm 3.1\%$  of S-Ag-specific lymphocytes migrating after 4 hr (Fig. 7a). The migration of these same antigen-specific lymphocytes across Lewis retinal EC, however, was different in that the migration of the PPD ( $44 \pm 2.8\%$ ), OA ( $38.6 \pm 5.6\%$ ), and S-Ag ( $40.2 \pm 4.4\%$ ) T cell lines were significantly greater (all  $P < 0.01$ ) (Fig. 7b).

There was no difference in the migration of the PPD

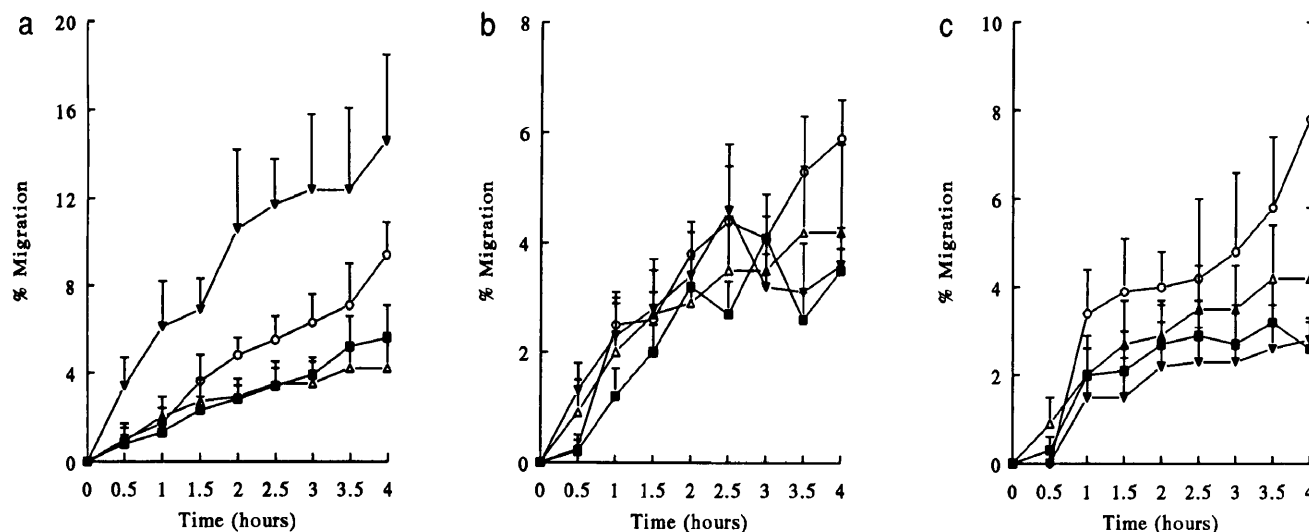
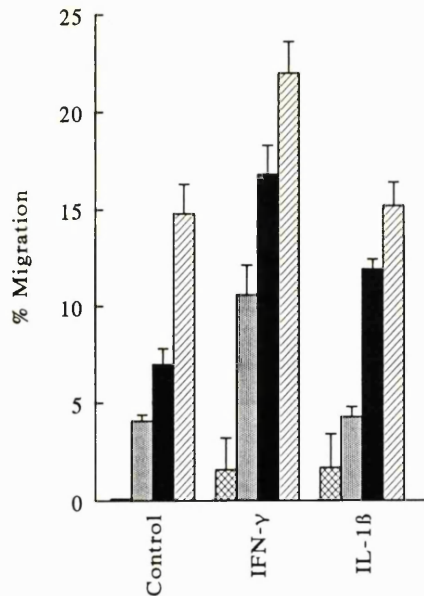


FIG. 5. The percentage migration of PLN cells under different activation conditions across RPE monolayers. (a) PLN cells activated with Con A for 24 hr (open triangles), activated with Con A for 24 hr, and then maintained in medium containing IL-2 for 24 hr (open circles), 48 hr (solid squares), and 72 hr (solid triangles). (b) PLN cells activated with Con A for 24 hr (open triangles), activated with Con A for 24 hr, and then maintained in the absence of exogenous IL-2 for 24 hr (open circles), 48 hr (solid squares), and 72 hr (solid triangles). (c) PLN cells activated with Con A for 72 hr (open triangles), activated with Con A for 72 hr prior to culture in medium with IL-2 for 24 hr (open circles), 48 hr (solid squares), and 72 hr (solid triangles).



