



The Blood-Brain Barrier in Normal and Pathological Conditions

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ABSTRACT

The blood-brain barrier (BBB) consists of a specialised endothelium which effectively prevents free exchange between the blood and the brain but selectively allows the transport of nutrients and maintains homeostasis in the central nervous system. Despite decades of research, many aspects of the BBB are still obscure. The current study investigated the BBB in normal and pathological conditions induced by intravascular and extravascular insults. Intravascular insult was induced by the administration of *Clostridium perfringens* (*Cl p*) type D epsilon prototoxin. Extravascular insult was induced by an impact acceleration model for closed head injury to induce traumatic brain injury (TBI). The integrity of the BBB was examined ultrastructurally and by its ability to exclude endogenous and exogenous tracers. The expression of two BBB specific proteins, the endothelial barrier antigen (EBA) and the glucose transporter 1 (GLUT1), was studied.

The normal BBB was impermeable to macromolecules (serum albumin and horseradish peroxidase (HRP)) and small molecules (ionic lanthanum). Ultrastructurally the cerebral endothelium was non-fenestrated and had a low density of cytoplasmic vesicles. Tight junctions sealed the interendothelial cleft and effectively prevented the passage of tracers. Endothelial cells (ECs) at the BBB strongly expressed EBA and GLUT1.

Breakdown of the BBB was observed in both the *Cl p* prototoxin and the TBI models. Leakage of tracers indicated the development of vasogenic brain oedema. Undulation of EC luminal membrane and increased cytoplasmic vesicles appeared

to be a common response to vascular injury in the two models. The alteration of tight junctions occurred mainly in traumatic brain injury.

A reduction in EBA immunoreactivity in brain vessels correlated with the disruption of the BBB in both models and appeared to be a sensitive indicator of the compromised BBB. The function of EBA at the BBB was further studied by *in vivo* immunological targeting of this antigen. Intravenous injection of anti-EBA led to rapid opening of the BBB and the intravenously injected anti-EBA was bound to ECs, suggesting the formation of antigen-antibody complexes *in vivo*.

GLUT1 expression showed dissimilar results in the two models. *Cl p* prototoxin injection did not affect the expression of GLUT1, while TBI evidently reduced GLUT1 immunoreactivity. It appears that TBI has a profound effect on the cerebral glucose metabolism.

This study increased our understanding of the pathobiology of the BBB. Targeting BBB-specific proteins may provide a useful tool for future research into the function of the BBB.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give my consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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PUBLICATIONS AND PRESENTATIONS

Publications:

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MN Ghabriel, **C Zhu**, PL Reilly, PC Blumbergs, J Manavis, JW Finnie (2000) Toxin-induced vasogenic cerebral oedema in a rat model. *Acta Neurochirurgica Supplementum* 76: 231-236

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Presentations:

C Zhu, MN Ghabriel, and G Hermanis (2000) The role of the endothelial barrier antigen in the blood-brain barrier: a light and electron microscopic study. *Proceedings of the second Asia Pacific Symposium on Neural Regeneration*, pp20

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Endothelial cell proteins are altered in toxin-induced cerebral oedema.
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ABBREVIATIONS:

ABC	avidin-biotin complex
ADC	apparent diffusion coefficient
AIDS	acquired immunodeficiency syndrome
Anti-RECA-1	anti-rat endothelial cell antigen-1
BBB	blood-brain barrier
BSA	bovine serum albumin
CAM	chorioallantoic membrane
CBF	cerebral blood flow
<i>Cl p</i>	<i>Clostridium perfringens</i>
DAB	3,3'-diaminobenzidine tetrahydrochloride
DPBS	Dulbecco's phosphate-buffered saline
EAE	experimental allergic encephalomyelitis
EBA	endothelial barrier antigen
EBs	embryoid body structures
EC(s)	endothelial cell(s)
EM	electron microscope
Gd-DTPA	gadolinium-diethylenetriamine penta-acetic acid
GM-CSF	granulocyte-macrophage colony stimulating factor
GFAP	glial fibrillary acidic protein
GGTP	γ -glutamyl transpeptidase
GLUT1	glucose transporter protein 1
HIV	human immunodeficiency virus
HIVE	human immunodeficiency virus encephalitis
HRP	horseradish peroxidase
ICP	intracranial pressure
Ig	immunoglobulin
IL-	interleukin-
IMVS	Institute of Medical and Veterinary Science
LM	light microscope

mAb	monoclonal antibody
MAGUK	membrane-associated guanylate kinase
MDR	multidrug resistant
min	minutes
MRI	magnetic resonance imaging
MS	multiple sclerosis
NHS	normal horse serum
PBS	phosphate-buffered saline
SAL-3	Anti-Salmonella bacterial antigen
TBI	traumatic brain injury
TER	transendothelial electrical resistance
TNF- α	tumour necrosis factor-alpha
TNF- β	tumour necrosis factor-beta

CHAPTER 1
INTRODUCTION

1 Introduction

1.1 What is the blood-brain barrier?

The central nervous system (CNS), apart from a few selected sites, is isolated from the blood by a barrier system that provides a stable and chemically optimal environment for neuronal function. The specialised microvasculature of the brain and spinal cord regulates the free exchange of solutes between blood and neural tissue, forming the blood-brain barrier (BBB) (Holash & Stewart, 1993a). The BBB significantly restricts entry from blood to brain of virtually all molecules, except those that are small and lipophilic (Rubin & Staddon, 1999). This physiological barrier protects the brain from high potassium levels and from neurotoxic substances present in the blood such as glutamate and glycine. In the meantime, the BBB possesses specific transport systems to facilitate the transportation of essential nutrients such as glucose and certain amino acids to the CNS.

1.2 Historical review of the blood-brain barrier

The existence of a barrier between the blood and the CNS was postulated by the German pharmacologist Paul Ehrlich more than 100 years ago through his classic dye injection experiments. He found that the intravenous injection of dyes resulted in colouration of most tissues except the brain. The term "blood-brain barrier" was introduced by Lewandowski in 1900 to describe this phenomenon (see review by Dermietzel & Krause, 1991). The concept of the BBB was further supported by the experiments of Goldmann who showed that intravenous injection of trypan blue stained all tissues except the brain and spinal cord but when the dye

was injected intrathecally, trypan blue stained the CNS (see review by Dermietzel & Krause, 1991). Although the idea of a barrier between the blood and the brain was accepted, the exact anatomical site of the BBB remained controversial. The German pathologist Spatz was the first to suggest the capillary endothelium as the barrier structure, in 1933 (see review by Crone, 1986). However, this view was largely ignored by the scientific community at that time. It was not until 1946 that Krogh further suggested that active transport processes occurred at the cerebral capillaries (see review by Crone, 1986). The idea that the BBB resides in the endothelium was doubted by some early electron microscopists because they could not detect any differences between the capillaries of the brain and capillaries of other tissues (Maynard *et al.*, 1957). Very little interstitial space in the brain was found by electron microscopy (Schultz *et al.*, 1957). It was suggested that the plasma membranes of neurons and glial cells were directly exposed to solutes in the blood plasma. The lack of staining of the CNS in the dye injection experiments was explained by poor penetration across the plasma membranes of neurons and glial cells (Schultz *et al.*, 1957). This view was strongly opposed by further research and it was demonstrated that extracellular tracers were distributed in the brain when injected into the brain and the rate of distribution depended on the diffusion coefficients of the tracers (Davson & Spaziani, 1959; Davson *et al.*, 1961; Rall *et al.*, 1962). The failure to detect the extracellular space was later proved to be due to inappropriate fixation techniques (Van Harreveld *et al.*, 1966; Van Harreveld & Malhotra, 1967). The argument of the exact BBB site was finally settled by Reese and Karnovsky (1967) and Brightman and Reese (1969). These authors demonstrated at the electron

microscopic (EM) level that intravenously injected horseradish peroxidase (HRP) was stopped by tight junctions between endothelial cells (ECs) of the brain. When injected intraventricularly, HRP was able to diffuse among glial cells and neurons but was stopped from entering the circulation by endothelial tight junctions. Thus, it was concluded that the endothelium of the CNS is the anatomical site of the BBB.

1.3 Structure of the blood-brain barrier

1.3.1 Tight junctions

Tight junctions that join adjacent ECs of blood vessels in the CNS form the fundamental basis of the BBB. The tight junction is formed of a belt of anastomosing strands of proteins and lipids (Kachar & Reese, 1982; Pinto da Silva & Kachar, 1982; Griep *et al.*, 1983; Cereijido *et al.*, 1998). It surrounds the lateral membrane of ECs and seals the outermost end of the intercellular space. Using freeze-fracture electron microscopy, tight junctions at the BBB appear as continuous, anastomosing, belt-like configurations, which are very similar to those located between epithelial cells (Nagy *et al.*, 1984). Although tight junctions at the BBB are epithelial-like, they are different from epithelial tight junctions in some aspects. In epithelial cells, the tight junction is at the most apical zone of contact. Adherens junctions are adjacent to the tight junction and are clearly separate from it. In brain ECs, adherens and tight junction components are intercalated. Cadherins are detected along the entire intercellular cleft in brain ECs (Schulze & Firth, 1993).

The hydrophobic cell membrane organisation of the tight junction, resulting from the fusion of cell membranes, restricts the lateral movement of hydrophilic molecules such as proteins between luminal and abluminal membranes of ECs across the junction. The tight junction imposes a passive, non-selective diffusion restraint on the BBB (Brightman & Tao-Cheng, 1993). Electron microscopy reveals that in normal conditions, the passage of proteins such as HRP (MW 40,000) (Reese & Karnovsky, 1967) and microperoxidase (MW 2000) (Reese *et al.*, 1971; Bundgaard & Cserr, 1981) from the blood to the brain or vice versa is prevented by tight junctions.

Brain EC tight junctions have very high electrical resistance, average $1870\Omega\text{cm}^2$, which is similar to a 'tight' epithelium (Crone & Olesen, 1982). The high transendothelial electrical resistance (TER) reflects low ion transport across the BBB. The BBB permeability to the major monovalent ions, Na^+ , Cl^- and K^+ and the divalent calcium is about 1.4, 0.9, 13.5×10^{-7} and $5 \times 10^{-8}\text{cm s}^{-1}$ respectively, which is up to 500-fold less than those at the choroid plexus epithelium (Smith & Rapoport, 1986; Tai *et al.*, 1986). Ionic lanthanum, a commonly used small molecular weight tracer with molecular radius 0.46 nm, does not cross cerebral capillary tight junctions (Bouldin & Krigman, 1975).

In the BBB, the tight junction imposes a polarity on the EC by asymmetrically segregating plasma membrane associated enzymes and transporters to either luminal or abluminal sectors of the ECs membrane (Betz *et al.*, 1980). Polarity provides the necessary vectoriality for substances to be transported across

epithelia towards or away from the lumen. Therefore, the polarity of the endothelium of the BBB implies that the cerebral endothelium has the potential for active transcellular transport. Ultrastructural cytochemical studies revealed that alkaline phosphatase is located essentially in the luminal membrane of ECs of capillaries, suggesting that the luminal membrane is the site of extensive processes of dephosphorylation, transphosphorylation, and probably transport of phosphate ions (Kreutzberg & Toth, 1983; Vorbrodt *et al.*, 1983). However, in precapillary arterioles, alkaline phosphatase activity appears in both the luminal and abluminal membranes (Vorbrodt *et al.*, 1986). It is believed that this phenomenon reflects the specific function of these segments of brain microvasculature. $\text{Na}^+\text{-K}^+\text{-ATPase}$ is evidently more concentrated in the abluminal membrane of brain ECs, indicating that transportation of Na^+ from the ECs to the brain interstitial fluid and removal of K^+ from brain interstitial fluid is more intense at the abluminal front of the blood vessels. This finding is consistent with the role of the BBB in protecting the neuropil against considerable variation of potassium level in the blood (Betz *et al.*, 1980; Sanchez del Pino *et al.*, 1995a).

The molecular structure of the tight junction is not fully understood. In recent years, several protein components of the tight junction have been identified. ZO-1 is the first tight junction associated-protein to be identified. This 220 kDa protein is present in both epithelial cells and ECs (See 1.5.4.2). ZO-2 is a ZO-1 associated-protein. It is also present at tight junctions of brain ECs (See 1.5.3.2). Occludin is the only transmembrane protein identified in the tight junction (See 1.5.3.1). Two additional proteins have been identified in the tight junction,

cingulin (140 kDa) and the 7H6 antigen (155 kDa) (Citi *et al.*, 1988; Zhong *et al.*, 1993). These two proteins are yet to be cloned and sequenced. Although it is almost certain that not all the proteins in the tight junction have been identified, a picture of how these proteins might be organised in the tight junction has been proposed. However, the exact role of these proteins in tight junction permeability regulation is not clear. The modification of adherens junctions can also alter the permeability of tight junctions. Adherens junctions are formed by a class of membrane proteins termed cadherins and their cytoplasmic plaque proteins α -, β - and γ -catenin. The cadherins/catenins system at the adherens junction is sensitive to calcium levels. Removal of Ca^{2+} leads to the disruption of adherens junctions and the opening of tight junctions (Pitelka *et al.*, 1983; Gumbiner & Simons, 1986). The level of cAMP in cultured brain ECs can also regulate the tight junction formation. Elevation of cAMP levels causes a striking elevation of tight junction resistance and reorganisation of the actin cytoskeleton (Rubin *et al.*, 1991).

1.3.2 Non-fenestrated ECs with a low density of vesicles

The non-fenestrated ECs of the BBB contain few pinocytotic vesicles compared with those in most other tissues, indicating a low rate of transendothelial transport. This is generally considered to be a morphological constituent of the physiological BBB. The low density of vesicles in cerebral ECs was first noted in the classic HRP study (Reese & Karnovsky, 1967). Quantitative studies of vesicles containing HRP confirmed their low density. The density of brain EC vesicles recorded by a number of studies varied from 3 to 14 per μm^2 , which is

much lower than that of other tissues. The number of pinocytotic vesicles in ECs of barrier vessels in the brain is three times lower than that in vessels of the area postrema and seven times lower than that of smooth muscle vessels (Coomber & Stewart, 1985). Using ultrathin serial sectioning and three-dimensional vesicle reconstruction, it was revealed that the densities of vesicular clusters and surface-connected vesicles were much higher in the area postrema, choroid plexus and skeletal muscle vessels than in BBB vessels (Coomber & Stewart, 1986). A more recent quantitative study comparing EC vesicular densities among nine tissues showed that vesicular density of brain capillaries was significantly lower than that of all tissues except the testis and ovary (Stewart, 2000). As both testis and ovary capillaries are highly permeable to protein, this study demonstrated a weak correlation between low vesicle density and protein permeability. Therefore, further studies are needed to establish the relationship between vesicle density and permeability of ECs.

1.4 Other components of the BBB

1.4.1 Pericytes

Pericytes are abundant in the microvasculature of the CNS and are an important cellular constituent of the BBB. They are located at the abluminal side of the endothelium, embedded in the basal lamina of ECs. A layer of the basal lamina separates the pericyte from the astrocyte endfeet. Pericytes cover 22-30% of the cerebral microvascular circumference (Frank *et al.*, 1987). The pericyte has a spherical cell body with a prominent nucleus (Lafarga & Palacios, 1975). Pericytes may have abundant electron dense lysosomal particles in their

cytoplasm (Farrell, C. R. *et al.*, 1987). They display considerable heterogeneity between the CNS and other tissues. Cultured bovine retinal and brain pericytes are different in their growth patterns, glucose dependency and morphology (Wong *et al.*, 1992). The *in situ* ratio of pericytes to ECs and the degree of the endothelial area covered by pericytes appears to be related to the tightness of the blood vessels and the level of microvascular blood pressure (Frank *et al.*, 1987; Shepro & Morel, 1993).

The function of pericytes at the BBB is not fully understood. Pericytes are thought to be contractile cells. Contraction of bovine retinal pericytes in culture was observed (Kelley *et al.*, 1987). *In vitro* studies showed that retinal pericytes in culture produce abundant α -smooth muscle specific-actin (DeNofrio *et al.*, 1989). However, in brain pericytes culture, only a small portion of pericytes (0-10%) was found to express α -smooth muscle actin (Verbeek *et al.*, 1994). *In vivo* studies failed to detect the expression of α -actin mRNA or α -actin isoform in capillary pericytes in the retina and brain (Nehls & Drenckhahn, 1991; Boado & Pardridge, 1994). Smooth muscle specific microfilaments are only detected in a minority of pericytes in the brain, although they are distributed widely in intestinal pericytes. More work especially *in vivo* is needed to confirm that pericytes in the brain also have contractile capacity. Pericytes also express receptors for endothelin (Dehouck *et al.*, 1997) and a variety of vasoactive amines, such as vasoactive intestinal peptide (Ferrari Dileo *et al.*, 1996), vasopressin (van Zwieten *et al.*, 1988) and angiotensin II (Ferrari Dileo *et al.*,

1996), indicating that pericytes may be involved in regulating the blood flow in the microcirculation of the CNS.

CNS pericytes have also been regarded as phagocytic cells and form the second line of defence of the BBB. It has been shown that cerebral capillary pericytes actively took up HRP after cold injury to the brain (Cancilla *et al.*, 1972). An electron microscopic study on human brain oedema associated with brain tumours, brain trauma and congenital malformation showed that brain pericytes exhibited phagocytic activity as well as remarkable oedematous changes (Castejon, 1984). Phagocytic pericytes contained ingested erythrocytes, suggesting that pericytes might play some role in oedema resolution (Castejon, 1984). Pericytes express enzymes such as γ -glutamyl transpeptidase (GGTP) (Frey *et al.*, 1991; Risau *et al.*, 1992), glutamyl aminopeptidase (Healy & Wilk, 1993), aminopeptidase N (Krause *et al.*, 1993), and butyrylcholinesterase (Gerhart & Drewes, 1987), indicating that pericytes are actively involved in metabolism at the BBB.

CNS pericytes are an important cytokine-producing cellular constituent of the CNS. Smooth muscle/pericytes have been reported to produce IL-1 β , IL-6, and GM-CSF (Fabry *et al.*, 1993). Pericytes may inhibit endothelial cell proliferation by producing TNF- β together with ECs (Antonelli-Orlidge *et al.*, 1989). Pericytes are also regarded as a potential site of antigen presentation in the brain. A recent study showed that pericytes expressed MHC II antigen when responding to inflammatory cytokines (Balabanov *et al.*, 1999). The expression of MHC II

antigen by brain pericytes in response to inflammation suggests that pericytes are involved in T-lymphocyte activation in inflammation. Brain pericytes express cell surface adhesion molecules, which may have costimulatory activity in MHC II dependent antigen presentation (Balabanov *et al.*, 1999). Brain pericytes may have important neuroimmune potential at the BBB (Balabanov *et al.*, 1999).

1.4.2 Astrocytes

Astrocytes cover 80-95% of the brain capillary circumference with their endfeet (Johanson, 1980). Astrocytic endfeet are only separated from the plasma membrane of ECs by the basal lamina. Developmentally, the arrival of astrocytic endfeet around brain capillaries occurs at about the same time the permeability barrier develops. Therefore, it was postulated that astrocytes play an important role in the induction and maintenance of the BBB. However, despite extensive *in vivo* and *in vitro* studies, investigators cannot reach agreement on whether astrocytes could induce and maintain barrier function and if they did, how they influence brain capillary ECs. The importance of the neural environment for induction of BBB function in cerebral capillary ECs was highlighted by Stewart and Wiley (1987). By transplanting quail embryonic brain fragments to the chick embryonic coelomic cavity and quail embryonic mesoderm fragments to the chick embryonic brain, they found that host blood vessels derived from the coelomic cavity vascularised the transplanted brain tissues and these blood vessels gained barrier function. Conversely, blood vessels derived from the brain, vascularised the transplanted mesodermal fragments and these blood vessels lost BBB features (Stewart & Wiley, 1981). Further direct evidence of astrocytes being able to

induce barrier features in non-neural ECs was documented by Janzer and Raff (1987). Purified type I astrocytes injected into the rat eye anterior chamber or the chick chorioallantoic membrane (CAM) formed aggregates on the surface of the iris, or grew on chick CAM. The astrocyte aggregates were able to exclude Evans blue, indicating that capillaries and venules in the aggregates were tight. They concluded that astrocytes were responsible for inducing non-neural ECs to form the BBB (Janzer & Raff, 1987). However, the interpretation of the results of this study was challenged by Holash *et al.* (1993). They argued that astrocytes implanted into the anterior chamber form poorly vascularised grafts and grafting of cells to the CAM induces an extensive inflammatory response which could affect the permeability of blood vessels. They repeated the CAM grafting experiments performed by Janzer and Raff but found astrocytes did not induce barrier features in CAM vessels (Holash & Stewart, 1993b). Another *in vivo* study reported that in transplanted superior cervical ganglia, the host astrocytes migration occurred much later than the host endothelial proliferation and re-perfusion, which indicated that astrocytes were not responsible for inducing barrier functions in ECs (Rosenstein *et al.*, 1989). Specific injury to astrocytes by a gliotoxin in rats in the first postnatal week did not compromise the permeability of the BBB to HRP, indicating that astrocytes could not be responsible for maintaining BBB function in rats after birth (Krum & Rosenstein, 1993). However, using the same toxin to induce astrocytes injury in adult rats, the authors found increased permeability of the BBB to protein in some areas in the grey matter (Krum, 1994).

