ENDOTHELIAL CELL ADHESION MOLECULES: EXPRESSION, UPREGULATION AND MODULATION OF HUMAN BRAIN MICROVESSEL ENDOTHELIAL CELL-LEUKOCYTE INTERACTIONS

by

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ABSTRACT

Endothelial cells (EC) lining the cerebral blood vessels are responsible for the formation and maintenance of a unique structural and functional barrier, the blood-brain barrier (BBB), that normally prevents the movement of white blood cells from blood into brain. Inflammatory and infectious diseases of the central nervous system (CNS), including multiple sclerosis, encephalitis, meningitis and infarction, are characterized by increased BBB permeability and infiltration of the brain by leukocytes. The mechanisms that regulate the traffic of leukocytes across the BBB have not yet been elucidated. A group of EC surface molecules called adhesion molecules, have been implicated in interactions between inflammatory cells and extracerebral EC. They include E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and platelet/endothelial cell adhesion molecule-1 (PECAM-1). Blocking these molecules with antibodies (Abs) reduces leukocyte traffic across EC. One of these molecules, ICAM-1, has been demonstrated in inflammatory CNS lesions.

The objective of this study was to investigate the expression and function of four EC adhesion molecules in an *in vitro* model of the human BBB. The results indicate that primary cultures of unstimulated human brain microvessel EC (HBMEC) form high resistance monolayers and express low levels of E-selectin, VCAM-1 and ICAM-1 that can be significantly upregulated following stimulation with cytokines and LPS. PECAM-1 is strongly expressed by all cells constitutively. Treatment of HBMEC with TNF increases monolayer permeability, and augments adhesion and migration of polymorphonuclear leukocytes (PMN) and T-lymphocytes across the monolayers. Adhesion of resting T-lymphocytes to activated endothelium is mediated by VCAM-1 and ICAM-1. ICAM-1, E-selectin and PECAM-1 participate in T-lymphocyte migration across HBMEC monolayers. Adhesion of PMN to activated HBMEC is mediated by ICAM-1 and E-selectin, while ICAM-1 is involved in the migration process.

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increased endothelial permeability secondary to leukocyte migration. These studies indicate that EC adhesion molecules, induced by cytokines, mediate cerebral EC-leukocyte interactions which, in association with a concurrent increase in endothelial permeability, may facilitate leukocyte traffic across the BBB in the early stages of CNS inflammation.

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LIST OF ABBREVIATIONS

Abantibody
AGEPC
ANOVA analysis of variance
BBB blood brain barrier
BBMEC bovine brain microvessel endothelial cells
CNS central nervous system
CSF
DAB
EAE experimental allergic encephalomyelitis
EC endothelial cell
EGFepidermal growth factor
ELAM endothelial leukocyte adhesion molecule
EM electron microscopy
fMLP formyl-methionyl-leucyl phenylalanine
fMLPformyl-methionyl-leucyl phenylalanineHBMEChuman brain microvessel endothelial cell
fMLP formyl-methionyl-leucyl phenylalanineHBMEC
fMLPformyl-methionyl-leucyl phenylalanineHBMEChuman brain microvessel endothelial cellHDMEChuman dermal microvessel endothelial cellHECAhuman endothelial cell antigen
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LFA
LPS
LTB ₄ leukotriene-B ₄
mAb monoclonal antibody
MECA mouse endothelial cell antigen
MHCmajor histocompatibility complex
MS multiple sclerosis
NK natural killer
PBMC peripheral blood mononuclear cells
PECAM platelet/endothelial cell adhesion molecule
PMN polymorphonuclear leukocyte
SEM scanning electron microscopy
TEM transmission electron microscopy
TNF
VAP vascular adhesion protein
VCAM vascular cell adhesion molecule
VLA very late activation antigen

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CHAPTER 1

INTRODUCTION

Inflammation is a set of dynamic events that occurs in living tissue in response to injury or infection. The term "inflammation" is derived from the Greek words <u>phlogosis</u> and <u>phlegmoné</u>, meaning "heat" and "the burning thing", respectively, and the Latin <u>flamma</u> meaning fire (Ryan and Majno, 1977; Rocha e Silva, 1978). This phenomenon has been recognized and studied from the earliest times.

1.1 HISTORY OF INFLAMMATION

Inflammation is a concept known as early as the Egyptian times. At approximately 1650 B.C., the <u>swnw</u> (physician) described the treatment for a gaping wound using the term "inflammation". At the time of the Sumerians in Mesopotamia, the chief physician to King Esharaddon (680-669 B.C.) referred to "inflammation" several times in his writing (Majno, 1975). By the time of Hippocrates (460-380 B.C.), the Greeks had reasoned that a possible site of disease is the venules/small veins which could allow blood to escape by sheer fatigue or falling into a "spasm". The latter would tear the venule apart. These theories are not completely out of line with present observations. Electron microscopy shows that irritated venules do, in a sense, tear themselves apart. Chemical mediators cause endothelial cells (EC) lining these vessels to retract and pull away from one another, allowing fluid to escape (Majno, 1975).

The clinical symptoms of inflammation were not formally defined until Cornelius Celsus (approximately 10-40 A.D.) stated in his book "De Medicina" that "the signs of an inflammation are four: rubor et tumor cum calore et dolore" (redness and swelling with heat and pain) (Majno, 1975). Long before that, the Hippocratic treatises mentioned that pain, local heat and swelling are characteristics of a particular type of pathologic alteration (Mettler, 1947).

The fifth cardinal sign of inflammation <u>functio laesa</u> or "disturbed function" is often attributed to Galenos (130 A.D.), although no such sign was mentioned in any of his works (Rather, 1971). It appears that the author of a German Handbook of Pathology incorrectly attributed this in 1919. This idea quickly became widely accepted and became general knowledge. It was, in fact, Rudolf Virchow who first added this sign in his book "Cellular Pathology" in 1858 (Majno, 1975).

With the invention of the microscope, Herman Boerhaave (1668-1738) observed blood vessels in inflamed tissue and concluded that the constriction of the finest vessels led to the generation of local heat and fever through the friction between blood and the vessels. The dominant role of the finest blood vessels is emphasized here (Eisen, 1977). Later, others found that dilated rather than constricted microvessels (arterioles, venules and capillaries) were present in inflamed tissue (Eisen, 1977).

In 1867, Samuel also concluded that inflammation centered around "a lesion of the vessels, by irritating agents, in such a way that the inflamed vessels were rendered more permeable to liquids and blood cells, in a passive way", thus, producing exudate that would be collected at the site of minor resistance, resulting in inflammatory tumor (Rocha e Silva, 1978). More observations on early vascular events were provided by Cohnheim and Metchnikoff. In addition to supporting the above view of the changes in the vessels, Cohnheim observed that the microvessels undergo a brief period of dilatation. The blood flow first accelerates, then slows down. Newly opened capillaries become visible. White blood cells then begin to line these vessels and eventually force themselves through the vessel wall into the extravascular spaces. In some vessels, flow stop completely and red blood cells are tightly packed together as if plasma has escaped because of the increased permeability of the vessels (Cohnheim, 1890).

Further support for these views came from Weigert who confirmed the blood origin of the cells in the inflammatory exudate and Arnold who demonstrated the process of diapedesis (Rocha e Silva, 1978). Metchnikoff, on the other hand, saw inflammation

not as a disease of the blood vessels but as a protective mechanism to bring phagocytes to the injured areas to ingest and digest damaged cells, bacteria and other particles to be removed, a process he called "phagocytosis" (Rocha e Silva, 1978). Concurrently, the discovery of antibodies (Abs) brought another role to the inflammatory process - to allow Abs to flood the infected tissue for local defense.

Thus, it is clear that the microcirculation is of paramount importance in inflammation.

1.2 HISTORY OF THE MICROCIRCULATION

The idea of microvessels extends far back to the Egyptian times. At about 250 B.C., an anatomist, Erasistratos believed that blood moves from veins into the arteries through invisible "synanstomoses". It is these finest branches of arteries and veins along with nerves and parenchyma that make up tissues. In 1628, William Harvey discovered the circulation of blood. He reasoned that microvessels are present only in order to keep the circulation going. Malpighi was the first to directly visualize capillaries under the microscope. He was observing the alveoli of frog lungs when he saw the network of blood vessels and blood flowing from arteries to capillaries then to veins. About thirty years later, Antoni van Leeuwenhoek marveled at the circulation of the blood in the gills of tadpoles, made visible by the passage of globules (blood cells). Some vessels were so small that the globules could pass only in single file. He called these "blood capillaries". In the 1800's, von Recklinghausen showed that capillaries are tubes with a lining of cells by staining the cell borders of EC using silver nitrate (Majno, 1992).

1.3.1 The microcirculation

Blood enters the microcirculation through the arterioles, vessels with a diameter of about 100µm. It travels to the metarterioles and capillaries which have diameters of 5 to 10µm and exits through thin walled venules with a diameter of 200µm (Hurley, 1983; Junqueira et al., 1989; Gartner and Hiatt, 1990). Blood flows through capillaries in spurts because of the intermittent contraction of the metarterioles. Red blood cells traverse capillaries in single file due to the small diameter of these vessels (Hurley, 1983). The velocity of blood flow is very slow, in the order of 1-2mm per second. Leukocytes, having a larger size than red blood cells, have a much longer transit time. They must first squeeze into the shape of a cylinder. This sometimes makes them slow down so much that they stop all together. In the lung, they have been observed to stop for 2sec to 20min (Majno, 1992).

In arterioles and venules, blood travels by laminar flow. The velocity is greatest at the center of the vessel, decreasing progressively towards the vessel wall. This causes the blood cells to accumulate in the center of the stream of flowing blood, producing axial flow. Outside this central stream of closely packed blood cells is a peripheral zone of cell-free plasma (Hurley, 1983).

1.3.2 Inflammation

At the earliest stage of inflammation, there is an initial constriction of the arterioles followed by dilatation of arterioles and venules. Many vessels that had received little blood now open up to increase the blood flow as much as ten fold. The initial rapid flow of blood through these vessels slows down gradually. The central stream of closely packed cells widens while the peripheral zone of plasma narrows. In some vessels, flow may cease completely. Even before this occurs, however, leukocytes

start to appear in the plasma zone as the flow initially slows. Some will adhere to the vessel wall and roll along, a portion will flow back into the blood stream, while others will become more firmly adherent. This process is called "margination". The luminal side of the vessel wall may eventually become covered with a layer of leukocytes. This is termed "pavementing". The adherent leukocytes extend pseudopodia into the vessel wall, soon breaking through into the extravascular space. A small protrusion of the leukocyte appears just outside the vessel and this grows larger as the whole leukocyte crosses the wall. Soon, the whole cell is outside the vessel and connected back by only a long thin process, which eventually also detaches. This emigration process is called "diapedesis". Adhesion always precedes movement across the vessel wall (Allison et al., 1955). Whether diapedesis occurs across junctions between EC or through the EC cytoplasm remains controversial. The endothelial gaps close up immediately behind emigrated leukocytes. Concurrently, an exudation of fluid occurs as the vascular permeability increases (Hurley, 1983). Observations in rat skin with carbon or ink particle labeling suggest that emigrating leukocytes do not contribute to this vascular leakage, showing how tightly leukocytes squeeze between EC. Other studies, however, have found the opposite (Ryan and Majno, 1977).

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Acute inflammation is characterized by a predominance of polymorphonuclear leukocytes (PMN). As the response becomes chronic, mononuclear leukocytes (macrophages and lymphocytes) become dominant. This is because of the short life span of PMN and a switch from chemotactic agents for PMN to those for mononuclear leukocytes. Chronic inflammation may evolve from acute inflammation or arise without an acute phase. The lesions of chronic inflammation have a predominance of mononuclear leukocytes as well as granulation tissue - connective tissue with an abundance of inflammatory cells, fibroblasts and newly formed capillaries that may be leaky (Ryan and Majno, 1977). Thus the early inflammatory response includes three processes:

- 1. Blood vessel dilation and changes in blood flow, responsible for "rubor".
- Increased vascular permeability leading to edema with inflammatory exudates ("tumor").
- 3. Movement of leukocytes from the blood through the vessel wall into the extravascular space.

"Calor" is due to the great increase in blood flow and stimulation of heat sensitive nerve endings in the skin. Release of endogenous chemicals, such as bradykinin, serotonin and some prostaglandins, in addition to a rise in tissue tension cause "dolor". "Functio laesa" results from reflex inhibition of muscular movement by the pain, limitation of movement by the swelling and death or damage of parenchymal cells (Hurley, 1983).

1.4 CELL ADHESION MOLECULES

A major development over the last fifteen years has been the realization that EC lining the blood vessels do not merely constitute a passive barrier to the movement of solutes and cells across the vascular wall, but are active participants in inflammation. A number of cell surface molecules that mediate EC-leukocyte interactions have been found on the EC surface and they are of particular importance since the specificity of the transendothelial movement of leukocytes seems to reside within an altered endothelium. Activated, as well as nonactivated, leukocytes preferentially migrate across endothelium that has been activated and altered in inflammation (Ward and Marks, 1989). The EC adhesion molecules that have been characterized to date include E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and platelet/endothelial cells adhesion molecule-1 (PECAM-1).

1.4.1 E-selectin

E-selectin (CD62E) is a surface glycoprotein of the selectin family. It is a 64kD core protein with eleven potential glycosylation sites to give 115kD. Three domains are present: a lectin-like binding domain, an epidermal growth factor-like domain, and six complement-regulatory protein regions. The cytoplasmic domain contains tyrosine residues that may mediate the internalization of this antigen. Its ligands bind the amino-terminal of the lectin domain and a portion of the epidermal growth factor-like domain (Pigott et al., 1991; Carlos and Harlan, 1994). E-selectin binds to ligands containing the Lewis x, sialyl-Lewis x (CD15S) or sialyl Lewis a blood group carbohydrates (Kerr and Stocks, 1992; Phillips et al., 1990; Foxall et al., 1992; Ohmori et al., 1993; Bevilacqua and Nelson, 1993; Nelson et al., 1991; Picker et al., 1991a; Carlos et al., 1991; Walcheck et al., 1993; Postigo et al., 1992; Benjamin et al., 1990; Weller et al., 1991).

L-selectin on leukocytes may be one of the ligands for E-selectin (Picker et al., 1991b). Analysis of how mutations at the lectin domain affect binding has further defined the region near the antiparallel beta sheets derived from the NH_2 and COOH terminus of the lectin domain, and two adjacent loops to be critical for carbohydrate recognition (Erbe et al., 1992).

A large body of evidence indicates that E-selectin is important in the initial rolling of PMN on the EC surface in the presence of shear stress. Under conditions of flow, the initial contact leading to the rolling of PMN on unstimulated human umbilical vein EC (HUVEC) at a rate of 10µm/sec can be blocked up to 90% by an anti-E-selectin Ab, while a 30% inhibition can be achieved following activation of HUVEC (Abbassi et al., 1993). Rolling under conditions of flow does not involve the ICAM-1/CD18 pathway (Lawrence et al., 1990). Adhesion via E-selectin does not require active metabolism, unlike that via the integrins, but the strength of adhesion can be just as great

and much less time is required for adhesion strengthening (Lawrence and Springer, 1993). Binding of E-selectin also enhances the activity of the Mac-1 integrin (CD11b/CD18), leading to firm adhesion (Lo et al., 1991).

Unstimulated HUVEC do not express E-selectin. Following activation with bacterial lipopolysaccharide (LPS), interleukin-1 (IL-1), or tumor necrosis factor-a (TNF- α), E-selectin expression reaches a maximal level after 4h, and declines back to unstimulated levels by 24-48h (Bevilacqua, 1993; Butcher, 1992; Springer, 1994). The adhesion of neutrophils, basophils, eosinophils, monocytes, a subset of lymphocytes and Plasmodium-infected erythrocytes is partially mediated by E-selectin since anti-E-selectin Abs can inhibit this interaction (Bochner et al., 1991; Graber et al., 1990; Walcheck et al., 1993; Postigo et al., 1992; Benjamin et al., 1990; Weller et al., 1991; Ockenhouse et al., 1992; Hakkert et al., 1991; Luscinskas et al., 1991; Kishimoto et al., 1991; Kyan-Aung et al., 1991b; Shimizu et al., 1991b; Picker et al., 1991a; Ockenhouse et al., 1992). Moreover, binding of E-selectin to its ligand on neutrophils seems to induce a physiologic upregulation of the adhesive activity of another group of adhesion molecules, the β 2 integrins, on the neutrophils (Lo et al., 1991; Kuijpers et al., 1991). However, it has likewise been reported that E-selectin is not involved in leukocyte transmigration (Hakkert et al., 1991; Kishimoto et al., 1991), or eosinophil adhesion to EC (Dobrina et al., 1991).

The adhesion of some tumors has also been found to be mediated partially through E-selectin. E-selectin expressing COS cells bind the human colon carcinoma cell line HT-29 (Hession et al., 1990). Melanoma (Rice and Bevilacqua, 1989), carcinoma (Dejana et al., 1992) and leukemia cells (Takada et al., 1993) bind to activated HUVEC, at least partially, through E-selectin.

E-selectin has been found *in vivo* on the post-capillary endothelium of many inflammatory disorders, including systemic sclerosis (Sollberg et al., 1992), late phrase allergic reactions to intradermal allergen (Leung et al., 1991; Munro et al., 1991),

dermatitis (Groves et al., 1991; Munro et al., 1992; Cotran et al., 1986), psoriasis (Groves et al., 1991; Munro et al., 1992), dermal infiltrates associated with benign, premalignant and malignant keratinocyte proliferation (Groves et al., 1991), uveitis (Whitcup et al., 1992), peritonitis (Engelberts et al., 1992), appendicitis (Munro et al., 1992; Etzioni et al., 1992), insect bite (Munro et al., 1992), reactive lymph nodes (Munro et al., 1992), sarcoidosis (Munro et al., 1992), tonsilitis (Munro et al., 1992; Etzioni et al., 1992), gingivitis (Moughal et al., 1992), septic shock (Redl et al., 1991), atherosclerotic lesions (van der Wal et al., 1992), rheumatoid and osteoarthritic synovial tissues (Koch et al., 1991), cardiac allografts (Briscoe et al., 1991), kidney transplants (Fuggle et al., 1993), Hodgkin's and T cell lymphoma (Cotran et al., 1986; Etzioni et al., 1992), acute granulomatous lymphadenitis (Cotran et al., 1986), and thyroiditis (Cotran et al., 1986). Patients with leukocyte deficiency type 2, due to the absence of the sialyl-Lewis x ligands for E-selectin have recently been described. Affected individuals have recurrent episodes of bacterial infections, high leukocyte counts and marked defect in neutrophil motility (Etzioni et al., 1992). In contrast, E-selectin-deficient mice show no changes in neutrophil trafficking in inflammation. It is possible that another selectin takes the place of E-selectin in the necessary pathways in these mice (Labow et al., 1994).

1.4.2 Vascular cell adhesion molecule-1 (VCAM-1)

VCAM-1 (CD106) is a member of the immunoglobulin (Ig) gene superfamily. It consists of two forms: one with six extracellular Ig-like domains (6D VCAM-1) and another with seven Ig-like domains (7D VCAM-1). The former is the result of the alternative splicing of the latter. EC express mostly 7D VCAM-1. A gene duplication of domains 1 to 3 or 4 to 6 seems to be involved in this form. A 81 kD core protein is glycosylated at seven potential sites to give a 110 kD surface protein. Its ligands bind to the amino-terminal first domain of 6D and 7D VCAM-1 as well as the fourth domain of 7D VCAM-1 (Cybulsky et al., 1991a, Polte et al., 1991; Hession et al., 1991; Cybulsky et al., 1991a, Polte et al., 1991; Hession et al., 1991; Cybulsky et al., 1991a, Polte et al., 1991; Hession et al., 1991; Cybulsky et al., 1991a, Polte et al., 1991; Hession et al., 1991; Cybulsky et al., 1991a, Polte et al., 1991; Hession et al., 1991; Cybulsky et al., 1991a, Polte et al., 1991; Hession et al., 1991; Cybulsky et al., 1991a, Polte et al., 1991; Hession et al., 1991; Cybulsky et al., 1991a, Polte et al., 1991; Hession et al., 1991; Cybulsky et al., 1991a, Polte et al., 1991; Hession et al., 1991; Cybulsky et al., 1991a, Polte et al., 1991; Hession et al., 1991; Cybulsky et al., 1991a, Polte et al., 1991; Hession et al., 1991; Cybulsky et al., 1991a, Polte et al., 1991; Hession et al., 1991; Cybulsky et al., 1991a, Polte et al., 1991; Hession et al., 1991; Cybulsky et al., 1991; Cybulsky et al., 1991a, Polte et al., 1991; Polte et al., 1991; Cybulsky et al., 1991; Polte et al., 1991; Pol

al., 1991b; Carlos and Harlan, 1994). VCAM-1 binds to the very late antigen 4 molecule $(\alpha_4\beta_1 \text{ integrin}, \text{VLA4}, \text{CD49d})$ (Elices et al., 1990), as well as the $\alpha_4\beta_7$ integrin (Ruegg et al., 1992; Chan et al., 1992) on lymphocytes and monocytes. VCAM-1 is minimally expressed by unstimulated HUVEC. IL-1, TNF- α , and LPS activated EC express VCAM-1 maximally after 4-6h, and its expression persists for 72h in the presence of the cytokines (Bevilacqua, 1993; Butcher, 1992; Springer, 1994). VCAM-1 is involved in the adhesion and migration of lymphocytes, monocytes, <u>Plasmodium</u>-infected erythrocytes, eosinophils and basophils but not neutrophils across the endothelium (Pober et al., 1990; Bochner et al., 1991; Carlos et al., 1991; Postigo et al., 1992; Kyan-Aung et al., 1991a). The adhesion of these cells can be partially inhibited by anti-VCAM-1 and anti-VLA4 Abs (Bochner et al., 1991; Sadahiro et al., 1993). It has also been suggested that additional receptors for VLA-4, other than VCAM-1, may be present on activated HUVEC (Vonderheide et al., 1992).

Van Kooyk et al. (1993) found that binding of activated T lymphocytes to endothelium is mainly mediated through LFA-1. VLA-4 is used only when LFA-1 is not expressed or not functional as in some T cell leukemia cell lines. However, Baron et al. (1993) demonstrated that VLA-4 may be crucial in allowing activated T lymphocytes to leave the blood and enter the brain and other tissues to clear infections. T lymphocyte clones expressing a high concentration of VLA-4 entered the mouse brain parenchyma in abundance and caused experimental allergic encephalomyelitis, while clones expressing low levels of VLA-4 do not enter the brain and are not encephalitogenic.

Expression of VCAM-1 by vascular endothelium has been observed in several pathological and experimental conditions: acute appendicitis, acute colonic diverticulitis, lymphadenitis, dermatoses (Rice et al., 1991; Rice et al., 1990; Briscoe et al., 1992; Orosz et al., 1992; Briscoe et al., 1991), cardiac allograft rejections (Carlos et al., 1992;

Taylor et al., 1992), liver and pancreas allograft rejection (Bacchi et al., 1993; Steinhoff et al., 1993; Cybulsky et al., 1991a; Koch et al., 1991).

1.4.3 Intercellular adhesion molecule-1 (ICAM-1)

ICAM-1 (CD54) is a member of the immunoglobulin (Ig) gene superfamily. It is synthesized from a single gene on chromosome 19. A 55kD protein is made with five extracellular Ig-like domains with different amounts of N-linked glycosylation in these domains to give a total of 76 to 114 kD. Its ligands bind to the amino-terminal first domain and the third Ig-like domain. The latter is affected by the amount of glycosylation. An eight amino acid portion of the cytoplasmic domain binds to the cytoskeleton, such as actin via α -actinin. This allows the localization of ICAM-1 to specific regions of the cell membrane (Carlos and Harlan, 1994). ICAM-1 binds to leukocyte function associated molecule 1 (LFA-1), a β_2 integrin composed of two portions: CD11a and CD18, on the neutrophil, lymphocyte and monocyte surface, and Mac-1 (CD11b/CD18, Mo-1) on neutrophils, monocytes and NK cells (Arnaout et al., 1988). Fibrinogen (Languino et al., 1993), hyaluronan (Mccourt et al., 1994), CD43 - a leukocyte cell surface molecule also called sialophorin, leukosialin, large sialoglycoprotein or gp115 (Rosenstein et al., 1991), Plasmodium falciparum infected erythrocytes (Berendt et al., 1989, 1992) and rhinovirus (Staunton et al., 1989; Greve et al., 1989) also bind ICAM-1. ICAM-1 is expressed at low levels on unstimulated HUVEC. After activation by IL-1, TNF- α , LPS or IFN- γ , ICAM-1 expression reaches maximal levels after 24h (Bevilacqua, 1993; Butcher, 1992; Springer, 1994). ICAM-1 mediates the adhesion and transendothelial migration of lymphocytes, monocytes, neutrophils, basophils and eosinophils (Albelda and Buck, 1990; Albelda, 1991; Bochner et al., 1991; Pober and Cotran, 1991; Shimizu et al., 1991a; Kyan-Aung et al., 1991b). Abs against ICAM-1 or its ligand, LFA-1, can block the adhesion of leukocytes in vitro (Bochner et al., 1991) and the extravasation of leukocytes in vivo (Albelda, 1991;

Issekutz et al., 1992; Sadahiro et al., 1993; Kurtel et al., 1992; Scheynius et al., 1993). Microspheres coated with recombinant soluble ICAM-1 (Welder et al., 1993) and synthetic peptides based on specific amino acid sequences of human ICAM-1 (Ross et al., 1992) can also inhibit lymphocyte adhesion *in vitro*. In fact, it has been observed that decreased leukocyte adhesion with anti-CD18 Abs is mediated by receptor internalization. This is not associated with cell activation (Rubin et al., 1992).

Mice deficient in ICAM-1 exhibit impaired neutrophil migration in inflammation and decreased contact hypersensitivity (Sligh et al., 1993). In another report, such animals were observed to have also elevated circulating leukocytes and diminished allogeneic T cell responses. Their resistance to the lethal effects of endotoxin and exotoxin is due to reduced leukocyte-EC interaction and decreased systemic release of inflammatory cytokines and hepatocyte necrosis (Xu et al., 1994). Recently, it was reported that ICAM-1-deficient mice have greater incidence of <u>Haemophilus influenzae</u> type b bacteremia but less mortality than wild-type mice. In contrast, mortality due to <u>Streptococcus pneumoniae</u> was greater in ICAM-1-deficient mice than wild type. Histology of the meninges was not different between the two types of mice (Tan et al., 1995).

1.4.4 Platelet/endothelial cell adhesion molecule-1 (PECAM-1)

PECAM-1 (CD31) is also a member of the immunoglobulin (Ig) gene family. It was independently discovered by several groups to be a glycoprotein expressed constitutively by all HUVEC (Simmons et al., 1990; van Mourik et al., 1985; Albelda et al., 1990a; Muller et al., 1989). This is a 130kD glycoprotein present on EC, platelets, most monocytes, some granulocytes and a subset of CD4+ T lymphocytes and CD8+ T lymphocytes, including all naive CD8+ T lymphocytes (Stockinger et al., 1990; Tanaka et al., 1992). The exons of its gene seem to have arisen through gene duplication and shuffling. Alternate splicing of the complex cytoplasmic domain appears to generate

multiple isoforms (Kirschbaum et al., 1994). A 80kD core protein has six extracellular Ig-like domains and nine possible N-linked glycosylation sites (Albelda et al., 1991; Carlos and Harlan, 1994). PECAM-1 binds to other molecules of PECAM-1 as well as other structures (Muller et al., 1992; DeLisser et al., 1993a, 1993b). The distribution of PECAM is restricted to the EC borders (Albelda et al., 1991) and has been suggested to be re-distributed in response to cytokines. It is highly expressed especially on endothelium-adherent lymphocytes undergoing transmigration (Bogen et al., 1992). This expression amplifies the leukocytes' adhesive properties by transiently inducing the adhesive function of β_1 (VLA) and β_2 (LFA-1, Mac-1, p150,95) integrins (Tanaka et al., 1992). Thus, in collaboration with other adhesion molecules, PECAM-1 functions in an "adhesion cascade" by amplifying integrin-mediated adhesion of PECAM-1+ leukocytes to EC. Anti-PECAM-1 Abs can block neutrophil accumulation in inflammatory sites *in vivo* (Vaporciyan et al., 1993). In addition, PECAM-1 plays a role in the control of EC migration (Schimmenti et al., 1992).