**FIG. 6.** The percentage migration of untreated PLN cells (cross-hatched bars), Con A-activated PLN cells (shaded bars), CD3-activated PLN cells (solid bars), and PPD-specific T cell line lymphocytes (hatched bars) through untreated, IFN- $\gamma$ -activated (100 U/ml) and IL-1 $\beta$ -activated (5 U/ml) RPE monolayers after a 4-hr coculture.

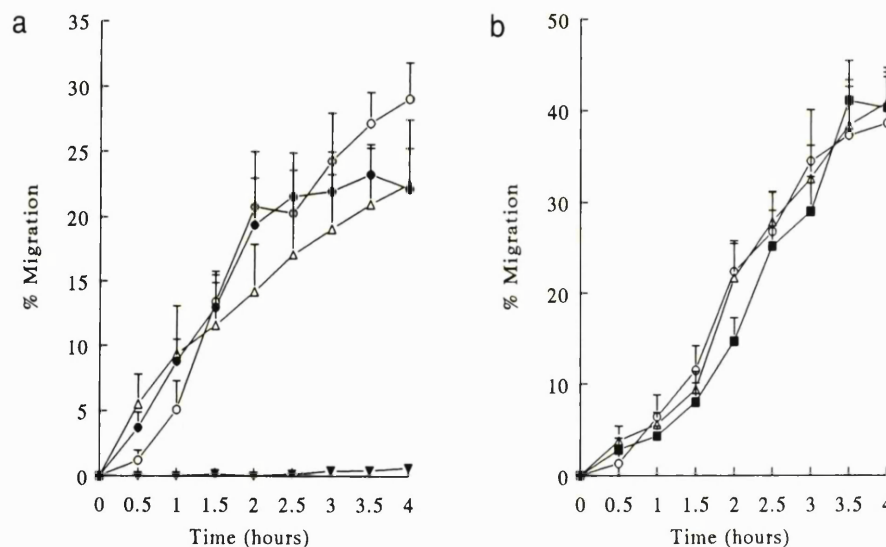
or OA T cell line lymphocytes across PVG RPE or retinal EC at the 4-hr time point, although the rate at which this migration occurred differed because at the 30-min time point the migration across RPE monolayers was significantly greater ( $P < 0.001$ ). The migration of the S-Ag-specific T cell line was greater across

RPE compared with retinal EC at all time points throughout the assay ( $P < 0.001$ ).