In contrast to *in vivo* studies, *in vitro* studies on the role of astrocytes in cultured cerebral capillary ECs are more consistent, although caution should be taken when applying the results of *in vitro* studies to the *in vivo* situation. Brain ECs in culture tend to lose barrier features. After several passages, brain capillary ECs lose tight junctions, and subsequently possess very low transendothelial electrical resistance. They also lose the expression of BBB specific markers such as GGTP and the glucose transporter 1 (GLUT1). It has been shown repeatedly that astrocytes could induce and/or enhance barrier properties of cultured cerebral ECs. When ECs are co-cultured with astrocytes, tight junctions are enhanced in length, width and complexity. In the meantime gap junctions are markedly diminished (Tao Cheng *et al.*, 1987). The enhancement of tight junctions could also be induced by adding astrocytes-conditioned medium to EC culture, indicating that astrocytes secrete factor(s) that influence the differentiation of brain ECs in culture (Arthur *et al.*, 1987). Treating brain ECs with astrocytes-conditioned medium, increased the TER by twofold, although the TER did not reach the level observed *in vivo* (Rubin *et al.*, 1991). Interestingly, increasing cAMP levels in endothelial cell culture already treated with astrocytes-conditioned medium, resulted in a significant change in cell morphology and a rapid increase in TER (Rubin *et al.*, 1991). C6 glioma cells, a commonly used substitute for astrocytes, could also increase the TER of brain ECs in culture by 200-440% (Raub *et al.*, 1992).

In addition to inducing the formation of tight junctions between ECs in culture, astrocytes and glioma cells have been shown to be able to induce the expression

of GGTP (Maxwell *et al.*, 1987; Raub *et al.*, 1992). GGTP is regarded as a BBB marker as it is abundant in ECs of the brain. It is not expressed by other ECs at a detectable amount. Astrocytes could increase the expression of GGTP by 34% when cultured with ECs (Maxwell *et al.*, 1987). Astrocytes-conditioned medium was shown to be able to induce the expression of HT7, a BBB marker for chick (see 1.5.1.2), in ECs of chorioallantoic vessels (Janzer *et al.*, 1993). However, some authors argued that most *in vitro* studies used ECs derived from adult bovine brain capillaries, which already possess barrier functions. Therefore the evidence of enhanced barrier properties induced by co-culturing with astrocytes could only demonstrate the maintenance role of astrocytes at the BBB (Holash *et al.*, 1993). A recent study showed that astrocytes are able to induce BBB properties in non-neural ECs (Kuchler-Bopp *et al.*, 1999). When co-cultured with astrocytes or treated with astrocyte-conditioned culture medium, human umbilical cord ECs expressed some BBB features such as the formation of tight junctions, increased TER, high activity of GGTP and expression of GLUT1 (Kuchler-Bopp *et al.*, 1999). It is not clear how astrocytes influence brain capillary ECs. Direct cell-cell contact seems unlikely since astrocytes are separated from ECs by a basal lamina. In the immature brain, blood vessels have only limited contacts with astrocytic endfeet (Saunders *et al.*, 2000). As mentioned above, astrocytes-conditioned medium is able to enhance BBB characteristics in cultured cerebral capillary ECs, which suggests that astrocytes may produce factors to influence ECs. However, so far nothing is known about the factor(s) secreted by astrocytes which may be responsible for the induction and maintenance of the BBB.

1.5 Molecular anatomy of the BBB

A number of proteins have been detected at the BBB by immunocytochemistry and histochemistry techniques. These proteins are considered as BBB markers. Some of these proteins such as EBA and OX-47 are of unknown function, some are enzymes such as GGTP, and some are receptors such as insulin and transferrin receptors. Other proteins act as transporters such as GLUT1 and P-glycoprotein or proteins related to the tight junctions such as occludin and ZO-1. Some of these proteins have been widely used in BBB studies and will be reviewed below.

1.5.1 Proteins of unknown function

1.5.1.1 Endothelial Barrier Antigen

The endothelial barrier antigen (EBA) is a protein selectively expressed by ECs of microvessels at the BBB. It is a protein triplet of 30, 25 and 23.5 kDa and can be detected by a monoclonal antibody raised in mice against rat brain homogenate (Sternberger & Sternberger, 1987). EBA is strongly expressed at the BBB, blood-retinal barrier, and blood-optic nerve barrier (Sternberger & Sternberger, 1987; Lawrenson *et al.*, 1995). In contrast, EBA is generally absent or only weakly expressed in ECs of fenestrated brain vessels such as those in the choroid plexus, area postrema, neurohypophysis and hypothalamus. It is absent in non-barrier vessels in other body organs (Sternberger & Sternberger, 1987). Thus, it appears that EBA is a 'barrier protein' of neural vessels. However, some reported aspects of the expression of EBA do not seem to be in full agreement with the above evidence. Firstly, in the eye, while the optic nerve, retina and iris have tight vessels, which according to the above interpretation, are expected to express EBA,

other areas in the eye with permeable vessels show staining for EBA. Examples are vessels of the choriocapillaris and some vessels in the ciliary body (Lawrenson *et al.*, 1995). Secondly, anti-EBA stains some ECs in the spleen, and is expressed by some cells in the skin, tentatively identified as Langerhans cells (Sternberger & Sternberger, 1987). However, from the predominant distribution of EBA in barrier vessels, the earlier interpretation of Sternberger and Sternberger of EBA as a barrier protein is corroborated.

The exact function of EBA is unknown. As EBA is expressed in the spleen and Langerhans cells, it is speculated that EBA may be involved in immune-related functions at the BBB. However, this suggestion has not been proven. The development of EBA immunoreactivity is a postnatal event. Some blood vessels are positive for EBA staining from postnatal day 3 (Sternberger & Sternberger, 1987) and by 20 days postnatal almost all vessels are positive for EBA (Sternberger & Sternberger, 1987; Cassella *et al.*, 1996). Therefore EBA represents a late phase marker with respect to BBB maturation and its expression occurs latest among the many BBB markers in the developmental sequence of BBB differentiation. In normal conditions, EBA is expressed by microvessels in the grey and white matter as well as in pial vessels on the brain surface. However, in the spinal cord, immunoreactivity for EBA is absent in the large posterior and anterior spinal arteries (Perdiki *et al.*, 1998). Also arterioles arising from the internal carotid and middle cerebral arteries are devoid of EBA immunoreactivity (Nishigaya *et al.*, 2000). The lack of EBA expression in ECs of pial and parenchymal arterioles may be related to their incomplete barrier characteristics.

Endothelial cells of arterioles contain more vesicles in the cytoplasm compared with capillaries and venules (Allt & Lawrenson, 1997). Developmentally, EBA is unlikely to be directly related to the presence of tight junctions between cerebral ECs, as tight junctions have been observed at 13 days gestation in the rat brain (Kniesel *et al.*, 1996). Moreover, EBA immunoreactivity is not associated with the junctional complex at the electron microscopic level (Rosenstein *et al.*, 1992). The evidence for the relationship between astrocytes and the presence of EBA is conflicting.

Astrocytes may play an important role in the induction of the BBB during development. The time course of the appearance of EBA immunoreactivity in brain ECs correlates well with the association of astrocytic end-feet with cerebral capillaries. In the foetal brain, few capillaries are covered by astrocyte end-feet but by postnatal day 10 over 90% of the capillary surface is covered by astrocytes. However, both pial and iris vessels which have physiological properties nearly identical to CNS vessels, also have strong EBA expression although they are not normally contacted by astrocytic end-feet. In experimental conditions, direct injury to neurons and astrocytes resulted in a rapid reduction of EBA expression during the first 3 weeks. However, the immunoreactivity of EBA began to recover by 4 weeks post-injury in areas that had minimal numbers of neurons and astrocytes (Krum *et al.*, 1997). Specific injury to astrocytes by injecting the anti-metabolite 6-aminonicotinamide induces severe degeneration of astrocytes but leaves microvessels intact. Despite the damage to astrocytes, ECs continued to express EBA (Krum, 1994). Similarly, blood vessels newly formed in the brain

after ischaemic injury showed EBA expression at day 28 but no glial fibrillary acidic protein (GFAP) staining, indicating that the expression of EBA in these blood vessels is not correlated with the formation of astrocytic/endothelial contacts (Nishigaya *et al.*, 2000). The influence of astrocytes may be important for the induction of EBA expression during development but not for its maintenance at the BBB.

Although the significance of EBA in the normal function of the BBB is unknown, EBA immunoreactivity appears to correlate with an intact BBB. In experimental allergic encephalomyelitis (EAE), the disruption of the BBB has been documented by numerous studies (Juhler *et al.*, 1984; Juhler *et al.*, 1986; Kato & Nakamura, 1989; Claudio *et al.*, 1990; Hawkins *et al.*, 1991). EBA expression was abolished in blood vessels surrounded by inflammatory cells during experimental allergic encephalomyelitis. EBA expression recovered along with the reconstitution of the BBB but not in residual inflammatory areas (Sternberger *et al.*, 1989). In brain angiogenesis induced by stab wound, regenerating vessels did not stain or only weakly stained for EBA even after 10 days of postoperative healing (Rosenstein *et al.*, 1992). In moderate or severe compression injury to the spinal cord, a remarkable reduction of EBA expression in the ECs of spinal cord microvessels at 4 days after the injury was reported (Perdiki *et al.*, 1998). A reconstitution of EBA immunoreactivity was observed 9 days after injury. In rat cerebral ischaemia, both qualitative and quantitative studies demonstrated a decrease of EBA expression in brain endothelium (Lin & Ginsberg, 2000; Nishigaya *et al.*, 2000). EBA immunoreactivity started to recover at day 14 after the injury

(Nishigaya *et al.*, 2000). The decrease and/or disappearance of EBA may be due to its degradation *in vivo*, alteration of EBA structure or reduced production of the protein induced by the injury.

1.5.1.2 OX-47

OX-47, or CE 9, is an integral membrane glycoprotein of unknown function expressed at the rat BBB. It is a member of the immunoglobulin superfamily (Fossum *et al.*, 1991) and homologous to the HT7 or neurothelin of the chick, basigin or gp 42 of the mouse, and M6 or EMMPRIN of the human (Kasinrerk *et al.*, 1992; Seulberger *et al.*, 1992). The chick protein HT7, which is much more extensively studied than the rat OX-47, is a specific marker for chick brain ECs. The amino acid sequence identity between OX-47 and HT7 is 94% (Seulberger *et al.*, 1992). In the posthatch chick, HT7 expression is found on ECs within the brain but not on those of the systemic vascular system (Schlosshauer & Herzog, 1990) and it is also absent in the ECs of the choroid plexus, subfornical organ, and pineal and pituitary glands, all of which lack BBB (Albrecht *et al.*, 1990). The epithelium of the pigment layer which forms a barrier between the outer layer of the eyeball and the retina and the epithelium of the choroid plexus which forms the blood-cerebrospinal fluid barrier also express neurothelin, indicating a role in the transport process (Unger *et al.*, 1993). *In vitro* studies of choroid epithelial cells showed that the expression of neurothelin was at the cell-cell site, indicating that the distribution of neurothelin was regulated by cell-cell interaction. An ontogenic study showed that HT7 is a very early BBB marker even when compared with the early embryonic brain endothelial cell markers, alkaline

phosphatase and transferrin receptor, which appear slightly later. HT7 is first detectable at embryonic day (E) 9-10 in chick development, and becomes more extensive from E11 onwards (Risau *et al.*, 1986b). The appearance of neurothelin coincides with the maturation of CNS ECs. Neurothelin is an inducible protein in ECs. The induction may be mediated by cell-cell interactions or by factors produced by astrocytes. Although the localisation of neurothelin strongly indicated its involvement in the barrier function, its expression by transplanted brain tumour cells has been reported (Papoutsi *et al.*, 2000). Four tumour cell lines were inoculated on the chorioallantoic membrane (CAM) of chick embryos. Neurothelin was expressed by ECs in all four tumours. As it is well known that BBB function is compromised in brain tumour blood vessels, this finding suggests that the expression of neurothelin does not correlate with a functional BBB. A study using knockout mice lacking the basigin gene (*Bsg*) showed that the BBB of the knockout mice [*Bsg* (-/-) mice] was impermeable to Evans blue, indicating a normal barrier function of the mutant mice (Igakura *et al.*, 1996).

OX-47 antigen, like HT7, is located on ECs of the BBB but it has a more extensive distribution. It is also found on most epithelial cells including the kidney, small intestine and liver. It is present at low levels on most thymocytes and bone marrow cells but increases markedly upon activation of both B and T cells. The cell types expressing the OX-47 antigen can be divided into four main groups: (a) immature, activated and dividing cells, e.g. bone marrow cells, cortical thymocytes, lymphoblasts, spermatogonia B, basal layers of epidermis and hair follicles, (b) epithelial cells involved in transepithelial transport, e.g. nephronic

tubular cells, salivary duct cells, hepatocytes, intestinal absorptive cells, brain endothelium, choroid plexus, perineural cells, (c) cells with excitable membranes, e.g. nerve and muscle cells and (d) other cells including endocrine cells such as Leydig cells and ovarian follicular cells (Fossum *et al.*, 1991). It is likely that the OX-47 antigen is part of a complex of surface proteins and it is possible that the other components vary between the different cell types. Although the function of OX-47 remains unclear, Nehme *et al.* (1995) reported remarkably increased levels of CE 9 mRNA and protein in brown adipose tissue, liver and the lymphoid organ after metabolic activation. They suggested that the level, and possibly also the localisation, of CE 9 may be correlated in a positive fashion with metabolic activity in a diverse array of cell types. The localisation of OX-47 on ECs and activated lymphocytes also indicated a function of cell transporter and cell-cell signalling.

1.5.2 Proteins of transport function

1.5.2.1 Glucose Transporter Protein 1

Under normal conditions, glucose is the primary metabolic fuel for the brain. Since the brain cannot store or synthesise glucose by itself, a constant supply is essential to maintain the normal cerebral development and function. Because glucose is a polar, water-soluble compound that cannot easily traverse the lipid bilayer of cell plasma membranes, glucose transporter molecules must exist in the plasma membranes of all cells that use glucose. To date, six genes encoding homologous but distinct proteins, and one pseudogene that does not encode protein, have been identified and designated GLUT1-7, based on the order in

which they were cloned (Bell *et al.*, 1993; Gould & Holman, 1993). Three proteins, GLUT 1, 3, 5, have now been identified as cell-specific transporters in the mammalian brain, and GLUT1 is found to be most abundant in the endothelium of brain vessels.

Crone (1965) first predicted the existence of a carrier-mediated facilitated transport system in brain capillary endothelium that facilitates the transport of glucose from blood to brain. It is now established that transport of glucose by the ECs of brain capillaries is saturable, stereospecific, non-concentrative, non-energy dependent, and not influenced by insulin or sodium. Because of the BBB, the brain endothelium, which accounts for less than 0.1% of the brain weight, must transport all the glucose that enters the brain. Thus, it was estimated that the brain endothelium transports about 10 times its weight of glucose per minute (Dick *et al.*, 1984; LaManna & Harik, 1985). From this, it would be reasonable to expect that brain microvessels are endowed with a high density of the glucose transporter protein (Harik *et al.*, 1993).

GLUT1 is the predominant, if not exclusive, facilitative glucose transporter in brain microvascular ECs. It is a 492 amino acid protein and is detected in whole brain as two molecular mass forms of 55 kDa and 45 kDa, which are encoded by a single gene but differ in their extent of glycosylation. In humans the gene is assigned to a chromosomal location at chromosome 1p33. The GLUT1 mRNA sequence is highly conserved in the different species studied, and shows a range of 93% to 78% similarity to the human sequence (Drewes, 1998). The density of

GLUT1 in the brain is congruent with the local cerebral glucose utilisation and capillary density (Zeller *et al.*, 1997). GLUT1 is detected in cerebral microvessel ECs, choroidal epithelium and ependymal cells but is absent from the endothelia of the circumventricular organs and choroid plexus which do not exert barrier properties (Dick *et al.*, 1984; Kalaria *et al.*, 1988; Gerhart *et al.*, 1989; Harik *et al.*, 1990; Pardridge *et al.*, 1990; Farrell, C. L. & Pardridge, 1991; Rahner Welsch *et al.*, 1995; Dobrogowska & Vorbrodt, 1999). GLUT1 is also expressed by some astrocytes and oligodendrocytes but not by microglial cells (Devaskar *et al.*, 1991; Maher *et al.*, 1994; McCall *et al.*, 1996). A recent study showed that it is the 45 kDa form of GLUT1 that is expressed in these parenchymal cells (Yu & Ding, 1998). The 45 kDa GLUT1 is distributed in the cytoplasm of astrocytes and oligodendrocytes. No GLUT1 was detected in the plasma membrane of these cells. Although the reason for, and consequence of the different glycosylation are not clearly understood, it was reported that when expressed on BBB and on choroid plexus, GLUT1 is subject to different N-linked glycosylation. The 55 kDa form of GLUT1 is expressed at the BBB while the lower molecular weight form of GLUT1 is detected in the choroid plexus epithelium (Kumagai *et al.*, 1994). The heterogeneity of GLUT1 glycosylation in different barriers in the brain, such as the BBB and the blood-CSF barrier (choroidal epithelium), is believed to have a functional role that may participate in regulating glucose transporter kinetic activity or differential subcellular localisation of the glucose transporter.

The immunoreactivity of GLUT1 protein is not evenly distributed on the cell surfaces of the tissue in which it is expressed. On the epithelium of the choroid plexus, it is restricted to the basolateral surfaces whereas on ependymal epithelium lining a region of the third ventricle, it is distributed on the apical surface. There are varied reports about the subcellular distribution in brain capillaries. Farrell *et al.* (1991) reported an asymmetrical distribution of GLUT1 on brain capillary endothelial luminal and abluminal membrane, with distribution of immunoreactivity on luminal membrane, abluminal membrane and cytoplasm averaging 12%, 48%, and 40%, respectively. The observations that about 40% of the endothelial glucose transporter is contained within the cytoplasmic compartment, and the distribution of transporter being asymmetric on luminal and abluminal membranes, are consistent with transporter recruitment; a mechanism postulated for acute modulation of glucose transport. The relocation of transporter molecules from a cytoplasmic compartment to the luminal plasma membrane (the transporter recruitment hypothesis) could produce a rapid up-regulation of BBB glucose transport. Similarly, It was reported that in rat brain capillaries, 50% of the transporter was abluminal, 36% luminal, and 14% cytoplasmic (Dermietzel & Krause, 1991). In rabbit brain capillary endothelia, a three-fourfold greater abundance of transporter molecules on the abluminal than on the luminal membranes of brain ECs was observed (Cornford *et al.*, 1993). A more recent quantitative immunogold study of GLUT1 distribution in mouse brain showed an approximately threefold higher immunolabelling density of the abluminal than the luminal plasma membrane of ECs (Dobrogowska & Vorbrodt, 1999). Mathematical modelling based on studies of BBB glucose transfer has

also suggested the possibility that glucose transporter activity *in vivo* may be asymmetric. In human brain, asymmetric distribution of GLUT1 has been documented, with the ratio of luminal to abluminal being 1:3 (Cornford *et al.*, 1994b). However, in the canine brain, a symmetric luminal to abluminal distribution of GLUT1 epitopes was reported (Gerhart *et al.*, 1989). A lower proportion of cytoplasmic epitopes (9%) was seen in the dog brain endothelia. In isolated bovine microvessel plasma membranes, the distribution of GLUT1 between the luminal and abluminal membranes is also symmetrical (Lee, W. J. *et al.*, 1997). The reason for these differences awaits further clarification.

Both *in vivo* and *in vitro* studies showed up-regulation of GLUT1 immunoreactivity during brain development in the rat and rabbit (Devaskar *et al.*, 1991; Cornford *et al.*, 1994a). Cornford and colleagues demonstrated that the up-regulation of the rabbit BBB glucose transporter was not accompanied by changes in transporter affinity. A 65% increase in glucose transporter activity was observed related to age increase. A postnatal study on the distribution of GLUT1 in rats showed a complete congruence between the regional relative capillary density and the density of GLUT1 staining in all BBB structures during postnatal development (Zeller *et al.*, 1996). Relative capillary density mainly increases between postnatal days (P) 10 and P20 in the grey matter. In contrast, investigation of the circumventricular organs and subfornical organ showed some GLUT1 staining at birth but the immunoreactivity decreased at P10 and P15, and had disappeared in the adult animals (Zeller *et al.*, 1996). An ontogenetic study of GLUT1 in the rat nervous system showed that by birth, GLUT1 immunoreactivity

in the CNS was restricted to the endothelium, the epithelium (but not the endothelium) of the choroid plexus, and tanycytes. This cellular distribution of GLUT1 did not change much between birth and senescence despite considerable postnatal brain development and the increase in brain capillary density. It was suggested that while a CNS factor(s) may not have a role in the induction of the high expression of GLUT1 in CNS endothelium, such a factor(s) is (are) probably important in maintaining the high level of GLUT1 in these endothelia (Harik *et al.*, 1993). Up-regulation of GLUT1 expression has also been observed in human brain development (Bertossi *et al.*, 1999). Detected by immuno-gold cytochemistry, the GLUT1 antigenic sites on the ECs in the human foetus telencephalon increased greatly from the 12th to the 18th week of gestation. The distribution of GLUT1 is higher on the abluminal membrane of ECs at both the 12th and the 18th gestational weeks. The ratio of luminal to abluminal GLUT1 expression changes from 1:2 at the 12th week to 1:3 at the 18th week, indicating regulation of the BBB glucose transporter activity during development (Bertossi *et al.*, 1999).