1.4.5 Vascular addressins

In lymphoid organs, such as lymph nodes, lymphocytes leave the blood through high endothelial venules. Lymphocytes bind to these high endothelial venules through "homing receptors" on the lymphocyte surface and "vascular addressins" on the high or flat endothelium in lymphoid organs, and chronically inflamed non-lymphoid tissues. HECA-452 antigen was originally suggested to be a potential vascular addressin in humans, and MECA-325 antigen is its counterpart in mice. HECA-452 antigen can be induced on HUVEC by IFN- γ (Albelda and Buck, 1990). A note of unpublished observation indicated that the epitope bound by the only Ab available against this antigen may not be involved in leukocyte binding (Duijvestijin, 1988). HECA-452 antigen was not found in vessels with various dermal inflammatory diseases (Jalkanen et al., 1990),

but is present on T lymphocytes, PMN and monocytes at cutaneous sites (Picker et al., 1990; Davis and Smoller, 1992; Horst et al., 1988).

1.4.6 Other endothelial cell adhesion molecules

ICAM-2 is another molecule in the Ig gene superfamily. It consists of a core protein of 29 kD with six N-linked glycosylation sites. ICAM-2 has only two Ig-like domains and has been suggested to bind LFA-1 but not Mac-1 (Carlos and Harlan, 1994). Its expression is not regulated by cytokines (Staunton et al., 1989; Nortamo et al., 1991).

Another selectin - P-selectin (CD62P) - is also inducible on EC. Initially, this molecule was found on platelets and called platelet-activation-dependent granule-external membrane protein (PADGEM) or granule membrane protein-140 (GMP-140) (Carlos and Harlan, 1994). The core protein of 86 kD has twelve potential N-linked glycosylation sites. A secreted and a membrane associated form of the molecule are produced (Johnston et al., 1990). P-selectin is present in Wiebel-Palade bodies in large vessel EC and α -granules in platelets (McEver et al., 1989; Stenberg et al., 1985). After activation by thrombin or histamine, P-selectin is rapidly redistributed to the cell surface (Stenberg et al., 1985). This surface molecule can then be utilized by EC to bind sialylated molecules, including L-selectin (Carlos and Harlan, 1994) on neutrophils and monocytes (Hamburger and McEver, 1990). It has been found to mediate the initial interactions between EC and leukocytes under conditions of flow, where leukocytes roll on the endothelium (Lawrence and Springer, 1991). P-selectin deficient mice exhibit elevated numbers of circulating neutrophils, loss of leukocyte rolling in venules and delayed neutrophil recruitment in inflammation (Mayadas et al., 1993). It appears that an intact E-selectin pathway is insufficient to completely make up the function.

New adhesion molecules continue to be discovered. Some examples are vascular adhesion protein-1 (VAP-1) (Salmi et al., 1992; Jalkanen and Salmi, 1993) and

lymphocyte-vascular adhesion protein-2 (L-VAP-2) (Airas et al., 1993). These molecules are beyond the scope of this study.

1.4.7 Adhesion molecule shedding

Of interest are reports that several forms of E-selectin (Leeuwenberg et al., 1992; Pigott et al., 1992; Jakobsen et al., 1994; Newman et al., 1993), VCAM-1 (Pigott et al., 1992; Hahne et al., 1994; Jakobsen et al., 1994), ICAM-1 (Seth et al., 1991; Leeuwenberg et al., 1992; Pigott et al., 1992; Clark et al., 1993; Rieckmann et al., 1993; Sharief et al., 1993b; Pizzolo et al., 1994; Jakobsen et al., 1994) and PECAM-1 (Goldberger et al., 1994) can be found in soluble form in culture media after EC activation, and in the circulation during inflammatory and immune reactions. In multiple sclerosis (MS) patients, VCAM-1 and E-selectin in the cerebrospinal fluid, as well as ICAM-1 and E-selectin in the serum are elevated (Dore-Duffy et al., 1995). Soluble ICAM-1 has been found to decrease lymphocyte adhesion to activated HUVEC *in vitro* (Hashimoto et al., 1994). For some adhesion molecules, this may influence the dynamics of leukocyte adhesion and diapedesis *in vivo*.

1.4.8 Leukocyte adhesion molecules

A number of receptors for endothelial cell adhesion molecules have been characterized on leukocytes. L-selectin (CD62L) is a member of the selectin family that is constitutively present on lymphocytes, neutrophils and monocytes (Bevilacqua, 1993). A 37 kD core protein is differentially glycosylated in different leukocytes at eight possible N-glycosylation sites (Carlos and Harlan, 1994). Like other members of the selectin family, L-selectin is employed during flow conditions in the rolling of leukocytes on EC. It binds sialylated structures, CD34 and the murine mucosal lymphoid addressin MAdCAM-1 (Carlos and Harlan, 1994). When leukocytes are activated, the activity of L-selectin is transiently upregulated before its shedding (Bevilacqua, 1993).

In L-selectin deficient mice, lymphocytes do not bind peripheral lymph node high endothelial venules and the number of lymphocytes are reduced in peripheral lymph nodes, mucosal lymph nodes, Peyer's patch and spleen. They also show defects in neutrophil migration to inflammatory sites and leukocyte rolling (Arbones et al., 1994). Any rolling present is mediated by P-selectin. No evidence of E-selectin involvement is observed (Ley et al., 1995).

The integrins are another major group of adhesion molecules on leukocytes. Each integrin consists of a heterodimer of noncovalently linked α and β chains. Integrins are separated into groups by the β chain used. The β 2 integrins, $\alpha_L\beta_2$ (CD11a/CD18, LFA-1), $\alpha_M\beta_2$ (CD11b/CD18, Mac-1) and perhaps $\alpha_X\beta_2$ (CD11c/CD18, P150,95) mediate binding to the ICAMs. LFA-1, on lymphocytes, neutrophils, monocytes and NK cells, binds ICAM-1 and ICAM-2. Mac-1, on neutrophils, monocytes and NK cells, binds ICAM-1 fibrinogen, factor X and complement fragments. P150,95, on neutrophils, monocytes and NK cells, binds complement fragments and plays only a minor role in leukocyte adhesion to EC. Mac-1 and p150,95, but not LFA-1, are present in vesicles / granules in neutrophils and monocytes. Activation of leukocytes and binding of leukocytes to E-selectin increase the binding activity of these integrins (Carlos and Harlan, 1994).

A lack of β_2 integrins (LFA-1, Mac-1, p150,95) or nonfunctional β_2 integrins on leukocytes causes a human disorder called leukocyte adhesion deficiency. There is abnormal leukocyte aggregation, margination, chemotaxis and phagocytosis, leading to a decrease in inflammation, poor wound healing, absence of pus, recurrent bacterial and fungal infections and persistent neutrophilia (Anderson et al., 1987).

The β_1 integrin VLA-4 ($\alpha_4\beta_1$, CD49d/CD29) and β_7 integrin $\alpha_4\beta_7$ bind VCAM-1, although the latter plays a minor controversial role (Bevilacqua, 1993). VLA-4 is present on lymphocytes, monocytes, eosinophil, basophil and NK cells but not neutrophils, and $\alpha_4\beta_7$ on lymphocytes. In addition to VCAM-1, VLA-4 bind the

bacterial outer membrane protein invasin, and extracellular matrix proteins fibronectin and thrombospondin. The binding site for VCAM-1 and fibronectin, at least, are different (Carlos and Harlan, 1994).

1.4.9 Adhesion molecule therapy

Anti-adhesion molecule Abs hold great promise as a therapy for inflammatory conditions. In animal models, anti-ICAM-1 Abs inhibit experimental allergic encephalomyelitis (EAE, an animal model for MS) induced by active immunization with myelin but not passive EAE induced by transfer of myelin basic protein-specific CD4+ T lymphocytes (Archelos et al., 1993; Willenborg et al., 1993; Cannella et al., 1993). Anti- $\alpha_4\beta_1$ integrin (VLA-4) Abs prevent passively transferred EAE (Yednock et al., 1992). CNS injury due to ischemia (Clark et al., 1991; Bowes et al., 1993) and dermal thermal injury (Mileski et al., 1992) are also reduced by anti-ICAM-1 Abs. Abs against ICAM-1 and its ligand LFA-1 can inhibit leukocyte accumulation in the postcapillary venules of rabbits treated with zymosan-activated serum (C5a source) to induce leukocyte adhesion (Argenbright et al., 1991). Anti-PECAM-1 Abs block leukocyte emigration in a murine model of peritonitis (Bogen et al., 1994). Anti-VCAM-1 Abs decrease the rejection of murine cardiac allografts (Pelletier et al., 1992) and, on occasion, induce sometimes indefinite cardiac allograft acceptance (Isobe et al., 1994). In addition to wound healing, a long list of diseases have been targeted, including inflammation, cancer, MS, viral infections, rheumatoid arthritis, osteoporosis, atherosclerosis and the common cold. The rhinovirus which causes 50% of all colds enters cells by binding to the adhesion molecule ICAM-1. Genetically engineered ICAM-1 fragments fused to antibody (Ab) fragments are being generated to block rhinovirus infections. ICAM-1, VCAM-1 and E-selectin, are being examined as potential treatments for ischemia and reperfusion injury and inflammation (Peake, 1993; Rothlein et al., 1993; Travis, 1993; McMillen et al., 1993; Seekamp and Ward, 1993; Seekamp et

al., 1994). Clinical trials with anti-ICAM-1 Abs have commenced. In cadaveric kidney transplantation, they decrease the incidences of delayed graft function and rejection (Haug et al., 1993). Marked or moderate improvements have also been seen in rheumatoid arthritis patients treated with anti-ICAM-1 Abs (Kavanaugh et al., 1992).

1.5 LIPOPOLYSACCHARIDE AND CYTOKINES

It is apparent from the discussion of adhesion molecule upregulation above that bacterial lipopolysaccharide (LPS) and cytokines, such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interferon- γ (IFN- γ) can be very important in inflammation. Cytokines are defined as non-antibody proteins that act as intercellular mediators. In addition to their role in the upregulation of adhesion molecules and movement of leukocytes into inflammatory lesions, they exhibit various proinflammatory and pro-thrombotic activities.

1.5.1 Lipopolysaccharide (LPS)

Gram negative bacteria produce a toxin called endotoxin which is a component of the bacterial cell wall and is released by living as well as dead or disintegrated bacteria. It has been defined and given the name lipopolysaccharide (LPS) (Abbas et al., 1991; Doran, 1992). LPS is composed of a variable region of repeating oligosaccharide units called o-specific polysaccharide; core polysaccharides made of an inner-core of D-glucose, D-galactose and N-acetyl-glucosamine, and an outer core of 2-keto-3-deoxyoxtonic acid, heptose, phosphate and phosphate-bound ethanolamine; and lipid A - the most conserved region made of long chain fatty acids attached to a phosphorylated glucosamine-disaccharide backbone (Doran, 1992). LPS activates EC to express adhesion molecules, produce cytokines and activates both the classical and alternate pathways of complement (actions of lipid A and the oligosaccharides, respectively) with generation of the anaphylotoxins C3a and C5a (Abbas et al., 1991;

Doran, 1992). However, most of the toxicity caused by LPS is due to the host's response. LPS stimulates T and B lymphocytes, mononuclear leukocytes, EC and fibroblasts to secrete colony stimulating factors that stimulate progenitors to differentiate into mature leukocytes. In addition, it stimulates monocytes / macrophages to secrete large amounts of TNF, IL-1, prostaglandins and leukotrienes which may be responsible for much of LPS's biological activities.

Endotoxin has been found to occur at <25 to 260μ g/ml in the plasma of meningitis patients, and 210 to $170,000\mu$ g/ml in septicemia, but tissue concentrations may be much higher (Brandtzaeg et al., 1989).

1.5.2 Tumor necrosis factor (TNF)

There are two species of TNF: TNF-α and TNF-β (lymphotoxin). They share 30% homology and are encoded by different genes, but have the same receptor and action. TNF is produced by lymphocytes, mast cells, PMN, keratinocytes, astrocytes, microglial cells, smooth muscle cells, intestinal paneth cells and tumor cells. By far the largest amount is produced by monocytes / macrophages activated by LPS, bacteria, viruses, fungi, parasites or tumor cells (Jattela, 1991; Vassalli, 1992). The actions of TNF are due to the activation of several nuclear-binding proteins / transcription factors (NF). In lymphocytes and monocytes, at least three of these NF-κB-like DNA binding proteins interact with κ B-like enhancement elements to synthesize IL-2 receptors and express class II major histocompatibility complex (MHC) proteins. Activation of the transcription factor IFN regulatory factor 1 induces the synthesis of IFN- β . Lastly, activation of the jun/AP-1 transcription factor activates collagenase in fibroblasts and class I MHC genes (Jattela, 1991).

TNF has many effects on many different cell types. Its most important action on EC is the induction/upregulation of adhesion molecules. In addition, it induces the production of IL-1, colony stimulating factors (to increase circulating leukocytes),

chemokines (IL-8, MCP-1), prostaglandins and class I MHC protein, and inhibits proliferation. Coagulation is also promoted by suppressing thrombomodulin synthesis to suppress EC surface anticoagulant activity, increasing tissue factor-like procoagulant activity, and decreasing plasminogen activator synthesis (Mantovani and Dejana, 1989; Vassalli, 1992).

In Kawasaki disease, up to 68U TNF/ml have been found in the patient's serum (Matsubara et al., 1990). A much higher concentration of up to 500U TNF/ml was found in the serum of patients with Guillain-Barré syndrome (Sharief et al., 1993a). The concentration of TNF rose to 15U TNF/mg cortex homogenate after fluid-percussion trauma of rat brains (Taupin et al., 1993).

1.5.3 Interleukin-1 (IL-1)

There are two species of IL-1: IL-1α and IL-1β. They are produced by different genes but share a related tertiary structure, bind the same receptor and generate the same biological activities. IL-1 is produced mainly by mononuclear leukocytes (Epstein, 1993). Most actions of TNF are shared by IL-1, including induction of adhesion molecules, chemokines, colony stimulating factors, prostaglandin, procoagulant activity, suppression of fibrinolytic activity and EC growth. The exceptions are its lack of effect on class I MHC protein expression and lack of cytotoxicity (Mantovani and Dejana, 1989; Abbas et al., 1991). Mechanical trauma in rats caused up to 3U IL-1/ml to be released into the brain as measured by a microdialysis technique (Woodroofe et al., 1991). IL-1 levels reached 18U IL-1/mg cortex homogenate following fluid-percussion trauma of rat brains (Taupin et al., 1993).

1.5.4 Interferon-γ (IFN-γ)

IFN- γ is a cytokine quite different from TNF and IL-1, and even from other interferons (α and β). Probably all cell types produce IFN- α/β , but IFN- γ is only

synthesized by T lymphocytes and natural killer (NK) cells after antigen and IL-2 stimulation, respectively. The actions of IFN-γ are mainly mediated by the activation of protein kinase C and intracellular calcium fluxes followed by *de novo* RNA and protein synthesis (Williams et al., 1993). IFN-γ induces synthesis of ICAM-1, class I and II MHC molecules and IL-1 on EC. It inhibits growth most likely by downregulating fibroblast growth factor receptors. In addition, it exhibits antiviral and antiprotozoal actions (Sen and Lengyel, 1992).

Up to 2.5U IFN- γ /ml have been found in the serum of patients with Kawasaki disease (Matsubara et al., 1990) and in human T lymphotropic virus type I-associated myelopathy (Kuroda and Matsui, 1993), while up to 3.5U IFN- γ /ml was found in the cerebrospinal fluid (CSF) of the latter.

1.6 ENDOTHELIAL CELL HETEROGENEITY

Most of the observations previously reported on cytokines and EC adhesion molecules were made using HUVEC. It is not clear how this fetal vascular tissue applies to the adult vasculature and whether results can be extrapolated particularly to specialized endothelium in other organs such as the brain. It is now well accepted that EC of different species and vascular beds have distinct phenotypes, functions and antigenic properties. There is already ample evidence that EC lining the cerebral microvessels are different from their counterparts in other organs. Cerebral capillaries contain the decarboxylation enzyme that converts L-dopa to dopamine while peripheral capillaries do not (Owman and Hardebo, 1988). *In vitro*, cerebral EC have greater γ -glutamyl transpeptidase and alkaline phosphatase activity than aortic EC (Gordon et al., 1991), and aortic and adrenal cortical EC have protein kinase C type III while cerebral EC have type II and III enzymes (Kobayashi et al., 1990). More importantly, lymphocyte adhesion to unstimulated cerebral endothelium is much lower than to aortic endothelium. IFN- γ induces two to three fold more class II MHC molecules on cerebral than aortic EC (Male
et al., 1990a). Another very important property of cerebral EC is their ability to form the blood-brain barrier (BBB).

1.7 THE BLOOD-BRAIN BARRIER (BBB)

The brain is vigorously maintained in a constant microenvironment for the proper functioning of the neurons by means of a structural and functional barrier, the bloodbrain barrier (BBB).

Two morphological features of the cerebral endothelium are responsible for the formation and maintenance of the BBB. Firstly, the presence of tight junctions between adjacent EC prevents the movement of ions, proteins, large lipid-insoluble nonelectrolytes and circulating blood cells from the blood into the brain paracellularly. Tight junctions form continuous belts made up of interconnected loops of strands around EC. At a junction, the two apposed plasma membranes come together and the outer leaflets fuse, obliterating the intercellular space. It has been hypothesized that the complexity of the tight junctional complexes correlates with the degree of protein impermeability (Claude and Goodenough, 1973), although it is uncertain how the anatomical subunits of tight junctions relate to their barrier function. EC junctions in normal intact brains do not appear to open and close since molecular tracers, such as horseradish peroxidase, and ionic and colloidal lanthanum are not found in any of the lacunae - intervening pockets formed by tandem junctions (Dorovini-Zis et al., 1983; Brightman, 1977). A second method to examine the efficiency (tightness) of the barrier involves measuring the electrical resistance across the endothelium. The average resistance of cerebral capillaries is $1900\Omega \cdot cm^2$ in frogs and $3000\Omega \cdot cm^2$ in rats. This is in the same order of magnitude as the tightest epithelium (Bradbury, 1993).

Secondly, the paucity of cytoplasmic vesicles restricts the transport of solutes across the EC cytoplasm. A distinction must be made between two types of cytoplasmic vesicles. Pinocytosis generates vesicles that fuse with lysosomes or multivesiculate

bodies, the contents are digested and the whole process ends inside the cell. In vesicular transport, vesicles transfer material from the fluid compartment at one side of the cell to the other side. These vesicles do not appear to any extent in cerebral microvessel EC, but are abundant in skeletal muscle and other EC (Brightman, 1977). Of the vesicles that are present in cerebral EC, most have been found not to be true vesicles but rather invaginations connected via narrow necks to the extracellular milieu or are clathrin-coated vesicles (Bradbury, 1993).

A number of specific transporters present on the luminal or abluminal EC membranes ferry biologically important substances across this efficient barrier. Many of these mechanisms are specific to the cerebral EC, such as the Glut-1 glucose transporter, γ -glutamyltranspeptidase, alkaline phosphatase and abluminal Na⁺-K⁺-ATPase (Janzer, 1993). Thus, in contrast to the extracerebral microvasculature, cerebral EC permit only a very specific, highly selective exchange between the brain and the rest of the body.

1.8 INFLAMMATION IN THE CENTRAL NERVOUS SYSTEM

1.8.1 General aspects

Inflammation in the CNS is characterized by increase in BBB permeability and influx of inflammatory cells into the brain.

Acute CNS inflammation is characterized by PMN infiltration while chronic inflammation by mononuclear inflammatory cells, especially lymphocytes and macrophages. In acute-types of inflammation, such as those caused by bacteria (abscess, meningitis), viruses (viral meningitis and encephalitis), and infarction, there is an initial (1-2 days) migration of PMN across dilated vessels. PMN surround these vessels to form "perivascular cuffs". This short lived process may be accompanied by edema, hemorrhage and necrosis in bacterial infections. PMN not only release hydrolytic enzymes and free radicals to combat invading organisms but also phagocytoze and

destroy them. Later, after 4-5 days, macrophages, lymphocytes and plasma cells replace PMN in the extravascular tissue. Plasma cells generate Abs that opsonize invading organisms. This facilitates phagocytosis by macrophages, which also present antigens of these invaders to CD4+ T lymphocytes. The latter secrete cytokines to aid the inflammatory and immune reactions, in response to antigen recognition. In infarcts, the initial infiltration by PMN is followed, 36 to 48h later, by macrophage infiltration. If necrosis occurs in the brain parenchyma, inflammatory cells will stream from the perivascular spaces to the necrotic brain tissue (Brierley and Graham, 1984; Harriman, 1984; Bromwell and Tomlinson, 1984). A more chronic-type of CNS inflammation occurs in MS. In acute MS, the entire lesion is intensely inflammatory. Small mononuclear cells (T lymphocytes and macrophages) form perivascular cuffs. In chronic MS, inflammatory cells are rare in inactive plaques, but in active plaques, lymphocytes, some macrophages and plasma cells form perivascular cuffs. Only a small number of PMN is present (Raine, 1991).

1.8.2 Adhesion molecules in the central nervous system

The mechanisms responsible for the recruitment of leukocytes to sites of CNS inflammation are largely unknown. Several lines of investigation indicate that expression or upregulation of EC adhesion molecules may play an important role during leukocyte-EC interactions in inflammation. There have been a few reports on the presence of adhesion molecules in CNS disorders *in vivo*. Sobel et al. (1990) reported that microvessels along with glia are ICAM-1 positive in MS plaques, viral encephalitis lesions, infarcts and some controls. Lymphocytes from MS patients induce human brain EC to express and release high concentrations of ICAM-1 *in vitro* (Tsukada et al., 1994). HECA-452 antigen was found in cerebral postcapillary venules in the peri-plaque area in a rapidly evolving case of MS (Raine et al., 1990a). The attachment and infiltration of lymphocytes in EAE was found to correlate with the appearance of MECA-325 and

ICAM-1 on the CNS vessels (Raine et al., 1990b; Cannella et al., 1990, 1991). Recently, we demonstrated that E-selectin and VCAM-1 are present in patients with encephalitis, CNS vasculitis, multiple sclerosis, meningitis, brain abscess and to a lesser extent, cerebral infarcts, but not in traumatic lesions or normal brain (Dorovini-Zis et al., 1992b; Dorovini-Zis et al., 1993). Positive staining of vessels is usually associated with leukocytes undergoing diapedesis and extensive inflammatory cell infiltration. ICAM-1 and VCAM-1 are also upregulated in the CNS of mice with EAE (Dopp et al., 1994; Barten et al., 1994; Steffen et al., 1994), while ICAM-1 is upregulated in baboons after focal brain ischemia and reperfusion (Okada et al., 1994). Akiyama et al. (1993) and Verbeek et al. (1994) found some intense vascular staining for ICAM-1 in Alzheimer's disease, especially in areas with a large number of senile plaques, and in amyotrophic lateral sclerosis.

1.8.3 Cytokines in central nervous system disorders

The cytokines that upregulate these adhesion molecules on HUVEC have also been found in CNS lesions in a variety of disorders: TNF- α in MS, bacterial meningitis, HIV infections and traumatic injury; IL-1 in MS, Alzheimer's disease and traumatic injury; and IFN- γ in MS (Benveniste, 1992; Morganti-Kossmann et al., 1992; Woodroofe and Cuzner, 1993). TNF expression is associated with astrocytes, macrophages, microglia, some EC and perivascular inflammatory cuffs in EAE (Renno et al., 1995), encephalitis, acute and chronic active MS lesions but not chronic inactive lesions (Hofman et al., 1989; Woodroofe and Cuzner, 1993; Rieckmann et al., 1995). Intracerebroventricular administration of LPS into rats also induces the expression of TNF in the CSF (Sanna et al., 1985). IL-1 is also present in perivascular cells in chronic active MS lesions (Hofman et al., 1989; Woodroofe and Cuzner, 1993), in macrophages and activated microglia throughout the course of EAE in mice and rats (Kennedy et al., 1992; Bauer et al., 1993), as well as in ischemic rat brain (Liu et al., 1993; Wießner C,

1993), and rat brain after intraperitoneal or intraventricular injection of LPS (Quan et al., 1994). IFN- γ is secreted by some lymphocytes, astrocytes and glia in EAE (Traugott and Lebon, 1988; Kennedy et al., 1992, Morganti-Kossmann et al., 1992; Woodroofe and Cuzner, 1993). All of these cytokines are also found in the CSF and brain of patients with meningitis and mice with lymphocytic choriomeningitis (Moller et al., 1991; Campbell et al., 1994).

1.9 HUMAN BRAIN MICROVESSEL ENDOTHELIAL CELLS (HBMEC) IN VITRO

There is increasing evidence that HBMEC and their interaction with cytokines and leukocytes play a key role in CNS inflammation. Experiments in vivo can document the site of the BBB breakdown, but cell-cell interactions are difficult to characterize because of the many complex factors involved. This has led to the establishment and use of in vitro models of the BBB that respond to experimental manipulation. Thus, cultures of bovine brain microvessel EC (BBMEC) share the morphological and barrier characteristics of their in vivo counterparts. Neither ionic lanthanum nor horseradish peroxidase penetrate the interendothelial tight junctions normally, but hyperosmotic arabinose and urea solutions open these junctions to let the tracers penetrate paracellularly (Dorovini-Zis et al., 1984, 1987a, 1987b). PMN can be stimulated by a gradient of formyl-methionyl-leucyl phenylalanine (fMLP), leukotriene- B_{Δ} (LTB_{Δ}) or acetyl-glyceryl-ether-phosphorylcholine (AGEPC) to adhere to and migrate across monolayers of BBMEC (Dorovini-Zis et al., 1992a). Isolation and culture of cerebral EC from human brains has permitted the study of these cells and their barrier function in vitro. HBMEC monolayers in primary culture retain essential features of the BBB: tight junctions between adjacent cells that restrict the paracellular movement of horseradish peroxidase and a paucity of cytoplasmic vesicles (Dorovini-Zis et al., 1991). Using this system, it has been demonstrated that IFN-y induces *de novo* expression of class II MHC molecules in these cells, changes their morphology and increases the monolayer

permeability to horseradish peroxidase (Huynh and Dorovini-Zis, 1993). IFN- β reverses these changes (Huynh, 1994). Expression of class II MHC molecules is associated with increase in the adhesion and migration of resting and anti-CD3 activated T lymphocytes across HBMEC (Huynh, 1994).

In the present study, the expression of adhesion molecules by HBMEC and their role in HBMEC-leukocyte interactions were examined using this *in vitro* model of the human BBB.

1.10 GENERAL AIM OF THIS STUDY

The overall aim of this study is to characterize ① the expression / upregulation of adhesion molecules by human brain microvessel EC, ② their role in the adhesion and migration of inflammatory cells across the endothelium and ③ the effect of EC-leukocyte interactions on the permeability of the blood-brain barrier (BBB).