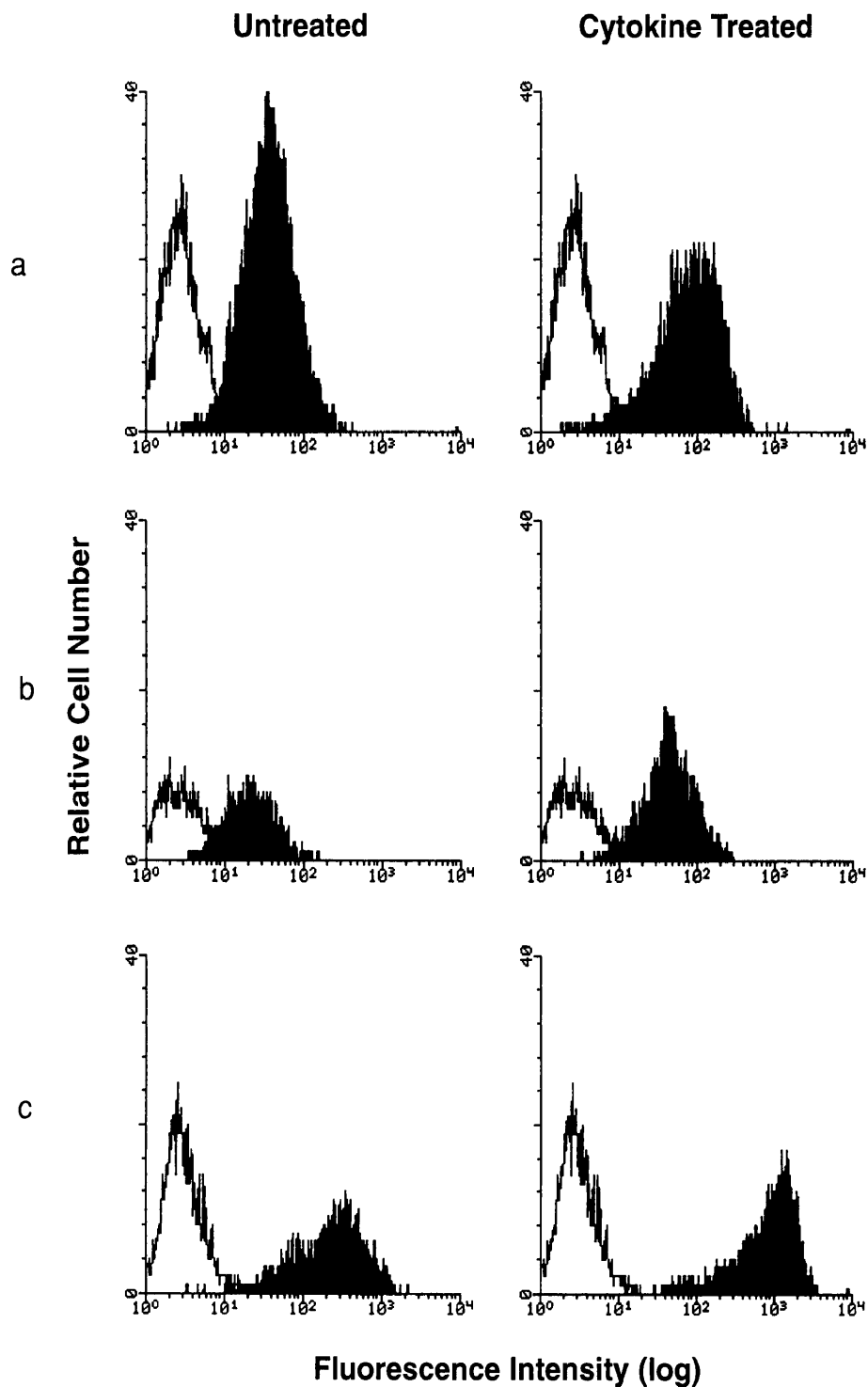
*ICAM-1 expression on PVG RPE and PVG and Lewis retinal EC.* All cells examined constitutively expressed ICAM-1 which was upregulated upon activation with cytokines. However, the expression was lower on PVG than Lewis retinal endothelium and the upregulation after IL-1 $\beta$  activation was less (Fig. 8). The increase in ICAM-1 expression on RPE cultures following cytokine stimulation was also less than with endothelium.

## DISCUSSION

These results demonstrate that, as previously reported with retinal endothelial cells (9, 10), the mode of activation of lymphocytes is critical in determining their ability to adhere to and migrate across RPE monolayers. Activation of PLN cells with Con A results in a small but significant increase in both adhesion and migration, an effect which was only seen with lymphocyte adhesion (4), and not migration through retinal EC monolayers (10). However, the level of adhesion and migration of Con A-activated PLN cells is very low in comparison to that found with antigen-specific T cell line lymphocytes. With the adhesion studies, however, caution must be taken in interpreting the results of the T cell lines because this assay does not discriminate between adherent and migrated cells and thus much of the apparent adhesion is likely to be due to cells that have already migrated. An additional potential



**FIG. 7.** The percentage migration over a 4-hr period of antigen-specific T cell line lymphocytes across untreated retinal endothelial cell monolayers. (a) Migration of PPD-specific (open triangles), OA-specific (open circles), and S-Ag-specific (solid circles) T cell line lymphocytes and Con A-activated (24 hr) PLN cells (filled triangles) through untreated retinal endothelial monolayers derived from PVG rats. (b) The migration of PPD-specific (open triangles), OA-specific (open circles), and S-Ag-specific (solid squares) T cell line lymphocytes through untreated retinal endothelial monolayers derived from Lewis rats.