Glucose transport at the BBB can be up-regulated or down-regulated by physiological alterations that cause either increased or decreased cerebral glucose metabolism. Altered glucose transport at the BBB and changes in the glucose transporter protein density were reported following chronic changes in blood glucose levels. Increased glucose transport into the brain has been reported uniformly by studies on chronic hypoglycaemia (Kumagai *et al.*, 1995; Simpson *et al.*, 1999). Consistent with enhanced glucose uptake, the expression of GLUT1

and GLUT1 mRNA at the BBB is also increased (Kumagai *et al.*, 1995). A more recent study on hypoglycaemia induced by insulin, demonstrated a 25-45% increase in regional BBB glucose uptake (Simpson *et al.*, 1999). Besides a 23% increase in the 55 kDa GLUT1 isoform expression, a greater increase in luminal GLUT1 was noted, which indicated a redistribution of GLUT1 to luminal membrane of ECs. GLUT1 mRNA also increased twofold. In contrast, no change was observed in the expression of the 45 kDa GLUT1 isoform. Unlike studies on hypoglycaemia, studies on the effects of hyperglycaemia/diabetes on the transport of glucose across the BBB have not been able to reach conclusive agreement. Increased glucose uptake has been reported in experimental hyperglycaemia by some researchers (Cornford *et al.*, 1995b; Duelli *et al.*, 2000). However, contrasting findings have been reported in the genetically diabetic *db/db* mouse (Vannucci *et al.*, 1997). Genetically diabetic *db/db* mice with hyperglycaemia and obesity show a significant reduction of cerebral glucose utilisation. Up-regulation of BBB GLUT1 expression and mRNA were reported by some studies (Maher *et al.*, 1993) but these results could not be confirmed by other studies. No changes of GLUT1 expression have been observed in these mice although mRNA level of GLUT1 was regionally increased. In contrast, decreased GLUT1 expression in chronic hyperglycaemia, accompanied with an increase in glucose utilisation was reported recently (Duelli *et al.*, 2000). Differing methodologies could explain some of the discrepancy. For example, earlier studies employed the brain uptake index technique, which has problems with tracer dilutions. However, studies using the same methodology have documented different results. More studies are needed to elucidate the effects of

hyperglycaemia on BBB glucose transport and GLUT1 expression. Studies on glucose transporter in human neurodegenerative diseases have shown a down-regulation of GLUT1 level in the brain. Patients with Alzheimer's disease have shown a marked decrease in the glucose transporter protein in the brain (Harik, 1992; Simpson *et al.*, 1994). The reduction of reactivity was selectively in the cerebral neocortex and hippocampus, regions that are severely affected in Alzheimer's disease, but not in the putamen or cerebellum, which are not affected to a major extent in this disease (Harik, 1992). Studies of cerebral glucose transporter expression in human immunodeficiency virus (HIV) infection, demonstrated that GLUT1 level in grey and white matter regions increased with human HIV infection without neuropathology when compared to normal controls, and decreased to baseline with HIV encephalitis (HIVE) (Kovitz & Morgello, 1997). These findings may parallel glucose hypermetabolism found in patients with early acquired immunodeficiency syndrome (AIDS) dementia complex and glucose hypometabolism in patients with late clinical stage dementia (Rottenberg *et al.*, 1987). An up-regulation of GLUT1 expression was reported in human brain resections from a patient surgically treated for seizures (Cornford *et al.*, 1994b). They postulated that the acute modulation of glucose transporter activity involved translocation of GLUT1 from cytoplasmic to luminal membrane sites. A further study showed that in patients with seizure, altered glucose transporter activity in the BBB was characterised by a bimodal GLUT1 distribution in which the smaller ECs displayed low GLUT1 immunoreactivity, whereas adjacent larger ECs showed five to tenfold greater expression of membrane GLUT1 transporter

protein (Cornford *et al.*, 1998b). The variation of GLUT1 immunoreactivity among capillaries may reflect certain disease conditions.

The GLUT1 glucose transporter level may also be altered in other pathological conditions. In rapidly growing CNS tumours such as glioblastomas, Guerin *et al.* (1990) have reported that the GLUT1 is down-regulated, with a loss of 80% of the immunoreactive capillary glucose transporter protein. The glucose transporter immunoreactivity was lacking in tumour microvessels but was retained in normal brain and brain tissue surrounding the tumours (Harik & Roessmann, 1991; Nagamatsu *et al.*, 1993). This finding suggested that in these pathological conditions, transportation of glucose by GLUT1 is not needed for tumour growth because blood vessels in these tumours are leaky and glucose exchange can occur freely. However, in a study of hemangioblastoma, an uncommon CNS neoplasm, high expression of GLUT1 reactivity was found in the central tumour mass which was composed solely of endothelial-lined channels (Cornford *et al.*, 1995a). These endothelia are highly permeable and devoid of astroglial contacts. This finding suggested that up-regulation of glucose transporter activity could occur concomitantly with diminished barrier operation in the brain. Similarly, it was shown that in cold injury of the brain, which resembles human cortical contusions, the endothelium of vessels which were permeable to protein, whether in the cold-injury site or in the perilesional area, all contained GLUT1 protein (Nag, 1996). Interestingly, in the process of neovascularization, regenerating ECs become immunoreactive for GLUT1 at 5 days and this coincided with the presence of tight junctions in these cells (Nag, 1996). These results suggested that during nervous

system development or endothelial regeneration, the presence of GLUT1 in cerebral endothelium coincides with the presence of an intact BBB to proteins and protein tracers. However, in pathological states, the presence of GLUT1 in the cerebral endothelium does not appear to correlate with an intact BBB to protein. Consistently, in human brain injury, the high GLUT1 density observed in capillaries of acutely injured brain, occurs concomitantly with compromised barrier function (Cornford *et al.*, 1996). Increased immunoreactivity of GLUT1 and the expression of its mRNA have been reported in cerebral ischaemia (McCall *et al.*, 1996; Urabe *et al.*, 1996). GLUT1 has been easily detected in neurons and astrocytes in brain ischaemia but under normal physiological conditions, GLUT1 is only detected in some astrocytes. The changes in GLUT1 expression in ischaemia may reflect the regulation of glucose metabolism in response to ischaemic injury to the brain.

1.5.2.2 P-glycoprotein

P-glycoprotein is a multidrug resistant (MDR) protein present in the BBB of all mammals investigated, including humans (Cordon-Cardo *et al.*, 1989; Cordon-Cardo *et al.*, 1990). It is regarded as a guardian of the brain by actively keeping potentially toxic compounds out of brain parenchyma. P-glycoprotein is *N*-glycosylated membrane protein of about 1280 amino acids (170 kDa). It belongs to the superfamily of ATP-binding cassette (ABC) transporters. In humans, there is only one P-glycoprotein gene that actually confers drug resistance; *MDR1*, but mice have two P-glycoprotein genes for drug transporting, *mdr1a* (also called *mdr3*) and *mdr1b* (also called *mdr1*) (Hsu *et al.*, 1989; Devault & Gros, 1990). P-

glycoprotein was first identified in Chinese hamster ovary cells selected for colchicine resistance and was demonstrated to mediate resistance to a wide range of anticancer agents (Juliano & Ling, 1976). Later the presence of P-glycoprotein in brain capillaries of humans, rats, mice, pigs and cattle was reported (Hegmann *et al.*, 1992; Tsuji *et al.*, 1992; Barrand *et al.*, 1995; Toth *et al.*, 1996). The subcellular location of P-glycoprotein in the BBB has been studied extensively and conflicting findings have been reported. Many studies using light and electron microscopic immunocytochemistry or confocal immunofluorescence microscopy demonstrated that P-glycoprotein is primarily localised to the luminal membrane of ECs in the BBB (Sugawara, 1990; Sugawara *et al.*, 1990; Tsuji *et al.*, 1992; Stewart *et al.*, 1996). The most convincing evidence to support this observation came from a well performed study by Beaulieu *et al.* (1997). The expression of P-glycoprotein was quantitatively studied in purified luminal membrane of ECs. Enrichment of P-glycoprotein in purified luminal membrane preparations, relative to isolated brain capillaries and whole brain membrane preparations were 17- and 400- fold respectively, indicating a high level of P-glycoprotein in the luminal membrane of brain ECs. The localisation of P-glycoprotein to the luminal membrane of ECs would enable P-glycoprotein to immediately extrude substrates that had diffused into the ECs, back to the bloodstream. However, one study showed that the expression of P-glycoprotein in human brain resections of patients with epilepsy was not limited to ECs but also occurred in astrocytes (Tishler *et al.*, 1995). In the latter study, astrocytes from normal brains did not stain for P-glycoprotein. The most conflicting findings of P-glycoprotein subcellular localisation were reported by Pardridge and

co-workers (Pardridge *et al.*, 1997; Golden & Pardridge, 1999). They demonstrated that the cellular origin of P-glycoprotein in isolated human brain microvessels was the astrocyte foot process, not the endothelium, using the anti-P-glycoprotein antibody MRK16 and double-labelling confocal fluorescent microscopy. In their studies, anti-GFAP antibody was used as a marker for astrocyte foot processes and anti-GLUT1 antibody was used as a marker for ECs. The localisation of anti-GFAP, anti-GLUT1 and MRK16 was detected by confocal fluorescence microscopy. The labelling patterns of anti-GFAP and MRK16 at isolated human brain capillaries were identically discontinuous. Double labelling by these two antibodies showed complete overlap. However, anti-GLUT1 antibody continuously labelled the capillaries. Double labelling with anti-GLUT1 and MRK16 showed that they were not colocalised at the capillaries. A possible limitation of the technique used in that study is the use of only one antibody to P-glycoprotein. It is reported that the epitope of human MDR1 P-glycoprotein for the MRK16 antibody can be completely shielded in some cell types by heavy N-glycosylation of P-glycoprotein (Cumber *et al.*, 1990). It has been suggested that at least two, and preferably three or more different antibodies be used to study the immunolocalisation of P-glycoprotein (van der Valk *et al.*, 1990). An in situ hybridisation study showed a similar discontinuous pattern of P-glycoprotein transcripts in the brain microvasculature (Qin & Sato, 1995). More work is needed to determine the subcellular localisation of P-glycoprotein as it is of great significance in determining the function of P-glycoprotein at the BBB.

The BBB has been considered as a lipid wall with special receptors and carrier systems for certain compounds. Hydrophobic compounds penetrate the BBB by passive diffusion. The identification of P-glycoprotein in the BBB revealed that active transportation of lipid soluble compounds occurs at the BBB. Early studies on the permeability of the BBB to hydrophobic compounds found that the permeability of compounds with a molecular weight less than 400 Da can be predicted by the relationship of octanol/water partition coefficient and molecular weight. However, for molecules between 400 and 700 Da, the penetration to the brain is much less than predicted. Molecules with high lipid solubility but molecular weight greater than 700 cannot enter the brain at a detectable level (Levin, 1980). It has been revealed recently that these molecules are substrates for P-glycoprotein. P-glycoprotein is able to actively transport a wide range of drugs, including anticancer drugs such as anthracyclines, taxanes (van der Kolk *et al.*, 1999), the immunosuppressive agent cyclosporin A (Tsuji, 1998) and several HIV protease inhibitors (Kim *et al.*, 1998). The varied structure and function of these drugs are unrelated and the molecular size of these chemicals, ranges from about 250 Da (cimetidine) to more than 1850 Da (Gamicidin D). The only common feature they share is that they are all somewhat hydrophobic and amphipathic in nature.

The importance of P-glycoprotein for drug resistance has been revealed through the use of knockout mice lacking *mdr1a* P-glycoprotein (*mdr1a* $^{-/-}$ mice). Although *mdr1a* $^{-/-}$ mice appear physically normal and fertile, they have a very low tolerance to neurotoxic substances such as ivermectin. A routine safety spray

of ivermectin to treat mite infestation accidentally resulted in the death of almost all *mdr1a* (-/-) mice. A further study showed that the accumulation of ivermectin was 100 fold greater in knockout mice than in normal wild-type mice (Schinkel *et al.*, 1994). Many more drugs have been shown to accumulate to higher extents in the brains of P-glycoprotein gene knockout mice (Schinkel *et al.*, 1995; Schinkel *et al.*, 1996; van Asperen *et al.*, 1997; Kim *et al.*, 1998). The results indicated that P-glycoprotein plays an important role in excluding substances from the brain compartment and is directly involved in the protection of the brain. A number of P-glycoprotein blocker or reversal agents have been discovered (Tsuruo *et al.*, 1981; Hyafil *et al.*, 1993; Dantzig *et al.*, 1996). The P-glycoprotein blockers can inhibit the drug transport effect mediated by P-glycoprotein without apparent cytotoxicity. Most of the reversal agents are actually substrates for P-glycoprotein. They may inhibit P-glycoprotein activity by competitive transportation. The cellular mechanism underlying P-glycoprotein transport function remains controversial as does its subcellular localisation. The localisation of P-glycoprotein at the luminal membrane of brain capillary ECs indicates that P-glycoprotein regulates blood-to-brain transport, while the localisation of P-glycoprotein at the astrocyte foot process indicates that P-glycoprotein mediates drug efflux across the astrocyte foot process membrane from the brain interstitial fluid into astrocytes. In the latter model, knocking out P-glycoprotein genes would result in increased drug distribution into brain cells such as astrocytes without significantly increasing BBB permeability (Pardridge *et al.*, 1997; Golden & Pardridge, 1999).

1.5.2.3 Gamma-Glutamyl Transpeptidase

Gamma-glutamyl transpeptidase (GGTP) is found in the epithelium of secretory tissues (Chiba & Jimbow, 1986; Hanigan & Frierson, 1996) and in many organ barriers, e.g. the BBB and the blood-cerebrospinal fluid barrier (Ogawa *et al.*, 1998; Lawrenson *et al.*, 1999). Since no other capillaries possess GGTP in detectable amounts, the activity of this enzyme is used as a biochemical marker for the BBB.

In the rat CNS, the activity of GGTP is mainly present in areas where the BBB is expressed, such as the cerebral cortex, cerebellum, striatum, and spinal cord. In areas where the BBB is absent, GGTP activity is not detectable or only a few blood vessels showed GGTP activity (Wolff *et al.*, 1992). GGTP activity varies significantly in different areas of rat brain. The percentage of GGTP-positive vessels in the brain ranged from 100% in the anterior commissure to only 8% in the optic nerve (Wolff *et al.*, 1992). These authors proposed that the regional difference of GGTP activity might be related to the influence of astrocytes. This view is supported by another study which showed that blood vessels in pia mater in rats lack GGTP activity while cortical vessels strongly express GGTP activity (Lawrenson *et al.*, 1999). As pial vessels do not regularly contact astrocytes, it seems that GGTP activity is astrocyte-dependant. *In vitro* studies also showed that astrocytes enhanced GGTP activity in brain EC culture (Maxwell *et al.*, 1987; Raub *et al.*, 1992).

The precise localisation of the GGTP at the BBB still remains controversial. Some investigators reported that GGTP localised at the luminal membrane of ECs (Ghandour *et al.*, 1980; Sanchez del Pino *et al.*, 1995a; Ogawa *et al.*, 1998). Other authors found the main activity of GGTP at the abluminal membrane of the capillaries and at higher concentration in pericytes (Frey *et al.*, 1991; Risau *et al.*, 1992). Further studies are needed to clarify the subcellular localisation of this enzyme at the BBB. The different subcellular localisation of GGTP may indicate a different role of GGTP at the BBB.

The function of GGTP at the BBB is still not clear. It is generally accepted that GGTP is involved in amino acid transport across the BBB (Wolf & Gassen, 1997). It was demonstrated that GGTP has a high affinity to cystine (Thompson & Meister, 1975, 1976). However, a recent study showed that inhibition of GGTP activity in isolated adult porcine brain vessels did not affect the uptake of cystine (Wolff *et al.*, 1998). Another possible function of GGTP at the BBB is detoxification (Wolf & Gassen, 1997). GGTP is involved in the mercapturic acid pathway. As brain ECs are not able to exclude lipophilic xenobiotic substances, the mercapturic acid pathway can detoxify these potentially harmful substances (Wolf & Gassen, 1997). More studies are needed to elucidate the role of GGTP at the BBB.

1.5.3 Junctional proteins

1.5.3.1 Occludin

Occludin is the only known transmembrane protein in the tight junction. It was identified initially from junction-enriched fractions of the chicken liver and was found to be present in the tight junctions of both epithelial and ECs (Furuse *et al.*, 1993). By immunogold labelling freeze-fracture techniques, occludin has been shown to localise precisely within the tight junction fibrils (Furuse *et al.*, 1993; Hirase *et al.*, 1997). A study using a Lac-inducible vector showed that the expression of chicken occludin in MDCK cells increased transepithelial electrical resistance by 30-40% (McCarthy *et al.*, 1996). In the meantime, the number of fibrils increased by 15%. Both *in vivo* and *in vitro* studies have shown that the expression of occludin is higher in brain ECs than in peripheral ECs (Hirase *et al.*, 1997). The same difference in expression of occludin, between brain and peripheral ECs has also been found at the mRNA level (Hirase *et al.*, 1997). The expression of occludin at the BBB is altered in several pathological conditions. In patients with cerebral malaria, the parasites do not enter the brain parenchyma but are able to induce a diffuse reversible encephalopathy. The BBB showed malfunction in these cases, as demonstrated by an increased permeability to plasma proteins in association with altered distribution of occludin (Brown *et al.*, 1999). The disruption of occludin was also observed in the brains of patients who have died with HIV. Fragmentation or absence of immunoreactivity for occludin is associated with perivascular accumulation of activated HIV-1-infected macrophages, serum protein extravasation, and marked astrogliosis (Dallasta *et al.*, 1999). In experimental neutrophil induced BBB breakdown, the loss of

occludin was accompanied by increased BBB permeability to HRP (Bolton *et al.*, 1998). It seems that occludin levels could determine tight junction permeability at the BBB. However, a developmental study of occludin in rats showed that occludin was hardly detected in brain ECs at P8 but was clearly detectable at P70 (Hirase *et al.*, 1997). Therefore it seems unlikely that occludin is responsible for the impermeability of the BBB to plasma proteins at the early stage of brain development. In brain endothelial cell culture, a high level of occludin was obtained but the electrical resistance of cultured ECs was relatively low. More surprisingly, occludin-deficient embryonic stem cells formed embryoid body structures (EBs) delineated by an outermost layer of epithelial cells in suspension culture just like wild-type embryonic stem cells do within 2-4d. Tight junctions formed between epithelial cells of occludin-deficient EBs were not different from tight junctions formed between epithelial cells of wild-type EBs and functioned normally as a barrier to a low molecular mass tracer (Saitou *et al.*, 1998). These studies suggest that occludin is not the major structural component of tight junctions. There are yet unidentified transmembrane junctional protein(s) that is (are) responsible for the barrier function.

1.5.3.2 The proteins ZO-1 and ZO-2

ZO-1 and ZO-2 are to date the most extensively studied proteins within the cytoplasmic plaque of the tight junction, which are present between adjacent epithelial cells (Stevenson *et al.*, 1986; Denker & Nigam, 1998; Mitic & Anderson, 1998), and in ECs (Rubin *et al.*, 1991; Hirase *et al.*, 1997). They are members of the membrane-associated guanylate kinase (MAGUK) homologue

protein family. All MAGUK proteins are associated with the plasma membrane, typically at sites of specialised cell-cell contact. Both ZO-1 and ZO-2 are expressed and co-localised with occludin in all cells that form tight junctions. ZO-1 has been shown to bind directly to the C-terminal cytoplasmic tail of occludin (Furuse *et al.*, 1994). ZO-2 and ZO-1 interact with each other (Gumbiner *et al.*, 1991; Itoh *et al.*, 1999). ZO-2 has been shown to form a complex with occludin and ZO-1 to establish tight junction domain (Itoh *et al.*, 1999). The disruption of ZO-1 is observed in human cerebral malaria and HIV. However, as both ZO-1 and ZO-2 are cytoplasmic, they cannot function as a permeability barrier (Rubin & Staddon, 1999). The exact role of these proteins in permeability control is not clear.

1.6 BBB transport

The BBB possesses specific transport systems to facilitate the uptake of certain essential nutrients such as some amino acids, glucose and iron into the brain. These substances are shuttled through the BBB by specific carrier systems or receptor mediated endocytosis.

1.6.1 Transport via carrier systems

1.6.1.1 Glucose transport

Glucose transport in the brain is facilitated by GLUT1. This issue is addressed in 1.5.2.1.

1.6.1.2 Amino acid transport

Amino acids play an important role in brain development and normal function. Many neurotransmitters in the brain are amino acids, such as glutamate, aspartate and glycine, and are synthesised locally in neurons (Smith & Stoll, 1998). The interstitial concentration of these neurotransmitters has to be strictly controlled to ensure normal neural function. In the mean time, most large neutral and basic amino acids including arginine, lysine and tryptophan cannot be synthesised in the brain and have to be transported from the blood to the brain. These amino acids are required for brain protein synthesis and are precursors for serotonin (tryptophan), nitric oxide (arginine) and the catecholamines (tyrosine) (Smith & Stoll, 1998). Currently, nine different amino acid transport systems have been identified at the brain capillary endothelium, and can be divided into three groups: sodium-independent systems, sodium-dependent systems and low activity systems.