1.11 SPECIFIC OBJECTIVES OF THIS STUDY

1.11.1 Phase I. Investigation of the expression of adhesion molecules by human brain microvessel endothelial cells in primary culture

1. To study the upregulation of E-selectin on human brain microvessel EC by cytokines and LPS.

2. To study the expression of VCAM-1 by human brain microvessel EC and its upregulation by cytokines and LPS.

3. To study the expression of ICAM-1 by human brain microvessel EC and the kinetics of its upregulation by cytokines and LPS.

4. To study the expression and change in distribution of PECAM-1 by human brain microvessel EC following activation with cytokines and LPS.

5. To study the upregulation of the high endothelial venule antigen - HECA-452 antigen on human brain microvessel EC by cytokines.

1.11.2 Phase II. Investigation of the role of endothelial cell adhesion molecules in leukocyte adhesion and transmigration across human brain microvessel endothelial cell monolayers

1. To investigate the role of these adhesion molecules in the adhesion and transmigration of PMN.

2. To study the modulation of T lymphocyte adhesion and migration across HBMEC monolayers by EC adhesion molecules.

1.11.3 Phase III. Investigation of the monolayer permeability after cytokine treatment and in the course of HBMEC-leukocyte interactions

1. To determine the role of TNF, as well as PMN and T lymphocyte migration and adhesion molecule blocking Abs on the permeability of confluent HBMEC monolayers.

CHAPTER 2 MATERIALS AND METHODS

2.1 EXPRESSION OF ADHESION MOLECULES

2.1.1 Human brain microvessel endothelial cells

Primary cultures of HBMEC were established by methods previously described (Dorovini-Zis et al., 1991). Briefly, cerebral cortex was obtained from temporal lobectomy specimens removed from patients with intractable seizure disorders and autopsy brains less than 12 hours postmortem. The clinical history at the time of postmortem examination was carefully screened to exclude brains with various neuropathological processes including inflammatory / infectious diseases, tumors, infarcts and cerebral anoxia. Cerebral cortices were transferred to the laboratory in medium 199 (M199, Gibco, Burlington, ON) with antibiotics at 4°C. Meninges and associated large vessels were peeled off before cutting the tissue into millimeter cubes with a pair of scalpels. This was then digested in 0.5% dispase (Boehringer Mannheim, Indianapolis, Indiana) in a shaking water bath at 37°C for 3h. Following centrifugation at 1000x g for 10min, pellets were centrifuged at 5800x g in 15% dextran (Sigma, St. Louis, Missouri) for 10min. The pellet containing the microvessels was washed and incubated with 1mg/ml collagenase/dispase (Boehringer Mannheim) at 37°C overnight (10-16h), in order to free the EC from pericytes and basement membrane. EC were separated from pericytes, erythrocytes and basement membrane by centrifugation in Percoll gradients (Sigma) for 10min at 1000x g. The band containing the EC was aspirated and washed. The isolated clumps of EC were seeded onto fibronectin-coated 24 or 96 well plates (Corning Plastics, Corning, N.Y.) and maintained in culture in M199 supplemented with 10% horse serum, endothelial cell growth supplement ($20\mu g/ml$), heparin (100µg/ml) (both from Sigma) and antibiotics (Gibco). Culture media were

changed every other day. EC reached confluency 7 to 9 days after plating and were used for studies on or after the tenth day (Fig. 1). The endothelial origin of the cells was determined by their positive staining for Factor VIII antigen (Fig. 2) and binding of Ulex europaeus agglutinin as previously described (Dorovini-Zis et al., 1991). Several primary cultures were used for each experiment.

2.1.2 Adhesion molecule induction by lipopolysaccharide and cytokines

Bacterial lipopolysaccharide (LPS from <u>E. coli</u> 055:B5, Sigma, St. Louis, MO), human recombinant interferon- γ (IFN- γ , Collaborative Research Inc., Bedford, MA), human recombinant tumor necrosis factor- α (TNF- α , Sigma) or human recombinant interleukin-1 β (IL-1 β , Boehringer Mannheim, Laval, PQ) were dissolved in complete growth media at a final concentration of 0.001, 0.01, 0.1, 1 μ g LPS/ml; 10, 50, 100, 500 U IFN- γ /ml; 1, 10, 100 U TNF- α /ml; or 0.1, 1, 10 U IL-1 β /ml. Ten to eleven day old cultures were incubated with different concentrations of LPS and cytokines for 4h, 12h, 24h and 48h. In addition, separate wells were incubated with various concentrations of TNF- α and IL-1 β (1 U TNF- α /ml + 0.1 U IL-1 β /ml; 10 U TNF- α /ml + 0.1 U IL-1 β /ml) or TNF- α and IFN- γ (1, 10, 100 U TNF- α /ml + 100, 500 U IFN- γ /ml) to examine the combined effects of these cytokines. Duplicate or triplicate wells were used for each treatment. Several primary cultures derived from different autopsy brains were utilized in these experiments.

The reversibility of ICAM-1 expression was studied by incubating confluent, eight to ten day old, monolayers with LPS (0.001, 0.01, 0.1, 1, 5µg/ml); IFN- γ (100, 500 U/ml); TNF- α (1, 10, 100 U/ml) or IL-1 β (0.1, 1, 10 U/ml) for 48h. At the end of the incubation period, the cultures were washed three times with M199 to remove the cytokines and LPS, placed in complete media and then returned to the incubator for 3 days prior to ICAM-1 detection.

2.1.3 Antibodies

Mouse monoclonal anti-human ICAM-1 OKT27, a gift from Dr. P. Rao (Ortho Diagnostics, Raritan, NJ) and RR1/1, a gift from Dr. T. Springer (Harvard Medical School, Boston, MA), anti-human VCAM-1 2G7 and anti-human E-selectin 3B7, gifts from Dr. W. Newman (Otsuka America Pharmaceutical Inc., Rockville, MD), anti-human PECAM-1 4G6, a gift from Dr. S. Albelda (University of Pennsylvania, Philadelphia, PA), and rat anti-human HECA-452 IgM, a gift from Dr. Duijvestijin (University of Limburg, Maastricht, The Netherlands) were used as the primary Ab. Anti-human pituitary follicle-stimulating hormone IgG (Biogenex Lab, CA) was used as an irrelevant Ab in place of primary Ab in immunogold silver staining. Goat anti-mouse IgG coupled to 5nm gold particles (Auroprobe[™] LM GAM IgG, Janssen / Cedarlane Labs Ltd., Hornby, ON) and goat anti-rat IgM coupled to 12nm gold particles (Jackson/ Bio Can, Mississauga, ON) were used as the secondary Ab for immunogold silver staining and goat anti-mouse IgG coupled to 5nm gold particles (Auroprobe[™] GAM IgG G5, Janssen / Cedarlane Labs Ltd., Hornby, ON) was used as the secondary Ab for immunogold EM. Horseradish peroxidase-conjugated goat anti-mouse Ab (Jackson Immunoresearch Laboratories, West Grove, PA) was used for ELISA.

2.1.4 Immunogold silver staining (IGSS) for light microscopy

Following treatment with cytokines or LPS, cultures were washed briefly with buffer containing phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA), 1% normal goat serum (NGS) and 0.05% sodium azide (PBS/BSA/NGS) (all from Sigma) and then incubated for 40 minutes at room temperature with the primary Ab at a final concentration of 2μ g/ml for anti-ICAM-1, 5μ g/ml for anti-VCAM-1 and anti-E-selectin, 1:10 dilution for anti-PECAM-1 and 1:20 dilution for anti-HECA-452 antigen in carrier buffer containing PBS with 5% BSA, 4% NGS and 0.05% sodium azide. The sodium azide was added to prevent internalization of antigen-Ab complexes.

At the end of the incubation period, the monolayers were washed twice with PBS/BSA/NGS, and then incubated with the secondary Ab, Auroprobe[™] LM GAM IgG, at 1:40 dilution or goat anti-rat IgM Ab at 1:20 dilution in carrier buffer for 1h at room temperature. Subsequently, cultures were washed with PBS/BSA/NGS, fixed in 9.25% formaldehyde and 45% acetone in PBS for 30 seconds, washed with distilled deionized water and incubated in silver enhancing solution (IntenSE M[™], Janssen / Cedarlane) for 22-26 minutes at 21°C. EC were counterstained with Giemsa. Except as noted above, all cultures were grown, fed, stained and counted the same way at the same time.

<u>Controls</u> included cultures incubated in growth media in the absence of cytokines or LPS, and monolayers incubated with 1) normal mouse IgG (Cedarlane) at the same concentration as the primary Ab, 2) irrelevant Ab and 3) carrier buffer instead of the primary Ab.

Immunostained cultures were examined under a Nikon Labophot light microscope. <u>Quantitation</u> of adhesion molecule expression was performed by counting cells as labeled or unlabeled in four peripheral and one central randomly selected fields with an ocular grid under 20X magnification. An average of 200 EC were present per optic field. All measurements were performed blindly by masking the identity of the wells prior to counting.

2.1.5 Enzyme-linked immunosorbent assay (ELISA)

Untreated as well as LPS or cytokine-treated monolayers were washed with PBS and fixed in 0.025% glutaraldehyde for 10 minutes. Following a rinse with PBS and three washes with PBS/BSA/NGS, cells were incubated for 60 minutes at room temperature with the primary Abs at the same concentration as for IGSS in carrier buffer containing PBS with 5% BSA and 4% NGS. At the end of the incubation period, the monolayers were washed with PBS/BSA/NGS and incubated with the secondary Ab (horseradish peroxidase conjugated goat anti-mouse IgG) at 1:5000 dilution in carrier

buffer for 1h. Subsequent washes in PBS were followed by incubation with 2mg/ml o-phenylenediamine with 0.015% hydrogen peroxide in 0.1M PBS for 45 minutes. The color development was stopped by the addition of 2M sulfuric acid or 3M HCl and the absorbance read on a ELISA microtiter plate reader at 490nm. Except as noted above, all cultures were grown, fed, stained and assayed the same way at the same time.

<u>Controls</u> included cultures incubated in growth media in the absence of cytokines and LPS, and monolayers incubated with normal mouse IgG (Cedarlane) at the same concentration as the primary Ab or carrier buffer instead of the primary Ab.

2.1.6 Immunogold electron microscopy (IGEM)

Nine day old subconfluent HBMEC monolayers were treated with 100U TNF- α /ml for 4, 18 or 24h for maximal upregulation of E-selectin, VCAM-1 and ICAM-1, respectively. For PECAM-1, EC were treated with 500U IFN- γ /ml for 48h. Cytokine treated as well as control untreated cultures were washed briefly with cold PBS/BSA/NGS and then incubated with the appropriate primary Ab at the same final concentration as IGSS in cold carrier buffer for 30 min at room temperature. The carrier buffer had the same composition as the one used in IGSS. At the end of the incubation period, the monolayers were washed three times with cold PBS/BSA/NGS and then incubated with the secondary Ab (5nm gold-conjugated goat anti-mouse IgG) at a 1:10 dilution in cold carrier buffer for 1h. Subsequently, cultures were washed with cold PBS/BSA/NGS, fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.2M sodium cacodylate buffer for 1h at 4°C, washed with 0.2M cacodylate buffer, post fixed with 1% osmium tetroxide for 1h at 4°C, and en bloc stained with uranyl magnesium acetate overnight at 4°C. Dehydration in graded series of methanol was followed by embedding in epon-araldite. Blocks cut from embedded cultures were re-embedded perpendicular to the growth area for cross sectioning. Controls included cultures grown in the absence of cytokine, and monolayers incubated with carrier buffer instead of primary Ab. Thin

sections were viewed unstained with a Philips EM 400. Except as noted above, all cultures were grown, fed, stained, processed and examined with the TEM the same way at the same time.

Quantitation. One hundred each of treated and untreated cells were photographed at standard magnification and the number of gold particles bound to the apical and basal surfaces were counted. The actual length of the cell membrane on each surface was determined by measuring the length on the electron micrograph, taking into account the magnification. The number of gold particles per μ m of EC membrane length was calculated using the above measurements.

2.1.7 Controls

Controls included cultures incubated in growth media in the absence of cytokines or LPS, and monolayers incubated with 1) normal mouse IgG (Cedarlane) at the same concentration as the primary Ab, 2) irrelevant Ab and 3) carrier buffer instead of the primary Ab.

2.1.8 Statistics

Statistics were performed using the computer program SigmaStat (Jandel Scientific, San Rafael, CA). ANOVA was first performed. Where significant differences were found, Student's t-test was applied to the data to determine the significance of the differences between cultures treated with cytokine or LPS and those untreated in the IGSS and ELISA data. Mann-Whitney test was employed to determine the significance of these differences in the IGEM data since they were not normally distributed. P<0.05 was taken as statistically significant for all analyses.

2.2 ROLE OF ADHESION MOLECULES IN PMN AND T LYMPHOCYTE ADHESION TO AND MIGRATION ACROSS HUMAN BRAIN MICROVESSEL ENDOTHELIAL CELL MONOLAYERS

2.2.1 Human brain microvessel endothelial cells

Primary cultures of HBMEC were established from brains as described in section 2.1.1. For the adhesion assays, cells were grown on fibronectin coated 96 well plates, and for migration assays, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) on permeable membranes of purified collagen (Cellagen discs, ICN, Cleveland, OH). Individual Cellagen discs were placed in separate wells of 24 well plates. Supports at the bottom of the Cellagen discs separate them from the wells so that the Cellagen discs form the upper chamber and the 24 well plate the lower chamber of a double chamber chemotaxis system (Fig. 3). HBMEC readily grow on collagen membranes and form confluent cultures. 9-10 day old confluent cultures were used. Several primary cultures from different autopsy brains were utilized.

2.2.2 Polymorphonuclear leukocyte (PMN) isolation

PMN were isolated from anti-coagulated peripheral blood of several healthy donors by centrifugation in a gradient of Lympholyte-poly (Cedarlane). By this method, we routinely obtained cell fractions containing 99% PMN as determined on Giemsa-stained smears. Viability was 99% by trypan blue exclusion test.

2.2.3 T lymphocyte isolation

Peripheral blood mononuclear cells (PBMC) were isolated from anti-coagulated peripheral blood of healthy volunteers by centrifugation in Histopaque (Sigma). T lymphocytes were separated by passage of PBMC through a "T cell recovery column" (Cedarlane). Monocytes and B lymphocytes were trapped in the column by size and by

the action of anti-immunoglobulin Abs on the column beads, respectively. By this method, greater than 90% of cells eluted were T lymphocytes by fluorescence-activated cell sorting (FACS) analysis and viability was 99% by trypan blue exclusion test. FACS analysis was carried out by incubating lymphocyte suspensions with mouse mAbs against CD14, CD45, CD2 or CD20 to detect monocytes, all leukocytes, T lymphocytes plus NK cells, and B lymphocytes, respectively. The anti-CD14 and anti-CD2 Abs were conjugated to phycoerythrin (Coulter Corp., Hialeah, FL). The anti-CD45 and anti-CD20 Abs were conjugated to fluorescein isothiocyanate (Becton Dickenson, Mississauga, ON). After a 30min incubation at 4°C, cells were washed with 2% fetal calf serum in TC 199 (Gibco) and fixed in 1% paraformaldehyde. Fluorescence was read on an Epic Profile I (Coulter Corp). The quadrants were set by using isotypic controls and dead cells were gated out.

2.2.4 Antibodies

The following Abs were used: Mouse anti-human E-selectin at 20µg/ml (3B7, a mouse IgG2a), and mouse anti-human VCAM-1 at 20µg/ml (2G7, a mouse IgG1) (gifts from Dr. W. Newman). Mouse anti-human ICAM-1 at 20µg/ml (RR1/1, a mouse IgG1, Biosource International / Immunocorp, Montreal, Quebec). Rabbit anti-human PECAM-1 at 50µg/ml (a polyclonal Ab from Dr. S. Albelda). All the above Abs have been shown to bind to EC and to be active in the inhibition of specific leukocyte-EC interactions in our system and other EC systems (Graber et al., 1990; Shimizu et al., 1991; Dustin and Springer, 1988; Liversidge et al., 1990; Muller et al., 1993). Isotype matched control Abs included: mouse anti-membrane co-factor protein at 20µg/ml (J4.48, a mouse IgG1), mouse anti-HLA-A,B,C at 20µg/ml (B9.12.1, a mouse IgG2a), and rabbit gammaglobulin at 50µg/ml (all from Dimension lab, Mississauga, Ontario). All Abs were free of preservatives and stabilizers. The Ab concentration giving the highest reading by ELISA was taken to be the optimal concentration for the assays.

Mouse anti-human leukocyte common antigen-HRP (Dako / Dimension Lab) was used to label leukocytes in the adhesion assay.

2.2.5 Adhesion assay

Each adhesion molecule was maximally upregulated on HBMEC by treatment with 100U TNF- α /ml for 4-24h (4h for E-selectin, 18h for VCAM-1, 24h for ICAM-1 and PECAM-1). Monolayers were then incubated with individual blocking Abs for 30min and PMN ($4x10^6$ cells/ml) or T lymphocytes ($2x10^6$ cells/ml) were added to the wells and incubated with HBMEC for 10, 20 or 30 min for PMN and 20, 30 or 60 min for T lymphocytes at 37°C. At the end of the incubation period, the supernatants with the nonadherent leukocytes were removed and the monolayers were gently washed four times, once at each of the lateral walls of the wells. Adherent leukocytes were fixed in 1:1 acetone:ethanol for 7 min at 4°C, and stained with the immunoperoxidase technique for leukocyte common antigen as follows. After a brief wash, endogenous peroxidase was blocked by treatment with 2.5% hydrogen peroxide in 100% methanol. Monolayers were incubated with mouse anti-human leukocyte common antigen-HRP for 1h, then with 0.05% 3,3'-diaminobenzidine (DAB) and counterstained with haematoxylin. Controls included untreated EC, EC treated with TNF but without anti-adhesion molecule Abs and EC incubated with isotype matched control Abs. Except as noted above, all cultures were grown, fed, processed and examined with the microscope the same way at the same time.

<u>Quantitation</u>. The number of leukocytes bound to the monolayers was determined by counting the number of adherent leukocytes per mm² in one central and four peripheral randomly selected fields by light microscopy. The peripheral fields chosen were uniformly one field diameter (1.2mm) from the edge of the well. This negates any effect from the possible gradual change of leukocyte density between the center and the edge of the well. The average number of adherent PMN per field varied from less than

one for unstimulated EC to sixty for EC treated with TNF for 24h. The number of adherent T lymphocytes per field varied from eight for unstimulated EC to six hundred for EC treated with TNF for 24h. The identity of the wells was masked before counting so that all counts were performed blindly (Dorovini-Zis et al., 1992a).

In addition, the percentage of adherent cells was calculated by dividing the number of cells adherent per well (calculated knowing the area of the well and number of cells adherent per mm²) by the number of cells added to each well and multiplying by one hundred.

Monolayers grown on cellagen discs and treated as above were processed for scanning EM (SEM) as previously described (Dorovini-Zis et al., 1991). Briefly, cells were fixed with 2.5% glutaraldehyde in 0.05M sodium cacodylate for 1h at 4°C, then in 1% OsO_4 for 1h and 1% tannic acid for 1h. After dehydration through a graded series of ethanol, monolayers were stained with 2% uranyl acetate in 70% ethanol overnight. Subsequently, cells were washed five times in 70% ethanol, dehydrated through 100% ethanol, critical point dried and coated with gold. Specimens were examined with a Cambridge Sterescan 250T SEM.

2.2.6 Migration assay

In order to distinguish between the process of adhesion and migration, leukocytes $(5x10^{6} \text{ PMN/ml or } 3x10^{6} \text{ T lymphocytes/ml})$ were allowed to bind to the HBMEC monolayer for 15min (PMN) or 30min (T lymphocytes) in the absence of Abs. This period of time is not long enough for migration to occur. Unbound cells were washed off and then blocking Abs were added. After a further 2h incubation for PMN and 30, 60 or 180min for T lymphocytes, the HBMEC with the migrated leukocytes were gently washed and then processed for EM as described for immunogold EM above. <u>Controls</u> include untreated HBMEC and EC treated with TNF without anti-adhesion molecule Abs. As an additional positive control for the PMN migration experiments, a standard

PMN chemotactic factor (10⁻⁷M N-formyl-methionyl-leucyl-phenylalanine, fMLP, Sigma) was placed in the outer chamber under the HBMEC monolayers to establish a chemotactic gradient before PMN were added in the inner chamber over the EC. Except as noted above, all cultures were grown, fed, processed and examined under the microscope the same way at the same time.

Quantitation. The number of leukocytes that crossed the monolayers was counted in one hundred and twenty 1µm thick sections taken 15µm apart and stained with toluidine blue. The length of the monolayer in each thick section was measured and the number of migrated leukocytes per mm of the monolayer length was calculated by dividing the total number of migrated leukocytes by the monolayer length in each section. The identity of the sections was masked before counting so that all counts were performed blindly.

In addition, in order to calculate the percentage of cells that adhered and subsequently migrated, the number of leukocytes present in the wash before the addition of the Abs (pre-Ab wash) and in the wash at the end of the migration period and before fixation (pre-fixation wash) was counted in a haemocytometer. The percentage equals: Cells at start of assay - (Cells in pre-Ab wash + Cells in pre-fixation wash)

Cells at start of assay - Cells in pre-Ab wash	X 100
Cens at start of assay - Cens in pie-Ab wash	

Selected treatments were repeated for scanning electron microscopy. Processing for SEM was performed as described in the adhesion assay above.

2.2.7 Controls

For all experiments, cells grown in the absence of TNF, monolayers incubated with TNF but without Abs and/or EC treated with isotype matched Abs served as controls.

2.2.8 Statistics

Statistics were performed using the computer program SigmaStat (Jandel Scientific). ANOVA was performed on all data. Where significant difference was found, Student's t-test was applied to examine the results between cultures treated with Abs and those untreated for the adhesion assay. The Mann-Whitney test was applied to examine the results between cultures treated with Abs and those untreated for the adhesion assay. The Mann-Whitney test was applied to examine the results between cultures treated with Abs and those untreated for the migration assay since the data were not normally distributed. P<0.05 was taken as statistically significant for all analyses.

2.3 DETERMINATION OF MONOLAYER PERMEABILITY FOLLOWING TNF-α TREATMENT AND IN THE COURSE OF LEUKOCYTE MIGRATION ACROSS THE MONOLAYERS

2.3.1 Permeability assay

The permeability of HBMEC cultures was determined by measuring the electrical resistance across confluent monolayers using a volt-ohm meter (World Precision Instruments, Sarasota, FL). HBMEC grown to confluence on Cellagen discs were transferred from the double chamber chemotaxis system to a chamber with an electrode firmly positioned in the center of the chamber. Another electrode was placed on top of the monolayers and resistance was measured. Resistance was measured at each media change and the experiments were performed when a high resistance (>90 Ω ·cm²) was reached. To assess the effect due to TNF treatment, HBMEC were treated with 100U TNF/ml for 4, 18 or 24h. The resistance was measured in these cultures as well as in unstimulated cultures. The effect of PMN and T lymphocyte migration was examined following leukocyte incubation with untreated or TNF treated HBMEC for 4, 18 and 24h and PMN migration in response to gradients of 10⁻⁷M fMLP. Resistance was measured at the end of TNF treatment prior to leukocyte addition, then again at the end of the

migration period. In addition, HBMEC treated with TNF as above were incubated with anti-adhesion molecule Ab before addition of leukocytes. Again, resistance was measured prior to leukocyte addition and then after leukocyte migration. Since the resistance is inversely proportional to the area of the tissue, it is standard practice to report the product of the resistance and the growth area so that comparisons with results in the literature can be made. This value is then independent of the area of the tissue. In this study, the resistance was multiplied by the growth area of the Cellagen disc (0.64cm²) before reporting. Except as noted above, all cultures were grown, fed and measured with the ohm-meter the same way at the same time. <u>Controls</u> included cultures grown without TNF, with TNF but without Abs and EC without the addition of PMN or T lymphocytes.

2.3.2 Controls

For all experiments, cells grown in the absence of TNF, monolayers incubated with TNF but without Abs and EC without the addition of leukocytes served as controls.

2.3.3 Statistics

Statistics were performed using the computer program SigmaStat (Jandel Scientific). ANOVA, followed by Student's t-test where there was significant difference were applied to the results from transendothelial resistance measurements. T-tests compared cultures activated with TNF to those unstimulated, and cultures treated with Ab to those untreated. P<0.05 was taken as statistically significant.

CHAPTER 3 RESULTS

3.1 HUMAN BRAIN MICROVESSEL ENDOTHELIAL CELL CULTURE (HBMEC)

HBMEC in primary culture form confluent, contact-inhibited monolayers made up of elongated cells. There are no differences in cell growth or morphology between HBMEC grown on fibronectin coated plastic wells (Fig. 1a) and Cellagen discs (Fig. 1b). The endothelial origin of the cells was demonstrated by the positive granular, perinuclear staining for Factor VIII related antigen (Fig. 2).

3.2 EXPRESSION OF ADHESION MOLECULES BY HBMEC IN PRIMARY CULTURE AND UPREGULATION BY LPS AND CYTOKINES

3.2.1 E-selectin

3.2.1.1 Immunogold silver staining (IGSS)

Both the unstimulated and stimulated expression of E-selectin were determined by counting the number of cells with positive and negative surface staining following immunogold silver staining. Only 7% of unstimulated cells were stained for E-selectin (Figs. 4-8). There was minimal variation in the unstimulated expression among different cultures. Cells expressing E-selectin displayed diffuse staining over the cell surface (Fig. 4a). Treatment with LPS, TNF- α or IL-1 β generated a great rise in the percentage of E-selectin expressing cells (P<0.001 by ANOVA for all three treatments). The staining was evenly distributed on the cell surface in the form of fine black, granular deposits. Overall, the intensity of labeling was greater in treated than in unstimulated cells (Fig. 4). No staining was observed in control cultures incubated with normal mouse IgG, irrelevant Ab or buffer instead of the primary Ab.

LPS induced the greatest upregulation of E-selectin expression (Figs. 4b, 5a). The level was highest after 4h of treatment for all concentrations, then dropped to a level not statistically different from the unstimulated level by 24h for the lower concentrations or 48h for the higher concentrations in the continuous presence of LPS. Treatment with the three highest concentrations of LPS (0.01, 0.1 and 1µg/ml) for 4h induced E-selectin expression in about 90% of cells, while a 80% expression was observed with 0.001µg LPS/ml. The subsequent drop in the number of immunostained cells to unstimulated levels by 48h was similar for the 0.1 and 1µg/ml concentrations. The decline to pre-stimulation levels occurred earlier with 0.01µg LPS/ml and even more so with 0.001µg/ml.

TNF- α was similarly effective in upregulating E-selectin (Fig. 4c). 91% of the cells showed positive staining after 4h of treatment with the highest concentration - 100U/ml (Fig. 6a). Lower concentrations also induced a maximal rise at 4h, which decreased to very low levels by 48h. Following the initial rise in expression with 100U TNF- α /ml, E-selectin levels decreased considerably after 48h to levels still statistically different from the unstimulated level. 10U TNF- α /ml increased E-selectin expression to a lesser extent, while 1U TNF- α /ml induced even lower levels of E-selectin which actually became statistically no different from unstimulated levels by 48h.

Upregulation of E-selectin following IL-1 β treatment was less impressive than that with LPS and TNF- α (Fig. 4d). The expression was maximal by 4h then decreased to unstimulated levels by 24 to 48h, similar to the other treatments (Fig. 7a). However, even with the highest concentration (10U IL-1 β /ml), only 58% of cells became E-selectin positive by 4h. 1U IL-1 β /ml induced levels slightly below those of 10U/ml, while the expression with 0.1U/ml was much lower than with the other two concentrations.