**FIG. 8.** Flow cytometric analysis of ICAM-1 expression. (a) Untreated and IFN- $\gamma$ -activated (100 U/ml 24 hr) RPE derived from PVG rats. (b) Untreated and IL-1 $\beta$ -activated (5 U/ml, 24 hr) retinal endothelial cells derived from PVG rats. (c) Untreated and IL-1 $\beta$ -activated (5 U/ml, 24 hr) retinal endothelial cells derived from Lewis rats. The controls (left-hand open histograms) were set using FITC-labeled RAMIG.

problem is the possibility that Con A bound to the surface of the lymphocyte is also binding to mannose residues on the RPE cell surface and producing higher levels of apparent adhesion. We have previously addressed this issue in adhesion and migration studies

with retinal endothelia (9, 10). Pretreatment of Con A-activated PLN cells with 0.1 M  $\alpha$ -methyl mannose to block the lectin binding sites did not affect the level of adhesion to untreated retinal EC ( $14.3 \pm 1.2\%$ ) compared with Con A-activated controls ( $11.3 \pm 1.1\%$ ). Fur-

thermore, in a separate study investigating lymphocyte adhesion to brain EC it was reported that Con A treatment of red blood cells did not cause an increase in binding (22).

A major technical problem associated with this study arises from the necessity of using RPE derived from pigmented PVG rats because visual identification of the RPE is required during the isolation procedure. The production and maintenance of PVG T cell lines, however, proved problematic because they consistently failed to respond to IL-2 and antigen stimulation after 2–3 passages and so T cell lines derived from Lewis rats were mainly employed. By demonstrating that there was no difference in the level of migration between PPD-specific T cells derived from either PVG or Lewis rats across PVG RPE monolayers suggests that the use of allogeneic T cells during this short assay is valid.

It has previously been reported that IFN- $\gamma$  is produced within the retina during EAU and that this cytokine is likely to play a significant role in the pathogenesis of the disease *in vivo* (24). Activation of both brain (25) and retinal endothelium (9) with IFN- $\gamma$  has been shown to enhance lymphocyte adhesion. However, studies investigating the adhesion of lymphocytes to cultured RPE cells have provided conflicting results. Human RPE cells activated with IFN- $\gamma$  increase their ability to bind lymphocytes (14, 15), whereas in the rat our results and those of Mesri *et al.* (13) indicate that IFN- $\gamma$  has no effect. The relevance of adhesion studies to cells that are not involved in capturing circulating cells is questionable. What these studies are likely to provide us with is an indication of whether cell/cell adhesive interactions exist between these two cell types and whether they are subject to modulation. The activation of RPE monolayers with IFN- $\gamma$  caused a significant increase in migration which was not observed with IFN- $\gamma$ -activated retinal EC (10). IL-1 $\beta$  activation, however, only increased the migration of untreated PLN cells and PLN lymphocytes activated via CD3 cross-linking. In both this and previous studies (12), IL-1 $\beta$  did increase the migration of antigen-specific T cells across retinal endothelium which may indicate a difference in the response of endothelial and epithelial cells. Indeed, similar differences have also been reported in a study comparing lymphocyte interactions with endothelium and epithelium from non-CNS tissue (26).