Sodium-independent systems include system L1 for large neutral amino acids (Oldendorf & Szabo, 1976) and system y^+ for basic (cationic) amino acids (Stoll *et al.*, 1993). These systems are located at both the luminal and abluminal membranes of ECs. Transport via these two carrier systems is saturable, stereospecific (preferring L to D), bidirectional, and sodium/energy independent. Their function is to deliver dietary essential neutral and basic amino acids. The L1 system transports nine or more amino acids including L-leucine, L-phenylalanine, L-tryptophan and it is heavily saturated (>95%) with amino acids in normal conditions. Therefore, transportation through the L1 system is very

sensitive to competition. Elevation of one large neutral amino acid in the plasma concentration will reduce brain uptake of others. System y^+ delivers three amino acids, L-lysine, L-arginine, and L-ornithine, across the BBB.

Sodium-dependent systems include systems A, $B^{\circ+}$ and ASC (Tayarani *et al.*, 1987; Sanchez del Pino *et al.*, 1995b). They are located at abluminal membranes of brain capillaries. System A and ASC show affinity to small neutral amino acids. The subcellular localisation of these systems indicates their role in actively transporting amino acids from brain extracellular fluid to ECs. Systems T, β , and X^- are high affinity and low capacity carriers (Smith & Stoll, 1998; Smith, 2000). System T transports thyroid hormones into the brain at a very low level. System β is sodium and chloride dependent and mediates active transport of β -amino acids. System X^- delivers anionic amino acids such as glutamate and aspartate into the brain at slow rates. System X^- may have a function in controlling glutamate concentration in the brain. A distinct transport system for L-glutamine has been identified recently (Ennis *et al.*, 1998; Lee, W. J. *et al.*, 1998). L-glutamine has a high affinity to system L but studies showed that the transportation of L-glutamine is actually by the sodium-dependent system N. However, whether the function of system N is to transport L-glutamine into or out of the brain is still controversial.

1.6.2 Transport via receptors

1.6.2.1 Transferrin receptor-mediated iron uptake

Iron is delivered into the brain by transferrin receptor-mediated transcytosis. Neuronal cells need a high level of iron supply for the mitochondrial respiratory

pathway. Iron is bound as Fe^{3+} to transferrin in plasma. Transferrin is a monomeric glycoprotein mainly found in serum and normally is only about 30% saturated with iron. The uptake of iron into cells is via the receptor-mediated endocytosis of iron-transferrin. Transferrin receptor was first identified in brain capillaries by Jefferies *et al.* (Jefferies *et al.*, 1984). Using a monoclonal antibody OX-26 against rat transferrin receptor, it was demonstrated that transferrin receptors are present on the luminal membrane of brain capillary ECs. There is no doubt that transferrin is endocytosed into brain ECs. However, the fate of transferrin after it is endocytosed has been controversial. Some researchers reported transcytosis of intact transferrin across the BBB (Fishman *et al.*, 1987; Descamps *et al.*, 1996). However, some authors believe that Fe^{3+} or another degraded complex is the transported species (Roberts *et al.*, 1993; Moos & Morgan, 2000). They suggested that iron is released from transferrin in the ECs and transported across the abluminal membrane either as Fe^{3+} or as a low-molecular weight complex. However, immuno-electron microscopic studies support the hypothesis that transcytosis of transferrin occurs at the BBB (Bickel *et al.*, 1994; Broadwell *et al.*, 1996). An immunogold electron microscopic study demonstrated the three steps of transcytosis of gold-OX-26 conjugates. Gold/OX-26 conjugates were observed at the luminal surface, in the cytoplasm and being exocytosed into interstitial fluid (Bickel *et al.*, 1994). A recent study using confocal microscopy showed transferrin receptors are also present on the abluminal membrane of ECs, which is consistent with exocytosis of transferrin at the abluminal membrane. A drug delivery study using transferrin receptors also

supported the concept that receptor mediated transcytosis occurs in brain capillary ECs (Lee, H. J. *et al.*, 2000).

1.6.2.2 Insulin receptor-mediated insulin uptake

Insulin and insulin receptors are found in abundance in the brain. Both *in vivo* and *in vitro* studies demonstrated that insulin receptors are present at the endothelium of brain capillaries and mediate active insulin transport across the BBB by transcytosis (Miller *et al.*, 1994; Pardridge *et al.*, 1995; Banks *et al.*, 1997). The signalling of insulin in the brain is not fully understood. Apparently, insulin has no effect on glucose transport across the BBB (Hasselbalch *et al.*, 1999; Knudsen *et al.*, 1999). Insulin receptors are widely distributed on neurons, indicating that insulin has a neuromodulatory role in the brain. It has been proposed that alteration of insulin level in the brain may relate to serious mental disorders (Holden, 1999).

1.7 Development of the BBB

It has been widely accepted that the BBB undergoes structural and functional changes during brain development. Immature brains seem to be more susceptible to viral infection, metal toxicity and other disorders occurring predominantly at an early stage of life, such as kernicterus. As early as the 1920s, it was documented that trypan blue could cross the BBB of immature mice relatively more easily than it did in the adult rat brain, which led to the conclusion that the BBB is not fully developed in young animals (Reviewed by Saunders *et al.*, 2000). Extensive studies have been performed on the ontogenetic development of the BBB ever

since. Despite many studies on brain development, the question of when the BBB reaches maturity in the developing brain is still controversial.

Information on permeability properties of embryonic brain capillaries is contradictory. Leakage of HRP in the brain of chick and mice embryos was observed after its intravenous injection (Wakai & Hirokawa, 1978). In chick embryos, it is at the 15th day of incubation that the barrier to HRP is fully established in the brain and spinal cord. The injection volume of HRP in this study was one tenth of the total body blood volume (Wakai & Hirokawa, 1978). In mice embryos, blood vessels in the telencephalon were found to be impermeable to HRP around day 16 (Risau *et al.*, 1986a). These findings have been used to demonstrate the immaturity of the developing BBB. However, the technique employed in these studies was questioned by Saunders and co-workers (Saunders *et al.*, 1991; Saunders, 1992; Saunders & Dziegielewska, 1997; Saunders *et al.*, 1999b). These authors argued that a large volume and high concentration of HRP were used in these studies, which could have damaged the vasculature of the brain and caused the leakage of HRP. Estimated by Saunders and co-workers, the plasma protein concentration in the chick embryos study was increased 50 to 100% while in the mice embryos study, the injected volumes doubled the blood volume. However, to prove whether high protein concentration would lead to a breakdown of the barrier, more direct evidence is needed. Saunders and co-workers provided evidence that in human developing brain, the BBB is tight enough to exclude serum proteins (Møllgård *et al.*, 1988). Employing a less traumatic technique, they identified 23 plasma-like proteins such

as α -fetoprotein, GC-globulin and albumin in the developing human brain (Møllgård *et al.*, 1988). Among them, mRNA of 13 proteins including albumin were demonstrated, indicating these proteins were synthesised in the brain and had not originated from the blood. The 23 proteins identified in the developing brain were found in the ECs, lumen of blood vessels and in some nerve cells. However, no perivascular protein was detected. It is postulated that the remaining 10 proteins identified in the brain, whose mRNA could not be found, enter the brain by the CSF or retrograde uptake via neuronal processes extending beyond the BBB. These investigators concluded that the BBB of the developing human brain is tight enough to exclude plasma proteins.

While the barrier function of the immature brain to large molecules remains controversial, the BBB of immature brains does appear more permeable than the adult brain to low molecular weight lipid-insoluble molecules such as sucrose and inulin. An early study performed by Stern and co-workers in 1920s examined the penetration of sodium ferrocyanide (MW 304) in newborn animals of different species. They found that more sodium ferrocyanide passed into the brain in newborn rats, dogs, rabbits and cats than in adult animals. However, they found no leakage of trypan blue in brains of newborn animals (Reviewed by Saunders, 1992). Later quantitative studies using sucrose or inulin confirmed that the immature brains of neonatal rats, chick embryos and foetal sheep are more permeable to small molecules than adult brains (Saunders, 1992; Saunders & Dziegielewska, 1997).

Morphological studies of the endothelium in immature brains suggest a maturational process occurring during development. The invasion of capillaries into the developing nervous system in mice embryos starts at day 9 (E9) and at E10 the first intraneural vascular plexus is established (Bauer *et al.*, 1993). At the first day of vascularisation, intraneural ECs lose fenestration and numerous junctions appear. It is clear that the morphological properties of the BBB are present in the very early stage of mouse brain development. From E10 to E17, the number of pinocytotic vesicles in the cytoplasm of ECs is greatly reduced and pericytes show a closer contact with ECs. However, studies on rat embryos found that the blood vessels in the parenchyma were fenestrated until E13 and in pial vessels, fenestration disappeared after E17 (Stewart & Hayakawa, 1994). Recent ultrastructural studies on tight junction formation in the developing rat brain demonstrated that tight junctions undergo morphological changes during development (Kniesel *et al.*, 1996). A morphometric study employing the technique of high power electron microscopy and goniometric tilting of ultrathin sections, demonstrated the maturation process of tight junctions during the BBB development in rats (Schulze & Firth, 1992). From E17 to the adult, membrane separations (>20 nm) at the tight junctional contact disappeared, the cleft length reached 500 to 900 nm and the ratio of the narrow to expanded parts of the cleft increased (Schulze & Firth, 1992). A freeze-fracture electron microscopic study revealed a progressive increase in tight junction complexity during rat brain development (Kniesel *et al.*, 1996). Although tight junctions were present as early as E13, the complexity of the junction was very low, as demonstrated by few particles being detected on both fracture faces. The overall complexity of the

tight junction network and P-face (protoplasmic fracture face) associated particles largely increased between E18 and P1. In epithelial tight junction systems, the extent of the tight junction particles' association with the P-face is correlated with paracellular barrier functions. The shifting of tight junction particles from the E-face (extracellular fracture face) to the P-face at the EC tight junction could suggest the maturation of barrier properties of the junction. Consistent with the developmental changes of tight junctions, the electrical resistance across the BBB increases during brain development (Butt *et al.*, 1990). It can be concluded from the above studies that tight junctions do appear very early in development and they undergo morphological changes. However, more studies are needed to establish the relationship between tight junction morphology and permeability properties in cerebral blood vessels, as no correlation was found in developing choroid plexus vessels (Møllgård *et al.*, 1976). Fundamental changes in blood vessel ultrastructure in the human foetus telencephalon from 12 to 18 weeks of gestation have also been documented. The endothelium-pericyte layer flattens out, endothelial tight junctions become extensive, and the pericyte sheath becomes more continuous.

It is unlikely that all the features of the BBB reach maturity simultaneously (Rubin & Staddon, 1999). Comparative studies showed sequential expression of BBB enzymes and proteins during development. Gamma glutamyl transpeptidase activity in the rat brain increases during the first three weeks after birth. The activity of Na-K-dependent ATPase in rat brain increases considerably after birth, particularly at the age of 10 to 12 days. Other BBB proteins appear at different

times during development of the brain. In mice, the first known endothelial cell differentiation marker, the multidrug resistance gene, appears at embryonic day 10.5 (E10.5), even before the presence of astrocytes (Qin & Sato, 1995). An ontogenetic study of four BBB specific proteins in rats showed that OX-47 was detectable at E16; EBA appears at 15 days postnatal (P15); S-laminin was present in microvessels at P7, but more strongly expressed from P15, and GLUT1 was strongly expressed from E16 (Cassella *et al.*, 1996).

1.8 The BBB in pathological conditions

Many pathological conditions of the CNS are related to altered BBB. The permeability of the BBB can be reduced or increased. Reduced permeability of the BBB reflects either enhanced barrier function in the restriction of exchange of substances or reconstitution of the BBB. In experimental cholestatic liver injury, the permeability of the BBB is largely decreased despite elevated levels of substances known to enhance vascular permeability such as bile acids, substance P and histamine (Wahler *et al.*, 1993). The decreased permeability of the BBB is attributed to a decrease in cerebral capillary endothelial cell membrane fluidity. Numerous factors have been reported to have the ability to enhance the barrier function of the BBB. Antenatal corticosteroid treatment can decrease BBB permeability in the ovine foetus which consequently reduces the incidence of intraventricular haemorrhage in premature lambs (Stonestreet *et al.*, 1999). Ifenprodil has been reported to be able to reduce infarction volume after cerebral ischaemia (Baskaya *et al.*, 1997b). In studies on cerebral oedema associated with brain tumours, dexamethasone and human corticotropin-releasing factor have

been shown to restore BBB integrity in brain tumour vessels (Tjuvajev *et al.*, 1996). Experimental allergic encephalomyelitis is used as an animal model for multiple sclerosis (MS). It is characterised by BBB disruption, cerebral oedema formation, lymphocyte infiltration, and demyelination. AN100226m, a monoclonal antibody to alpha 4 integrin has been reported to block leukocyte-endothelial adhesion at the BBB and reverse the clinical and histological signs of EAE (Kent *et al.*, 1995). A further study showed that AN100226m reduced the BBB permeability, prevented accumulation of inflammatory oedema and resolved pre-existing oedema. Some cytokines can also affect BBB integrity. Systemic injection of tumour necrosis factor-alpha (TNF- α) largely reduced BBB permeability while administration of interleukin-2 and interleukin-6 had the opposite effect (Saija *et al.*, 1995).

The direct consequence of increased BBB permeability is vasogenic brain oedema, which is a great problem in brain tumours, ischaemia, and head injury (Lobato *et al.*, 1996; Castejon *et al.*, 1997; Schlaug *et al.*, 1997; Castejon, 1998). Brain oedema is also present in neurodegenerative diseases such as Alzheimer's disease and MS (Stone, *et al.*, 1995; Claudio, 1996; Wada, 1998; Berzin *et al.*, 2000), infectious diseases such as malaria and bacterial meningitis (Ashwal *et al.*, 1992; Brown *et al.*, 1999; Ozates *et al.*, 2000), hypertension and acute hepatic failure (Watanabe *et al.*, 1992; Schwartz *et al.*, 1998). In experimental conditions, disruption of the BBB can also be induced by infusion of chemotoxic solutions such as contrast media used in radiology (Wilson *et al.*, 1991; Wilson & Sage,

1994) and hyperosmolar solutions such as hyperosmolar mannitol and L⁽⁺⁾arabinose [Nagy, 1984 #268; (Vorbodt *et al.*, 1994).

According to Klatzo, “brain oedema is an abnormal accumulation of fluid within the brain parenchyma producing a volumetric enlargement of brain tissue” (Klatzo, 1994). In 1967, Klatzo classified brain oedema into two types, vasogenic and cytotoxic depending on the primary source of excess fluid (Klatzo, 1967). Vasogenic oedema is characterised by extravasation of proteins due to increased vascular permeability. Oedema fluid is basically a plasma filtrate, containing serum proteins. In cytotoxic oedema, the primary injury occurs in cerebral parenchyma, the BBB permeability is relatively intact and there is no extravasation of plasma proteins. Oedema fluid in this case is basically a plasma ultrafiltrate, which is high in K⁺ ions and low in protein. The main characteristic is a cellular swelling without measurable BBB breakdown. Now it is recognised that the astrocyte is the major cell type showing swelling in cytotoxic oedema (Kimelberg, 1995). As Klatzo emphasised, it is unlikely that a pure form of either types of oedema exists.

In vasogenic brain oedema, water and solutes pass through the compromised BBB through several pathways. Increased pinocytic vesicles in endothelial cell cytoplasm has been observed in several pathological conditions such as brain trauma, brain cold injury, and brain tumours, indicating increased vesicular transportation (Hurter, 1984; Vorbodt *et al.*, 1993; Vaz *et al.*, 1998). An immuno-gold electron microscopic study in experimental brain cold injury

demonstrated that albumin-gold particles cross the BBB by vesicular transport (Vorbodt *et al.*, 1993). Morphological alteration of tight junctions has not been observed in either human or experimental brain trauma (Vaz *et al.*, 1997; Vaz *et al.*, 1998). Also, in osmotic opening of the BBB, disruption of tight junctions is a characteristic feature, resulting in markedly increased diffusion and bulk fluid flow across the tight junction (Rapoport, 2000). Increased tight junction permeability was also observed in the early phase of incomplete continuous ischaemia. Astrocyte swelling is also noted in vasogenic brain oedema due to uptake of the extravasated plasma proteins. Pericytes may also show pathological changes such as increased vesicles and vacuoles, suggesting a pathway for blood-borne solutes. Thickening of the basal lamina and increased perivascular space are often present in vasogenic brain oedema.

In cytotoxic oedema, cell swelling is due to abnormal uptake of water by injured cells. The most common cytotoxic oedema is observed in cerebral ischaemia (Kimmelberg, 1995). The swelling of astrocytic processes has been observed at an early stage of ischaemia. Morphological changes of astrocytes were also observed in human brain trauma (Castejon *et al.*, 1997; Castejon, 1998). Massive astrocytic swelling was documented at three hours to three days after injury. Neurons may also show oedematous changes, featured by irregular enlargement of rough endoplasmic reticulum, nuclear envelope and Golgi apparatus (Castejon, 1998). Several mechanisms may underlie the occurrence of cytotoxic oedema. In ischaemia, the interruption of energy supply to the brain leads to ionic pump and osmoregulation failure, resulting in intracellular accumulation of water and

sodium. Glutamate, or the glutamate analogue kainic acid, may also play an important role in inducing the cytotoxic oedema.

Two pathological conditions accompanied by brain oedema will be addressed in detail in this project.

1.8.1 Traumatic brain injury

Traumatic brain injury resulting from motor vehicle accidents is the major cause of morbidity and mortality in young adults in the Western world. Head injury causes 1%-2% of all deaths from all causes, and one third to one half of all traumatic deaths are due to head injury (Graham *et al.*, 2000). Increased intracranial pressure (ICP) due to brain swelling after traumatic brain injury is the most common cause of death in patients with head injury. Studies have shown that cerebral blood volume is reduced after trauma (Kita & Marmarou, 1994; Qian *et al.*, 1996). Therefore, oedema, not vascular engorgement, is the major cause of brain swelling and ICP rise. It is well recognised that vasogenic brain oedema occurs following mild, moderate, or severe head injury. Leakage of macromolecular tracers, such as endogenous plasma proteins and/or exogenous protein has been observed by numerous studies on experimental brain injury (Povlishock *et al.*, 1978; Schmidt & Grady, 1993; Lammie *et al.*, 1999) and human brain injury (Liu & Sturmer, 1988; Cornford *et al.*, 1996). The opening of the BBB occurs as early as several minutes after the injury. Different experimental models present different time courses of cerebral oedema. A biphasic opening of the BBB was observed after cortical impact brain injury in

rats (Baskaya *et al.*, 1997a). Recent studies have shown that cytotoxic oedema may play a major role in the development of brain oedema (Ito *et al.*, 1996; Barzó *et al.*, 1997). Based on the measurement of the apparent diffusion coefficient (ADC) by diffusion-weighted imaging, Barzó and co-workers (1997) reported a transient opening of the BBB and a prolonged cytotoxic oedema after experimental brain injury. Apart from vasogenic and cytotoxic oedema, neurotoxic oedema is also considered to occur after traumatic brain injury. Significant increases in extracellular potassium and excitatory amino acids such as glutamate have been reported in experimental traumatic injury (Katayama *et al.*, 1990 a and b). Therefore, at least three forms of oedema, vasogenic, cytotoxic, and neurotoxic may contribute to brain oedema formation following trauma.

The subcellular passage-way for large molecules through the BBB following traumatic brain injury is not fully understood. There is little evidence to indicate that disruption of tight junctions occurs after traumatic brain injury. Examined ultrastructurally, tight junctions appear to remain intact after head injury in humans (Castejon, 1998) and experimental animals (Foda & Marmarou, 1994). However, there is abundant evidence which demonstrates increased pits and vesicle density in ECs in both human and experimental traumatic brain injury, indicating activation of ECs (Castejon, 1998). When exogenous tracer HRP was used in experimental brain injury model, vesicles were shown to contain HRP following injury (Povlishock *et al.*, 1978). However, the concept of vesicles playing a role in transendothelial transportation remains controversial (Broadwell, 1989).

While most studies on traumatic brain injury focused on the passage of tracers across the BBB, little is known about changes to BBB proteins during the course of injury. Cornford *et al.* studied GLUT1 expression in two cases of human brain injury (Cornford *et al.*, 1996). The majority of vessels showed high GLUT1 immunoreactivity except that vessels in areas of severe haemorrhage were devoid of GLUT1 immunoreactivity. Quantitative analysis revealed that 10% of blood vessels showed sectors of low expression of GLUT1. GLUT1 activity was greater on the luminal membrane in the high-density sectors but in low-density sectors, GLUT1 activity was greater on the abluminal membrane. Extravasation of human serum albumin was observed in regions where GLUT1 activity was elevated, indicating that enhanced GLUT1 activity had occurred concomitantly with compromised BBB function.