Amongst all the treatments applied, IFN- γ was the only one that was not effective in raising the level of E-selectin expression even at the high concentration of 500U/ml (P=0.548 by ANOVA) (Fig. 8a).

3.2.1.2 Enzyme-linked immunosorbent assay (ELISA)

The overall expression of E-selectin in confluent HBMEC monolayers was quantitated by ELISA. The results are similar to the counts obtained by immunogold silver staining. No increase in absorbance was seen in control wells incubated with buffer or normal mouse IgG instead of primary Ab. A low level of E-selectin expression was observed with unstimulated HBMEC (Figs. 5b-8b). LPS, TNF- α and IL-1 β all significantly upregulated E-selectin (P=0.014, <0.033 and <0.001, respectively by ANOVA). The treatment that generated the greatest E-selectin upregulation was, again, incubation with LPS (Fig. 5b). For all concentrations used, there was a great increase in E-selectin expression by 4h, the maximal levels being quite similar for all concentrations. With 0.01 and 0.001µg LPS/ml, the E-selectin levels rose even slightly higher at 12h. There was a rapid drop by 24h, and by 48h, there was no statistically significant difference from the unstimulated levels at all concentrations (Fig. 5b).

TNF- α , at all concentrations tested, induced a maximal E-selectin expression at 4h (Fig. 6b). This returned promptly to unstimulated levels by 24h for 1U TNF- α /ml, and 48h for 10 and 100U TNF- α /ml.

The effectiveness of IL-1 β was intermediate, while the pattern of E-selectin upregulation was similar to that of LPS and TNF- α (Fig. 7b). There was maximal expression at 4h, which then declined to levels not statistically different from the unstimulated ones by 12h for 0.1U IL-1 β /ml, 24h for 1U IL-1 β /ml, and 48h for 10U IL-1 β /ml.

E-selectin expression was not affected by IFN- γ even at high concentrations (500U/ml, P=0.333 by ANOVA) (Fig. 8b).

3.2.1.3 Immunogold electron microscopy (IGEM)

Ultrastructural localization of E-selectin was performed by immunogold labeling following maximal upregulation with TNF- α . Staining was present on the surface of

both unstimulated and TNF treated HBMEC (Figs. 9 and 10). A significantly greater amount of labeling was present on the apical surface compared to the basal surface of all EC. TNF treatment dramatically increased the number of gold particles on the apical surface and, to a lesser but still significant extent, on the basal surface of HBMEC (P<0.001) (Fig. 10). Gold particles were absent along intercellular contacts and at tight junctions. Finger-like cytoplasmic processes on the apical surface were more prominently decorated (Fig. 9c) than adjacent flat surfaces. A small number of gold particles was observed infrequently within pits / vesicles some of which appeared to be clathrin-coated invaginations of the apical cell membrane in TNF treated cells and rarely in unstimulated cells. No labeling was observed on cells incubated with buffer without primary Ab.

3.2.2 Vascular cell adhesion molecule-1 (VCAM-1)

3.2.2.1 Immunogold silver staining (IGSS)

Control HBMEC monolayers cultivated in the absence of LPS and cytokines exhibited minimal expression of VCAM-1 (13-18%) (Figs. 11a, 12a-15a). The degree of VCAM-1 expression in unstimulated cells was constant in a given culture, although varying slightly between cultures originating from different individuals. Growth in the presence of LPS, TNF- α or IL-1 β induced marked upregulation of VCAM-1 expression (P=0.008, <0.001, =0.022, respectively, by ANOVA) as indicated by the large number of VCAM-1 positive cells (Fig. 11b-d). The intensity of labeling varied among individual cells of the same culture, the largest cells generally being more darkly stained. Cells expressing VCAM-1 exhibited distinct, finely granular, brown-black staining diffusely throughout the cell surface (Fig. 11b-d). Unstimulated EC showed less intense surface staining with a tendency for localization at the periphery of the cell (Fig. 11a). Control

wells incubated with normal mouse IgG or buffer without primary Ab showed no labeling.

The greatest upregulation of VCAM-1 was observed following treatment with LPS. Incubation with 1µg/ml was associated with an increase to 50% by 4h (Fig. 12a) and a maximal level of 83% by 12h that remained at a plateau for up to 24h and then declined to a level not statistically different from the unstimulated level by 48h. 0.1µg/ml elicited a similar pattern of VCAM-1 upregulation, though the levels of expression were slightly lower. The increase in VCAM-1 induced by 0.01µg/ml was similar up to 12h. However, VCAM-1 levels started to fall by 24h and by 48h, they were no longer statistically different from the unstimulated levels. With 0.001µg/ml, there was significant upregulation to 45% by 12h followed by a decline close to unstimulated levels by 24h.

TNF- α caused the second highest level of VCAM-1 expression. Treatment with 100U/ml induced a maximal VCAM-1 level of 74% at 24h, which declined to 53% by 48h (Fig. 13a). 10U/ml generated a similar increase up to 12h but the level then declined to 48% and plateaued. A modest increase was noted with 1U/ml at 12h and 24h with levels remaining steady thereafter.

The extent of VCAM-1 upregulation was relatively less impressive following treatment of the monolayers with IL-1 β (Fig. 14a). 10U/ml raised the VCAM-1 level to 33% by 4h, and 60% by 12h. Longer periods of incubation resulted in declines to 49% and 36% at 24h and 48h, respectively. 1U/ml was not associated with any statistically significant difference in VCAM-1 levels at 4h. The level increased dramatically to 60% by 12h and then declined at a much faster rate than in cultures treated with 10U/ml. Cultures exposed to 0.1U/ml expressed VCAM-1 levels that paralleled those induced by 1U/ml except that the levels of expression were lower. Coincubation with the lowest concentrations of TNF- α and IL-1 β for 48h failed to upregulate VCAM-1 (P=0.812 by

ANOVA) (Fig. 16a). IFN- γ did not increase VCAM-1 expression to any great extent even at the high concentration of 500U/ml (P=0.1 by ANOVA) (Fig. 15a).

3.2.2.2 Enzyme-linked immunosorbent assay (ELISA)

Quantitation by ELISA generated results similar to those obtained by immunogold silver staining. The relative amount of VCAM-1 expressed was measured by this method. Control cultures incubated with normal mouse IgG or buffer only and no primary Ab showed no change in absorbance. Significant increases in expression were induced by LPS (P=0.02 by ANOVA), TNF (P=0.014) and IL-1 (P=0.013). LPS induced the highest levels of VCAM-1 expression, peaking at 12h at all concentrations tested and declining to a level not statistically different from the unstimulated expression (Fig. 12b). 1 and 0.1μ g/ml elicited the greatest upregulation while 0.01 and 0.001μ g/ml resulted in successively modest increase. TNF- α was second in effectiveness to LPS. Upregulation of VCAM-1 expression was maximal at 12h (Fig. 13b), followed by a less steep decline, resulting in higher levels of VCAM-1 expression at the end. IL-1 β was less effective than TNF- α in upregulating VCAM-1 (Fig. 14b). Maximal expression was observed by 12 to 24h followed by a decline. The increase in expression caused by 0.1U/ml was not statistically significant and did not peak until 24h. Treatment with IFN-y was not associated with any significant changes in VCAM-1 expression (P=0.198 by ANOVA) (Fig. 15b).

3.2.2.3 Immunogold electron microscopy (IGEM)

Immunogold electron microscopy revealed that VCAM-1 was readily detectable on the surface of the EC. Labeling usually occurred in clumps of 3 or more gold particles. Both unstimulated and TNF- α treated cells showed a statistically significant (p<0.05) increase in labeling on the apical surface compared to the basal surface (Figs. 17-18). Labeling was not observed in coated pits, along interendothelial contacts or at

tight junctions (Fig. 17d). There was a tendency for gold particles to localize on or near finger-like cytoplasmic projections rather than flat apical surfaces especially on TNF- α treated cells (Fig. 17c). Compared to unstimulated EC, cells incubated with TNF- α exhibited several folds more labeling on both the apical and basal surfaces (p<0.05), the apical surface being consistently more densely decorated with gold particles than the basal surface. Control cultures incubated with buffer instead of primary Ab were unlabeled.

3.2.3 Intercellular adhesion molecule-1 (ICAM-1)

3.2.3.1 Immunogold silver staining (IGSS)

In control cultures, 20-40% of EC expressed ICAM-1 (Figs. 19-23). Treatment of HBMEC with LPS, IFN- γ , TNF- α , and IL-1 β led to significant upregulation of ICAM-1 expression (P=0.001, =0.027, =0.002, <0.001, respectively by ANOVA). Positively stained cells displayed diffuse surface staining in the form of finely granular, dark-brown to black deposits (Fig. 19). There was some variability in the intensity of labeling among individual EC, the plumper cells being decorated with the most dense reaction product. The overall intensity of staining was higher in experimental than in control cultures (Fig. 19). LPS was the most effective inducer of ICAM-1 upregulation (Figs. 19b, 20). 5 μ g LPS/ml resulted in expression of ICAM-1 by 95% of the cells within 12h (p<0.05), and this level remained constant up to 72h in the continuous presence of LPS. The high level of ICAM-1 induced by 1 μ g and 0.1 μ g LPS/ml seemed to decline slightly after 24h. 0.01 μ g LPS/ml produced an intermediate rise in ICAM-1 which dropped slowly after 12h. A statistically significant (p<0.05) rise in ICAM-1 (40%) was not observed with 0.001 μ g LPS/ml until after 48h.

In contrast to the large increase in ICAM-1 expression observed with LPS, treatment with IFN-γ induced only a slight increase (Figs. 19c, 21), with maximal levels

of expression reaching only 58%. By 12h, a statistically significant level of upregulation to 47% was observed with all concentrations (p<0.05), but at longer incubation times, the relatively larger variance made any increases in expression not to be statistically significant.

The extent of ICAM-1 upregulation by both TNF- α (Figs. 19d, 22) and IL-1 β (Figs. 19e, 23) was intermediate between that of LPS and IFN- γ . By 24h, 85% of cells showed positive staining (p<0.05), after which there was a general trend of slight decline. The increased staining induced by 10U and 100U TNF- α /ml was statistically significant. The increase caused by 1U TNF- α /ml was not statistically significant until 12h, and after incubation for 72h, the increase declined to non-significant levels. The upregulation of ICAM-1 elicited by IL-1 β reached maximal levels at 24h (84% for 10U, 76% for 1U, and 67% for 0.1U, all p<0.05), and then declined.

Incubation of EC with TNF- α and IFN- γ combined resulted in marked increase in ICAM-1 expression that was even greater than that induced by LPS (Figs. 19f, 24). The number of immunostained cells ranged from 99 to 100% with 500U IFN- γ /ml plus 1U to 100U TNF- α /ml and reached 97% with 100U IFN- γ /ml plus 100U TNF- α /ml. No labeling was observed in cultures incubated with normal mouse IgG or buffer instead of primary Ab.

3.2.3.2 Reversibility of ICAM-1 expression

Stimulation of HBMEC with LPS or cytokines for 2 days followed by withdrawal and culture of cells in regular growth media for 3 days was associated with variable degrees of reversal of ICAM-1 upregulation following LPS and TNF- α incubation, and lack of reversal following IL-1 β and IFN- γ treatment (none statistically significant). These differences were due to cytokine and LPS removal since cultures incubated with cytokine or LPS for 5 days expressed high levels of ICAM-1 (Figs. 25-28). There was a decrease (10-30%) in the number of cells expressing ICAM-1 at all concentrations of

LPS (Fig. 25). Reversibility of expression was minimal when $5\mu g/ml$ LPS was used. Reversibility of upregulation was not observed with IFN- γ at 100U/ml, while 500U/ml was associated with a 19% increase instead (Fig. 26). Activation followed by withdrawal of TNF- α resulted in decrease in ICAM-1 expression ranging from 6% to 23%. TNF- α concentration of 100U/ml was associated with the smallest decrease while 1U/ml induced the greatest (Fig. 27). Paradoxically, incubation of EC in IL-1 β -free media for 3 days following activation resulted in increase in the number of labeled cells ranging from a low of 1% with 10U IL-1 β /ml to 17% with 0.1U/ml (Fig. 28).

3.2.3.3 Immunogold electron microscopy (IGEM)

ICAM-1 was expressed on the surface of both unstimulated and TNF treated HBMEC. There was significantly more labeling on the apical surface compared to the basal surface (Fig. 29 and 30). Indeed, gold particles were rarely observed on the basal surface. Labeling of cytoplasmic pits and vesicles and intercellular contacts was not observed. Gold particles were especially concentrated on finger-like cytoplasmic projections on the apical cell surface. TNF treatment greatly increased the apical and to a lesser extent the basal expression of ICAM-1. Control cultures incubated without primary Ab were unlabeled.

3.2.4 Platelet/endothelial cell adhesion molecule-1 (PECAM-1)

3.2.4.1 Enzyme-linked immunosorbent assay (ELISA)

PECAM-1 was constitutively expressed by HBMEC (Figs. 31-35). Treatment with 0.01-1µg LPS/ml (Fig. 32), 1-100 U TNF- α /ml (Fig. 33), 0.1-10 U IL-1 β /ml (Fig. 34) or 50-500 U IFN- γ /ml (Fig. 35) for 4 to 48h did not increase its expression quantitatively (P=0.063, 0.091, 0.723, 0.211, respectively by ANOVA) or change its distribution (Figs. 32, 33). In both unstimulated and activated EC, PECAM-1 staining appeared as fine black grains mostly concentrated at the borders between adjacent cells but also distributed diffusely, though less abundantly, over the cell surface (Fig. 31).

3.2.4.2 Immunogold electron microscopy (IGEM)

The presence of PECAM-1 was readily detectable on the surface of EC in the form of gold particles occurring singly or in small groups. Labeling was most prominent at the apical surface of HBMEC and, unlike ICAM-1, VCAM-1 and E-selectin, gold particles did not have the tendency to concentrate on finger-like projections at the apical cell surface (Figs. 36-38). There was no apparent difference in labeling between untreated and IFN- γ treated cells. Although gold particles were not observed along intercellular contacts, the most peripheral processes of the cytoplasm particularly in the vicinity of cell to cell contact, were constantly decorated with gold particles.

3.2.5 HECA-452 antigen

The HECA-452 antigen was not expressed by unstimulated HBMEC and could not be induced *de novo* in cells treated with 5µg LPS/ml, 10-100 U TNF- α /ml, 1-10 U IL-1 β /ml or 100-400 U IFN- γ /ml alone or in combination with IL-1 β for 1, 2, 3, 4, 6, or 7 days. Positive staining of high endothelial venule cells in control frozen sections of tonsils confirmed that this Ab was functional.

3.3 ROLE OF ADHESION MOLECULES IN PMN AND T LYMPHOCYTE ADHESION TO AND MIGRATION ACROSS HUMAN BRAIN MICROVESSEL ENDOTHELIAL CELL MONOLAYERS

3.3.1 Adhesion of polymorphonuclear leukocytes to human brain microvessel endothelial cells

A small number of PMN (approximately 0.06%) adhered to unstimulated HBMEC. Pretreatment of HBMEC with TNF- α for 4, 18 or 24h progressively increased the adhesion of PMN to EC (P<0.001 by ANOVA). This increase was directly related to the length of TNF treatment and the duration of EC-PMN incubation (Figs. 39-41). Thus, 4h of TNF treatment increased adhesion to 0.1-0.2%, 18h to 0.2-0.4% and 24h to 0.4-0.7%. Similarly, the number of adherent PMN increased from 11/mm² after 10 min incubation to $34/\text{mm}^2$ after 30 min incubation with EC (Fig. 41). None of the anti-adhesion molecule mAbs decreased PMN adhesion to unstimulated HBMEC (P>0.124 by ANOVA) (Figs. 42-44). Adhesion to TNF stimulated HBMEC was inhibited by Abs ($P \le 0.001$ by ANOVA). After 10, 20 and 30 min incubation of PMN with TNF-treated HBMEC, the anti-E-selectin mAb significantly blocked adhesion by 54, 40 and 38%, respectively. This level of adhesion is not significantly different from that obtained with unstimulated HBMEC. Anti-ICAM-1 mAb blocked adhesion by 37% at 10min, 47% at 20min and 45% at 30min (Figs. 42-44). The anti-PECAM-1 mAb decreased adhesion by 10 to 30%, but this was not large enough to be statistically significant. The anti-VCAM-1 mAb did not significantly decrease adhesion. Isotype matched control Abs did not have any effect. A preliminary experiment with the $F(ab')_{2}$ fragments of the anti-VCAM-1 and anti-E-selectin Abs showed the same results as experiments with intact Abs. It appears that the Fc receptor on peripheral blood PMN do not bind IgG strongly. Thus, all subsequent experiments were performed with intact Abs.

Scanning electron microscopy (SEM) showed that only a few PMN adhered to unstimulated EC (Fig. 39a). Pretreatment of the monolayers with TNF markedly increased the number of adherent PMN (Fig. 39b). PMN first adhered by extending pseudopodia to the surface of HBMEC while still retaining their spherical shape. They

tight junctional complexes between HBMEC overlying the migrated PMN appeared intact.

3.3.3 T lymphocyte adhesion

A small number of T lymphocytes (2%) adhered to unstimulated HBMEC (Figs. 51-53). Pretreatment of EC with TNF- α significantly upregulated adhesion of T lymphocytes following 20, 30 and 60 min incubation with HBMEC (16-23%, P<0.001 by ANOVA) (Figs. 51-53). Ab treatment decreased adhesion to unstimulated and activated EC (P<0.005 by ANOVA). The anti-ICAM-1 mAb was the only one that significantly blocked adhesion to unstimulated HBMEC by 60, 55 and 63% after 20, 30 and 60 min of T lymphocyte incubation, respectively (Figs. 54-56). However, when HBMEC were pretreated with TNF, both anti-VCAM-1 and anti-ICAM-1 mAbs significantly blocked adhesion: anti-VCAM-1 mAb by 32, 33 and 29%, and anti-ICAM-1 mAb by 47, 57 and 75% at 20, 30 and 60 min of T lymphocyte incubation, respectively (Figs. 54-56). The anti-E-selectin and anti-PECAM-1 Abs did not block adhesion (Figs. 54-56). Paradoxically, the anti-PECAM-1 Ab increased adhesion (20-100%) especially at 60 min (100% increase, Figs. 54-56). A 90% increase in adhesion was also observed when anti-PECAM-1 Ab was applied to unstimulated HBMEC prior to incubation with T lymphocytes for 60min (Fig. 56). Isotype matched control Abs did not have any effect.

SEM showed that only few T lymphocytes adhered to untreated EC (Fig. 51a). HBMEC treatment with TNF resulted in significant increase in the number of adherent lymphocytes (Fig. 51b-d). Adherent T lymphocytes appeared as small, roughly spherical cells that established contact with EC by extending long pseudopodia to their apical surface (Figs. 51, 57-61). They adhered to the surface of HBMEC (Fig. 62) and also aligned themselves singly or in groups along adjacent EC (Fig. 51).

3.3.4 T lymphocyte migration

Very few T lymphocytes migrated across unstimulated HBMEC even after 180min of incubation with EC (Figs. 63-64). Pretreatment of HBMEC with TNF significantly increased migration only after prolonged (180min) EC-lymphocyte incubation (P<0.001 by ANOVA) (Figs. 63-64). Thus, a 30 fold increase in transmigration was observed when T lymphocytes were incubated for 180min with EC pretreated with TNF for 24h, while only a modest 7 and 8 fold increase was recorded for the same incubation time but with shorter TNF pretreatment (4h and 18h, respectively). Anti-adhesion molecule Abs had significant effect on migration at prolonged (180min, $P \le 0.002$ by ANOVA) but not short (30 or 60min, P>0.08 by ANOVA) incubations (Figs. 65-66). Thus, anti-PECAM-1 Ab blocked migration by 75% and anti-ICAM-1 mAb by 93% (Figs. 67). In fact, the anti-ICAM-1 mAb brought migration across activated HBMEC down to levels obtained with unstimulated HBMEC. Anti-E-selectin mAb blocked migration by 40%, while the 20% decrease with anti-VCAM-1 is not statistically significant.

Examination by SEM showed that, following their initial adhesion to the apical surface of the endothelium (Fig. 68a), lymphocytes proceeded with crossing the monolayers by moving between adjacent EC or by direct penetration of the EC cytoplasm (Fig. 68b, c). In the initial stages of transcellular migration, T lymphocytes were seen within deep invaginations of the HBMEC surface and appeared to fit tightly the size of these invaginations (Fig. 68c). Examination by TEM showed that following the initial firm contact between pseudopodia of lymphocytes and the apical surface of the endothelium (adhesion), lymphocytes began inserting foot-like processes into the endothelial cytoplasm. One or more such areas of penetration by the same lymphocyte were in tight contact with the EC cytoplasm. Occasionally, the most advancing tip was preceded by a variably sized cytoplasmic "opening" or gap (Figs. 69-70), which

progressively receded and enlarged to allow for the remaining cytoplasm and nucleus of the lymphocyte to go through the EC into the subendothelial region (Fig. 72). Once across the monolayers, lymphocytes became elongated and remained between the underlying collagen membrane and the overlying EC while maintaining close contact with the latter (Fig. 73). At the end of the migration period, the monolayers resumed their continuity and the junctional complexes between adjacent EC appeared intact.

3.4 DETERMINATION OF MONOLAYER PERMEABILITY FOLLOWING TNF-α TREATMENT AND IN THE COURSE OF LEUKOCYTE MIGRATION ACROSS THE MONOLAYERS

Lastly, we examined the effect of TNF treatment, leukocyte migration, and anti-adhesion molecule Abs (that block adhesion and/or migration) on the barrier properties of HBMEC monolayers by measuring the electrical resistance across monolayers treated with different combinations of the above. A drop in resistance indicated an increase in permeability, thus a decrease in barrier function. Unstimulated HBMEC grown on Cellagen discs had an average resistance of $108\Omega \cdot \text{cm}^2$ (Table 1). TNF treatment progressively decreased the resistance to $68\Omega \cdot \text{cm}^2$ by 18h, then further down to $63\Omega \cdot \text{cm}^2$ by 24h (P<0.001) (Table 1). An additional drop in resistance was observed following PMN migration across the monolayers. This was especially evident when fMLP was placed in the lower chamber to attract PMN to migrate $(44\Omega \cdot cm^2)$ compared to monolayers without chemotactic gradient (99 Ω ·cm²). PMN migration across HBMEC pretreated with TNF for 4 and 18h decreased the resistance by 25% while migration across HBMEC preincubated with TNF for 24h decreased it by 43%. The presence of anti-adhesion molecule Abs did not prevent the drop in resistance following PMN migration (P=0.11 by ANOVA) (Table 2). T lymphocyte migration across 4, 18 or 24h TNF pretreated HBMEC also decreased resistance by an average of about 25% (P=0.002). This drop in resistance was not prevented by the presence of

anti-adhesion molecule Ab (duplicate wells of one experiment, P=0.082 by ANOVA) (Table 3).

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CHAPTER 4

DISCUSSION

4.1 EXPRESSION OF ADHESION MOLECULES

4.1.1 E-selectin

In the present study, we found that 7% of untreated HBMEC express E-selectin. This expression can be upregulated on HBMEC by LPS, TNF- α and IL-1 β . Positively stained cells display an intense black granular reaction product evenly distributed throughout the cell surface. The maximal staining intensity is much greater in HBMEC than in HUVEC where the reaction product is concentrated mainly at the peri-nuclear region (Varani et al., 1992). E-selectin expression is maximal 4-6h after LPS or cytokine treatment of HBMEC, HDMEC (Sepp et al., 1994), HUVEC (Bevilacqua et al., 1989; Luscinskas et al., 1989; Pober et al., 1986a, 1986b; Leeuwenberg et al., 1989; Leeuwenberg et al., 1990a, 1990b; Bevilacqua et al., 1987; Graber et al., 1990; Wellicome et al., 1990; Doukas et al., 1990) and mouse endothelioma cells (Hahne et al., 1993). The concentrations required to generate a maximal expression of E-selectin on HUVEC are mostly higher than those for HBMEC (Wellicome et al., 1990; Luscinskas et al., 1989). IFN- γ , at a concentration that upregulates ICAM-1, is not effective in either cell type (Graber et al., 1990).

After maximal expression, E-selectin declines to unstimulated levels by 48h in all *in vitro* systems examined thus far including HBMEC, HDMEC (Sepp et al., 1994), HUVEC (Bevilacqua et al., 1989; Luscinskas et al., 1989; Pober et al., 1986a, 1986b; Leeuwenberg et al., 1989, 1990a, 1990b; Bevilacqua et al., 1987; Graber et al., 1990; Wellicome et al., 1990; Doukas and Pober, 1990) and mouse endothelioma cells (Hahne et al., 1993). Such declines in staining after 48h does not seem to be due to cytotoxicity on HBMEC since no cell retraction or necrosis is seen under light microscopy, SEM or TEM after 48h of LPS or cytokine treatment. Indeed, EC are still able to maintain their
high ICAM-1 expression at this time. However, a factor recently found in human serum causes E-selectin and its mRNA to be persistently expressed, at least up to 48h (Sepp et al., 1994). The effectiveness of treatments that increase E-selectin expression is different between HBMEC and HUVEC. For HBMEC, LPS and TNF- α are most effective followed by IL-1 β , while for HUVEC, the order is reversed (Wellicome et al., 1990).

	HBMEC	HUVEC	HDMEC
Unstimulated			
Expression			
(% cells labelled)	7%	None	-
Time of maximal			
expression	4h	4-6h	4-6h
Concentration	0.1 µg LPS/ml	1 ug LPS/ml	~
giving maximal	10 U IL-1β/ml	10 U IL-1/ml	-
expression	100U TNF-α/ml	320U TNF/ml	-
Level post	Declines to	Declines to	Declines to
maximal increase	unstimulated	unstimulated	unstimulated
	levels by 48h.	levels by 48h.	levels by 48h.
Relative effectiveness	LPS/TNF-α>IL-1β.	IL-1β>TNF/LPS	-
of cytokines/LPS	IFN-γ not effective.		

Summary of E-selectin expression on EC

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It is unlikely that difference in staining found are due to different culture conditions between the published reports and the present studies since reports from different laboratories with different growth conditions are quite consistent.

The greater unstimulated level of E-selectin expression and greater sensitivity to cytokine and LPS suggest that E-selectin may be utilized to a greater extent in the brain than extracerebral organs. It is used in both PMN adhesion and T lymphocyte migration across HBMEC. Although E-selectin expression declines to unstimulated levels by 48h, indicating its use is short lived, if a human serum factor could maintain a high level of E-selectin on HBMEC, then it could be used in the long term. If not, then there is also the possibility that after a refractory period, E-selectin may be induced on these EC again and HBMEC in areas around the center of the inflammatory site may become progressively activated and express E-selectin stepwise.

The greater expression of E-selectin on the apical surface of HBMEC observed ultrastructurally may have functional significance regarding leukocyte adhesion to EC. Prominent expression at the finger-like projections of EC may facilitate initial interactions between leukocytes and EC at these points of contact during the "rolling" of leukocytes. The upregulation of the low level of E-selectin expression at the basal surface by TNF may point to participation of this molecule in leukocyte migration across the endothelium. The use of subconfluent monolayers ensured that the Abs still reach the basal EC surface from the edge of the colony or between non-contacting EC. E-selectin was observed within cytoplasmic vesicles or pits in HBMEC, although infrequently. This presence may permit adherent leukocyte pseudopods to begin penetration of the EC for transmigration. We have shown that E-selectin acts in T lymphocyte migration, perhaps in this way. The presence of E-selectin in cytoplasmic vesicles has been reported for HUVEC (von Asmuth et al., 1992; Kuijpers et al., 1994). It was suggested that this is a consequence of endocytosis of these receptors, in order to remove E-selectin as well as free forms of its ligand, L-selectin, that have been shed into the circulation with eventual degradation in the lysosomal compartment. E-selectin labeling has also been localized in multivesicular bodies - a member of this compartment (von Asmuth et al., 1992).