Antigen specificity has previously been shown to be irrelevant in determining the ability of lymphocytes to cross CNS endothelium (10, 11), although our results suggest that migration through RPE monolayers is dependent on antigen specificity. The higher levels of migration observed by the S-Ag T cell line across RPE compared to that of either the PPD or OA T cell line is unlikely to be due to the ocular specificity of the S-Ag T cell line. We believe that a more plausible explanation is that this is a function of the greater antigenicity

of S-Ag, resulting in lymphocyte lines that are more activated and hence more migratory. Indeed, the cytokine profile of the S-Ag T cell line differs from that of the PPD T cell line in that it produces greater amounts of IFN- $\gamma$  (V. Calder, personal communication). The reason why a differential ability to migrate is not observed with the endothelial monolayers is that the rate-limiting factor may be due to functional aspects of the endothelium which limits the degree of migration. On the RPE these factors might not be rate limiting thus allowing for differences in the migratory ability of the T cell lines to be observed. For example, retinal endothelial cells express lower levels of ICAM-1 than RPE and as this molecule is central to the process of migration across retinal EC (12), it may be one of the limiting factors involved.

To ascertain whether PLN cells could be activated to become as migratory as antigen-specific T cell line cells, we stimulated PLN cells in a variety of ways. In a separate study we have shown that IL-2 activation is important in the migration of T cell line cells across brain endothelial cell monolayers (unpublished data). Because Con A activation alone failed to induce a marked increase in PLN cell migration across RPE monolayers, we investigated whether further stimulation with exogenous IL-2 would enhance their migration. Despite using several combinations of Con A and IL-2 stimulation we were unable to elicit an increase in migration to a level comparable with the T cell lines. Thus it appears that at least one other signal is required to trigger lymphocyte migration.

It has been shown that stimulation of Con A lymphocytes with PMA increases their adhesion to brain microvascular endothelium due to the enhanced avidity of LFA-1 and VLA-4 (27). However, this did not increase the migration of Con A-activated PLN cells across RPE monolayers. We also considered the possibility that stimulating PLN cells via the CD3 receptor may act more like antigenic stimulation and subsequently increase their ability to migrate. However, there was no significant difference in the ability of PLN cells to migrate when they were activated with Con A or via the CD3 receptor. This implies that activation via the CD3 complex under these conditions is also unable to deliver all the signals required to trigger lymphocyte migration.

The level of lymphocyte migration across PVG retinal endothelium was much lower than that observed with Lewis retinal endothelium. A number of factors have previously been suggested for the observed differences in susceptibility of animal strains to the experimental model for uveitis, experimental autoimmune uveoretinitis (EAU), and the experimental model for multiple sclerosis, experimental allergic encephalomyelitis, (EAE). These include differences in the inducibility of MHC class II on nonimmune cells (28–31) and their ability to produce inflammatory cytokines (32). This



study indicates for the first time that there is also a difference in the capacity of lymphocytes to migrate across endothelium from different strains which correlates with the responsiveness of the strain to EAU. Thus, a lower level of migration was observed with PVG retinal EC, a strain which is a medium responder, than with the Lewis retinal EC which is a high responder. These differences also correlate with lower constitutive expression of ICAM-1 on PVG compared with Lewis retinal endothelium, a finding that has also been reported for brain endothelium (30).

The main difference in lymphocyte migration across RPE compared with retinal EC is the rate at which migration occurs. Large numbers of lymphocytes cross the RPE monolayer during the first 30 min of coculture, reaching a plateau by 2 hr. Migration across the retinal EC, however, is slower, with few cells migrating during the first 1.5–2 hr, but increasing during the remainder of the assay. This may be due to the requisite molecules on the RPE already being present and in the correct configuration for migration whereas with retinal EC, T-cell-derived cytokines may be required to induce, upregulate, or bring about qualitative changes in these molecules.

Work carried out in our laboratory has shown that the migration of T cell line cells across both brain (manuscript in preparation) and retinal microvessel endothelium (12) is predominantly ICAM-1/LFA-1 dependent. Both human (14, 15) and rat (13) RPE cells have been shown to constitutively express ICAM-1 which can be upregulated with IFN- $\gamma$ . It is possible, therefore, that ICAM-1 may also be involved in mediating and controlling lymphocyte migration through the cellular barrier formed by the RPE.

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