1.8.2 Toxaemia

Clostridium perfringens (*Cl p*) is a normal inhabitant of the duodenum of ruminants. It occasionally causes gas gangrene and food poisoning in humans and is an opportunistic infectious bacteria for patients with AIDS (Caumes *et al.*, 1998; Rood, 1998; Tallis *et al.*, 1999). It is also associated with outbreaks of fatal toxaemia in domestic animals (McDonel, 1986). *Clostridium perfringens* type B and D produce epsilon toxin, which is the most potent clostridial toxin after the botulinum and tetanus neurotoxins. The epsilon toxin is produced initially in an inactive form, epsilon prototoxin. The epsilon prototoxin is activated by cleavage of 13 amino acids by treatment with proteases such as trypsin, chymotrypsin and a

zinc metalloprotease (Bhown & Habeeb, 1977; Minami *et al.*, 1997). The potent toxin causes increased mucosal permeability facilitating its own absorption. Several early studies employing *Cl p* epsilon toxin showed that the major pathological changes caused by the toxin were observed mainly in the brain. The toxin produces diffuse cerebral vasogenic oedema in both domestic and experimental animals (Nagahama & Sakurai, 1991; Finnie & Hajduk, 1992; Nagahama & Sakurai, 1992; Barker *et al.*, 1993). Intravenous injection of epsilon toxin in mice results in extensive disruption of the BBB, as indicated by a wide distribution of HRP in brain parenchyma and the accumulation of labelled bovine serum albumin in the brain (Buxton, 1976; Nagahama & Sakurai, 1991). In the rat, treatment with epsilon toxin causes BBB breakdown as detected by the leakage of serum albumin (Finnie & Hajduk, 1992). The epsilon toxin shows a high binding affinity to the brain as shown by the accumulation of ^{125}I -labelled toxin, after its intravenous injection in mice (Nagahama & Sakurai, 1991). It was concluded that ECs are the primary sites of action for the epsilon toxin and that the toxin may bind to certain receptor sites on ECs.

In mice, prior injection of the inactive *Cl p* prototoxin offers transient protection to the brain against the toxin. Previous studies employing injections of epsilon prototoxin prior to the treatment with the toxin increased the length of survival time of the animals and reduced the binding of labelled toxin to the brain (Nagahama & Sakurai, 1991). Tracer studies showed that the epsilon prototoxin injection prior to the toxin could reduce the accumulation of labelled bovine serum albumin and prevent HRP leakage (Buxton, 1976; Nagahama & Sakurai,

1991). A 90% decrease in ^{125}I -labelled toxin in mice brains was detected with prior injection of prototoxin (Nagahama & Sakurai, 1991). The protective effect extended up to 100 min but was less effective by 750 min (Buxton, 1976). It was suggested that such protection may be due to the prototoxin competitively binding to the same receptor on ECs, thus averting the action of the toxin on the animals. Using the immunoperoxidase method, it has been conclusively demonstrated that the prototoxin binds to luminal membranes of brain ECs (Buxton, 1978).

1.9 Summary

The blood-brain barrier has been extensively studied over the last century and enormous knowledge has been gained. The application of electron-dense tracers such as HRP in the 1960s was a milestone for BBB research, which established the CNS endothelium being the anatomical site of the BBB. It is now well recognised that tight junctions and the non-fenestrated ECs possessing a low density of pinocytotic vesicles, form the morphological and physiological barrier at the blood-brain interface. Tight junctions seal the interendothelial cell space and produce a very high transendothelial electrical resistance pathway, similar to that of the epithelium. As a result, the paracellular passage of ions and proteins is highly restricted. In addition, the very low density of pinocytotic vesicles restricts transendothelial transportation. The BBB is capable of excluding almost all molecules except those that are small and hydrophobic. Even some hydrophobic substances have a low transport rate across the BBB because the BBB is also capable of actively transporting substances back to the blood using proteins such as the P-glycoprotein. The BBB effectively provides homeostasis for the CNS which is essential for its functions. The BBB is also essential for CNS development. Although some aspects concerning the appearance of an effective BBB remain controversial, it is now agreed that the BBB appears before birth and gradually reaches full maturation after birth.

It is now recognised that the BBB is much more than a rigid barrier between the blood and the CNS. Active transport occurs at the BBB. The BBB possesses specific transport systems for facilitating the transport of essential nutrients such

as amino acids, glucose and iron into the brain. Among those transport systems, the glucose transporter protein 1 (GLUT1) has been given much attention, because glucose is the sole fuel for the brain in normal conditions. The activity of GLUT1 can be modulated by physiological changes in brain metabolism and in some pathological conditions. However, the significance of the changes of GLUT1 expression that occur are not fully understood, especially in disease states such as traumatic brain injury.

The cerebral capillary ECs that possess a barrier function express some proteins that are not found or are not detectable in non-barrier endothelia. These proteins are defined as BBB markers. A number of proteins have been reported in recent years such as GLUT1, EBA and OX-47. Most of them are of unknown function except GLUT1. EBA has been regarded as a barrier marker for mature BBB, because its distribution correlates with the classic BBB. Little is known about its function at the BBB although changes in EBA expression have been observed in some pathological conditions.

Many pathological conditions are accompanied by disruption of the BBB. The most common consequence of an abnormal BBB is cerebral oedema. Cerebral oedema in return can cause severe secondary damage to the brain that worsens the primary injury, the consequence could be fatal. Despite decades of studies on brain oedema, the development of cerebral oedema is not fully understood. Understanding the changes that occur in BBB markers such as EBA and GLUT1

in pathological conditions is of great importance as it leads to an understanding of the mechanisms underlying the formation of brain oedema.

CHAPTER 2

AIMS

2 Aims of the Thesis

The aims of this thesis are to study the normal structural and molecular characteristics of brain endothelial cells and the changes that may occur in pathological states. Two experimental models will be used to induce intravascular and extravascular insults to the brain. The intravascular insult will be produced by employing a toxin model for induction of brain oedema. The agent to be used is *Clostridium perfringens* epsilon prototoxin, which induces vasogenic brain oedema. The extravascular insult to the BBB will be induced by a model of traumatic brain injury, which mimics closed head injury in humans. The methods employed will include immunocytochemistry, histochemistry, electron microscopy, immuno-electron microscopy and the application of protein and ionic tracers as a functional assessment of barrier properties of brain microvessels.

Special goals are to:

1. understand the ultrastructure of the BBB in normal and pathological conditions
2. investigate the permeability of the BBB to macromolecules and small molecules/ions in normal and pathological conditions
3. study the expression of BBB markers, namely EBA and GLUT1 in normal and pathological conditions
4. study the function of EBA
5. compare the BBB to another known biological barrier, namely the blood-retinal barrier

CHAPTER 3
MATERIALS AND METHODS

3 Materials and methods

3.1 Materials

3.1.1 Animals

3.1.1.1 *Clostridium perfringens* type D epsilon prototoxin-induced oedema model

Hooded Wistar rats of both sexes (23 female and 20 male) aged 7-9 weeks (140-220g) were used in this study. Animals were supplied and maintained under standard conditions by the Animal Houses of the University of Adelaide and Institute of Medical and Veterinary Science (IMVS). The experiment was approved by the Animal Ethics Committees of the University of Adelaide (S/56/98) and IMVS (80/98) and conducted under the guidelines of the NH&MRC of Australia.

3.1.1.2 *Traumatic brain injury model*

Thirty-one adult male Sprague-Dawley rats (380g-400g) were used. Animals were supplied and maintained under standard conditions by the Animal House of the University of Adelaide. The experiment was approved by the Animal Ethics Committee of the University of Adelaide (M/24/98) and conducted under the guidelines of the NH&MRC of Australia.

3.1.1.3 *In vivo immunological targeting of EBA model*

Thirty-six adult male Sprague-Dawley rats (350g-380g) were used. Animals were supplied and maintained under standard conditions by the animal house of the University of Adelaide. The experiment was approved by the Animal Ethics

Committee of the University of Adelaide (M/59/99) and conducted under the guidelines of the NH&MRC of Australia.

3.1.2 *Clostridium perfringens* type D epsilon prototoxin

Clostridium perfringens type D epsilon prototoxin was obtained from the Commonwealth Serum Laboratories Ltd, Victoria, Australia, and was supplied as inactive culture supernatant.

3.1.3 Antibodies and antisera

3.1.3.1 Primary antibodies and antisera

(1) Mouse anti-EBA antibody

Anti-EBA antibody (SMI 71) was purchased from Sternberger Monoclonal Inc., USA. The antibody is a mouse anti-rat, IgM monoclonal antibody (mAb), supplied as a high titre mouse ascites fluid. This antibody is species-specific for rat, used at a dilution of 1:3000 in 1% normal horse serum.

(2) Rabbit anti-GLUT1 antiserum and goat anti-GLUT1 antibody

Rabbit anti-GLUT1 antiserum was provided by Dr. F Maher, University of Melbourne. This antiserum was raised in rabbit against the C-terminal sequence of GLUT1. It recognises rat, mouse and human GLUT1, used at a dilution of 1:1000 in 1% normal goat serum.

Goat anti-GLUT1 polyclonal antibody (SC1605) was purchased from Santa Cruz Biotechnology, Inc. It is an affinity purified goat polyclonal antibody raised

against a peptide mapping within the carboxy terminal, cytoplasmic domain of the GLUT1 precursor of human origin (identical to corresponding rabbit sequence), used at a dilution of 1:2000 in 1% normal horse serum.

(3) Goat anti-rat albumin antiserum

Anti-rat albumin antiserum was purchased from ICN Pharmaceuticals Inc., USA. This antibody is raised in goat, recognises rat endogenous albumin and has been widely used in immunocytochemistry studies. It was used at a dilution of 1:20,000 in 1% normal horse serum.

(4) Mouse anti-rat endothelial cell-specific antigen 1 (Anti-RECA-1) antibody

Mouse anti-rat RECA-1 antibody was purchased from Serotec Ltd., UK. Mouse anti-RECA-1 is a pan-endothelial cell specific IgG₁ mAb, clone HIS52, which recognises a surface membrane protein on ECs of the rat (Duijvestijn *et al.*, 1992). It was used at a dilution of 1:20 in 1% normal horse serum.

(5) Mouse anti-human cytokeratin antibody

Mouse anti-human cytokeratin antibody was obtained from Dako Ltd, USA. It is an IgM mAb, clone 35 β H11, raised in mice against human cytokeratin 8, and supplied as culture supernatant.

(6) Mouse anti-Salmonella antibody (SAL-3)

Mouse anti-Salmonella antibody (SAL-3) was a generous gift from Dr LK Ashman, IMVS, Adelaide. It is an IgM mAb against Salmonella bacterial antigen, supplied as culture supernatant.

3.1.3.2 Secondary antibodies

Biotinylated anti-mouse IgG, biotinylated anti-rabbit IgG, and biotinylated anti-goat IgG were purchased from Vector Laboratories (Burlingame, CA, USA).

Gold conjugated anti-mouse IgM was purchased from ICN Pharmaceuticals Inc., USA. Gold particles were 10 nm in diameter.

3.1.4 Biochemical reagents

3.1.4.1 Immunochemicals

The avidin-biotin-horseradish peroxidase complex (ABC kit) and streptavidin conjugated peroxidase were obtained from Vector Laboratories.

Normal horse serum, normal rabbit serum, and normal goat serum were obtained from Vector Laboratories.

3.1.4.3 BBB tracers

Horseradish peroxidase type II (HRP, MW 40,000) was purchased from Sigma, USA. HRP is a glycoprotein which is widely used as a macromolecular tracer for barrier studies.

Lanthanum chloride was purchased from Sigma, USA. Lanthanum chloride, MW 374, is widely used as a small molecular tracer for barrier studies.

3.1.4.4 Resins

TAAB resin was obtained from TAAB Laboratories Equipment Ltd, UK. LR white resin was purchased from ProSci Tech Ltd, Australia.

3.2 Methods

3.2.1 Animal models

3.2.1.1 *Clostridium perfringens* type D epsilon prototoxin-induced oedema

Hooded Wistar rats (23 females and 20 males, 7-9 weeks old, 140-220g) were used in this study. Experimental rats were injected intraperitoneally with epsilon prototoxin diluted in physiological saline (4ml of 1:500, n = 9 or 1:400, n = 27). The control rats were either not injected (n = 5) or injected with an equal volume of the vehicle (n = 2). After the injection, daily consumption of water and food by the animals was monitored. At certain time points after the injection, 1h (1:400, n = 3, 1:500, n = 1), 6h (1:400, n = 3, 1:500, n = 1), 9h (1:500, n = 1), 12h (1:400, n = 3, 1:500, n = 1), 18h (1:500, n = 1), 24h (1:400, n = 4, 1:500, n = 1), 48h (1:400, n = 3, 1:500, n = 1), 4d (1:400, n = 3, 1:500, n = 1), 7d (1:400, n = 4, 1:500, n = 1), and 14d (1:400, n = 4), the rats were deeply anaesthetised with pentobarbitone (Nembutal, 60mg/Kg, Rhone Merieux Australia) and killed by transcardiac perfusion. Brain tissues removed from the 1:500 group animals were used for both light (n = 9) and electron (n = 9) microscope immunocytochemical studies. In the 1:400 group, one animal from each time interval was used for

ultrastructural study only (n = 8). The brain tissues from the other animals in the 1:400 group were used for both light (n = 19) and electron (n = 19) microscope immunocytochemical studies.

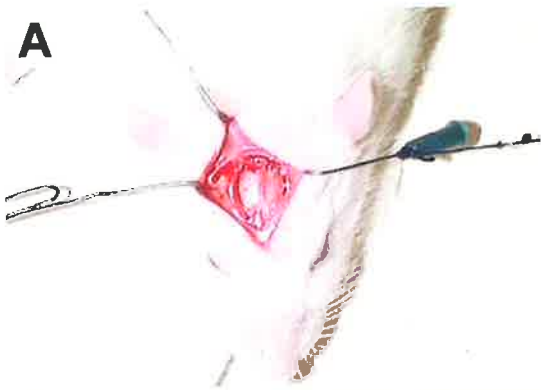
3.2.1.2 Traumatic brain injury

The traumatic injury model used in this study was first described by Marmarou and co-workers (Foda & Marmarou, 1994; Marmarou *et al.*, 1994). First, Sprague-Dawley rats (380-450g) were deeply anaesthetised by inhalation of isoflurane. Then an endotracheal tube was inserted into the trachea and connected to a Harvard rodent ventilator (model 683). Animals were mechanically ventilated with 1-1.5% isoflurane in oxygen throughout the experiment and the rectal temperature was kept at 36.5-37.5°C by keeping the animals under a heating lamp. Femoral arterial blood pressure was monitored in some experimental animals before and after the injury. A midline incision was performed in the scalp to expose the skull between the coronal and lambdoid sutures (Fig 3.1 A). The periosteum covering the vertex was reflected. After thoroughly drying the skull, a stainless-steel disc was fixed to the central portion of the skull vault of the rat between the coronal and the lambdoid sutures (Fig 3.1 B). The endotracheal tube was disconnected from the ventilator and the animal was quickly transferred to the trauma device (Fig 3.1 C). The injury was induced by allowing a metal rod weighing 450 g to fall freely from a height of 1.5 meters inside a guide tube onto the protective disc fixed to the animal's skull (Fig 3.1 D). After the trauma, the animal was removed from the device and the endotracheal tube reconnected to the ventilator. Experimental animals were allowed to survive

for 1h (n = 8), 6h (n = 5) and 12h (n = 3) before sacrifice by perfusion. Experimental animals were divided into three groups. Group 1 was used for ultrastructural study (1h, n = 1; 6h, n = 1; 12h, n = 1). Group 2 was used for the lanthanum permeability study (1h, n = 2; 6h, n = 2). Group 3 was used for the light and electron microscope immunocytochemical study (1h, n = 5; 6h, n = 2, 12h, n = 2). The controls included sham (n = 9) and normal non-operated controls (n = 7). In the sham controls, animals underwent the same endotracheal intubation and surgical procedure. After fixing the protective disc on the skull of the animal, the endotracheal tube was disconnected and the animal transferred to the trauma device for a similar time, but without the impact injury. The animal was then removed from the device and the endotracheal tube reconnected to the ventilator, as described previously. Sham control animals were divided into three groups. Group 1 was used for the ultrastructural study (1h, n = 1; 6h, n = 1). Group 2 was used for the lanthanum permeability study (1h, n = 1; 6h, n = 1). Group 3 was used for the light and electron microscope immunocytochemical study (1h, n = 2; 6h, n = 2; 12h, n = 1). In normal controls, animals were anaesthetised and perfused with fixative without intubation or surgery. Normal control animals were also divided into three groups: group 1 was used for the ultrastructural study (n = 1), group 2 for lanthanum study (n = 2), and group 3 was used for the light and electron microscope immunocytochemical study (n = 3).

FIG. 3.1. Surgical procedures of the traumatic brain injury model.

- (A) The skull between the coronal and the lambdoid sutures was exposed and the periosteum was reflected in experimental and sham control animals.
- (B) A stainless-steel disc (10mm in diameter and 3mm in thickness) was fixed to the central portion of the skull vault.
- (C) The animal was disconnected from the ventilator and transferred to the trauma device.
- (D) A metal rod weighing 450g was allowed to fall freely within a guide tube from a height of 1.5 meters onto the metal disc.



3.2.1.3 *In vivo immunological targeting of EBA*

Thirty-six Sprague-Dawley rats (350-380g) were used in this study. Animals were anaesthetised by intraperitoneal injection of pentobarbitone sodium ($n = 20$, 60mg/kg), or Urethane ($n = 16$, 1.3g/kg, Sigma, USA). Under anaesthesia, experimental animals were intravenously injected with 20 μ l of anti-EBA antibody in 0.4 ml phosphate-buffered saline (PBS) through the femoral vein ($n = 14$). Control animals received intravenous injections of either: (a) the same amount of the vehicle ($n = 5$), (b) 20 μ l of anti-human cytokeratin 8 in 0.4 ml PBS ($n = 5$), (c) 20 μ l of SAL-3 in 0.4 ml PBS ($n = 5$), or (d) 20 μ l of anti-RECA-1 in 0.4 ml of PBS ($n = 7$). The injection times ranged from 5 to 12 minutes (min) with a further circulation time of 5 min. In each group, some animals (anti-EBA, $n = 9$; PBS, $n = 3$; SAL-3, $n = 2$; anti-cytokeratin, $n = 2$; anti-RECA-1, $n = 5$) were injected intravenously with HRP 0.5 ml (200mg/kg in physiological saline, pH 7.0), a circulation time of 15 min was allowed before perfusion. Animals were sacrificed by transcardiac perfusion.

3.2.2 *Animal perfusion*

Animals were sacrificed by transcardiac perfusion. Under deep anaesthesia, the thoracic cage was quickly opened. A wide bore needle was inserted into the left ventricle and the right atrium was cut. Under constant pressure of 90 mm Hg, the vascular bed was washed with 37°C Dulbecco's phosphate buffer (containing 1% Minimum Essential Medium (Sigma) and gassed with 95%O₂ : 5%CO₂) for 30 seconds, followed by perfusion with cold fixative. For the animals used for

immunocytochemistry, the fixative contained 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). For the animals used in the ultrastructural study, the fixative contained 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The rate of perfusion was adjusted to allow a fast flow for the first 300 ml of fixative (5 min), followed by 400 ml at a slow rate (30 min), with a total perfusion time of 35 min.

In the traumatic brain injury model, lanthanum chloride was used as a small molecular tracer for the BBB. As lanthanum chloride could be precipitated by contact with phosphate buffer, sodium cacodylate buffer was used (cacodylic acid, Prosci Tech, Australia). The animals used for the lanthanum tracer study were perfused with physiological saline (pH 7.4), oxygenated with pure medical oxygen, followed by fixation with 300 ml 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Then 300 ml of the fixative containing 20 mM lanthanum chloride in 0.1 M sodium cacodylate buffer (pH 6.9) was perfused. After the perfusion, the brain was removed and immersed in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 hours at 4°C to precipitate the lanthanum *in situ*.

3.2.3 Tissue preparation

After perfusion, the brains were removed and the eyes were removed in the *in vivo* immunological targeting of EBA model. Tissues were immersed in the same fixative used in perfusion (except in lanthanum tracer study, see 3.2.2) for 3 hours at 4°C.

3.2.3.1 Brain

Brains were divided in the median plane and tissues were prepared according to the following protocol.

- 1) For immunocytochemistry, a 2 mm thick sagittal block of the brain was embedded in paraffin. Paraffin sections of 7 μm thickness were cut and mounted on silane coated slides. Sections of 50 μm thickness were cut from the other half of the brain using a Vibratome (Lancer Laboratory Supply, USA). Vibratome sections were floated in PBS.
- 2) For the ultrastructural study and lanthanum tracer investigation, small blocks taken from different parts of the brain were post-fixed with 1% osmium tetroxide for 1 hour, followed by graded alcohol dehydration and were embedded in TAAB resin. Ultrathin sections were cut using a diamond knife on a Leica Ultracut microtome. Sections were counterstained with uranyl acetate and lead citrate or only lightly counterstained with uranyl acetate for the lanthanum tracer study.

3.2.3.2 Eyes

The retina was removed as a whole-mount from one eye, according to a method described before (Chan Ling, 1997). The eyeball was transferred to a petri dish containing PBS after removal from the orbit. An incision was made in the cornea to allow the release of aqueous humor. The cornea was removed by cutting circumferentially along the limbus. After careful removal of the iris, lens and

vitreous body, radial cuts were made through all three tunics of the eye and the whole retina was carefully removed from the choroid and sclera.

The other eye was cut into two halves and embedded in paraffin. Sections of 7 μm thickness were cut and mounted on silane coated slides.