E-selectin, a member of the selectin family, is expressed on LPS or cytokine treated EC (Bevilacqua and Nelson, 1993). Molecular biologic analysis demonstrated that interaction of TNF with the well characterized 55KDa TNF receptor on HUVEC can induce the expression of E-selectin (Throp et al., 1992). The induction by TNF, IL-1 or LPS involves the PKC-independent activation of NF- κ B binding to the E-selectin enhancer/promoter sequence in HUVEC (Montgomery et al., 1991; Whelan et al., 1991; Read et al., 1994). Protein synthesis inhibitors can augment this NF- κ B binding activity and increase the mRNA level by stabilization (Ghersa et al., 1992). E-selectin expression can be conferred to nonmyeloid cell lines by transfection of a human fucosyltransferase cDNA. Thus, this enzyme may regulate adhesion of E-selectin by modulating the cell surface expression of sialyl-Lewis x (Lowe et al., 1990). The expression of E-selectin itself is inhibited by the binding of glucocorticoid to its receptor, giving rise to a decreased level of E-selectin mRNA eventually (Cronstein et al., 1992). Cyclic AMP has been suggested to be an inhibitor but not a physiological regulator (Pober et al., 1993).

In vitro, E-selectin, is endocytozed by HUVEC during the induction of its expression (von Asmuth et al., 1992). However, soluble E-selectin has also been shown to be released by activated HUVEC (Leeuwenberg et al., 1992), and soluble E-selectin is found in the serum of patients with septic shock (Newman et al., 1993).

Only one report has dealt with the expression of E-selectin in CNS disorders *in vivo* (Dorovini-Zis et al., 1992b). The findings include the demonstration of E-selectin on capillaries, arterioles and venules in all cases of encephalitis, meningitis and brain abscess studied. Focal staining was seen in cases of infarcts. Neither larger arteries and veins, nor endothelium of normal brain or traumatic lesions stained. A positive correlation between E-selectin expression and the degree of inflammation was observed.

Studies on the expression of E-selectin in cultured EC monolayers have been performed mainly on HUVEC and human dermal microvessel EC (HDMEC). Unstimulated HUVEC do not express E-selectin or express it at minimal levels (Varani et

al., 1992; Bevilacqua et al., 1989; Luscinskas et al., 1989; Pober et al., 1986a, 1986b; Leeuwenberg et al., 1989; Leeuwenberg et al., 1990b; Bevilacqua et al., 1987; Graber et al., 1990; Wellicome et al., 1990; Doukas and Pober, 1990). There has only been one brief report with a mouse endothelioma cell line established from polyoma-induced hemangioma in the brain (Hahne et al., 1993) and one report dealing with induction by endothelin on human brain microvascular EC (McCarron et al., 1993c).

Our results indicate that activation of HBMEC with LPS, TNF and IL-1β, but not IFN-γ, greatly upregulate the expression of E-selectin on HBMEC. Maximal upregulation is reached by 4h, followed by a rapid decline to unstimulated levels by 48h in the continuous presence of LPS or cytokines. Comparing our data with those reported on HUVEC by other investigators, it appears that HBMEC are more sensitive to these treatments than HUVEC with respect to the upregulation of E-selectin expression. E-selectin is largely expressed on the apical surface of HBMEC. TNF treatment increases its level of expression by many folds, especially at finger-like cytoplasmic projections. E-selectin is also occasionally present within cytoplasmic vesicles, but absent at tight junctions.

4.1.2 Vascular cell adhesion molecule-1 (VCAM-1)

The present study demonstrates that HBMEC in primary culture express low levels of VCAM-1 that can be upregulated by activation with LPS, TNF- α and IL-1 β in a time and concentration dependent manner. Previous *in vitro* studies have demonstrated a lack or minimal constitutive expression of VCAM-1 on passaged HUVEC and HDMEC (Wellicome et al., 1990; Swerlick et al., 1992; Graber et al., 1990). In the CNS, VCAM-1 has not been observed in neurons, glia or EC in the absence of inflammation (Rice et al., 1991), however Abs against its ligand, VLA-4, have been shown to inhibit lymphocytes and monocytes from binding to cryostat sections of rat brain with

experimental allergic encephalomyelitis (EAE) and prevent the development of EAE *in vivo* (Yednock et al., 1992). Similarly, VCAM-1 expression was not observed in microvessel preparations isolated from normal human brains at autopsy (Washington et al., 1994), but could be upregulated on human brain microvascular EC by endothelin (McCarron et al., 1993c).

Previous studies on VCAM-1 induction on HUVEC have demonstrated that VCAM-1 mRNA is already present 2h following treatment with IL-1 or TNF- α , peaks at 4h and is maintained for at least 72h (Swerlick et al., 1992; Osborn et al., 1989). Surface expression of VCAM-1 peaks from 4 to 8h after cytokine treatment (Rice and Bevilacqua, 1989; Wellicome et al., 1990; Swerlick et al., 1992; Carlos et al., 1990), then either stays constant with TNF and LPS (Wellicome et al., 1990; Swerlick et al., 1992) or declines gradually to unstimulated or near unstimulated levels after 48h to 72h (Rice and Bevilacqua, 1989; Wellicome et al., 1990; Swerlick et al., 1992). Activation of HDMEC with TNF- α induces early increase in VCAM-1 mRNA after 4h followed by a decline to undetectable levels by 24h (Swerlick et al., 1992). Surface expression rises 16h after treatment with cytokines and declines rapidly by 24h. On a mouse endothelioma cell lines, VCAM-1 becomes maximally expressed 16h after TNF treatment, then drops slightly by 24h (Hahne et al., 1993). In contrast, surface expression of VCAM-1 on HBMEC becomes maximal 12-24h after cytokine or LPS treatment and drops back to nearly unstimulated levels by 48h.

	HBMEC	HUVEC	HDMEC
Unstimulated			
Expression			
(% cells labelled)	15%	Low	None
Time of maximal			
expression	12h	4-8h	16h
Concentration	0.1 μg LPS/ml	0.1-1 μg LPS/ml	0.01µg LPS/ml
giving maximal	10 U IL-1β/ml	10 U IL-1/ml	-
expression	100U TNF-α/ml	500U TNF-α/ml	500U TNF/ml
Level post	Declines to	Plateaus or	Drops fast by
maximal increase	unstimulated	drops slowly.	24h, then
	levels by 48h.	Does not reach	slowly, but not
		unstimulated	to
		levels.	unstimulated
			levels by 72h.
Relative	LPS>TNF-α	TNF>IL-1β>LPS	TNF>LPS
effectiveness	>IL-1β		IL-1 not
of cytokines/LPS	IFN-γ not effective.		effective.

Although surface expression of VCAM-1 was induced by LPS, TNF- α and IL-1 β , upregulation was maximal with LPS, followed by TNF- α and IL-1 β in that order. Regulation of VCAM-1 expression by cytokines on HBMEC is different from that previously reported for HUVEC (Swerlick et al., 1992; Graber et al., 1990) and HDMEC (Swerlick et al., 1992). IFN- γ , at a concentration that upregulates ICAM-1, failed to upregulate VCAM-1 on HBMEC. This is in contrast to dermal microvessel EC which show significant upregulation of VCAM-1 following intradermal injection of IFN- γ (Groves et al., 1993). The concentrations of LPS, TNF- α and IL-1 required for induction of maximal VCAM-1 expression on HBMEC are comparable to those inducing maximal upregulation on HUVEC by some investigators (Wellicome et al., 1990) but not by others (Swerlick et al., 1992). In contrast, much higher concentrations of TNF- α and lower concentrations of LPS are required for maximal induction of VCAM-1 on HDMEC (Swerlick et al., 1992). Activated HUVEC release VCAM-1 in a soluble form (Pigott et al., 1992), but VCAM-1 is not found in endocytotic vesicles in EC (Kuijpers et al., 1994). These differences in the kinetics of VCAM-1 upregulation in the three *in vitro* systems may either reflect diverse culture and experimental conditions among different laboratories, or, possibly indicate inherent differences in cytokine responsiveness between EC derived from different vascular beds.

The present findings correlate with previous *in vitro* immunocytochemical studies demonstrating preferential localization of VCAM-1 on the apical surface of IL-1 activated HUVEC, while expression was less prominent on the basal cell surface and absent from interendothelial contacts (Oppenheimer-Marks, 1991). The polarized expression of VCAM-1 may be of functional significance considering cerebral EC-leukocyte interactions. The fact that VCAM-1 is present predominantly on the apical surface of activated HBMEC and not along intercellular contacts may indicate a possible role of VCAM-1 primarily in EC-leukocyte adhesion rather than in transendothelial migration. Thus, VCAM-1 / VLA-4 binding could augment the adhesion of leukocytes to HBMEC prior to their subsequent transendothelial migration, since the increase in labeling is greater on the apical than the basal surface. The high concentration at or near thin cytoplasmic processes, the supposed points of contact with leukocytes, could facilitate adhesion. The minimal expression on the basal cell surface and its upregulation

by TNF- α suggest a possible utilization of VCAM-1 at the final steps of the migration process or during post-migration events.

In the CNS, VCAM-1 has been reported in AIDS encephalitis induced in macaques by the Simian Immunodeficiency virus (Sasseveille et al., 1992), and, recently, in human cerebral microvessels isolated from postmortem brains of patients with MS (Washington et al., 1994).

Recently, the mechanism of VCAM-1 expression on glial cells has been linked to the transcription factor NF- κ B (Meynagh et al., 1994; Shu et al., 1993; Neish et al., 1992) and PKC activation (Mattila et al., 1992; Deisher et al., 1993). Cyclic AMP does not appear to be a physiological regulator but rather an inhibitor of VCAM-1 expression (Pober et al., 1993).

The present studies demonstrate that unstimulated HBMEC express low levels of VCAM-1 which are significantly increased following activation with LPS, TNF- α or IL-1 β , but not IFN- γ and return to unstimulated levels after 48h in the continuous presence of LPS or cytokines. Ultrastructurally, VCAM-1 is localized mostly on the apical surface of EC and is not present along intercellular contacts or at junctional complexes. The induced upregulation of VCAM-1 is associated with increased expression mostly at the apical surface.

4.1.3 Intercellular adhesion molecule-1 (ICAM-1)

Intact, unstimulated EC constitutively express low levels of ICAM-1. Incubation with LPS, TNF- α , IL-1 β and to a lesser extent IFN- γ , results in upregulation of ICAM-1 expression which persists in the continuous presence of mediators in the culture.

The pattern of ICAM-1 expression in inflammatory disorders of the nervous system was recently investigated in guinea pigs and mice during the acute and chronic phases of EAE. O'Neill et al. (1991) observed the upregulation of MALA-2 (the murine

homologue of ICAM-1) on EC and mononuclear infiltrates in spinal cord lesions during the active phase of chronic relapsing EAE in the Biozzi AB/H mouse. Recent studies on adoptively transferred EAE in the SJL/J mouse have shown that attachment and extravasation of mostly LFA-1 positive lymphocytes correlated with the appearance of MALA-2 on CNS vessels and the onset of clinical signs (Raine et al., 1990b). In fact, the expression of MALA-2 fluctuated according to the clinical phase of EAE, with upregulation occurring with each relapse and down-regulation during remissions (Cannella et al., 1991). In both studies, endothelial and glial cells of normal mice showed low levels of ICAM-1. Similarly, the normal spinal cord vasculature of guinea pigs has only a few ICAM-1 positive EC, while ICAM-1 was present on the endothelium of both lesion- and non-lesion-associated blood vessels of animals during the acute phase of EAE (Wilcox et al., 1990). In the same study, cultured EC isolated from guinea pig brains did not express ICAM-1 unless incubated in a lymphocyte-conditioned medium. The above animal studies suggest that upregulation of ICAM-1 may be important in the initial influx of lymphocytes into the brain in autoimmune demyelinating CNS disorders. The role of ICAM-1 in orchestrating leukocyte traffic across the BBB in the course of inflammatory diseases of the human CNS has not yet been fully investigated. In a recent neuropathologic report, based on the examination of post-mortem material from normal and pathological brain tissue, only a small number of microvessels were ICAM-1 positive within the normal CNS. In contrast, numerous ICAM-1 positive vessels were observed in MS plaque edges, viral encephalitis lesions and infarcts (Sobel et al., 1990).

In vitro, ICAM-1 is released in a soluble form by activated HUVEC (Leeuwenberg et al., 1992; Pigott et al., 1992) but not endocytozed during the induction of its expression (von Asmuth et al., 1992; Kuijpers et al., 1994).

Summary	of	ICAM-1	expression	on	EC
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	HBMEC	HUVEC	HDMEC
Unstimulated			
Expression			
(% cells labelled)	20-40%	<11%	11-19%
Time of maximal			
expression	12-24h	10h or 24h	24h-6d
Amount of maximal	95% (LPS)	-	-
expression	58% (IFN-γ)	-	93% (IFN-γ)
(% cells labelled)	85% (IL-1β)	-	-
	85% (TNF-α)	-	100% (TNF-α)
	99% (TNF+IFNγ)	-	100% (TNF+IFNγ)
Concentration	1 ug LPS/ml	0.1 µg LPS/ml	_
giving maximal	1-500U IFN-y/ml	-	10^{3} U IFNv/ml
expression	10 U II -18/ml	>10 U U -1/ml	
expression	1000 IL-IP/III	100UTNE a/ml	- 104UTNE/m1
	500U IFNγ/ml +	10000 IFN-γ/ml +	1000 IFNγ/mi
	100U TNF-α/ml	100U TNF-α/ml	+10 ^o U TNF/ml
Level post	Plateaus for	Plateaus for	Plateaus for
maximal increase	at least 72h.	at least 24h.	at least 24-72h.
Relative	LPS	TNF/IL-1>LPS	TNF/IL-1>LPS
effectiveness	>TNF-α/IL-1β		>IFN-γ
of cytokines/LPS	>IFN-γ		

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The upregulation of ICAM-1 induced by LPS and TNF- α on HBMEC is very drastic even by 4h, and is maximal by 12h, while IL-1 β did not induce a maximal level until 24h. IFN-y led to a slight but statistically significant increase in expression. In HUVEC and HDMEC, LPS induced a rise in ICAM-1 that did not peak until 24h (Swerlick et al., 1991; Wellicome et al., 1990). Swerlick et al. (1991) and Pober et al. (1986b) observed a similar rise of ICAM-1 in HUVEC and HDMEC following treatment with TNF- α and IL-1 β , in contrast to Wellicome et al. (1990) who showed that TNF and IL-1 induced a peak in ICAM-1 expression on HUVEC by 10h. The slight upregulation of ICAM-1 observed after treatment of HBMEC with IFN- γ is in keeping with previous reports indicating that IFN-y is a weak inducer of ICAM-1 upregulation on extracerebral endothelium. Thus, IFN-y did not induce a noticeable change in ICAM-1 levels in HDMEC until 72h (Swerlick et al., 1991), and caused a "slower" increase than IL-1 and TNF in HUVEC (Pober et al., 1986b). It is evident that the highest levels of ICAM-1 induced by LPS, TNF- α and IFN- γ appear earlier in HBMEC than in HDMEC and some HUVEC cultures. The time course of ICAM-1 expression generated by IL-1 β is similar in all three culture systems.

The optimal concentration of cytokines and LPS required for the upregulation of ICAM-1 is different in cerebral and extracerebral endothelial cultures. Thus, a higher concentration of LPS (Wellicome et al., 1990) and lower concentration of TNF- α (Detmer et al., 1990; Pober et al., 1987) and IFN- γ (Ruszczak et al., 1990) are needed for HBMEC compared to HUVEC. Human retinal capillary EC, on the other hand, showed maximal induction of ICAM-1 upregulation with the low concentration of 5U IFN- γ /ml (Liversidge et al., 1990). IL-1 was required in a concentration greater than 10U/ml for maximal effect on HUVEC (Pober et al., 1986b; Wellicome et al., 1990), while 10U/ml induced highest levels on HBMEC. HBMEC are thus more sensitive to cytokines and less sensitive to LPS than HUVEC and HDMEC with respect to ICAM-1 upregulation.

The staining intensity of HBMEC varied even within monolayers derived from a single isolation. As a rule, the labeling of larger EC was stronger than that of actively proliferating cells. Such heterogeneity in ICAM-1 expression has also been reported in cultured human saphenous vein EC and HUVEC (Dustin and Springer, 1988) and in cultured fibroblasts (Dustin et al., 1986).

The unstimulated expression of ICAM-1 in HBMEC is higher than that in human retinal capillary EC (Liversidge et al., 1990) and in HDMEC (Ruszczak et al., 1990), which in turn are higher than the expression in HUVEC (Swerlick et al., 1991).

The maximal levels of ICAM-1 expression on HBMEC and HDMEC (Detmer et al., 1990; Ruszczak et al., 1990) are also different. HBMEC express much lower levels of ICAM-1 than HDMEC when activated with LPS or cytokines. Hess et al. (1994) demonstrated by ELISA that ICAM-1 upregulation by LPS and cytokines is much greater on HBMEC than HUVEC. Following incubation of HBMEC with a combination of TNF- α and IFN- γ , 99 to 100% of the cells became labeled for ICAM-1. This combined effect has also been observed in HDMEC (Detmer et al., 1990) and HUVEC (Doukas and Pober, 1990). In the latter study, it was also found that IFN- γ does not significantly enhance the expression of ICAM-1 induced by IL-1 β . It has also been shown that endothelin and cytokines upregulate ICAM-1 on human brain microvascular EC (McCarron et al., 1993c). TNF, IL-1 and IFN increase ICAM-1 expression on murine cerebrovascular EC (McCarron et al., 1993b, 1993c). Shear stress has also been shown to upregulate ICAM-1 expression on HUVEC (Nagel et al., 1994). Other cell types within the brain respond much differently from EC to cytokine activation. ICAM-1 expression on oligodendrocytes and astrocytes was markedly increased by IFN-y, while TNF- α , IL-1 α and LPS were less effective (Satoh et al., 1991). One study with a mouse endothelioma cell line reported that ICAM-1 was only very weakly upregulated in these cells (Hahne et al., 1993).

We found that removal of cytokines and LPS from the culture media was associated with variable expression of ICAM-1 after 3 days. Thus, there seemed to be a slight decrease in ICAM-1 with LPS (10-30%) and TNF- α (6-23%), and a slight increase with IFN- γ (0-19%) and IL-1 β (1-17%). Dustin and Springer (1988) observed that ICAM-1 expression by HUVEC increased following stimulation with IL-1 and TNF, but returned to unstimulated levels when the mediators were washed off. It would seem that HBMEC maintain their ICAM-1 expression much longer than HUVEC.

Ultrastructurally, ICAM-1 is present on the luminal but not abluminal surface of high endothelial venules. High concentrations are found on the "microvilli" of these EC (Brown et al., 1993; Sasaki et al., 1993). Similarly, ICAM-1 is expressed mainly on the apical surface of HBMEC, especially on finger-like projections. TNF treatment significantly upregulates the expression of this molecule, however, little labeling is observed on the basal surface while intercellular contacts remain unlabeled. This may suggest a role of ICAM-1 in the firm adhesion and perhaps initial migration of leukocytes across these monolayers but little utilization once the barrier is crossed. In contrast to HBMEC and high endothelial venules, ICAM-1 is prominent on the apical and basal surfaces of HUVEC, with high staining at the apical surface. It is also present at the tight junctions and at contact sites between T lymphocytes and the apical and basal surfaces of HUVEC (Oppenheimer-Marks et al., 1991). Thus, ICAM-1 may function in adhesion and migration of leukocytes across extracerebral EC, but its role in the HBMEC may be more limited.

The mechanism(s) of ICAM-1 induction by cytokines is not well defined. Lane et al. (1990) and Sung et al. (1994) concluded that IL-1, TNF- α and LPS activate HUVEC expression of ICAM-1 in part by activation of protein kinase C (PKC), since PKC inhibitors decrease subsequent ICAM-1 upregulation by these agents. Myers et al. (1992a, 1992b) observed that ICAM-1 upregulation by phorbol myristate acetate, but not by cytokines or LPS, involved PKC activation. On the other hand, Ritchie et al. (1991)

found that TNF does not activate HUVEC through the PKC pathway (Ritchie et al., 1991) and PKC activation is capable of but not essential for ICAM-1 induction by cytokines and LPS (Myers et al., 1992a, 1992b). Renkonen et al. (1990a) found that IL-1 operates via cAMP but not via PKC, and IFN- γ functions through PKC and calcium dependent pathways. In a subsequent paper Renkonen et al. (1990b) showed that IFN- γ induced a rapid activation of phospholipase C, leading to a breakdown of phosphoinositol diphosphate into diacylglycerol (DAG) and inositol triphosphate (IP₃). DAG is a natural activator of the PKC pathway. PKC inhibitors abolished the IFN- γ effect, while PKC stimulators mimicked the effects of IFN- γ . Elevations of IP₃ levels led to activation of the calcium-dependent pathway. A calmodulin inhibitor decreased the IFN- γ induced ICAM-1 expression. Recently, the transcription factor NF- κ B has also been suggested to be involved in ICAM-1 expression by glial cells (Maynagh et al., 1994).

Our results indicate that HBMEC, compared to other EC, tend to express a higher unstimulated level of ICAM-1, are more sensitive to cytokines, and less sensitive to LPS, express ICAM-1 earlier but at a lower level when induced, and fail to reverse its expression within a short time upon removal of these agents. ICAM-1 is expressed mainly on the apical surface of HBMEC and on finger-like projections, and is greatly upregulated by TNF. Taken together, the relatively high level of ICAM-1 expression on unstimulated HBMEC and its fast maximal upregulation with relatively low concentrations of cytokines, to levels lower than those obtained in other EC *in vitro* systems, indicate different kinetics of ICAM expression and upregulation in HBMEC which may be relevant to unique responses of these cells in CNS inflammation.

4.1.4 Platelet/endothelial cell adhesion molecule-1 (PECAM-1)

PECAM-1 is especially concentrated at the EC cell borders where adjacent cells contact each other and tight junctions are formed. Cytokines do not alter PECAM-1 expression on HUVEC but it was suggested that they induce its redistribution from the

cell border evenly throughout the cell surface (Romer et al., 1991). The presence of this molecule at the cell borders has led to speculations that it may be involved in EC-EC interactions and EC-leukocyte interactions after cytokine activation of EC (Albelda et al., 1990b; Romer et al., 1991). The constitutive expression of PECAM-1 and lack of change after EC activation on HBMEC are consistent with the reports on HUVEC. Interestingly, PECAM-1 was not redistributed on HBMEC by LPS or cytokines, unlike the reported changes in HUVEC. This suggests it may act mainly in the migration of leukocytes instead of adhesion, unlike HUVEC.

Consistent with the observations of Leach et al. (1993) on human placental microvessels, we found that, on HBMEC, PECAM-1 is present mainly on the apical surface. Labeling was prominent at the thin cytoplasmic areas close to the tight junctions but not along intercellular contacts or in junctions themselves. This may be due either to the absence of this molecule from the junctions or restriction of the Ab from penetrating the tight junctions. Staining after the junctions are opened by hyperosmotic solutions or post-embedding staining would ensure that the Ab can reach all areas of the tight junctions. Using HUVEC exposed to buffer without divalent ions to retract EC, Muller et al. (1989) found that PECAM-1 staining is prominent in the intercellular junctions between adjacent EC. Of interest is the observation that in subconfluent HBMEC cultures where EC are separated by a short space, the EC surfaces that would eventually contact the adjacent EC are heavily labeled. It seems likely that leukocytes can especially use PECAM-1 in these areas to migrate across the endothelium.

4.1.5 HECA-452 antigen

Horst et al. (1988) reported that 10-100 U IFN- γ /ml induced HECA-452 antigen expression on over 80% of HUVEC. Labeling was apparent after 6h, became maximal after 24h and remained high after 48h. Only cytoplasmic staining was observed, unlike the *in vivo* situation where membranes were also labeled. In contrast, we have not been

able to induce HECA-452 antigen on HBMEC with any of the cytokines including IFN-γ at 400U/ml for 1 to 7 days. Positive staining of high endothelial venule cells on control frozen sections of tonsils confirmed that this Ab is functional. Thus far, only one Ab, a mouse IgM, has been produced against this antigen and has been applied in all studies mentioned. In a strange twist of events, HECA-452 antigen, also known as cutaneous lymphocyte antigen, has been found to be the receptor for E-selectin on EC and may be a lymphocyte homing receptor for skin homing lymphocytes (Bos et al., 1993; Berg et al., 1991). 80-90% of T lymphocytes in inflammatory skin diseases are HECA-452+ and bind to E-selectin+ EC. Abs against either molecule can decrease adhesion (DeBoer et al., 1994). Although it is possible that HBMEC, being phenotypically distinct from EC of other vascular beds, do not express HECA-452 antigen, it is also possible that other treatments, not examined here, may induce this molecule. In addition, different epitopes of this antigen or different vascular addressins, not recognized by the present Ab, may be expressed by HBMEC. In any case, the expression of this antigen is controlled differently between HBMEC and extracerebral EC.

The current dogma states that leukocytes roll on EC using P- and E-selectin. This initial adhesion is followed by firm adhesion via ICAM-1 and VCAM-1. Migration follows using ICAM-1 and sometimes, VCAM-1. In this study, it was found that maximal E-selectin expression on HBMEC occurs at 4h, followed by a quick decrease. VCAM-1 and ICAM-1 are not maximally expressed until 12 to 24h. However, between 12 and 24h, all three molecules are expressed to a certain extent. Moreover, even at 4h, ICAM-1 and VCAM-1 levels have begun to rise quite rapidly. There may be a sufficient amount for both adhesion and migration. In addition, ICAM-2 and PECAM-1 are both constitutively expressed and may be utilized even when ICAM-1 and VCAM-1 are not yet upregulated.

	ICAM-1	VCAM-1	E-selectin
Unstimulated			
expression			
(% cells labelled)	20-40%	15%	7%
Location	Border	Border	Surface
Time of maximal			
expression	12h-24h	12h-24h	4h
Amount of maximal	95% (LPS)	83% (LPS)	90% (LPS)
expression	85% (IL-1β)	60% (IL-1β)	58% (IL-1β)
(% cells labelled)	85% (TNF-α)	62% (TNF-α)	91% (TNF-α)
Concentration for	1µg LPS/ml	0.1µg LPS/ml	0.1µg LPS/ml
maximal expression	10U IL-1β/ml	$10U$ IL-1 β/ml	1U IL-1β/ml
-	100U TNF-α/ml	100U TNF-α/ml	100U TNF-α/ml
Relative effectiveness	LPS>	LPS>TNF-α>	LPS/TNF-α>
of cytokines/LPS	TNF-α/IL-1β>	IL-1β.	IL-1β>
-	IFN-γ	IFN-γ -no effect.	IFN-γ -no effect.
Level post	Plateaus at	Drops to unstimula	ited
maximal increase	high level.	levels by 48h.	