3.2.4 Immunocytochemistry

3.2.4.1 Light and electron microscope immunocytochemistry for EBA

The indirect immunoperoxidase method was employed. Paraffin sections were deparaffinised and hydrated. Both paraffin and Vibratome sections were rinsed in PBS. To eliminate endogenous peroxidase activity, sections were incubated in 0.3% hydrogen peroxide in distilled water for 30 min. After washing in PBS, sections were incubated in 10% normal horse serum (NHS) to reduce non-specific protein binding (60 min for Vibratome sections and 30 min for paraffin sections). Sections were then incubated in the anti-EBA monoclonal antibody diluted 1:3000 in 1% NHS, at 4°C overnight with agitation. Thereafter, sections were washed in PBS and incubated in a biotinylated horse anti-mouse IgG secondary antibody diluted 1:200 in 1% NHS, 60 min for Vibratome sections and 30 min for paraffin sections. Following thorough washing in buffer, the bound secondary antibody was detected by one of the following two methods.

- 1) Avidin-biotin peroxidase method (ABC kit, Vector Laboratories). Sections were incubated in ABC complex for 60 min for Vibratome sections and 30 min for paraffin sections. The peroxidase reaction product was visualised by using 3,3'-diaminobenzidine tetrahydrochloride as chromogen (DAB, Sigma).

Sections were incubated in 0.05% DAB in Tris buffer (pH 7.4) for 10 min then in 0.05% DAB containing 0.009% H_2O_2 for 5-10 min. Nickel enhancement using the glucose oxidase method was employed in some sections (Shu *et al.*, 1988). The reaction was stopped by washing the sections in PBS.

- 2) Peroxidase conjugated streptavidin method. Sections were incubated in peroxidase conjugated streptavidin diluted 1:1000 in 1% NHS for 60 min. After washing, sections were incubated in 0.05% DAB in 0.1 M Tris buffer (pH 7.6) containing 0.009% H_2O_2 for 7 min. The reaction was stopped by washing the sections in PBS.

Controls for the specificity of the immunostaining consisted of staining some sections without primary antibody and others without both the primary and secondary antibodies.

After being thoroughly washed in PBS, paraffin sections were dehydrated, cleared in Histo-clear and covered with coverslips. Some sections were counterstained lightly with haematoxylin.

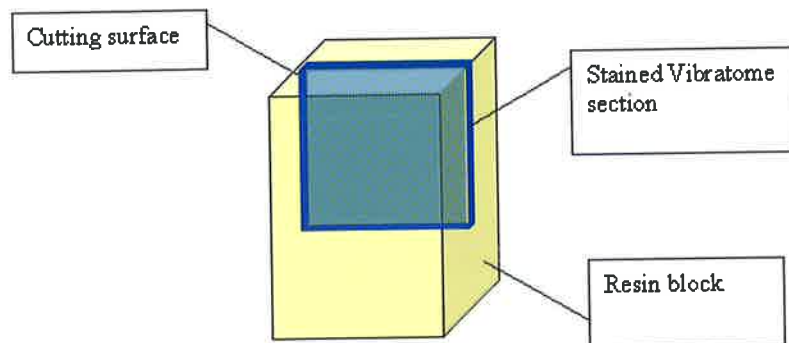
Vibratome sections used for light microscope (LM) examination were mounted on gelatine coated slides. After air-drying overnight, sections were dehydrated, cleared and covered with coverslips. Vibratome sections used for electron microscopic (EM) examination were osmicated, dehydrated and processed in TAAB resin. Before polymerisation small rectangles 1×2 mm were cut out from

representative areas of the brain: grey matter and white matter of cerebral hemispheres, cerebellum, diencephalon and brainstem. These tissue blocks were embedded in TAAB resin and polymerised. Sections were embedded vertically in the block (Fig 3.2) using a method described previously (Ghabriel *et al.*, 1994). Ultrathin sections (approximately 90 nm) were cut. Sections, counterstained with uranyl acetate for 3 min or without counterstaining, were viewed in a Philips CM 100 electron microscope.

FIG. 3.2.A. Diagram showing a small rectangular piece from immunostained Vibratome sections vertically embedded in resin block. Ultrathin sections were then cut at a plane perpendicular to that of the Vibratome section.

FIG. 3.2.B. 1 μm section obtained from resin-embedded immunostained Vibratome section. Arrows indicate blood vessels in the section. Toluidine blue stain. Bar, 100 μm .

A



B



3.2.4.2. Light and electron microscope immunocytochemistry for GLUT1

The indirect immunoperoxidase method was employed. Paraffin sections were deparaffinised and hydrated. Both paraffin and Vibratome sections were rinsed in PBS. To eliminate endogenous peroxidase activity, sections were incubated in 0.3% hydrogen peroxide in 50% ethanol in distilled water for 30 min. After several washes in PBS, paraffin sections were microwaved in citrate buffer (pH 6) for 10 min. After the sections had cooled down to room temperature, they were washed in PBS. For Vibratome sections, the microwaving procedure was omitted. After washing, sections were incubated in 10% normal goat serum (NGS) or 10% NHS to reduce non-specific protein binding (60 min for Vibratome sections and 30 min for paraffin sections). Then, sections were incubated in the anti-GLUT1 (1:1000 in 1% NGS for anti-GLUT1 antiserum or 1:2000 in 1% NHS for anti-GLUT1 polyclonal antibody) at room temperature overnight with agitation. Thereafter, sections were washed in buffer and incubated in a biotinylated goat anti-rabbit IgG secondary antibody diluted 1:200 in 1% NGS, or biotinylated horse anti-goat IgG secondary antibody (60 min for Vibratome sections and 30 min for paraffin sections). Following thorough washing in buffer, the bound secondary antibody was detected by methods described in 3.2.4.1. Paraffin and Vibratome sections were prepared for LM and EM examination as described in 3.2.4.1.

3.2.4.3 Light and electron microscope immunocytochemistry for endogenous albumin

The indirect immunoperoxidase method was employed. Paraffin sections were deparaffinised and hydrated. Both paraffin sections and Vibratome sections were rinsed in PBS. To eliminate endogenous peroxidase activity, sections were incubated in 0.3% hydrogen peroxide in distilled water for 30 min. After washing in PBS, sections were incubated in 10% NHS to reduce non-specific protein binding, 60 min for Vibratome sections and 30 min for paraffin sections. Then, sections were incubated in the goat anti-rat albumin 1:2000 in 1% NHS, for paraffin sections, or 1:20,000 for Vibratome sections at room temperature overnight with agitation. Thereafter, sections were washed in buffer and incubated in a biotinylated horse anti-goat IgG secondary antibody diluted 1:500 in 1% NHS, 60 min for Vibratome sections and 30 min for paraffin sections. Following thorough washing in buffer, the bound secondary antibody was detected by methods described in 3.2.4.1.

After immunostaining, paraffin and Vibratome sections were prepared for LM and EM examination as described in 3.2.4.1.

3.2.4.4 Post-embedding immunogold staining for EBA

Four Hooded wistar rats were used for the immunogold study for EBA. Animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Small blocks from the cortex were dehydrated and embedded in LR white resin. Ultrathin sections were cut and collected on nickel coated grids. Indirect

immunogold method was employed. Sections were placed in a moist chamber on a drop of 10% normal goat serum to block non-specific binding for 30 min. Then sections were incubated in the primary antibody, anti-EBA, diluted 1:100 in 1% normal goat serum overnight at 4°C. Sections were washed with the following solutions: (1) 0.05 M Tris buffer (pH 7.4), 5×1 min, (2) Tris buffer with 0.1% bovine serum albumin (BSA, pH 7.4), 3×1 min, (3) Tris buffer containing 1% BSA (pH 8.2) for 15 min. Sections were then incubated in gold (10 nm) conjugated goat anti-mouse secondary antibody diluted 1:20 in Tris buffer containing 1% BSA (pH 8.0) for 1 hour. Sections were thoroughly washed in Tris buffer (Tris buffer with 0.1% BSA, pH 7.4, 3×1 min followed by Tris buffer without BSA, pH 7.4, 3×1 min) and distilled water (5×1 min). Sections were lightly counterstained with uranyl acetate for 3 min and examined in a Philips CM 100 electron microscope. Control sections for the specificity of the immunostaining were stained with gold conjugated anti-mouse antibody only.

3.2.5 Histochemistry for HRP study

Some animals in the *in vivo* immunological targeting of EBA model were intravenously injected with HRP. Vibratome sections from the brain and wholemount retinas from the eyes were reacted with DAB to detect HRP leakage.

- 1) Brain sections were incubated in 0.05% DAB for 10 min then incubated in 0.05% DAB containing 0.009% H₂O₂ for 5-10 min. Some brain sections were incubated in 0.05% DAB containing nickel ammonium sulphate, and the glucose oxidase method was used to visualise the reaction. After the reaction, sections for LM were mounted on gelatine coated slides, air dried overnight,

dehydrated, cleared and covered with coverslips. Sections for EM were osmicated, dehydrated and embedded in TAAB resin.

- 2) Whollemount retinas were washed in 0.1 M Tris buffer (pH 7.4) for at least 1 hour before being incubated in DAB solution in Tris buffer for 30 min. Then the retinas were incubated in 10 ml DAB solution in Tris buffer containing 100 μ l 3% H₂O₂ for 10-15min. After washing in Tris buffer, the retinas were mounted on gelatine coated slides, air dried overnight, dehydrated, cleared and covered with coverslips.

3.2.6 Quantitative assessment of EBA immunoreactivity

Quantitative assessment of EBA immunoreactivity was performed at the LM and EM levels for traumatic brain injury model and at the EM level for *Cl p* prototoxin-induced oedema model.

3.2.6.1 Quantitative assessment of EBA immunoreactivity at the LM level

Non-counterstained sections were viewed in an Olympus microscope equipped with an Olympus digital camera. Five microscopic fields from the frontal cortex and five fields from the brainstem were photographed using 20 $\times$ objective lens. The digital images (final magnification $\times$ 140) were transferred to a computer screen, enlarged and analysed using an image analysis system (Measure Master, Leading Edge Technology, SA, Australia). A scale bar was added to the image according to the final magnification. Then the image analysis system was calibrated according to the scale bar. The field area of each image was 0.145 mm². The number of EBA-immunoreactive blood vessels contained in this area

was counted. The results from the five fields in each region were pooled and the Mean $\pm$ SD per mm² was obtained.

3.2.6.2 Quantitative assessment of EBA immunoreactivity at the EM level

Ultrathin sections immunostained for EBA and without heavy metal counterstaining were viewed in a Philips CM100 transmission electron microscope. Blood vessels were photographed. An image analysis system (analySIS, Soft Imaging System GmbH, Germany) was used to quantify the percentage of length of EC luminal membranes, which showed immunoreactivity for EBA in relation to vessel luminal circumference. The image analysis system was calibrated according to the scale bars printed on the negatives automatically by the electron microscope which show the final magnification of the image.

Percentage of immunoreactive luminal membrane length = stained length of EC luminal membrane/perimeter of the blood vessel $\times 100$

The percentage of immunoreactive luminal membrane length was plotted for individual vessels. The results from all vessels in each animal were pooled and compared to the results of the control animals.

3.2.7 Quantitative assessment of GLUT1 immunoreactivity

Quantitative assessment of GLUT1 immunoreactivity was performed at the LM level for *Cl p* type D epsilon prototoxin-induced oedema model and at both the LM and EM levels for the traumatic brain injury model.

3.2.7.1 Quantitative assessment of GLUT1 immunoreactivity at the LM level

For the *Cl p* type D epsilon prototoxin-induced oedema model, paraffin brain sections were viewed at 64× in an Olympus microscope. LM images from four areas, frontal cortex, inferior colliculus, cerebellum and brainstem, were taken. Immunoreactive blood vessels were counted from the developed negatives using a dissecting microscope and an overlaid graticule. For each brain area, the number of immunoreactive blood vessels per mm² was plotted against the time interval following *Cl p* prototoxin injection.

For GLUT1 quantitative assessment in the traumatic brain injury model, the same method employed for EBA in traumatic brain injury was used. For details see 3.2.6.1.

3.2.7.2 Quantitative assessment of GLUT1 immunoreactivity at the EM level

The same method used to analyse EBA immunoreactivity at the EM level was employed to assess the GLUT1 immunostaining at the EM level for the traumatic brain injury model (see 3.2.6.2).

CHAPTER 4

RESULTS

4 Results

4.1 Clostridium perfringens type D epsilon prototoxin-induced BBB opening

4.1.1 General conditions of experimental animals

All experimental animals survived the injection of *Cl p* prototoxin until electively sacrificed at various time intervals. Following the injection animals sustained a mild drop in weight (5-15g) for two days, after which they steadily gained weight (~10g/day). Control animals injected with the vehicle showed a mild drop in weight for one day (~5g). Experimental animals maintained their interest in the environment. Their eating and drinking habits and self-grooming were similar to those of the controls throughout the experiment.

4.1.2 Ultrastructural changes of the BBB in prototoxin-induced BBB opening

In control animals, non-fenestrated ECs lined the wall of brain capillaries (Fig. 4.1.1). Few vesicles were observed in the cytoplasm of ECs. No pits or indentations were noted. The cytoplasm of endothelial cells appeared evenly electron dense. Tight junctions were seen between endothelial cells. Occasionally, no tight junction was seen in apparently seamless capillaries. Pericytes were often seen embedded in the basal lamina of endothelial cells. Astrocytic endfeet covered ECs. No obvious extravascular space was observed.

In experimental animals, morphological changes in brain microvessels were observed by electron microscopy as early as 1h after *Cl p* prototoxin injection (Fig. 4.1.1). A few capillary profiles showed increased EC indentations, microvilli and

vesicles compared to control material. These changes were pronounced at 6h and 12h (Fig. 4.1.2). The most dramatic changes occurred at 24h and 48h (Fig. 4.1.2). Many ECs were swollen, depleted of organelles and had electron lucent cytoplasm (Fig. 4.1.2 F). Large vacuoles, endothelial deep craters, and occasional EC channels were present. Subendothelial clefts, which detached ECs from the basal lamina were seen (Fig. 4.1.2 C). The basal lamina of many ECs was thickened (Fig. 4.1.2 F). A few interendothelial tight junctions showed focal widening, although the majority of the junctions appeared intact (Fig. 4.1.2 D). The pericytes displayed similar pathological changes with many large vacuoles (Fig. 4.1.2 E). At 4d and 7d after the injection, in some areas, ECs were denuded and short stretches of the wall were formed only by the basal lamina forming the wall of the blood vessel (Fig. 4.1.3 B). Perivascular astrocytic processes appeared swollen (Fig. 4.1.3). Perivascular space was increased in some blood vessels, indicating oedema formation. At 14d post-injection, the ultrastructure of ECs had not normalised (Fig. 4.1.3 D). Many of the vessels examined showed increased cytoplasmic vesicles. Experimental animals injected with 1:400 dilution of prototoxin showed more severe pathological changes than the animals injected with 1:500 dilution of prototoxin at each time interval.

FIG. 4.1.1 (A-E). Electron micrographs of cerebral cortical microvessels from a normal control animal (**A-C**) and an experimental animal (**D** and **E**) at 1h after the injection of 1:400 epsilon prototoxin, showing ultrastructural changes.

(A and B) In normal conditions, ECs are thin and their cytoplasm is evenly electron dense, with a few vesicles. Pericytes (Per, **B**) are embedded in the EC basal lamina. Tight junctions (**A**) seal the interendothelial space.

(C) A high magnification shows a tight junction between ECs (arrows, luminal and abluminal ends of the tight junction).

(D) One hour after the injection of epsilon prototoxin, numerous indentations on the luminal membrane of ECs are seen (arrows).

(E) At 1h after the injection, a blood vessel shows increased microvilli (arrows) and vesicles [V].

BL, basal lamina; EC, endothelial cell; ICJ, intercellular junction; N, endothelial cell nucleus; Per, pericyte.

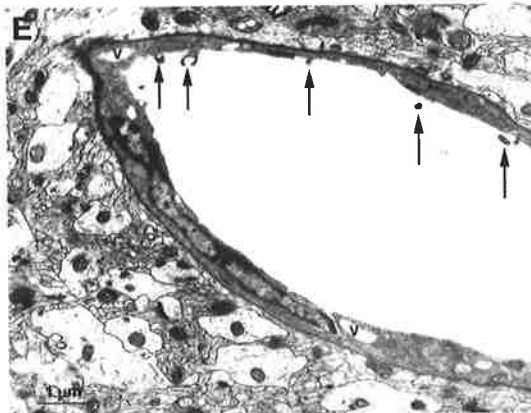
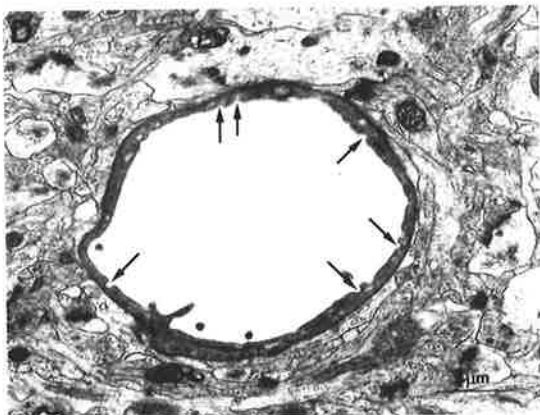
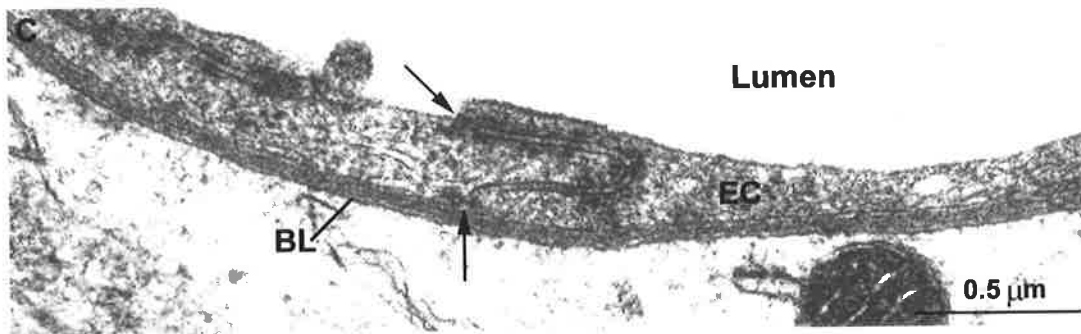
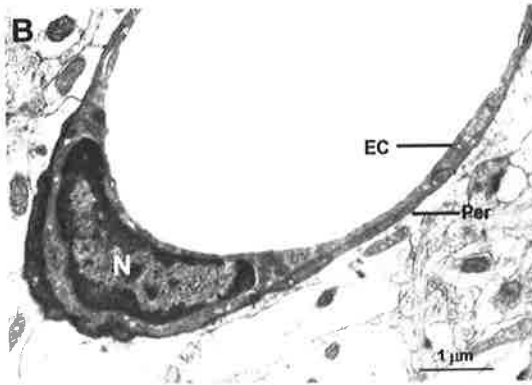
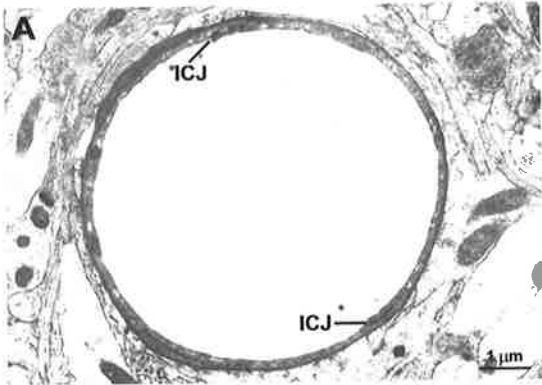


FIG. 4.1.2 (A-F). Electron micrographs of cerebral cortical microvessels from experimental animals with survival times of 12h (**A, B and D**), 24h (**C**) and 2 days (**E and F**) following the injection of 1:400 dilution of *Cl p* epsilon prototoxin, showing ultrastructural changes.

(A) At 12h after the injection, a vessel shows numerous vesicles (arrows) in the cytoplasm and microvilli (arrowheads) on the luminal membrane.

(B) Twelve hours after the injection, some ECs show translucent cytoplasm.

(C) At 24h after the injection, a blood vessel shows increased vesicles (arrows) and a subendothelial cleft (large arrow) in the EC.

(D) A tight junction shows focal widening (arrows) at 12h after the injection.

(E) At 2 days after the injection, large vacuoles [V] are seen in the cytoplasm of ECs and pericytes. A pericyte nucleus (Per N) appears homogeneously electron dense.

(F) At 2 days after the injection, a blood vessel shows translucent cytoplasm, due to the degeneration of organelles (asterisks). The basal lamina appears thickened (white arrows).

EC, endothelial cell; ICJ, intercellular junction; M, mitochondria; N, EC nucleus; Per, pericyte; Per N, pericyte nucleus; RBC, red blood cell; V, vacuole.

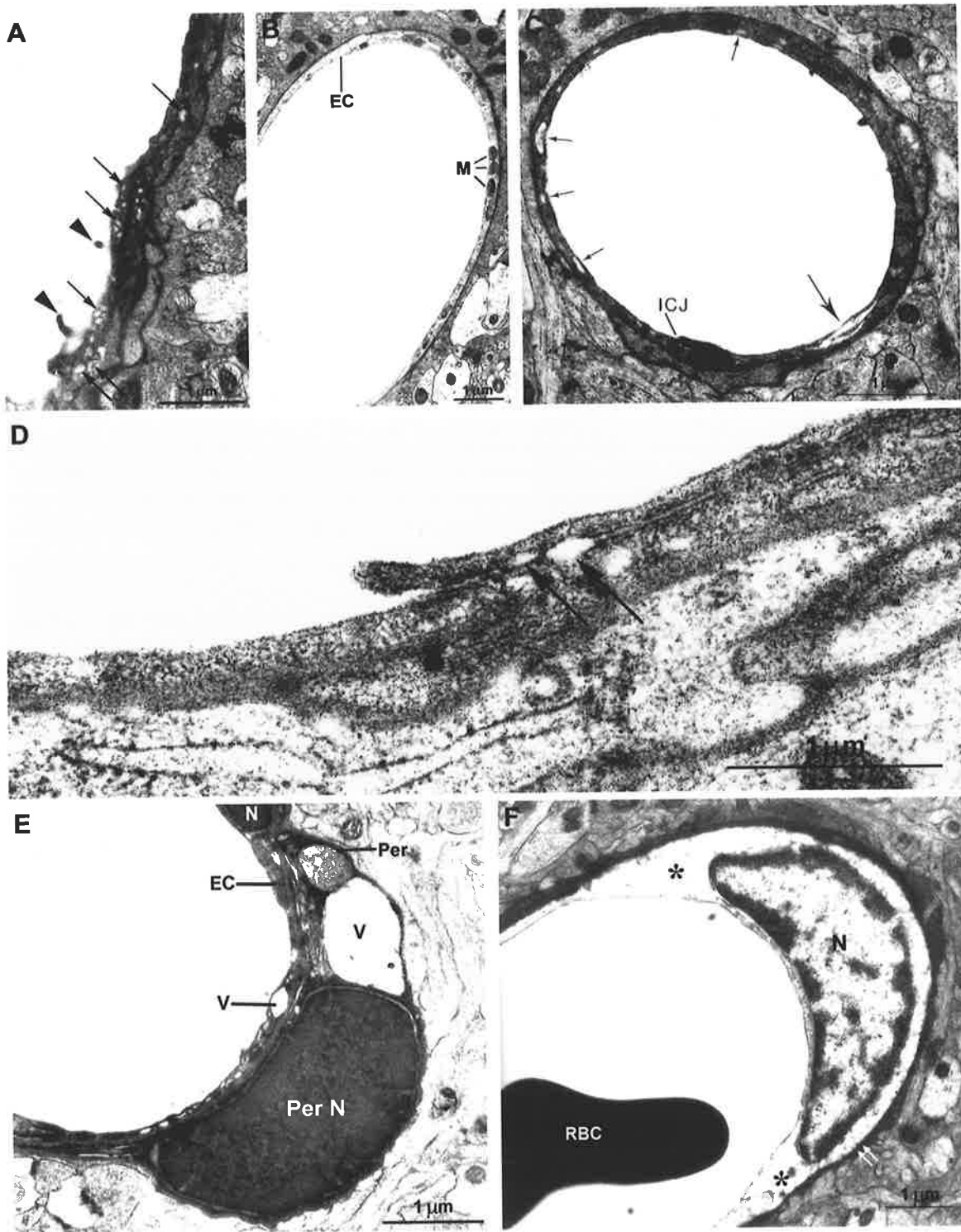


FIG. 4.1.3 (A-D). Electron micrographs of cerebral cortical microvessels from experimental animals with survival times of 4 days (**A**), 7 days (**B** and **C**), and 14 days (**D**) following the injection of 1:400 dilution of *Cl p* epsilon prototoxin, showing ultrastructural changes.

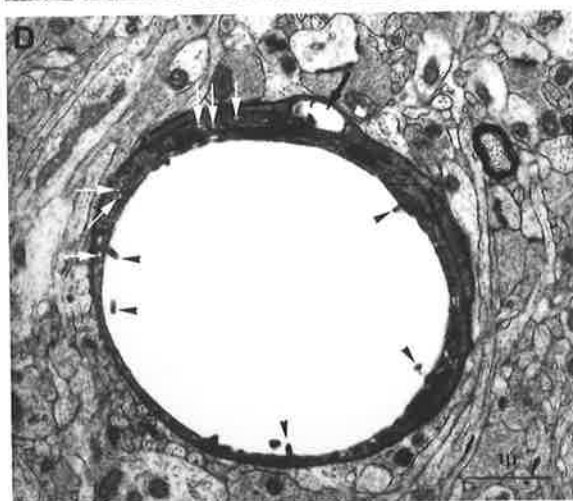
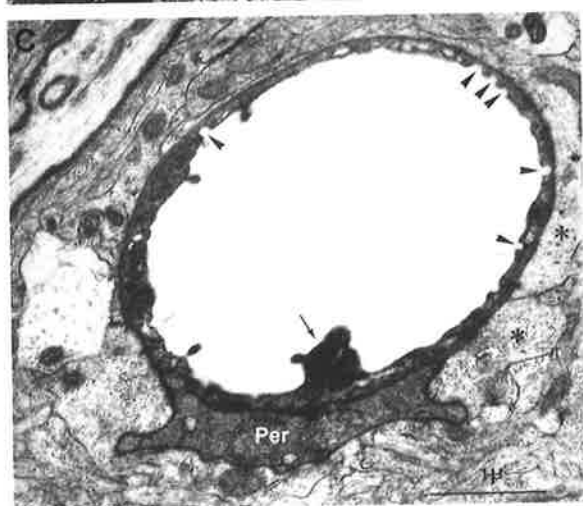
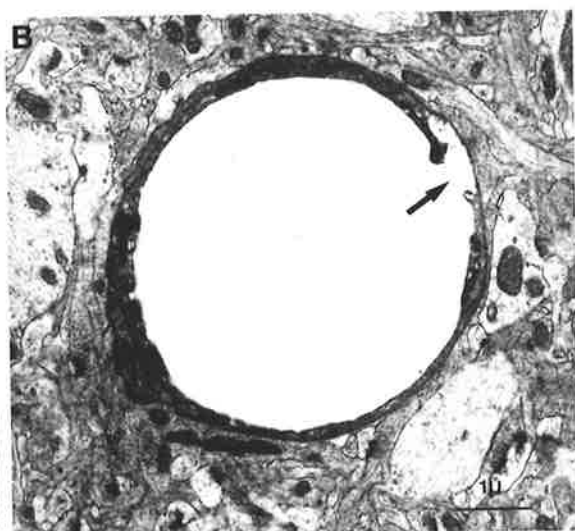
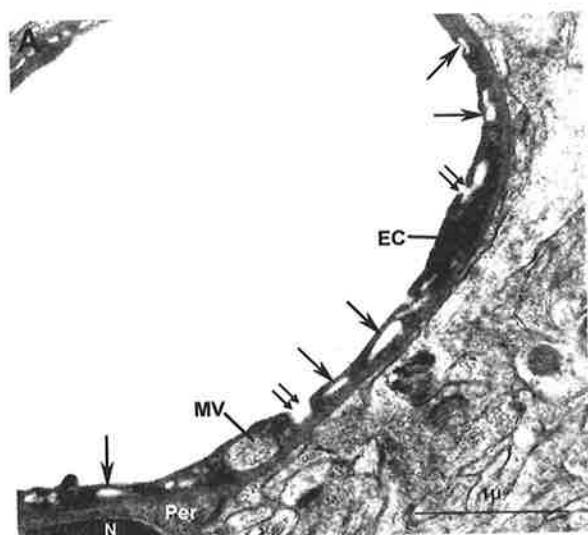
(**A**) At 4 days after the injection, several vesicles (arrows) and a multivesicular body (MV), presumably representing degenerated organelle are present in the cytoplasm of ECs. Some vesicles communicate with the lumen (double arrows).

(**B**) At 7 days after the injection, disruption of EC lining is seen (large arrow).

(**C**) At 7 days after the injection, a blood vessel shows many indentations on the luminal membrane (arrowheads) and luminal projection (arrow).

(**D**) At 14 days after the injection, a blood vessel shows increased microvilli (arrowheads) and vesicles (white arrows). A pericyte shows a large vacuole in the cytoplasm (arrow).

EC, endothelial cell; N, pericyte nucleus; Per, pericyte.



4.1.3 Permeability of the BBB in prototoxin-induced BBB opening

Permeability of the BBB was investigated by the presence of endogenous serum albumin in the parenchyma. Light microscopy of control brains showed no albumin immunoreactivity except in the choroid plexus, hypothalamus, and periventricular zones (Fig. 4.1.4). In these regions, albumin staining appeared as black reaction product. In some areas such as the superior cerebellar peduncles and some foci in the brainstem, higher immunoreactivity was seen. In occasional vessels the walls were highlighted by immunoreactivity. In general the white matter appeared to have faint immunoreactivity compared to the grey matter.

Light microscopy of experimental material demonstrated a dose and time dependant albumin leakage (Fig. 4.1.4). One hour after prototoxin injection, Vibratome and paraffin sections examined by light microscopy showed some stained blood vessels in the frontal cortex, brainstem and at the depth of cerebellar fissures, indicating intramural albumin. Brain sections from animals sacrificed at 6h, 9h, 12h, and 18h showed increased immunoreactivity to albumin. Perivascular areas were stained, indicating albumin leakage into the parenchyma. Many neurons showed immunoreactivity in the cytoplasm, but their nuclei were not stained. At 24h and 48h, albumin leakage plateaued at its maximum level (Fig. 4.1.4 C). The parenchyma throughout the brain showed reaction product with the presence of multiple foci of higher immunoreactivity in the cerebral cortex, brainstem and corpus callosum. Four days after injection, albumin leakage was still present but the intensity of immunoreactivity was reduced markedly. At 7d and 14d post injection, the staining was similar to that of control animals,

except for occasional foci seen in the cerebrum and brainstem. In animals injected with the higher concentration (1:400 dilution) of the prototoxin, albumin staining had a similar pattern but higher staining intensity.

The electron microscopic study of albumin distribution confirmed the results observed by light microscopy. In the electron microscope, albumin immunoreactivity appeared as electron dense deposits (Figs. 4.1.5 and 4.1.6). The lumina of microvessels were clear of any reaction product or blood cells in both control and experimental materials because of the perfusion fixation. Control material showed albumin deposits at the luminal surface of some ECs, but no albumin was seen in EC vesicles, cytoplasm or intercellular junctions (Fig. 4.1.5 A). Occasionally a small amount of reaction product was seen in the brain parenchyma. In experimental animals, albumin was noted in the cytoplasm of some ECs (Fig. 4.1.6). In severe cases, albumin was observed throughout the whole thickness of the EC (Fig. 4.1.6 C). Albumin was also observed in some vesicles in the cytoplasm of ECs although many vesicles were clear of albumin (Figs. 4.1.5 E and 4.1.6 A). Occasionally albumin was seen in intercellular junctions (Figs. 4.1.5 C and 4.1.6 A, B). Albumin was also present in the parenchyma around some blood vessels (Figs. 4.1.5 D, E and 4.1.6 C, D). The basal lamina appeared to be heavily infiltrated with albumin (Fig. 4.1.5B). In the parenchyma albumin was never seen as continuous electron dense deposits permeating all the extracellular space, even at the time intervals of maximal leakage. The reaction product in the parenchyma only circled some profiles of nerve fibres in the field.

FIG. 4.1.4 (A-F). Light micrographs of sagittal sections of paraffin-embedded brains from control (A) and experimental animals (B-F) injected with 1:400 dilution of *Cl p* epsilon prototoxin. The sections show immunostaining for endogenous albumin with nickel enhancement, but with no H & E counterstaining.

- (A) No albumin immunoreactivity is seen in the section from a control animal.
- (B) At 1h postinjection slight immunoreactivity is seen especially in the corpus callosum.
- (C) At 2 days after the injection, albumin immunoreactivity is visible as multiple foci of peroxidase reaction product in the corpus callosum and the cerebral cortex (arrows).
- (D and E) At 4 days (D) and 7 days (E) albumin immunoreactivity is still higher than the control (A) especially in the white matter (arrow).
- (F) At 14 days postinjection only few foci of immunoreactivity were detected (arrow).

CC, corpus callosum; CX, cortex. Bar, 200 μ m.

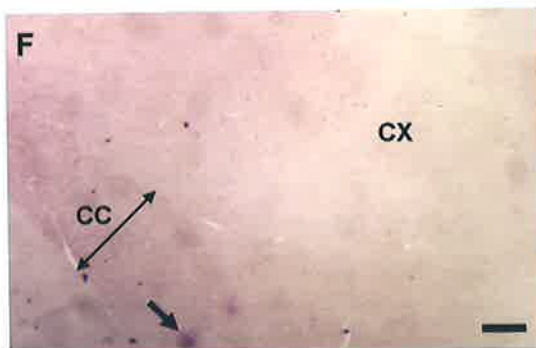
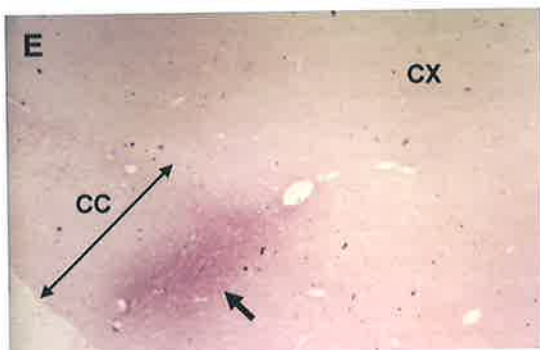
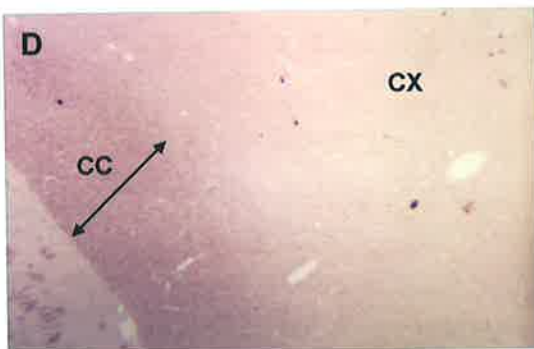
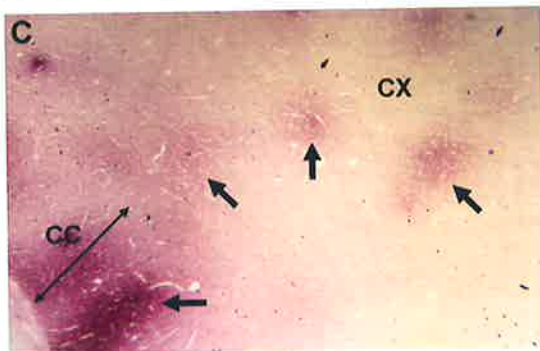
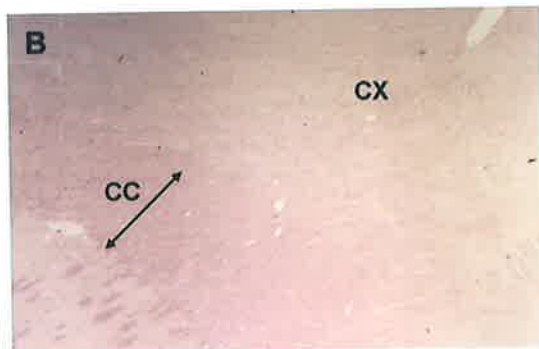
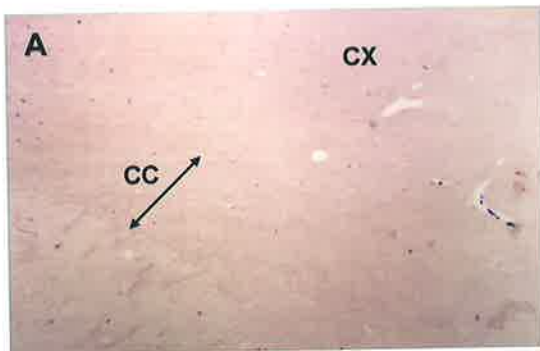


FIG. 4.1.5 (A-E). Electron micrographs of cerebral cortical vessels from normal control (**A**) and experimental animals (**B-E**) after the injection of 1:400 dilution of *Cl p* epsilon prototoxin, showing pre-embedding immunoperoxidase labelling for endogenous albumin.

(A-E) Electron dense deposits, representing albumin labelling, are seen at the luminal surface of ECs in all vessels (small black arrows).

(A) In the control animal, no labelling is seen in the parenchyma.

(B) At 6h after the injection, albumin labelling is seen in the parenchyma (arrowheads). The basal lamina appears thickened and immunoreactive to albumin (large black arrows).

(C) A high magnification shows that albumin infiltrates part of a tight junction (white arrow) and the basal lamina (large arrows) at 6h after the injection.

(D) At 12h after the injection, albumin labelling is seen in the perivascular space and parenchyma (large black arrows).

(E) Albumin immunoreactivity is seen in a cytoplasmic vesicle [V] and the parenchyma (large arrows) at 12 h after the injection.

BL, basal lamina; ICJ, intercellular junction.

Micrographs **A**, **D** and **E** are from grids not counterstained with heavy metals.

Micrographs **B** and **C** are from grids lightly stained with uranyl acetate.