Dummary of D-beleening CAMP-1 and ICAM-1 CAPTebbion on HDM112	Summary of E-selectin	. VCAM-1	and ICAM-1	expression	on HBMEC
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Thus, these results lead to the possibility that early in the course of inflammation, initial rolling on E-selectin is followed by firm adhesion using the moderate amount of VCAM-1 and ICAM-1 upregulated and ICAM-2 that is constitutively expressed. Some time later, rolling may be decreased due to the lower level of E-selectin expression, but

the presence of leukocytes already firmly attached to the surface and / or transmigrating across the endothelium may allow these leukocytes to interact with nonadherent leukocytes to form aggregates, which may also arrest them in the blood stream, ready for firm adhesion and transmigration. It is also possible that, in the human body, some substance in the serum may cause EC to maintain a high expression of E-selectin once activated. The close proximity of VCAM-1 and ICAM-1 on the finger-like projections to E-selectin, which is also prominently localized in these structures, facilitates the proposed sequence of events from rolling to firm adhesion. PECAM-1 may not be involved in these events since it is concentrated at the cell borders. This strategic location could reflect its possible role in transmigration. The presence of other adhesion molecules in these areas also suggest their role in this movement across the endothelium. The lack of adhesion molecule expression on the basal surface of HBMEC may imply that leukocytes do not cross back from the brain to the blood, or use other molecules for this purpose.

4.2 ROLE OF ADHESION MOLECULES IN PMN AND T LYMPHOCYTE ADHESION TO AND MIGRATION ACROSS HUMAN BRAIN MICROVESSEL ENDOTHELIAL CELL MONOLAYERS

4.2.1 PMN adhesion

Adhesion of PMN to intact, as well as cytokine-treated HBMEC is very low. Only 0.04-0.07% of PMN adhered to unstimulated HBMEC and 0.34-0.7% to activated HBMEC. PMN adhesion to unstimulated HUVEC is considerably higher - 1 to 5% in most reports (Wankowicz et al., 1988; Morzycki et al., 1990; Bevilacqua et al., 1985; Bochner et al., 1991), up to 12% (Hakkert et al., 1991) or 15% (Smith et al., 1988) in others. Cytokine activation of HUVEC raises the adhesion rate to 10-30% in most reports (Wankowicz et al., 1988; Morzycki et al., 1990; Bevilacqua et al., 1985; Bochner

90% decrease. Other investigators using different mAbs reported a 40 to 90% blocking of PMN adhesion to activated HUVEC (Kuijpers et al., 1991; Kishimoto et al., 1991; Benjamin et al., 1991; Leeuwenberg et al., 1990a; Luscinskas et al., 1989; Shimizu et al., 1991b; Bochner et al., 1991; Benjamin et al., 1990). There is a general consensus that E-selectin is involved in PMN adhesion. This is also supported by the work of Hahne et al. (1993) who observed a 20 to 30% decrease in PMN adhesion to an activated mouse brain endothelioma cell line with an anti-E-selectin Ab.

Ab	Untreated HBMEC	Activated HBMEC	Untreated HUVEC	Activated HUVEC
anti-E-selectin Ab	0%	38-54%	-	40-90%
anti-VCAM-1 Ab	0%	0-20%	-	3-5%
anti-ICAM-1 Ab	0-10%	37-45%	70%	30-60%
anti-PECAM-1 Ab	0%	10-40%	-	-

Ab blocking of PMN adhesion across EC monolayers (%)

Adhesion of PMN to activated HBMEC appears to be mediated, in addition, by ICAM-1, since it was significantly blocked (37 to 45%) by the anti-ICAM-1 mAb. Using the same anti-ICAM-1 Ab on cytokine activated HUVEC, Smith et al. (1988) found a 50% inhibition of adhesion. Other studies employing different Abs have reported a 30 to 60% decrease in PMN adhesion (Luscinskas et al., 1991; Bochner et al., 1991; Smith et al., 1988, 1989). PECAM-1 does not appear to have any apparent role in PMN adhesion to activated HBMEC, since Abs against this adhesion molecule did not affect PMN adhesion. At present, there are no other studies on the role of PECAM-1 in PMN adhesion to endothelium, although one study found that neutrophils, blocked from migration by anti-PECAM-1 Abs, remain attached to the EC surface. This is suggestive

of a lack of utilization in adhesion but no quantitation or statistical comparison to untreated EC was performed (Muller et al., 1993).

There have only been two reports on the role of VCAM-1 in PMN adhesion to activated EC. Activated HUVEC were used in both studies and both found that anti-VCAM-1 Abs had no effect on adhesion (Barber et al., 1990; Bochner et al., 1991). Our results with HBMEC are consistent with these findings. It is generally believed that PMN cannot utilize the VCAM-1 adhesion pathway because their expression of VLA-4 (the ligand for VCAM-1) in very low (Lund-Johansen and Terstappen, 1993).

Although E-selectin has been proposed to function under conditions of flow, during ischemia and inflammation stasis occurs in the microvascular bed, therefore, the findings using the present model would be physiologically relevant. Examination of the role of these adhesion molecules under flow conditions is beyond the scope of this study.

Our results show that TNF treatment upregulates PMN adhesion. None of the anti-adhesion molecule Abs studied block PMN adhesion to unstimulated HBMEC. Both E-selectin and ICAM-1 appear to mediate PMN adhesion to activated HBMEC, however, none of the other molecules can be entirely ruled out, since epitopes on these molecules, not blocked by the Abs used in the present experiments, may conceivably operate in these interactions.

4.2.2 PMN migration

Although many published reports tend not to distinguish between adhesion and migration of leukocytes in the design of experiments, these are two separate processes. Adhesion is followed by migration, but adherent cells do not necessarily migrate. Moreover, different sets of adhesion molecules are employed. Thus, in these studies, equal amounts of adhesion was permitted in all wells and nonadherent cells aspirated before the addition of Abs to assess migration.

Migration of PMN across unstimulated HBMEC monolayers was minimal (0.2 PMN/mm length of monolayer). Treatment of HBMEC with 100U TNF/ml significantly upregulated migration. Following 2h incubation of PMN with HBMEC, migration across 4h TNF pretreated monolayers increased by 4 fold (0.9 PMN/mm), while 12h pretreatment led to 12 fold increase (2.5 PMN/mm) and 24h pretreatment induced a 15 fold increase (3.1 PMN/mm). However, when the number of adherent PMN is taken into account, a large percentage of adherent cells migrated across both unstimulated HBMEC (79 to 88%) and activated HBMEC (81 to 94%). Our findings are consistent with previous reports in which a 3 fold increase in PMN migration across HUVEC treated with TNF for 1-2h was observed (Moser et al., 1989; Morzycki et al., 1990). Longer periods of TNF treatment (4h) increased PMN migration across HUVEC by 16 fold (Furie et al., 1989). Treatment of HUVEC with IL-1 β for 4h similarly led to a 3 fold increase in PMN transmigration (Hakkert et al., 1991; Luscinskas et al., 1991). In an in vivo study, intracisternal, but not intravenous, administration of 800 U TNF- α into the brain of rats, raised the number of leukocytes in the CSF maximally ten fold after 4h of treatment (Kim et al., 1992).

The present study shows that none of the anti-adhesion molecule Abs inhibited PMN migration across resting HBMEC. No other studies have been reported on this subject using *in vitro* EC systems, though Furie et al. (1991) observed that PMN migration across unstimulated HUVEC in response to a chemotactic gradient is blocked by 55% by an anti-ICAM-1 Ab. It should be clarified that the majority of assays used to examine transendothelial migration actually involve both adhesion to as well as migration across EC. The anti-adhesion molecule Abs are added to EC monolayers first, then the PMN or other leukocytes are added and allowed to migrate. This way, the Abs can inhibit both adhesion and migration, so that the two processes cannot be clearly separated. Results interpreted as inhibition of migration may actually have been overinterpreted.

Аb	Untreated HBMEC	Activated HBMEC	Untreated HUVEC	Activated HUVEC
anti-E-selectin Ab	33%	0%	-	0-90%
anti-VCAM-1 Ab	16%	4%	-	-
anti-ICAM-1 Ab	33%	75%	55%	47-97%
anti-PECAM-1 Ab	17%	15%	-	80%

Ab blocking of PMN migration across EC monolayers (%)

The anti-E-selectin, anti-VCAM-1 and anti-PECAM-1 Abs had no effect on the migration of PMN across activated HBMEC. Two studies on activated HUVEC found a similar lack of effect of an anti-E-selectin Ab (Kishimoto et al., 1991; Hakkert et al., 1991), in contrast to the findings of Luscinskas et al. (1991), who, using the same mAb as in the present study, observed a 90% decrease in migration. However, considering the fact that the migration assay was performed in such a way that the mAbs actually inhibited both adhesion and migration, the degree of inhibition may not be so high. Thus far, there have been no reports on the role of VCAM-1 on PMN migration across activated EC, as it is generally believed that PMN do not employ the VCAM-1/VLA-4 pathway, since they do not bear the VLA-4 molecule (Lund-Johansen and Terstappen, 1993). Only one study is available on the effect of anti-PECAM-1 Abs on PMN migration. Muller et al. (1993), using the same mAb as the one in the present study, found a 80% blocking of PMN migration and similar results were obtained with soluble PECAM. A different anti-PECAM-1 Ab inhibited migration only by 40%. Here again, the assay involved inhibition of both adhesion and migration since the blocking Ab was added to HUVEC cultures at the same time with the PMN. The distribution of PECAM-1 on HBMEC is different from HUVEC as discussed in a previous section.

HBMEC PECAM-1 is not as concentrated at the periphery, where adjacent cells contact, as in HUVEC. Moreover, unlike HUVEC, the distribution of PECAM-1 does not change with EC activation. It is notable that "PECAM-1 is expressed in high density on all early myeloid cells but is down-regulated during postproliferative maturation" (Lund-Johansen and Terstappen, 1993). It has also been reported that activation of granulocytes leads to further downregulation of PECAM-1 expression (Stockinger et al., 1990). Taken together, the above observations suggest another reason for the lack of use of the PECAM-1 pathway in PMN adhesion and migration due to the small amount of PECAM-1 expressed by peripheral blood PMN.

The anti-ICAM-1 Ab was the only one that significantly blocked PMN migration across activated HBMEC (75% decrease). A similarly significant blocking effect has also been observed with activated HUVEC (97% by Luscinskas et al., 1991 and 70% by Kishimoto et al., 1991). These figures probably include inhibition of adhesion also, as discussed above.

In most chemotaxis experiments, a chemotactic gradient is used for induction of PMN transmigration. In the present study, a considerable number of PMN migrated across TNF treated HBMEC in the absence of a chemotactic gradient. Previous studies indicate that immediately upon contact with activated EC, PMN become activated, as measured by increases in intracellular calcium levels (Kuijpers et al., 1991). Possibly as a result of contact with chemoattractants bound to the EC surface. Contrary to these observations, Adams and Shaw (1994) reported that integrins on circulating PMN do not adhere well to the endothelium unless they are activated. It is likely, therefore, that, in our studies, only a subset of possibly activated PMN was able to adhere to cytokine stimulated HBMEC. It has been reported that IL-1 or TNF stimulate HUVEC generate the chemoattractants platelet-activating factor (PAF) and IL-8 (Kuijpers et al., 1992; Sticherling et al., 1993). Anti-IL-8 Abs inhibit migration (Smart et al., 1994), while simultaneous blocking of both pathways with antagonists and Abs can completely

prevent migration (Kuijpers et al., 1992). IL-8 and PAF present on the EC surface can transiently activate PMN integrins, especially Mac-1, thus promoting PMN adhesion (Rot, 1992; Huber et al., 1991; Tanaka et al., 1993). The increase in surface expression of integrins is not involved but rather a change in the conformation of integrins resulting in the "expression" of "activation epitopes" (Springer, 1990). It has been proposed that this dynamic regulation of integrin adhesiveness permits deadhesion and retraction of the trailing edge of migrating PMN (Springer, 1994). Binding of PMN to E-selectin also activates the activity of Mac-1 (Lo et al., 1991). Whether these mechanisms are functional in the present culture system and whether the adherent PMN were activated or not is not known since we did not conduct studies to address this issue.

The scanning electron micrographs in the present study showed that PMN migrate between HBMEC through the tight junctions. Early studies indicated that all or most inflammatory cells migrate across the endothelium paracellularly, that is between EC and through the tight junctions. This included rat mesentery EC (Marchesi and Florey, 1960; Marchesi, 1961), rabbit ear EC (Florey and Grant, 1961), pig EC (Beesley et al., 1979) and cultured HUVEC (Moser et al., 1989). The present findings are in agreement with the work of Dorovini-Zis et al. (1992a) who showed by SEM and TEM that PMN migration across primary cultures of bovine brain microvessel EC occurs paracellularly. However, other studies suggest that not all PMN cross the endothelial barrier paracellularly. Some authors believe that PMN migration occurs transcellularly through the cytoplasm of cat brain EC (Faustman and Dermietzel, 1985), human, cat and rat lymph nodes (Azzali et al., 1990a) and other human and animal EC (Hammersen et al., 1988).

The findings in this study show that none of the adhesion molecules studied regulate migration of PMN across unstimulated HBMEC. ICAM-1 mediates PMN migration across TNF-treated HBMEC.

4.2.3 T lymphocyte adhesion

The discussion here will be restricted to peripheral blood resting T lymphocytes. The percentage of T lymphocytes that adhere to HBMEC monolayers is higher than that of PMN. 0.64 to 3% T lymphocytes adhered to unstimulated HBMEC, while 16 to 23% adhered to activated HBMEC. These percentages are slightly lower than those reported on mouse brain EC where 30 and 50% of T lymphocytes adhered to unstimulated or activated, respectively, murine cerebrovascular EC (McCarron et al., 1993b, 1993c). 4 to 10% of lymphocytes bind unstimulated, while 6 to 30% of lymphocytes adhere to activated rat brain EC (Hughes et al., 1988; Male et al., 1990b). In contrast, 50% of CD4⁺ T lymphocytes adhere to activated or unstimulated human retinal capillary EC (Liversidge et al., 1990). Adhesion to HUVEC is similarly high with 5-35% of T lymphocytes binding to unstimulated EC and 20-50% to activated HUVEC (Bereta et al., 1993; Yu et al., 1986; Cavender et al., 1986; Shimizu et al., 1991a; Yu et al., 1985).

Treatment of HBMEC with TNF increased T lymphocyte adhesion by 300 to 450%. Longer treatments (18 and 24h) caused a slightly greater increase than shorter (4h) incubations, however, there is no actual difference in the degree of T lymphocyte adhesion between 18 and 24h treatments. Activation of rat and mouse brain EC with TNF, IL-1 or IFN- γ produced much less dramatic results with 20 to 100% (Hughes et al., 1988; Male et al., 1990b) and 20 to 80% increases in lymphocyte adhesion (McCarron et al., 1993b, 1993b), respectively. Activation of bovine brain EC increases lymphocyte adhesion by 500% (de Vries et al., 1994), which is more comparable to HBMEC. Surprisingly, activation of human retinal capillary EC does not increase CD4⁺ T lymphocyte adhesion. The effect of HUVEC activation on T lymphocyte adhesion varies among different laboratories, ranging from only 4 to 28% increase after 1h of cytokine treatment (Yu et al., 1986), to 100-400% increase after 3-6h (Bereta et al., 1993; Cavender et al., 1986; Shimizu et al., 1991a, Rice et al., 1990) and down to 60-100% after 24h of activation (Yu et al., 1985).

Ab	Untreated HBMEC	Activated HBMEC	Untreated HUVEC	Activated HUVEC
anti-E-selectin Ab	0%	0-10%	-	20-40%
anti-VCAM-1 Ab	0-30%	29-32%	0-9%	30-60%
anti-ICAM-1 Ab	55-63%	47-75%	50-62%	8-70%
anti-PECAM-1 Ab	0%	0%	-	-

Ab blocking of T cell adhesion across EC monolayers (%)

With the exception of anti-ICAM-1, which blocked adhesion by 55 to 63%, none of the other anti-adhesion molecule Abs significantly decreased T lymphocyte adhesion to unstimulated HBMEC. These results are in keeping with three other studies in which the use of the same anti-ICAM-1 Ab decreased T lymphocyte adhesion to unstimulated HUVEC by 50 to 62% (Oppenheimer-Marks et al., 1991; Liversidge et al., 1990; Oppenheimer-Marks and Lipsky, 1993). Oppenheimer-Marks and Lipsky (1993) also found that an anti-VCAM-1 Ab had no effect on T lymphocyte adhesion to unstimulated HUVEC. Similarly, Bereta et al. (1993) found that an anti-VCAM-1 Ab had no effect on the adhesion of splenocytes to unstimulated murine cerebrovascular EC.

T lymphocyte adhesion to TNF-treated HBMEC was not blocked by the anti-E-selectin mAb. Applying the same mAb, Graber et al. (1990) and Shimizu et al. (1991b) found a 20 to 40% inhibition of the adhesion of T lymphocytes to activated HUVEC. Analysis using subsets of T lymphocytes is beyond the scope of this study, so comparisons are not possible. The VCAM-1 blocking Ab significantly decreased adhesion of T lymphocytes to activated HUVEC by 29 to 32%. A similar rate of inhibition (34 to 60%) was also observed with activated HUVEC using the same Ab as in this study (Graber et al., 1990; Shimizu et al., 1991a; Oppenheimer-Makes et al., 1993)

as well as with another Ab (Oppenheimer-Marks et al., 1991). A VCAM-1 blocking Ab also decreased the adhesion of splenocytes to activated murine microvascular EC (Bereta et al., 1993).

The effect of the anti-ICAM-1 Ab was even greater, resulting in 47 to 75% blocking of T lymphocyte adhesion to activated HBMEC. The mAb used in this study also blocked adhesion to activated HUVEC by 34% (Liversidge et al., 1990). Another anti-ICAM-1 Ab had an even greater effect (70% decrease) on activated HUVEC (Shimizu et al., 1991a). Oppenheimer-Marks et al. (1991) found only a 8% decrease, but the HUVEC used were stimulated for only 4h, a time when ICAM-1 expression is still low.

In addition, Abs to CD11a, CD18 and VLA-4, ligands to ICAM-1 and VCAM-1, respectively, decrease the adhesion of peripheral blood lymphocytes to bovine brain EC (de Vries et al., 1994). An anti-ICAM-1 Ab decreases the adhesion of lymphocytes to rat high endothelial venule EC (Tamatani and Miyaska, 1990). In contrast, mononuclear cells from MS patients show enhanced adhesion to unstimulated and activated HBMEC that does not appear to be dependent upon ICAM-1 (Tsukada et al., 1993).

There have been no reports on the effect of anti-PECAM-1 Abs on T lymphocyte adhesion. Following TNF activation of HBMEC, the presence of anti-PECAM-1 in the assay increased T lymphocyte adhesion by 14 to 123%. Actually, a 90% increase was also observed with unstimulated HBMEC, though the actual numbers are not statistically significant. This effect has been examined in several reports. Binding of PECAM-1 by anti-PECAM-1 Abs activates the β 1 integrin, especially VLA-4, the ligand for VCAM-1, and to a lesser extent the β 2 integrin LFA-1, the ligand for ICAM-1 on lymphocytes, monocytes, neutrophils and hemopoietic progenitor cells (Tanaka et al., 1992; Piali et al., 1993; Leavesley et al., 1994; Berman et al., 1995). In the present study, when the anti-PECAM-1 Ab was removed prior to the addition of T lymphocytes, the number of adherent lymphocytes was not different from that observed when HBMEC were not

treated with the anti-PECAM-1 Ab. Thus, PECAM-1 is likely not involved in the adhesion of T lymphocytes.

This study shows that none of the Abs used blocked any epitope utilized in adhesion of resting T lymphocytes to unstimulated HBMEC. TNF activation of EC upregulates this adhesion by a mechanism involving VCAM-1 and ICAM-1.

4.2.4 T lymphocyte migration

Migration of T lymphocytes across unstimulated HBMEC monolayers was extremely low (0.02 T lymphocyte/mm length of monolayer). Taking into account the initial adhesion step, 4 to 10% of adherent T lymphocytes migrated across unstimulated HBMEC and 0.25 to 17.5% migrated across TNF-treated HBMEC. These data are consistent with those presented in a recent report on rat retinal microvessel EC showing that 0.8% of peripheral lymph node lymphocytes and 52% of soluble retinal antigen specific T lymphocytes migrated across rat retinal EC regardless of the activation status of the endothelium (Greenwood and Calder, 1993).

TNF activation of HBMEC significantly increased T lymphocyte migration by 10 fold (0.2 T lymphocyte/mm) after 4 or 18h of pretreatment and 50 fold (1 T lymphocyte/mm) after 24h of pretreatment. In contrast, activation of rat retinal microvessel EC with IFN- γ did not change the migration of lymphocytes or soluble retinal antigen specific T lymphocytes (Greenwood and Calder, 1993). This may be due to the low efficiency of IFN- γ as an inducer of EC adhesion molecules, or an inherent difference between EC from different tissues. An increase of 0.2 to 0.7 fold in T lymphocyte migration has been reported after HUVEC activation with IFN- γ (Oppenheimer-Marks and Ziff, 1988) and a 0.5 fold increase after HUVEC activation with IL-1 (Oppenheimer-Marks and Lipsky, 1993).

Ab	Untreated HBMEC	Activated HBMEC	Untreated HUVEC	Activated HUVEC
anti-E-selectin Ab	0%	40%	-	-
anti-VCAM-1 Ab	0%	25%	0-4%	0-90%
anti-ICAM-1 Ab	0%	93%	50-60%	40-53%
anti-PECAM-1 Ab	0%	75%	-	-

Ab blocking of T cell migration across EC monolayers (%)

None of the anti-adhesion molecule Abs significantly blocked the migration of T lymphocytes across unstimulated HBMEC. Similarly, anti-VCAM-1 Abs do not decrease T lymphocyte migration across unstimulated HUVEC (Oppenheimer-Marks et al., 1991; Oppenheimer-Marks and Lipsky, 1993). Surprisingly, an anti-ICAM-1 Ab reduced T lymphocyte migration across unstimulated HUVEC by 50-60% (Oppenheimer-Marks et al., 1991; Oppenheimer-Marks and Lipsky, 1993). Since the migration assay used in the present study is the same as in these reports, these differences may reflect inherent differences between these two types of EC. Muller et al. (1993) found that an anti-PECAM-1 Ab decreases peripheral blood mononuclear cell migration across unstimulated HUVEC by 80% in contrast to our results. However, Bird et al. (1993) has reported that PECAM-1 is not necessary for migration of peripheral blood lymphocytes across unstimulated HUVEC or rat high endothelial venule EC, in support of our results.

At the present time, there are no studies on the effects of anti-E-selectin Ab on T lymphocyte migration across unstimulated EC.

Following TNF activation of HBMEC, the anti-E-selectin Ab marginally decreased T lymphocyte migration by 40%. The anti-VCAM-1 Ab had no effect, in

keeping with results previously reported on activated HUVEC (Oppenheimer-Marks et al., 1991; Oppenheimer-Marks and Lipsky, 1993). The anti-ICAM-1 Ab significantly blocked transmigration by 93% unlike the 40 to 53% decrease reported with 4h activated HUVEC (Oppenheimer-Marks et al., 1991; Oppenheimer-Marks and Lipsky, 1993). Inadequate upregulation of ICAM-1 after only 4h treatment with cytokines would most likely account for this difference. Interestingly, both the anti-ICAM-1 and anti-VCAM-1 Abs have been found to be involved in the migration of T lymphocytes from peripheral lymph nodes across rat brain EC (Male et al., 1992). A 75% decrease in migration across activated HBMEC was obtained with the anti-PECAM-1 Ab. A similar (70-90%) blocking of monocyte migration with anti-PECAM-1 Abs has been observed across activated HUVEC (Muller et al., 1993).

In a manner similar to PMN, integrins on circulating lymphocytes have low adherence unless they are activated (Adams and Shaw, 1994). Since 10% of peripheral blood T lymphocytes are activated (Hannet et al., 1992), it is conceivable that lymphocytes adhering to HBMEC in the present assays, were among this subset of activated T lymphocytes. Lymphocytes that adhere to EC are found to be activated within a short period of time. This involves G-protein associated receptors (Bargatze and Butcher, 1993). Chemokines, such as MIP-1 β can activate the β 1 integrins on the lymphocyte surface (Tanaka et al., 1993), thus, contact with MIP-1 β or other chemokines on the EC surface could activate lymphocytes and their integrins to permit greater adhesion and migration. Since expression of MIP-1 β by cerebral EC has not yet been reported, this mechanism remains speculative.

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Earlier studies showed that inflammatory cells migrate across the endothelium between adjacent EC (Wisniewski and Lossinsky, 1991). Recent reports indicate that lymphocytes cross transcellularly, through the EC cytoplasm in the microvessels of mice with EAE (Lossinsky et al., 1991), mouse lung EC (Fujitsu et al., 1991), high endothelial venules (DeBruyn and Cho, 1990), human, cat and rat lymph vessel cells (Azzali et al.,

1990), rat lacteal lymphatic EC (Azzali, 1990) and mouse mesenteric lymph nodes (Cho and DeBruyn, 1981). In the present study, it appeared that some lymphocytes migrated through the cytoplasm of HBMEC, while others preferred the paracellular route. These results along with those of PMN migration suggest that the route of transendothelial migration may depend both on the type of the inflammatory cell and the type of the microvascular bed.

Cross and Raine (1991) found that "finger-like fronds" project from the luminal surface of the involved brain microvessels of mice with EAE. Projections arise at interendothelial junctions and in animals with acute lesions (less than 24 to 48h clinical signs). Lossinsky et al. (1991) also noted these projections and called them "microvillar projections". Such structures were frequently seen on the apical surface of our TNF treated HBMEC and direct contacts between these finger-like extensions of the EC cytoplasm and pseudopodia of PMN and lymphocytes were often observed. Cross and Raine (1991) also observed that PMN contacted these fronds, while small lymphocytes rarely acted this way.

Approximately 10% of T lymphocytes in the peripheral blood are activated (Hannet et al., 1992). Presumably, this was the case with the T lymphocytes used in the present study. Activation of lymphocytes *in vitro* by pharmacological agents increases their adherence to HUVEC (Shimizu et al., 1991a; Greenwood and Calder, 1993; Dustin and Springer, 1988), rat brain microvessel EC (Hughes et al., 1988; Tanaka et al., 1993) and HBMEC (Huynh, 1994) by 2 to 12 fold. Their utilization of the endothelial adhesion molecules is also altered. Binding of activated T lymphocytes to unstimulated HUVEC is inhibited by an anti-ICAM-1 Ab to a small degree and does not involve VCAM-1. Binding to activated HUVEC is not inhibited by anti-VCAM-1 or anti-ICAM-1 Abs (Oppenheimer-Marks et al., 1991). This is in contrast to resting T lymphocytes which use ICAM-1 to adhere to unstimulated EC and VCAM-1 for adhesion to activated EC (Oppenheimer-Marks et al., 1991). Male et al. (1994) also found that anti-ICAM-1 Abs

do not decrease adhesion of either activated or resting lymphocytes to IFN-γ activated rat brain microvessel EC. The adhesion of memory and activated synovial T lymphocytes from rheumatoid arthritis patients to recombinant VCAM-1 and E-selectin is greatly increased compared to peripheral blood T lymphocytes. Migration of memory T lymphocytes across HUVEC is also enhanced more than 100% over naive T lymphocytes (Oppenheimer-Marks and Lipsky, 1993).

Other subsets of peripheral blood lymphocytes are the B lymphocytes and natural killer (NK) cells. Compared to peripheral blood T lymphocytes, the percentage of NK cells that adhere to and transmigrate across unstimulated HUVEC is 3 fold higher and 2 fold higher across activated HUVEC. Both the ICAM-1 and VCAM-1 pathways are employed in these processes (Bianchi et al., 1993). NK cells also preferentially bind to human foreskin microvascular EC (Bender et al., 1987). B lymphocytes bind more strongly to murine brain microvessel EC than T lymphocytes (Pryce et al., 1991). It is apparent that, *in vitro*, subsets of lymphocytes in peripheral blood possess different abilities to adhere to and migrate across vascular endothelium from different systems.

Work done *in vivo* on mice or rats with EAE demonstrated that sialyl residues (ligands for E-selectin) are important in lymphocyte accumulation in CNS inflammation (Simmons and Cattle, 1992). The majority of T lymphocytes infiltrating the CNS are activated (Hickey et al., 1991; Skundric et al., 1994) and memory cells (Skundric et al., 1994). From studies in which immunostaining for activated T lymphocytes is performed on sections of CNS lesions, it is not possible to determine whether only activated cells enter the CNS, or resting T lymphocytes entered and became activated in the extravascular tissue. Hickey et al. (1991) showed that when T lymphocytes from one strain of rat were injected into another strain, ten times more T lymphocytes were found in the spinal cord if the T lymphocytes were first activated. However, conclusions drawn must be made with care since, normally, in the blood, there are ten times more resting T lymphocytes than activated (Hannet et al., 1992). Thus, the mathematics would show

that the same number of activated and resting T lymphocytes will eventually wind up in the CNS.

CD4⁺ helper/inducer T lymphocytes are more abundant in inflammatory CNS infiltrates than CD8⁺ suppressor/cytotoxic T lymphocytes. Most of these cells are of the $T_H 1$ subset and synthesize IFN- γ and TNF- α (Skundric et al., 1994). Pryce et al. (1991) found that CD8⁺ T lymphocytes bind more abundantly than CD4⁺ T lymphocytes to murine brain microvessel EC in culture, but transendothelial migration was not assessed and may not necessarily follow the pattern of adhesion. EAE can be transferred from an affected animal to a syngeneic unaffected recipient by transferring helper T lymphocytes from the former to the latter. In the lesions that form in the recipient animal, the majority of infiltrating cells are derived from the recipient animal and are not CNS specific. The transferred CNS-specific helper T lymphocytes remain exclusively in perivascular cuffs and disappear from the CNS within two weeks of chronic illness (Cross et al., 1990).

The results from this study show that the baseline, unstimulated migration of resting T lymphocytes across HBMEC is minimal and not mediated by any of the EC adhesion molecules studied. TNF activation of EC upregulates migration largely via the E-selectin, ICAM-1 and PECAM-1 pathways. It has generally been surmised that E-selectin is only involved in adhesion under conditions of flow. This is the first study in which a role for E-selectin in transmigration of T cells is demonstrated. Although the contribution of E-selectin is definite, it is not as prominent as that of ICAM-1 and PECAM-1.

The current dogma on leukocyte-EC interactions, based on studies utilizing HUVEC, indicates that under conditions of flow, leukocytes initially roll along EC using the selectins. At the same time, leukocytes come in contact with chemoattractants and chemokines that activate these blood cells and the integrins on their surface. The leukocytes then firmly adhere to VCAM-1, ICAM-1 and PECAM-1 on the EC, move toward the tight junctions between EC and migrate across transcellularly or

paracellularly, along a chemotactic gradient, using these same molecules. The present results with activated HBMEC generally support this theory, except that PECAM-1 does not seem to be involved in the migration of PMN, or adhesion of PMN and T lymphocytes although it is involved in the migration of T cells across activated HBMEC. E-selectin now takes on a new role in facilitating T cell migration. In unstimulated HBMEC, ICAM-1 does not participate in the adhesion of PMN or the migration of PMN and T lymphocytes, and VCAM-1 is not used in the migration of T lymphocytes in contrast to HUVEC. This may account for the small number of leukocytes that eventually migrate across unstimulated HBMEC, consistent with the formation of a barrier under normal conditions.

Anti-adhesion molecule treatment has been studied in vivo during CNS inflammation. Administration of Abs against the $\alpha 4\beta 1$ (VLA-4) integrin, the ligand for VCAM-1, prevents the accumulation of leukocytes in the CNS of rats and the induction of EAE in these animals (Yednock et al., 1992). Abs against other molecules, including the β 2 integrins, are ineffective. However, an anti-ICAM-1 Ab has been found to inhibit active (inoculation with CNS myelin plus adjuvant) or adoptively transferred (injection of CNS-specific T lymphocytes) EAE in rats and reduce CNS inflammation (Archelos et al., 1993), as well as reduce ischemic injury in rabbits (Clark et al., 1991). In contrast, other authors, using the same Ab, observed no inhibitory activity in TNF induced CNS inflammation or adoptively transferred EAE, but some protection in active EAE in a small fraction of animals (Willenborg et al., 1993). The use of a different anti-ICAM-1 Ab in another study did not have a significant effect on mice with adoptively transferred EAE (Cannella et al., 1993). The reasons for this discrepancy are unknown. How this applies to the human demyelinating disease, MS, or other inflammatory CNS lesions, and how different Abs against human ICAM-1 or other adhesion molecules will fare, all await future investigations.

The present results indicate that depending on the type of inflammation, the use of different combinations of Abs may be necessary. For example, in the early stages of acute inflammation in meningitis and encephalitis, when PMN infiltrate the brain, anti-E-selectin, anti-ICAM-1 and perhaps anti-PECAM-1 may be used. Several days later, anti-ICAM-1, anti-VCAM-1 and perhaps anti-PECAM-1 and anti-E-selectin could be used to inhibit lymphocyte infiltration. In more chronic types of inflammation, such as MS, where mononuclear leukocytes are more prominent than PMN, a combination of all these Abs should be used. It may be necessary to administer these Abs during remission so that Abs are always available to bind adhesion molecules whenever they may be upregulated. Soon after the inflammatory reaction is in full swing during a relapse and a large number of lymphocytes have crossed the BBB, it would be too late to use anti-adhesion molecule Abs. Unless, perhaps, injected or otherwise brought into the brain to prevent leukocytes from binding to and adversely affecting glia or neurons in the parenchyma.

Two clinical trials using anti-ICAM-1 Abs in kidney transplants and rheumatoid arthritis seem to point toward some beneficial effects in these conditions (Haug et al., 1993; Kavanaugh et al., 1992). Of concern are the high immunogenicity of murine Abs, low yield of human Abs and reduced antigen affinity as well as low yield of humanized murine Abs (Peake, 1993). Different Abs block different epitopes of the antigen to a different degree. It is known that several epitopes on each adhesion molecule may be used to different extents. It may be necessary to employ a large number of Abs in an attempt to maximally inhibit binding. This may also be the reason why studies using different Abs report contrasting results. The findings by Welder et al. (1993) hold great promise. It was demonstrated that soluble ICAM-1 coated polystyrene microspheres can competitively inhibit cell adhesion that utilizes the ICAM-1 - LFA-1 pathway, whereas simple monovalent soluble ICAM-1 is ineffective. Moreover, all the epitopes are

conceivably blocked with soluble ICAM-1. Also worth consideration would be drugs that pharmacologically inhibit the expression of these adhesion molecules.

Molecules other than adhesion molecules are also involved in EC- leukocyte interactions. These include the class II major histocompatibility complex (MHC) molecules and their receptor, the T cell receptor. The inhibition of specific T cell receptors and class II MHC antigen have also been shown to reduce T lymphocyte migration across EC *in vitro* and *in vivo* (Huynh, 1994; Kuchroo et al., 1994).

4.3 DETERMINATION OF MONOLAYER PERMEABILITY FOLLOWING TNF-α TREATMENT AND IN THE COURSE OF LEUKOCYTE MIGRATION ACROSS THE MONOLAYERS

The low permeability of the cerebral endothelium can be variably demonstrated by the low hydraulic conductivity, high electrical resistance and exclusion of tracer molecules (Abbott and Revest, 1991). The electrical resistance of cerebral microvessels has been previously determined in amphibians and rats. The average resistance of frog pial microvessels is $1870\Omega \cdot \text{cm}^2$, while that of rat pial vessels ranges from $918\Omega \cdot \text{cm}^2$ to 1490 Ω ·cm² (Crone and Olesen, 1982; Abbot and Revest, 1991). These values are indicative of a tight endothelial barrier with virtually impermeable tight junctions. There are no reports on the in situ electrical resistance of the primate brain microvessels. Studies on brain EC isolated from human and bovine brains have reported electrical resistances across monolayers ranging between $10-50\Omega \cdot \text{cm}^2$ (Rubin et al., 1991). Interestingly, the low resistance values could be increased up to an average $500\Omega \cdot \text{cm}^2$ when EC were cultured in the presence of agents that increase cyclic AMP levels in the cell (Rubin et al., 1991). Growth in the presence of astrocyte condition medium increased resistance only to 70-115 Ω ·cm². However, serially passaged bovine brain EC cultured on collagen gel can attain resistances of 150 to $800\Omega \cdot \text{cm}^2$ depending on the culture, in the absence of cAMP or astrocytes (Rutten et al., 1981). Endothelial
monolayers from other organs have significantly lower resistance. For example, bovine adrenal cortical microvessel EC have resistances of $70\Omega \cdot \text{cm}^2$ (Furie et al., 1984), HUVEC (Burke-Gaffney and Keenan, 1993), bovine aortic endothelium (Rutten et al., 1987; Territo et al., 1984; Albelda et al., 1988) and rabbit aortic endothelium (Navab et al., 1986) have resistances between 13-18 $\Omega \cdot \text{cm}^2$. Bovine and porcine pulmonary artery EC have resistance from 4 to $6\Omega \cdot \text{cm}^2$ (Shasby and Shasby, 1986; Turner, 1992) and rat epididymal pad EC as low as $0.45\Omega \cdot \text{cm}^2$ (Rutten et al., 1987). Since voltage equals current divided by resistance, difference ohm-meters employing different current may slightly affect measurements made by different investigators. However, this may not extend beyond the background noise. Differences in culture conditions, medium, temperature, etc. may also account for a small part of the differences between HBMEC and HUVEC in published reports.

The average resistance of HBMEC, grown in the absence of astrocytes, astrocyte conditioned media or cyclic AMP in the present study, was $108\Omega \cdot cm^2$ and as high as $142\Omega \cdot cm^2$. These values are certainly higher than those reported in extracerebral EC, lower than some of the reported resistances of bovine brain EC, but considerably higher than the resistance of human and some bovine brain EC grown without cyclic AMP or astrocyte conditioned media. Although the factors required for the formation and maintenance of physiologically "tight" tight junctions are presently unknown, culture of HBMEC in artificial media and the presence, in a given culture, of EC from different segments of the microvascular bed, including venules that have much less "tight" tight junctions, would contribute, at least in part, to the present resistance values. In addition, it has been suggested that, sometimes large, ranges of resistance in monolayers of the same type of endothelium may be due to small (0.5-2µm) gaps between 5 to 10% of EC (Albelda et al., 1988). It should be emphasized that the relevance of the resistance values obtained in our system would be more meaningful if compared to the *in situ* electrical resistance of the human brain microvessels which is presently unknown.

TNF treatment significantly decreased the electrical resistance across HBMEC monolayers by about 10% after 4h of treatment. The decrease was maximal by 18h with a 40% drop. Previous studies have shown that TNF treatment of HUVEC for 24h increases I¹²⁵-albumin clearance rate by 4 fold (Burke-Gaffney and Keenan, 1993). In contrast, no significant change in I¹²⁵-albumin clearance was observed following TNF treatment of HUVEC for 4h (Furie and McHugh, 1989; Moser et al., 1989). Treatment of bovine pulmonary microvessel EC with 100-10000 U TNF/ml for 24h or bovine pulmonary artery EC with 200 U TNF/ml for 18h increased permeability to I¹²⁵-albumin by 3 to 7 fold (Partridge et al., 1992, 1993; Wheatley et al., 1993). The mechanism of TNF-induced increased EC permeability seems to involve the degradation of some extracellular matrix components, such as fibronectin, laminin, collagen and gelatin, by a metalloproteinase (Partridge et al., 1993; Wheatley et al., 1993) leading to remodelling of the extracellular matrix and decrease EC-extracellular matrix contact (Partridge et al., 1992). Among other inflammatory mediators, LPS at 10µg/ml causes a 25% increase in the rate of equilibration of ³H-water, 40% increase in I^{125} -albumin clearance and a 25-50% increase in hydraulic conductance of bovine pulmonary artery endothelium with maximal effect by 3-4h (Meyrick et al., 1986).

IFN-γ treatment of epithelial cell cultures decreases the resistance maximally to less than 25% by day 3. However, permeability to horseradish peroxidase (HRP) did not change (Adams et al., 1993). An alteration in the tight junctions (paracellular movement) rather than cell necrosis or transcellular movement was observed. Results from these different systems seem to suggest that longer cytokine treatments are required to increase the permeability of tissues that form a tighter barrier, such as HBMEC and epithelium, than those that are leaky. The different nature of the tight junctions may be involved.

In vivo, intracisternal administration of 800 U TNF into the rat CNS increased I^{125} -albumin penetration into the CSF, maximally (4 to 7 fold) by 3h, while intravenous administration lacked any effect (Kim et al., 1992). LPS, IL-1 and to a lesser extent,

TNF, injected intradermally in rats, induce leakage of colloidal carbon from postcapillary venules maximally after 3 to 4h, (Yi and Ulich, 1992). However, when IL-1 α and β are injected intraventricularly or intravenously in the mouse, the integrity of the BBB, as measured by I¹²⁵-albumin movement, is not affected after 2h. It is difficult to compare these results because of the different routes of cytokine administration and different concentrations injected.

There have been very few reports on changes in endothelial permeability due to cytokines and leukocyte migration and none reporting the decrease in resistance due to both processes as shown in the present study for HBMEC. The small increase in permeability due to leukocyte migration across unstimulated HBMEC is greatly increased after TNF treatment which not only increases permeability on its own, but also upregulates adhesion molecules to significantly increase leukocyte migration. Both of these processes combine to generate a large increase in permeability. In vitro, it has been reported that the migration of PMN across unstimulated or TNF (200U for 1 to 6h) activated bovine pulmonary artery EC causes a 20% increase in albumin permeability (Gibbs et al., 1990). Migration of activated memory T lymphocytes but not naive T lymphocytes increases endothelial permeability to albumin (Dalme and Doyle, 1990). Whether this is due to the migratory process itself is unknown. In animal models, the leakage of the tracer HRP across rat and mice cerebral microvessels in EAE has been variably attributed to increased tight junctional permeability, increased interendothelial space, increased numbers of cytoplasmic vesicles and tubular profiles or leakage alongside migrating leukocytes (Claudio et al., 1990; Lossinsky et al., 1989). The migration of leukocytes across mesenteric venules of cats subjected to ischemia and reperfusion has also been found to contribute to the leakage of HRP (Oliver et al., 1991). Even though the addition of an anti-CD18 (ligand to ICAM-1) Ab inhibited the adhesion and migration of leukocytes in this model, some HRP leakage was still evident across the blood vessels (Oliver et al., 1991). These data are consistent with our observations.

Migration of PMN and T lymphocytes variably decreased the resistance of monolayers by 20 to 70%. The decrease in resistance of unstimulated HBMEC following migration of PMN in response to chemotactic gradients of fMLP is especially great. This may conceivably be due to the greater number of PMN migrating per mm of EC monolayer than T lymphocytes. Although certain anti-endothelial adhesion molecule Abs decreased the adhesion and migration of PMN and T lymphocytes, they did not reverse the drop in resistance. This may be due to the lack of complete inhibition by the Abs. The small number of leukocytes still able to migrate may be sufficient to increase the permeability. Alternatively, EC-leukocyte contact may induce the leukocyte to release some fast acting mediator(s) on the EC with increase in permeability by some unknown mechanism.

It is clear that leukocyte migration can increase the permeability of the BBB. It seems unlikely that the increase in HBMEC permeability caused by TNF treatment affects migration by itself since blocking ICAM-1 by an Ab greatly decreases T lymphocyte migration, but does not affect the increase in permeability due to TNF treatment. Thus, although the use of anti-adhesion molecule Abs *in vivo* may suppress inflammatory cell infiltration, the presence of cytokine, such as TNF, will increase the BBB permeability independent of leukocyte migration. It would be necessary, therefore, to counteract the effect of these cytokines perhaps by using Abs directed against them or their synthetic soluble receptors (Mohler et al., 1993).

The effect of leukocyte migration on epithelial permeability has been documented by several laboratories. Peritoneal exudate cells decrease epithelial resistance by 70% after 90min of incubation (Evans et al., 1983), while PMN cause a 80 to 100% drop after 30 to 60 min in one report (Nash et al., 1987, 1988) and a 46% drop after 40 min in another (Milks et al., 1986). 10⁻⁷M fMLP was present under the epithelium in these experiments. Although PMN migration across HBMEC in response to a gradient of 10⁻⁷M fMLP was prominent, the resistance never dropped to such low levels. It is interesting to note that upon removal of PMN that are on top of the epithelium, the

resistance recovered by 20 to 60 min (Evans et al., 1983; Milks et al., 1986). The drop in epithelial resistance has been further dissected by Parkos et al. (1992) who noted that this is a biphasic response. The initial decrease in permeability, maximal 5 to 13min after transmigration begins, is due to chloride secretion by the epithelial cells and not to changes in tight junctional permeability. Further decrease in resistance after 60min of migration is due to increased junctional permeability. Whether this also occurs in HBMEC is not known. It should also be kept in mind that resistance measurements represent the movement of a current (electrons) across the tissue. Even though electrons may be able to penetrate a tight junction in some instances, other physiologically relevant molecules that are larger, such as ions (Na⁺, K⁺, Cl⁻), water and proteins may not. Thus, it would be more informative to complement electrical resistance measurements with tracer studies utilizing tracers such as HRP, I¹²⁵-albumin, ³H-H₂O and ionic lanthanum to further dissect this event. Indeed, morphological techniques employing HRP, lanthanum and autoradiography can clearly distinguish between the transcellular and paracellular pathways of tracer leakage.

The present study shows that HBMEC monolayers have relatively high electrical resistance which can be progressively decreased by TNF treatment. Transendothelial migration of PMN and T lymphocytes further decreases the resistance. Although anti-adhesion molecule Abs decrease PMN and T lymphocyte migration, they do not affect the drop in resistance due to this process.

CHAPTER 5 CONCLUSIONS

5.1 SUMMARY AND SIGNIFICANCE

The aim of this study was to 1) examine the expression, distribution and upregulation of the adhesion molecules E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), platelet/endothelial cell adhesion molecule-1 (PECAM-1) and HECA 452 antigen in primary cultures of HBMEC. 2) Elucidate their role in polymorphonuclear leukocyte (PMN) and T lymphocyte adhesion to and transmigration across the endothelium. 3) Determine the effects these events have on endothelial permeability. We hypothesized that in the initial stages of central nervous system (CNS) inflammation, lipopolysaccharide (LPS) and cytokines can upregulate and / or change the distribution of adhesion molecules on HBMEC. This "activation" of EC can then initiate leukocyte adhesion and transendothelial migration, with a concomitant increase in the permeability of the endothelial barrier. Use of antibodies against these adhesion molecules can counteract some of these processes.

Our findings show that a small percentage of cells in primary cultures of HBMEC express low levels of ICAM-1, VCAM-1 and E-selectin, the percentage of expression decreasing in that order. PECAM-1 is constitutively expressed by all HBMEC with a preferential localization at the cell borders. Activation of HBMEC with LPS, tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) dramatically upregulates the expression of E-selectin, VCAM-1 and ICAM-1. LPS is most effective in this respect, followed by TNF, then IL-1. IFN- γ weakly upregulates ICAM-1 but not the other adhesion molecules. Activation of EC bears no effect on the expression of PECAM-1. E-selectin is maximally upregulated 4h after activation. Its expression then drops rapidly and by 24 to 48h, is not different from that in unstimulated cultures. VCAM-1 becomes

maximally expressed 12 to 24h after activation and returns to unstimulated levels by 48h with most treatments. ICAM-1 expression becomes maximal 24h after HBMEC activation and remains high for at least 72h. Growth in the absence of LPS and cytokines for 3 days after maximal activation does not reduce the expression of ICAM-1. Ultrastructurally, E-selectin, VCAM-1 and ICAM-1 are expressed predominantly on the apical surface on unstimulated HBMEC. TNF treatment increases the apical expression to a greater extent than the basal expression. Labeling is particularly dense at finger-like cytoplasmic projections.

Cytokine activation of HBMEC greatly increases the number of adherent and migrated PMN and T lymphocytes. The minimal unstimulated adhesion and transmigration of PMN and T lymphocytes across unstimulated HBMEC is not affected by adhesion molecule blocking Ab with the exception of anti-ICAM-1 mAb that significantly decreases T lymphocyte adhesion. It is possible that some epitope of ICAM-1 is involved in this adhesion. Blocking mAb, however, effectively block the augmented adhesion and migration of PMN and T lymphocytes across cytokine treated HBMEC monolayers. Thus, both E-selectin and ICAM-1 are involved in PMN adhesion and ICAM-1 is utilized during PMN migration. VCAM-1 and ICAM-1 are required for T lymphocyte adhesion. Lastly, E-selectin, ICAM-1 and PECAM-1 all play an active role in T lymphocyte migration.

Unstimulated monolayers of HBMEC have a high electrical resistance indicative of their low permeability. Treatment with TNF increases the permeability progressively. Migration of PMN and T lymphocytes across TNF-treated HBMEC further increases the permeability. Although anti-adhesion molecule Abs effectively block migration of these leukocytes, they do not prevent the increase in permeability.

In summary, based on our findings in primary cultures of HBMEC, we hypothesize that cerebral EC *in situ* constitutively express high levels of PECAM-1, relatively low levels of ICAM-1 and very low levels of E-selectin and VCAM-1.

ICAM-1 would be involved in the adhesion of an extremely small number of T lymphocytes to intact HBMEC under normal conditions. Viral infections would lead to release of viral proteins and secretion of cytokines by mononuclear phagocytes and T cells that would in turn upregulate the expression of E-selectin, VCAM-1 and ICAM-1. Bacterial infections would similarly upregulate adhesion molecule expression via secretion of bacterial products and LPS. Upregulation of these adhesion molecules would then facilitate leukocyte adhesion and transendothelial migration. It is evident from the present studies that PMN utilize E-selectin and ICAM-1 for initial adhesion and ICAM-1 for migration, while T lymphocytes use VCAM-1 and ICAM-1 for adhesion and E-selectin, ICAM-1 and PECAM-1 for migration. In the course of these early events in the evolution of the inflammatory response, the permeability of the BBB will be compromised both by the direct action of cytokines and transendothelial migration of leukocytes. Abs against EC adhesion molecules can, at least partially, block the corresponding interactions but cannot prevent the increase in endothelial permeability secondary to leukocyte migration.

The significance of this study lies in the utilization of a new cell culture system, not previously accessible, that models the human blood-brain barrier (BBB). It is apparent from the findings presented herein that some conclusions drawn from other models, such as HUVEC and EAE are applicable to the human BBB while others are not. For example, ICAM-1 expression does not reverse as readily as in HUVEC, VCAM-1 expression drops more rapidly and PECAM-1 is not affected by cytokines unlike HUVEC. The adhesion molecules that regulate PMN and T lymphocyte adhesion and migration differ in some instances. For example, the anti-E-selectin Ab, unlike its effect on HBMEC, was found to inhibit the adhesion of a subset of T lymphocytes and the migration of PMN across HUVEC. Some molecules have not been previously examined in certain interactions, such as E-selectin in T lymphocyte migration and PECAM-1 in

PMN and T lymphocyte adhesion. Similarly, permeability changes of the cerebral endothelium during leukocyte migration have not been previously studied. The results of these studies extend our understanding of the mechanisms involved in the inflammatory process in the CNS. Therapeutic interventions utilizing mAbs against adhesion molecules or even soluble forms of adhesion molecules may prove useful in altering the course and severity of inflammation in the CNS.

5.2 FUTURE PROSPECTS

Three interesting projects are apparent from this immediate study. Firstly, expression of E-selectin and VCAM-1 return to unstimulated levels 24 to 48h after HBMEC activation. However, in patients, these molecules can be detected many days after the initiation of inflammation in the CNS. This may be because areas peripheral to the initial lesion are being activated in sequence, but Sepp et al. (1994) have also found a factor in human serum that prolongs the expression of E-selectin by HDMEC at least for 48h. It would be important to see if this is restricted to HDMEC and if this or other factors in human blood can otherwise modulate adhesion molecule expression on HBMEC.

Secondly, as mentioned in the discussion, leukocytes may bind to several different epitopes on each adhesion molecule, while each mAb can only block one epitope. It cannot be derived that an adhesion molecule is not employed by simply using only one mAb. Since it is very difficult to produce EC that are genetically altered not to produce specific adhesion molecules, the soluble adhesion molecule coated microspheres that Welder et al. (1993) used would be ideal for experiments to block all the epitopes on any one molecule.

Thirdly, the permeability experiments done in this study only measure the movement of current (electrons) across the monolayer. Movement of larger molecules such as ions, water and albumin also need to be examined. This can be done using

electron dense tracers such as ionic lanthanum and HRP for electron microscopy, and 3 H-H₂O and I¹²⁵-albumin in physiological experiments.

Other interesting aspects have not been mentioned during the discussion, but would be just as important to examine. Other than using Abs and soluble adhesion molecules, it may also be possible to inhibit the synthesis of adhesion molecules. Drugs that can perform this way would be very important in therapy. Bennett et al. (1994) produced oligonucleotides that inhibit the expression of ICAM-1, VCAM-1 and E-selectin on treated HUVEC. Other agents, such as nitric oxide, inhibit EC-leukocyte interactions by unknown mechanisms. These could be other avenues for therapy. It is also known that tumor cells adhere to blood vessel walls via adhesion molecules. Experiments with these cells along the same line as the leukocytes in this study could elucidate the mechanisms employed by tumor cells in metastasis. It would be especially important to examine the differences between EC of different sources to determine the differences that lead to preferential metastasis to specific organs, as well as differences between tumor and normal vessels. Therapy can be produced to inhibit adhesion of tumor cells to these organs and to encourage lymphocytes to infiltrate tumor vessels. Other than adhesion molecules, chemokines / chemoattractants are also important in the recruitment of leukocytes to inflammatory sites. Many chemokines such as monocyte chemoattractant protein 1 and interleukin-8 have not been examined in CNS inflammation. Literally a life-time's worth of studies remain to be done in this fast-paced field.

Figure 1. Confluent culture of human brain microvessel endothelial cells grown on (a) fibronectin coated plastic wells for adhesion molecule expression assays and adhesion assays,
(b) Cellagen discs for migration assays, scanning electron microscopy and electrical resistance measurements. X270.

Figure 1.



Figure 2.Primary cultures of human brain microvessel endothelial
cells show positive staining for Factor VIII antigen,
confirming their endothelial origin. X280.





Figure 3. Illustration of a Cellagen disc in a well of a 24 well plate. Human brain microvessel endothelial cells are grown in this system for migration, scanning and transmission electron microscopy.

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Figure 4. (a) HBMEC expression of E-selectin without LPS or cytokines, detected by immunogold silver staining. (b) HBMEC expression of E-selectin after incubation with 1µg LPS/ml for 4h, detected by immunogold silver staining. (c) HBMEC expression of E-selectin after incubation with 100U TNF-α/ml for 4h, detected by immunogold silver staining. (d) HBMEC expression of E-selectin after incubation after incubation with 10U IL-1β/ml for 4h, detected by immunogold silver staining. X270.

Figure 4.



Figure 5. Quantitation of E-selectin expression on HBMEC by (a) immunogold silver staining (IGSS) and (b) ELISA after incubation with LPS for 0 □, 4 □, 12 □, 24 □, or 48h ⊠
Values represent mean ± standard error of triplicate (IGSS) or duplicate wells (ELISA) from three experiments.

* p<0.05 compared to expression at 0h.





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b

Figure 6. Quantitation of E-selectin expression on HBMEC by (a) immunogold silver staining (IGSS) and (b) ELISA after incubation with TNF-α for 0 □, 4 □, 12 □, 24 □, or 48h
☑. Values represent mean ± standard error of triplicate (IGSS) or duplicate wells (ELISA) from three experiments.
* p<0.05 compared to expression at 0h.





Figure 7. Quantitation of E-selectin expression on HBMEC by (a) immunogold silver staining (IGSS) and (b) ELISA after incubation with IL-1β for 0 □, 4 □, 12 □, 24 □, or 48h
☑. Values represent mean ± standard error of triplicate (IGSS) or duplicate wells (ELISA) from three experiments.
* p<0.05 compared to expression at 0h.





Figure 8. Quantitation of E-selectin expression on HBMEC by (a) immunogold silver staining (IGSS) and (b) ELISA after incubation with IFN-γ for 0 □, 4 □, 12 □, 24 □, or 48h
☑. Values represent mean ± standard error of triplicate (IGSS) or duplicate wells (ELISA) from three experiments. No statistically significant difference from expression at 0h was found by ANOVA.

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Figure 9. (a) HBMEC expression of E-selectin without LPS or cytokines detected by immunogold EM. (b) HBMEC expression of E-selectin after treatment with 100U TNF-α/ml for 4h detected by immunogold TEM. (c) Note the higher amount of gold particles at the finger-like projections. X38,000.



Figure 10.Immunogold electron microcopy quantitation of E-selectin
expression on HBMEC apical \Box and basal \Box surfaces after
incubation with 100U TNF- α /ml for 4h or left
unstimulated. Values represent mean ± standard error of
one hundred cells for each treatment.

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Figure 10.



Number of gold particles $/\mu m$ cell membrane

Figure 11. Immunogold silver staining of HBMEC monolayers for VCAM-1. (a) Unstimulated expression of VCAM-1 is limited to a small number of cells in confluent cultures. Staining appears most dense at the periphery of the cell. (b-d) Upregulation of VCAM-1 expression following treatment with 1µg LPS/ml for 24h (b); 100U TNF-α/ml for 24h (c); 10U IL-1β/ml for 24h (d). a-d, X140.

Figure 12. Quantitation of VCAM-1 expression on HBMEC by (a) immunogold silver staining (IGSS) and (b) ELISA after incubation with LPS for 0 □, 4 ☑, 12 ⊒, 24 ☑, or 48h ☑
Values represent mean ± standard error of duplicate wells from three (IGSS) or two (ELISA) experiments. * p<0.05 compared to expression at 0h.







Figure 13. Quantitation of VCAM-1 expression on HBMEC by (a) immunogold silver staining (IGSS) and (b) ELISA after incubation with TNF-α for 0 □, 4 ○, 12 □, 24 □, or 48h
☑. Values represent mean ± standard error of duplicate wells from three (IGSS) or two (ELISA) experiments.

* p<0.05 compared to expression at 0h.

Figure 13.



Figure 14. Quantitation of VCAM-1 expression on HBMEC by (a) immunogold silver staining (IGSS) and (b) ELISA after incubation with IL-1β for 0 □, 4 □, 12 □, 24 □, or 48h
☑. Values represent mean ± standard error of duplicate wells from three (IGSS) or two (ELISA) experiments.

* p<0.05 compared to expression at 0h.






Figure 15. Quantitation of VCAM-1 expression on HBMEC by (a) immunogold silver staining (IGSS) and (b) ELISA after incubation with IFN-γ for 0 □, 4 □, 12 □, 24 □, or 48h
☑. Values represent mean ± standard error of duplicate wells from three (IGSS) or two (ELISA) experiments. No statistically significant difference from expression at 0h was found by ANOVA.





Figure 16.Quantitation of VCAM-1 expression on HBMEC by
immunogold silver staining after incubation with 0.1U/ml
 \Box or 0U/ml \Box IL-1 β and 10U/ml, 1U/ml and 0U/ml TNF- α
for 48h. Values represent mean ± standard error of
duplicate wells from three experiments. No statistically
significant difference from expression without (0h) TNF or
IL-1 was found by ANOVA.

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Figure 17. Immunogold labeling for VCAM-1 in HBMEC. (a) Small number of gold particles indicating the presence of VCAM-1 decorate the apical cell surface of a small population of untreated HBMEC. Labeling is minimal or absent at the basal cell surface. X44,000. (b-d) Treatment of HBMEC monolayers with TNF- α (100U/ml) for 18h significantly increases the number of gold particles at the apical and to a lesser extent the basal cell surfaces indicating upregulation of VCAM-1 expression. Labeling is most intense near or at finger-like cytoplasmic projections of the apical surface (c) and is not present along intercellular contacts (between arrow heads) or at tight junctions (arrows) between adjacent EC. b, X52,000; c, X50,000; d, X50,000.



Figure 11.



Figure 18. Quantitation of VCAM-1 expression on the apical
and basal
and surfaces of unstimulated and TNF-α (100U/ml for 18h) treated HBMEC following immunogold labeling. Values represent mean ± standard error of one hundred unstimulated and one hundred TNF-α treated cells.



Figure 18.

Number of gold particles / μm cell membrane

Figure 19. (a) HBMEC expression of ICAM-1 without LPS or cytokine, detected by immunogold silver staining. (b) HBMEC expression of ICAM-1 after incubation with 0.1µg LPS/ml for 24h, detected by immunogold silver staining. (c) HBMEC expression of ICAM-1 after incubation with 500U IFN-γ/ml for 24h, detected by immunogold silver staining. (d) HBMEC expression of ICAM-1 after incubation with 10U TNF-α/ml for 24h, detected by immunogold silver staining. (e) HBMEC expression of ICAM-1 after incubation with 10U TNF-α/ml for 24h, detected by immunogold silver staining. (f) HBMEC expression of ICAM-1 after incubation with 10U IL-1β/ml for 24h, detected by immunogold silver staining. (f) HBMEC expression of ICAM-1 after incubation with 100U TNF-α/ml and 500U IFN-γ/ml for 24h, detected by immunogold silver staining. X250.

Figure 19.



Figure 20. Quantitation of ICAM-1 expression on HBMEC by immunogold silver staining after incubation with LPS for 0
□, 4 □, 12 □, 24 □, 48 ⊠, or 72h □. Values represent mean ± standard error of results from three experiments.
* p<0.05 compared to expression at 0h.

Figure 20.



Figure 21. Quantitation of ICAM-1 expression on HBMEC by immunogold silver staining after incubation with IFN-γ for 0 □, 4 □, 12 □, 24 □, 48 ⊠, or 72h □. Values represent mean ± standard error of results from three experiments. * p<0.05 compared to expression at 0h.</p>

Figure 21.



Figure 22. Quantitation of ICAM-1 expression on HBMEC by immunogold silver staining after incubation with TNF-α for 0 □, 4 ○, 12 ⊡, 24 ⊇, 48 ≥, or 72h □. Values represent mean ± standard error of results from three experiments. * p<0.05 compared to expression at 0h.</p>





Figure 23. Quantitation of ICAM-1 expression on HBMEC by immunogold silver staining after incubation with IL-1β for 0 □, 4 □, 12 □, 24 □, 48 ⊠, or 72h □. Values represent mean ± standard error of results from three experiments. * p<0.05 compared to expression at 0h.</p>





Figure 24.Quantitation of ICAM-1 expression on HBMEC by
immunogold silver staining after incubation with 500U/ml \boxdot , 100U/ml \bigtriangledown , or 0U/ml \bigcirc IFN-γ and 100U/ml, 10U/ml,
or 1U/ml TNF- α for 24h. Values represent mean \pm
standard error of duplicate wells in one experiment. No
difference from cultures treated with only TNF- α without
IFN- γ was found by ANOVA.

Figure 24.



Figure 25. Reversibility of ICAM-1 expression on HBMEC induced by incubation with LPS for 2d then 3d without LPS □, with LPS for 2d □, with LPS for 5 days □, or without LPS □. Values represent mean ± standard error of results from three experiments. No significant difference was found by statistics.





Figure 26. Reversibility of ICAM-1 expression on HBMEC induced by incubation with IFN- γ for 2d then 3d without IFN- $\gamma \Box$, with IFN- γ for 2d \Box , with IFN- γ for 5 days Ξ , or without IFN- $\gamma \Box$. Values represent mean ± standard error of results from three experiments. No significant difference was found by statistics.

Figure 26.



Figure 27. Reversibility of ICAM-1 expression on HBMEC induced by incubation with TNF- α for 2d then 3d without TNF- α , with TNF- α for 2d \square , with TNF- α for 5 days \square , or without TNF- α \square . Values represent mean ± standard error of results from three experiments. No significant difference was found by statistics. Figure 27.



Figure 28. Reversibility of ICAM-1 expression on HBMEC induced by incubation with IL-1 β for 2d then 3d without IL-1 β \Box , with IL-1 β for 2d Δ , with IL-1 β for 5 days Ξ , or without IL-1 β \Box . Values represent mean ± standard error of results from three experiments. No significant difference was found by statistics.





Figure 29. (a) HBMEC expression of ICAM-1 without LPS or cytokines detected by immunogold EM. (b, c) HBMEC expression of ICAM-1 after treatment with 100U TNF-α/ml for 24h detected by immunogold TEM. Note the presence of gold particles at the apical surface and its finger-like projections. X38,000.

Figure 29.



Figure 30.Immunogold electron microcopy quantitation of ICAM-1.expression on HBMEC apical \Box and basal \Box surfaces after
incubation with 100U TNF- α /ml for 24h or left
unstimulated. Values represent mean ± standard error of
one hundred cells for each treatment.



Figure 30.

Number of gold particles / µm cell membrane

Figure 31. (a) HBMEC expression of PECAM-1 without LPS or cytokine, detected by immunogold silver staining. (b) HBMEC expression of PECAM-1 after incubation with 1µg LPS/ml for 24h, detected by immunogold silver staining. (c) HBMEC expression of PECAM-1 after incubation with 100U TNF-α/ml for 24h, detected by immunogold silver staining. (d) HBMEC expression of PECAM-1 after incubation with 10U IL-1β/ml for 24h, detected by immunogold silver staining. X183.




Figure 32. Quantitation of PECAM-1 expression on HBMEC by
ELISA after incubation with LPS for 48 ⊠, 24 ⊇, 12 ⊒, 4
□, or 0h □. Values represent mean ± standard error of
duplicate wells from three experiments. No statistically
significant difference was found between unstimulated (0h)
and treated cultures.

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Figure 32.

Figure 33. Quantitation of PECAM-1 expression on HBMEC by
ELISA after incubation with TNF-α for 48 Ø, 24 Ø, 12 □
, 4 Ø, or 0h □. Values represent mean ± standard error of
duplicate wells from three experiments. No statistically
significant difference was found between unstimulated (0h)
and treated cultures.

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Figure 33.



Figure 34. Quantitation of PECAM-1 expression on HBMEC by
ELISA after incubation with IL-1β for 48 ⊠, 24 ☑, 12 ⊟,
4 ☑, or 0h □. Values represent mean ± standard error of duplicate wells from three experiments. No statistically significant difference was found between unstimulated (0h) and treated cultures.



Figure 34.

Figure 35. Quantitation of PECAM-1 expression on HBMEC by
ELISA after incubation with IFN-γ for 48 ⊠, 24 ☑, 12 ☑,
4 ☑, or 0h □. Values represent mean ± standard error of
duplicate wells from three experiments. No statistically
significant difference was found between unstimulated (0h)
and treated cultures.

Figure 35.



Figure 36. Expression of PECAM-1 on unstimulated HBMEC (a-c). PECAM-1 is expressed on the apical surface of HBMEC and to a much lesser extent on the basal surface (a). Gold particles are concentrated on flat surfaces rather than on finger-like cytoplasmic projections (b). Gold particles are present on the surface immediately adjacent to the intercellular contacts but are absent along intercellular contacts and tight junctions (arrows) (c). a-b, X53,000; c, X67,000.

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Figure 37. Expression of PECAM-1 on unstimulated HBMEC (a-d).
PECAM-1 is especially concentrated at the periphery of the EC. In these areas, prominent labeling at the basal surface can be seen (a, b). In subconfluent areas of the monolayers, the advancing thin process of EC are heavily decorated with gold particles (c, d). Labeling on HBMEC activated with 500U IFN-γ/ml for 48h (e) is not significantly different from unstimulated EC. X53,000.



Figure 38. Immunogold electron microcopy quantitation of PECAM-1 expression on HBMEC apical ∑ and basal ∠ surfaces after incubation with 500U IFN-γ/ml for 48h or left unstimulated. Values represent mean ± standard error of one hundred cells for each treatment.

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Number of gold particles / μm cell membrane

Figure 39.Scanning electron micrographs of PMN adhesion to (a)
unstimulated and (b) TNF-treated (100U/ml for 24h)
HBMEC. No adhesion molecule blocking Ab was used.
X670.



Figure 39.

Figure 40.

Light micrographs of PMN adhesion to unstimulated (a) and TNF-treated (100U/ml) HBMEC for 4h (b), 18h (c), 24h (d). No adhesion molecule blocking Ab was used. PMN were stained for leukocyte common antigen using the immunoperoxidase method. HBMEC were counter-stained with haemotoxylin. X630.

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Figure 40.



 Figure 41.
 Light microscopy quantitation of PMN adhesion to TNF-α

 activated and untreated HBMEC.
 Values represent

 means±SEM of two experiments, each performed in
 triplicate wells.

 *Significant (p<0.05) increase in adhesion</th>
 compared to untreated HBMEC.

Figure 41.



Figure 42.Effects of adhesion molecule blocking Ab treatment on
PMN adhesion to TNF- α treated and untreated HBMEC.
PMN were incubated for 10min with HBMEC. Values
represent means±SEM of two experiments, each performed
in triplicate wells. *Significant (p<0.05) decrease in
adhesion compared to HBMEC not treated with Abs.

Figure 42.



Figure 43. Effects of adhesion molecule blocking Ab treatment on PMN adhesion to TNF-α treated and untreated HBMEC.
 PMN were incubated for 20min with HBMEC. Values represent means±SEM of two experiments, each performed in triplicate wells. *Significant (p<0.05) decrease in adhesion compared to HBMEC not treated with Abs.

Figure 43.



Figure 44.Effects of adhesion molecule blocking Ab treatment on
PMN adhesion to TNF-α treated and untreated HBMEC.PMN were incubated for 30min with HBMEC.Values
represent means±SEM of two experiments, each performed
in triplicate wells. *Significant (p<0.05) decrease in
adhesion compared to HBMEC not treated with Abs.

Figure 44.



Figure 45. Light micrographs of PMN migration across TNF-α treated and untreated HBMEC on 1µm thick plastic sections stained with toluidine blue. (a) Unstimulated EC, (b) 4h TNF, (c) 18h TNF, (d) 24h TNF. X980.

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 Figure 46. Quantitation, by light microscopy, of PMN migration across TNF-α activated HBMEC. Values represent means±SEM of 120 sections per treatment of two experiment. *Significant (p<0.05) increase in the number of migrated PMN compared to untreated HBMEC.

Figure 46.



Figure 47.Quantitation, by light microscopy, of PMN migration
across TNF- α activated HBMEC treated with anti-adhesion
molecule Abs following 2h incubation with EC. Values
represent means±SEM of greater than 100 sections per
treatment of two experiment. *Significant (p<0.05)
decrease in the number of migrated PMN compared to EC
not treated with Abs.

Figure 47.



Figure 48. Scanning electron micrographs of PMN interactions with HBMEC during transendothelial migration. PMN cluster at the contact points between EC (a, X5,100), then squeeze into a flattened shape as they move between endothelial cells in order to cross the endothelium (b, X5,100; c, X3,200).

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Figure 49. Transmission electron micrographs of PMN interactions with HBMEC during transendothelial migration. PMN migrate at the periphery of the cells, close to or at tight junctions. X21,000.


Figure 50.Transmission electron micrographs of PMN interactions
with HBMEC during transendothelial migration. (a) PMN
that have migrated under the EC monolayer appear slightly
flattened. The overlying monolayer remains intact.
X3,700. (b) Close contacts are present at several points
between migrated PMN and HBMEC Note the intact tight
junction (arrow heads) above the migrated PMN. X15,000.





Figure 51.Scanning electron micrographs of T lymphocyte adhesion
to unstimulated (a) or activated HBMEC, 100U TNF- α /ml
for 4h (b), 18h (c), 24h (d). No adhesion molecule
blocking Ab was used. X320.

Figure 51.



Figure 52. Light micrographs of T lymphocyte adhesion to unstimulated (a) or activated HBMEC, 100U TNF-α/ml for 4h (b), 18h (c), 24h (d). No adhesion molecule blocking Ab was used. X550.

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Figure 52.

Figure 53. Quantitation, by light microscopy, of T lymphocyte adhesion to TNF-α activated and untreated HBMEC.
 Values represent means±SEM of three experiments, each performed in triplicate wells. *Significant (p<0.05) increase in adhesion compared to untreated HBMEC.

Figure 53.



Figure 54.Effects of adhesion molecule blocking Ab treatment on T
lymphocyte adhesion to TNF-α treated and untreated
HBMEC. T lymphocytes were incubated for 20min on
HBMEC. Values represent means±SEM of three
experiments, each performed in triplicate wells.*Significant (p<0.05) decrease in adhesion compared to
HBMEC not treated with Abs.

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Figure 54.



Figure 55.Effects of adhesion molecule blocking Ab treatment on T
lymphocyte adhesion to TNF-α treated and untreated
HBMEC. T lymphocytes were incubated for 30min on
HBMEC. Values represent means±SEM of three
experiments, each performed in triplicate wells.*Significant (p<0.05) decrease in adhesion compared to
HBMEC not treated with Abs.

Figure 55.



Figure 56. Effects of adhesion molecule blocking Ab treatment on T lymphocyte adhesion to TNF-α treated and untreated HBMEC. T lymphocytes were incubated for 60min on HBMEC. Values represent means±SEM of three experiments, each performed in triplicate wells.
 *Significant (p<0.05) decrease in adhesion compared to HBMEC not treated with Abs.





Figure 57. Transmission electron micrograph of T lymphocyte adhering to a human brain microvessel endothelial cell.
Adherent T lymphocyte appear as a small roughly spherical cell. X3,400.





Figure 58. Transmission electron micrograph of T lymphocyte adhering to a human brain microvessel endothelial cell.
Adherent T lymphocyte appear as a small roughly spherical cell that established contact to EC by extending pseudopodia to the apical surface of EC. X18,000.





Figure 59.Transmission electron micrograph of T lymphocyte
adhering to a human brain microvessel endothelial cell.Adherent T lymphocyte appear as a small roughly spherical
cell that established contact to EC by extending
pseudopodia to the apical surface of EC. X22,000.



Figure 59.

Figure 60. Transmission electron micrograph of T lymphocyte
 adhering to a human brain microvessel endothelial cell.
 Adherent T lymphocyte appear as a small semi-spherical
 cell. X18,000.





Figure 61.Transmission electron micrograph of T lymphocyte
adhering to a human brain microvessel endothelial cell.Adherent T lymphocyte appear as a small semi-spherical
cell. X25,000.



Figure 61.

Figure 62.Transmission electron micrograph of T lymphocyte
adhering to a human brain microvessel endothelial cell.Adherent T lymphocyte form close contacts with the apical
surface of HBMEC. X35,000.

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Figure 62.

Figure 63. Light micrographs of T lymphocyte migration across
TNF-α treated and untreated HBMEC. These 1µm sections were stained with toluidine blue. (a) Unstimulated EC, (b) 4h TNF, (c) 18h TNF, (d) 24h TNF. X1,500.



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Figure 64.Quantitation, by light microscopy, of T lymphocyte
migration across TNF-α activated HBMEC. Values
represent means±SEM of greater than 100 sections per
treatment of one experiment. *Significant (p<0.05)
increase in the number of migrated T lymphocytes
compared to untreated HBMEC.

Figure 64.



Figure 65.Quantitation, by light microscopy, of T lymphocyte
migration for 30min across TNF-α activated HBMEC
treated with anti-adhesion molecule Abs. Values represent
means±SEM of greater than 100 sections per treatment of
one experiment.

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Figure 65.



Figure 66.Quantitation, by light microscopy, of T lymphocyte
migration for 60min across TNF-α activated HBMEC
treated with anti-adhesion molecule Abs. Values represent
means±SEM of greater than 100 sections per treatment of
one experiment.

Figure 67. Quantitation, by light microscopy, of T lymphocyte migration for 180min across TNF- α activated HBMEC treated with anti-adhesion molecule Abs. Values represent means±SEM of greater than 100 sections per treatment of one experiment. *Significant (p<0.05) decrease in the number of migrated T lymphocytes compared to EC not treated with Abs.

Figure 67.



Figure 68. Scanning electron micrographs of T lymphocyte interactions with HBMEC during transendothelial migration. T lymphocytes gather at the periphery of EC before migration (a, X2,000). Most T lymphocytes squeeze into a flattened shape between endothelial cells in order to cross the endothelium (b, X6,800), others move through the cytoplasm of endothelial cells (c, X5,300).




Figure 69.Transmission electron micrographs of T lymphocyte
interaction with HBMEC. Foot-like processes are inserted
by the lymphocyte into the endothelial cytoplasm.
X47,000.





Figure 70.Transmission electron micrographs of T lymphocyteinteraction with HBMEC. Foot-like processes are insertedby the lymphocyte into the endothelial cytoplasm.X27,000.



Figure 70.

Figure 71.Transmission electron micrographs of T lymphocyteinteraction with HBMEC. Foot-like processes are insertedby the lymphocyte into the endothelial cytoplasm.X43,000.

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Figure 71.

Figure 72. Transmission electron micrographs of T lymphocyte migration through HBMEC. A cytoplasmic "opening" or gap in the HBMEC progressively enlarges to allow the lymphocyte to pass through. X13,000.

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Figure 72.

Figure 73. Transmission electron micrographs of T lymphocyte migrated underneath the HBMEC. The lymphocyte become elongated and remain between the underlying collagen membrane and the overlying EC, maintaining close contact with the latter. X13,000.



could be found throughout the surface of the HBMEC but usually aggregated at the areas of contact between EC (Fig. 39).

3.3.2 Polymorphonuclear leukocyte (PMN) migration

Transendothelial migration of PMN across unstimulated HBMEC was minimal after 2h of incubation (Figs. 45-46). Pretreatment of EC with TNF- α increased the number of migrated PMN 2 to 10 fold (P<0.001 by ANOVA). The largest number of PMN under the monolayers was observed in cultures treated with TNF for 24h (Figs. 45-46). The increase in adhesion by TNF was not as dramatic as the one obtained in response to chemotactic gradients of 10⁻⁷M fMLP placed under the unstimulated monolayers when a 30 fold increase in migration was observed (Fig. 46). MAb had no effect on PMN migration across unstimulated HBMEC (Fig. 47). However, Ab treatment decreased migration across activated EC (P<0.001 by ANOVA). Preincubation with anti-ICAM-1 mAb significantly decreased the numbers of PMN migrating across TNF-treated cultures by 75% (Fig. 47). Other mAbs did not significantly block transmigration (Fig. 47). Thus, the anti-PECAM-1 Ab blocked adhesion by a modest 15% and anti-VCAM-1 by 4% only. MAb against E-selectin had no effect on PMN migration.

Observations with the SEM demonstrated that, following adhesion, PMN positioned themselves between adjacent EC (Fig. 48a) and eventually proceeded to migrate by moving between EC. During this process, PMN became elongated and flattened in their attempt to "squeeze" between the closely associated HBMEC (Fig. 48b, c). Examination by TEM revealed that PMN migrated between thin processes of adjacent HBMEC, possibly at the site of tight junctions (Fig. 49). PMN that had migrated under the HBMEC monolayer appeared flattened and remained between overlying EC and the underlying collagen membrane in close contact with the EC at several points (Fig. 50). The overlying monolayers showed no signs of disruption and

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et al., 1991; Hakkert et al., 1991). Much higher rates of 70% (Smith et al., 1988) and 90% (Kishimoto et al., 1991) have also been reported with similar activation regimes.

Cytokine treatment of EC has consistently been found to increase PMN adhesion to HUVEC and a mouse brain endothelioma cell line. Treatment with TNF, IL-1 or LPS, seems to upregulate adhesion to a similar extent. TNF treatment of HBMEC upregulates PMN adhesion by 81-683%. Two to four hours of HUVEC activation increases PMN adhesion to a much greater extent than in HBMEC (Wankowicz et al., 1988; Luscinskas et al., 1989; Luscinskas et al., 1991). The large range of reported values is due to the variable duration of PMN incubation with the EC and perhaps dissimilar culture conditions. These differences may also account, to some extent, for the differences between HBMEC and HUVEC in the literature. One report indicated that 24h treatment of HUVEC increased PMN adhesion by 400%, only half of the increase following 4h of activation (Luscinskas et al., 1989). This increase is in line with the 443 to 683% found after 24h treatment of HBMEC with TNF. Treatment of a mouse brain endothelioma line with TNF raised PMN adhesion by 500 to 1000% (Hahne et al., 1993), which is considerably higher than the one observed in HBMEC.

None of the blocking Abs used affected PMN adhesion to unstimulated HBMEC. In the only other similar study published, Smith et al. (1988) were able to inhibit PMN adhesion to unstimulated HUVEC with an anti-ICAM-1 blocking Ab by 70%. Their anti-ICAM-1 Ab was different from the one used in this study and the percentage of PMN adherent to HUVEC was 500 times greater than in our study. A possible explanation for this difference could be that an epitope, not blocked by the mAb used in the present study, may be involved in this binding.

The anti-E-selectin mAb significantly blocked PMN adhesion to TNF treated HBMEC by 38 to 54%. Luscinskas et al. (1991), working with HUVEC and the same anti-E-selectin mAb as the one we used, found a similar rate of inhibition (50%). However, Graber et al. (1990), using the same mAb on activated HUVEC, found a 80 to

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Figure 66.



Table 1.Effects of TNF treatment of HBMEC on the permeability
of the monolayer determined by electrical resistance
measurements across the monolayers. *Significant
(p<0.05) decrease in resistance compared to EC not treated
with TNF.

HBMEC treatment	Resistance ($\Omega \cdot cm^2$)
No TNF	108 ± 14
100U TNF/ml, 4h	$98^* \pm 1$
100U TNF/ml, 18h	$68^* \pm 9$
100U TNF/ml, 24h	$63^* \pm 4$

Table 2.Effect of PMN migration with and without adhesion
molecule blocking antibodies on the permeability of
HBMEC cultures determined by electrical resistance
measurements across the monolayers.

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	% change in resistance
HBMEC treatment	after PMN migration
No TNF, no Ab, no fMLP	-7
No TNF, no Ab, 10 ⁻⁷ M fMLP	-56
4h TNF, no Ab	-26
4h TNF, α -E-selectin	-44
18h TNF, no Ab	-41
18h TNF, α-VCAM	-50
24h TNF, no Ab	-43
24h TNF, α -ICAM-1	-73
24h TNF, α -PECAM	-44

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Table 3.Effect of T lymphocyte migration with and without
adhesion molecule blocking antibodies on the permeability
of HBMEC cultures determined by electrical resistance
measurements across the monolayers.

	% change in resistance
HBMEC treatment	after T lymphocyte migration
4h TNF, no Ab	-25 ± 10
4h TNF, α -E-selectin	-69 ± 10
18h TNF, no Ab	-24 ± 3
18h TNF, α-VCAM	-25 ± 23
24h TNF, no Ab	-26 ± 10
24h TNF, α-ICAM-1	-32 ± 12
24h TNF, α -PECAM	-24 ± 7

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