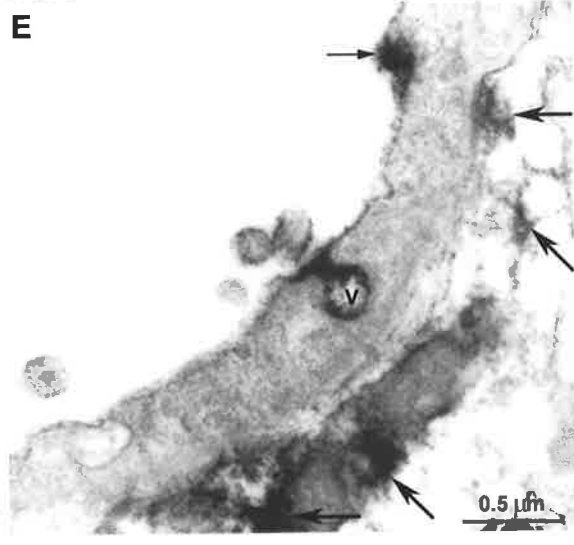
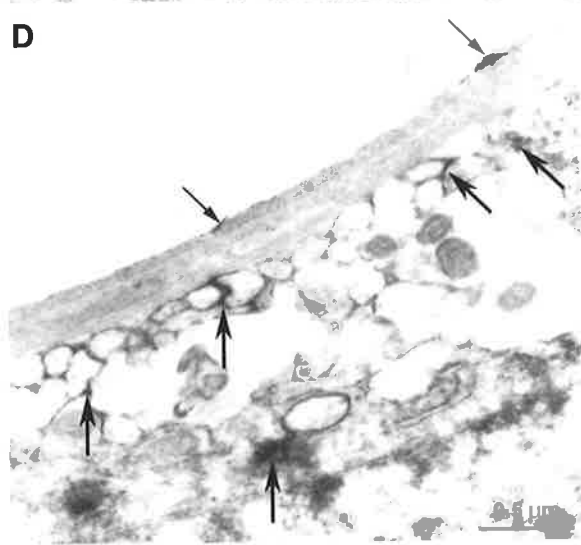
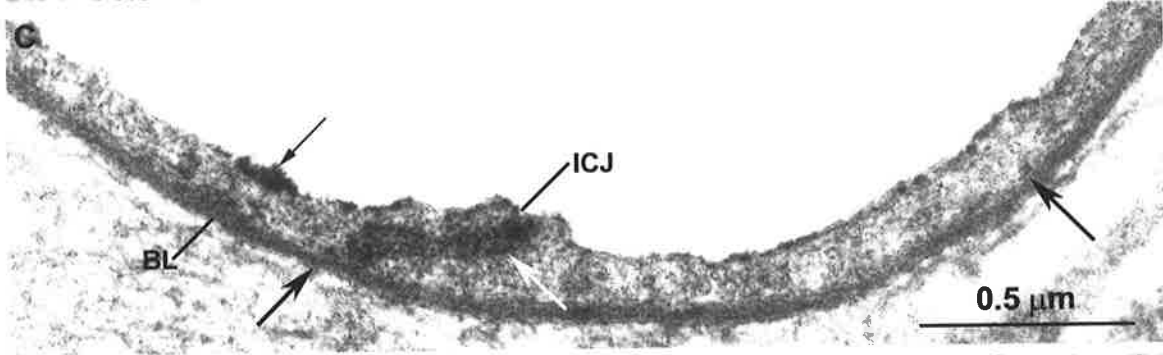
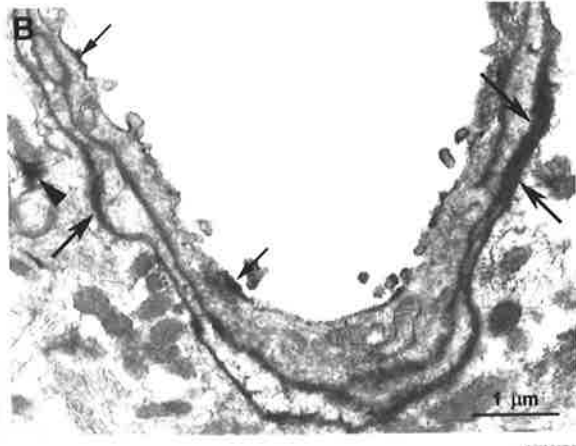
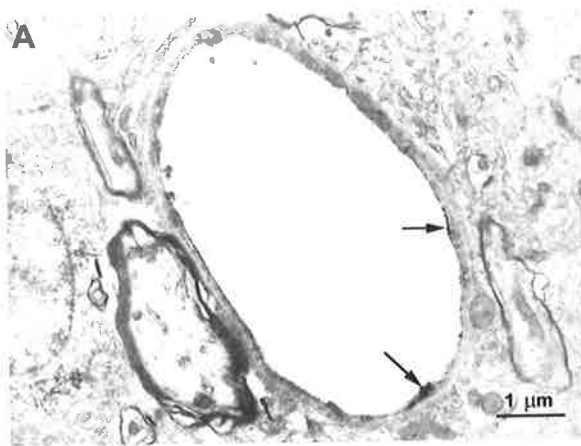
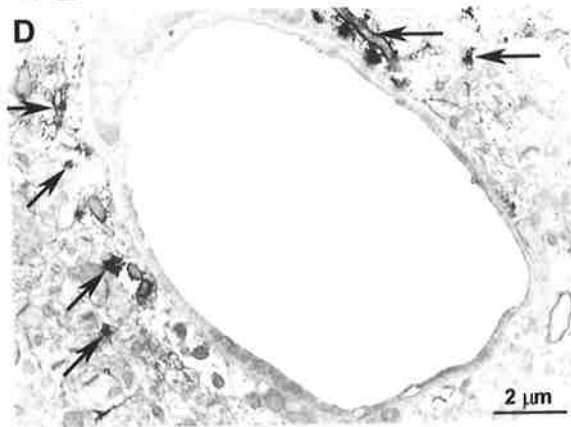
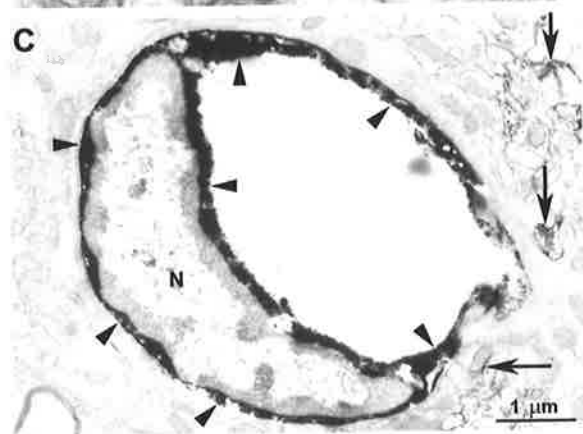
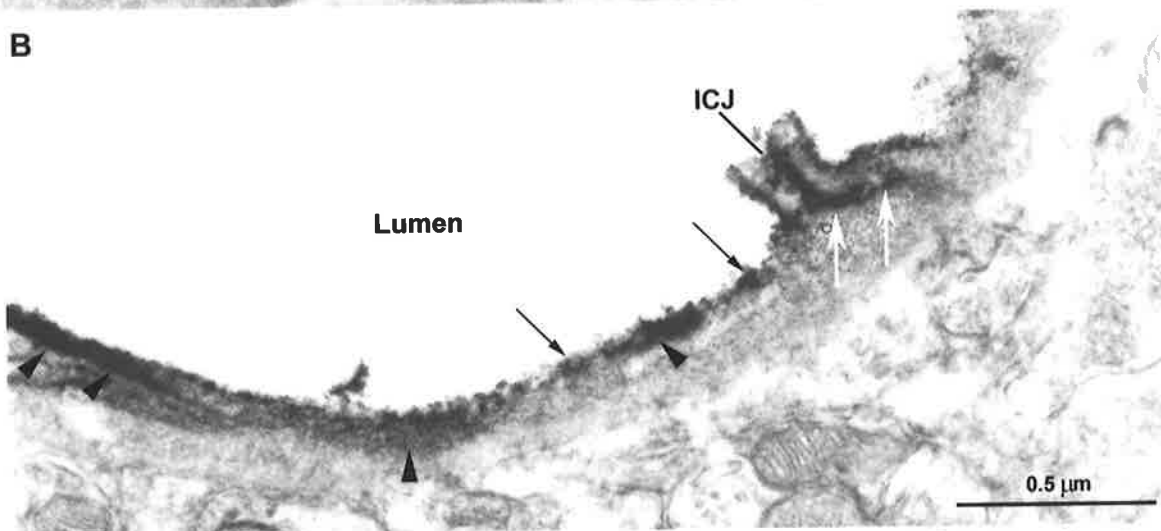
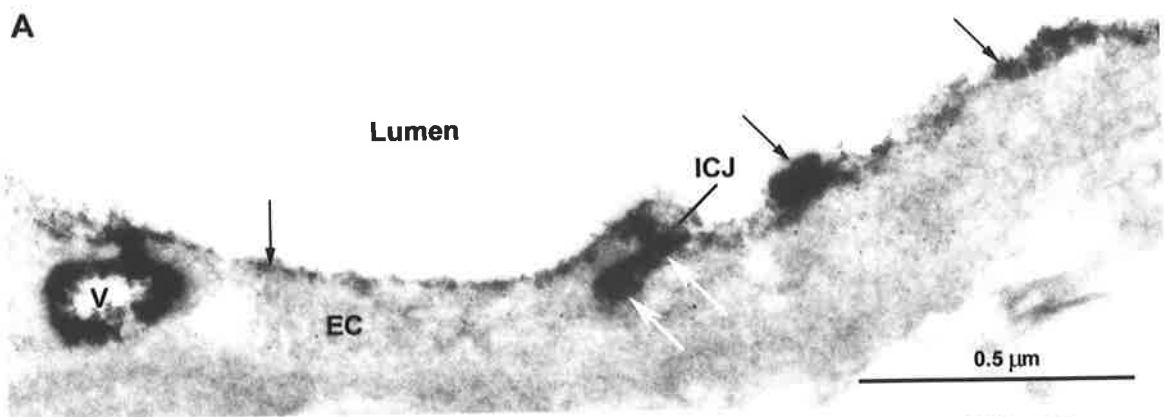


FIG. 4.1.6 (A-D). Electron micrographs of cerebral cortical vessels from experimental animals 24h (**A**), 2 days (**B** and **C**) and 14 days (**D**) after the injection of 1:400 dilution of *Cl p* epsilon prototoxin, showing pre-embedding immunoperoxidase labelling for endogenous albumin.

- (**A**) At 24h after the injection, albumin labelling is seen at the luminal surface (small arrows), within the luminal part of a tight junction (white arrows) and in a vesicle [V] of ECs.
- (**B**) At 2 days after the injection, albumin is seen at the luminal surface (small arrows) and in the cytoplasm (arrowheads) of ECs as diffuse labelling. White arrows indicate labelling in an intercellular junction.
- (**C**) At 2 days after the injection, a vessel shows albumin infiltration in the cytoplasm (arrowheads), indicating severely injured ECs. Albumin leakage in the parenchyma is also present (large arrows).
- (**D**) At 14 days after the injection, albumin is still seen in the parenchyma (large black arrows).

EC, endothelial cell; ICJ, intercellular junction; N, EC nucleus

All micrographs are from grids not counterstained with heavy metals.



4.1.4 EBA expression in prototoxin-induced BBB opening

4.1.4.1 EBA expression in controls

LM of paraffin and Vibratome sections from normal animals showed immunoreactivity in almost all vessels throughout the grey and white matter of the cerebrum, diencephalon, cerebellum and brainstem. Immunoreactive microvessels in the brain parenchyma and the pia mater, at the surface of the brain, were stained homogeneously dark brown (Fig. 4.1.7 A). Microvessels were seen in various orientations: transverse, oblique and longitudinal, with many vessels showing branching. Medium-sized vessels in the parenchyma and pia mater showed immunoreactivity at the plane of the intima. Larger vessels in the subarachnoid space were mostly unstained, but some showed discontinuous staining of the intima. Occasional vessels in the choroid plexus and neurohypophysis showed weak staining. The neuropil was clear and no staining of neurons or glial cells was noted. EBA negative controls, where the primary or the primary and secondary antibodies were omitted, showed no labelling of blood vessels.

EM of tissues immunostained for EBA showed electron dense peroxidase reaction product, which was especially apparent in sections not stained with heavy metals, due to better contrast (Fig. 4.1.8). The reaction product was essentially located at the luminal membrane of ECs. In most vessels the reaction product was seen along the entire circumference. However, the density of the reaction was not uniform in all ECs around the vessel circumference. A minority of ECs showed focal absence of the reaction product. In addition to luminal membrane staining,

the cytoplasm of many ECs showed staining. The staining involved the full thickness of the cytoplasm between the luminal and the abluminal membranes, or showed a gradient of staining fading away towards the abluminal membrane. EC vesicles were not stained and appeared electron lucent against the darkly stained cytoplasm. Some EC nuclei showed immunoreactivity on the side of the nucleus facing the lumen.

Post-embedding immunogold staining for EBA was performed on brain tissues from 4 normal animals. In normal brains, microvessels were observed to be highly immunoreactive to EBA. Immunogold particles were predominantly localised to luminal surface of brain microvessels (Fig. 4.1.9 A and B). To a less extent, gold particles were localised in the endothelial cell cytoplasm (Fig. 4.1.9 A and B). No immunogold particles were observed in the vesicles and within the tight junctions. Few gold particles were noted in the pericytes and brain parenchyma. Controls for the staining procedure where the primary antibody was omitted showed no gold particles in relation to brain blood vessels (Fig. 4.1.9 C).

4.1.4.2 EBA expression in experimental animals

In experimental animals, none of the time intervals examined showed complete elimination of EBA immunoreactivity in brain vessels (Fig. 4.1.7). At 1h post-prototoxin injection, brain EC immunoreactivity for EBA was similar to control material. However, some blood vessels were less reactive or non-reactive to EBA staining, as indicated by luminal membrane staining being limited to part of the circumference or completely absent throughout the circumference (Fig. 4.1.7 B).

At 6h to 2 days after the injection, more blood vessels were partially or weakly stained for EBA (Figs 4.1.7 C-E). Electron microscopy confirmed that at 1h post-injection, a number of blood vessels partially or completely lost EBA immunoreactivity. Electron dense reaction product of EBA staining was observed along part of the circumference of ECs or was absent throughout the circumference (Figs 4.1.10 A-C). At 6-18h, an increasing number of blood vessels showed reduction in EBA immunoreactivity (Fig 4.1.10 D). At 1 and 2d post-injection the reduction was apparent, with long stretches of luminal membranes devoid of the reaction product in most vessels (Fig 4.1.11 A). At 4 and 7d luminal membrane staining was still reduced (Fig. 4.1.11 B). At 14d although by LM immunoreactivity of vessels was similar to controls (Fig. 4.1.8F), EM showed many vessels with reduced staining (Fig 4.1.11 C) and some with full luminal staining (Fig. 4.1.11 D). Experimental animals injected with 1:500 dilution of the prototoxin showed similar changes of EBA immunoreactivity in the brain but the reduction of EBA immunoreactivity was less obvious at early time intervals.

4.1.4.3 Quantitative assessment of EBA expression in prototoxin-induced BBB opening

Quantitative assessment of EBA expression at the EM level was carried out in two groups of experimental animals, injected with 1:400 and 1:500 dilution of the prototoxin, and one control animal in each group. The experimental group injected with 1:400 dilution of the prototoxin included one animal killed at 1h, 6h, 12h, 24h, 2d, 4d, 7d, and 14d postinjection. The percentage of luminal membrane

length showing EBA immunoreactivity was calculated in relation to the total length of luminal membrane for each vessel. In control animals most vessels had 100% staining of their luminal membrane. Only occasional vessels did not show reaction product and some vessels lacked reaction product along part of their circumference. In experimental animals most vessels were either unstained or had values less than 100%. The results from all vessels in each animal were pooled and a mean value was obtained for each animal (Fig. 4.1.12). The control animal in this group had a value of 91% (Appendix A.1). An obvious reduction in EBA labelling was observed in all experimental animals in this group compared to the control, with the percentage of luminal membrane staining in experimental animals ranged from 35% to 66% (Appendix A.1). The reduction in EBA expression was more severe in this group compared to the second group, which received 1:500 dilution of the prototoxin.

The second group, which received 1:500 dilution, included one animal at 6h, 12h, 18h, 2d, and 7d. Except in the two experimental animals killed at 12 h and 2 d, all experimental animals showed many vessels with reduced percentage of luminal membrane staining. The control animal in this group showed 96% staining of EC luminal membranes (Appendix A.2). The percentages of luminal membrane staining were reduced to 81%, 68% and 62% in experimental animals killed at 6h, 18h and 7d respectively (Appendix A.2). The two animals killed at 12h and 2d did not show obvious changes in the percentage of luminal membrane staining, with the values being 93% and 99% (Appendix A.2) respectively. On the whole

the animals injected with 1:500 dilution showed a milder and delayed response to the prototoxin injection (Fig. 4.1.12).

FIG. 4.1.7 (A-F). Light micrographs of paraffin sections showing cerebral cortices from control (A) and experimental animals with survival times of 1h (B), 6h (C), 24h (D), 2 days (E), and 14 days (F) post-intraperitoneal injection of 1:400 dilution of *Clp* epsilon prototoxin.

(A-F) EBA-immunoreactive vessels (arrows) are seen in various orientations. Pia mater vessels [PV] on the surface of the cortex (A) are stained.

(B-E) Partially- or weakly-labelled vessels (arrowheads) are seen in experimental animals.

(F) At 14 days the labelling appears similar to that of the control (A).

Immunoperoxidase, with nickel enhancement. Bar, 100 μ m.

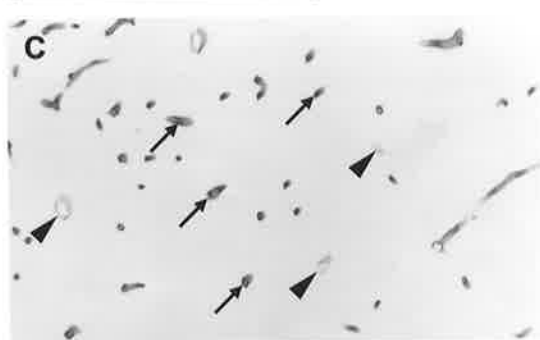
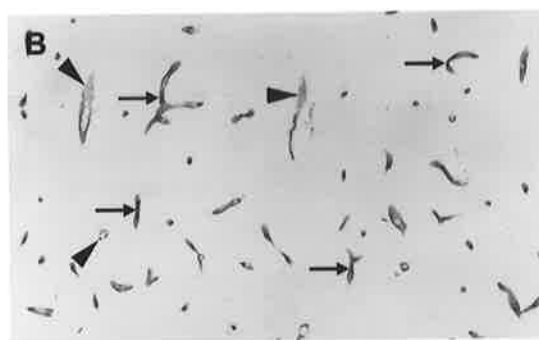


FIG. 4.1.8 (A-D). Electron micrographs of cerebral cortical microvessels from control (non-injected with prototoxin) animals, showing pre-embedding immunoperoxidase labelling for EBA, with nickel enhancement.

(A-D) The labelling appears as electron dense reaction product along the entire length of luminal membrane (small arrows) of endothelial cells. Neurons and nerve fibres in the neuropil (asterisks) are non-reactive.

(B-D) Endothelial cells show immunoreactivity in the cytoplasm involving the full thickness between the luminal and abluminal membranes (between curved arrows). The cytoplasmic staining is unlikely to be due to over development of the reaction product or a smearing from the luminal staining since cytoplasmic vesicles [V] appear unstained (**A, C and D**).

(A, B and D) show endothelial cells nuclei [N] which display immunoreactivity in the marginal chromatin located deep to the nuclear membrane on the luminal side.

(C) The astrocyte [As] and its nucleus [AsN] are not immunoreactive for EBA.

All micrographs are from grids not counterstained with heavy metals. Bar, 1 μm .

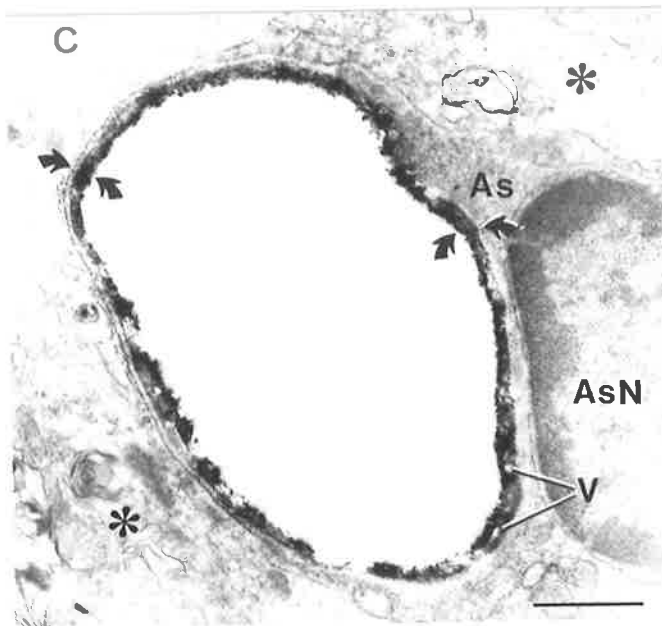
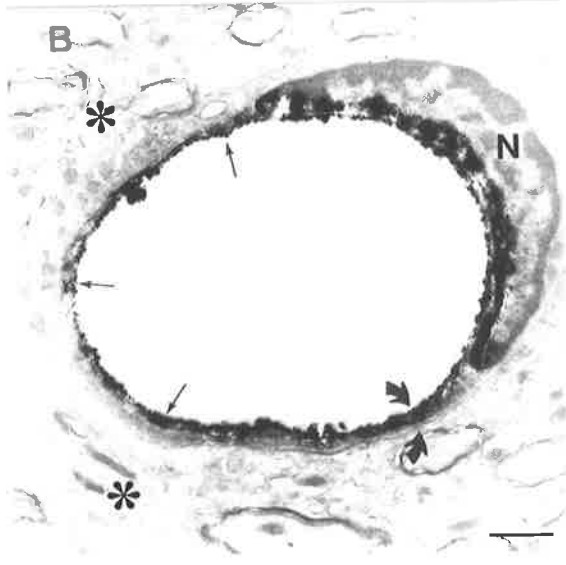
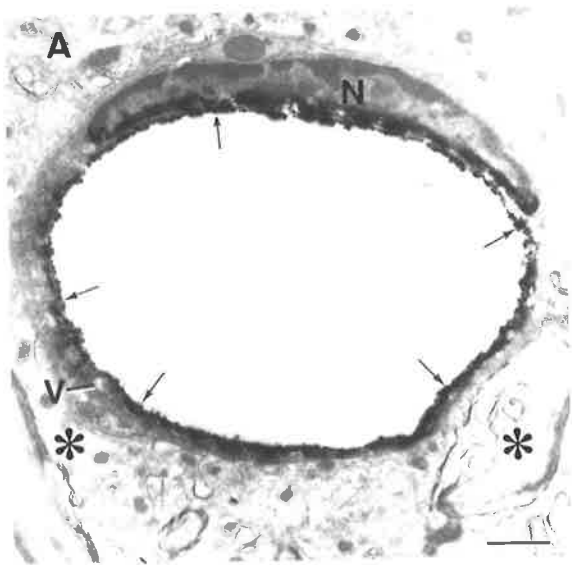


FIG. 4.1.9 (A-C). Electron micrographs of cerebral cortical microvessels from normal animals, showing post-embedding immunogold labelling for EBA in LR white sections.

(A and B) In sections stained with anti-EBA, the gold particle labelling, representing EBA immunoreactivity, is mainly at the luminal membrane of ECs (black arrows). Some gold particles are localised to the cytoplasm of ECs (white arrows). A few gold particles are observed at the abluminal membrane of ECs (arrowheads).

(C) No labelling is seen when the primary antibody was omitted.

ICJ, intercellular junction. Bar, 0.5 μm .

Sections were lightly stained with uranyl acetate.

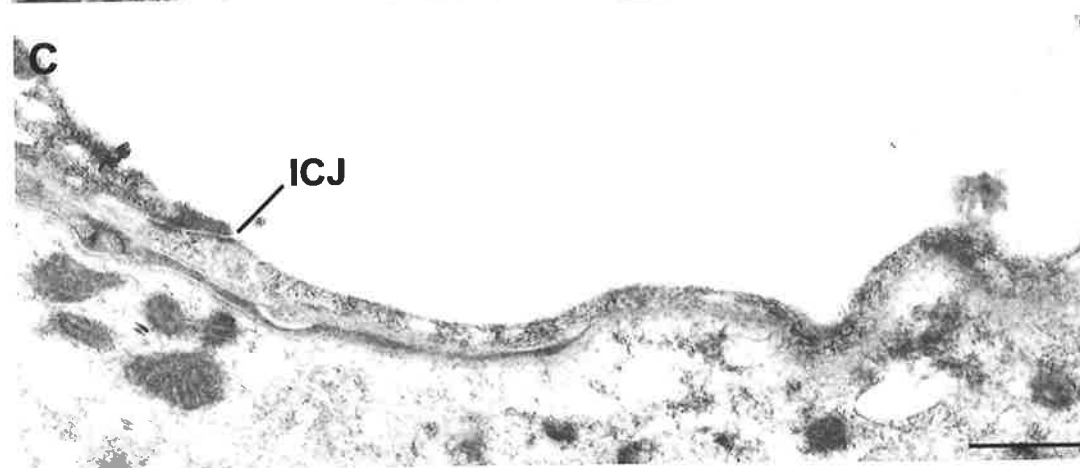
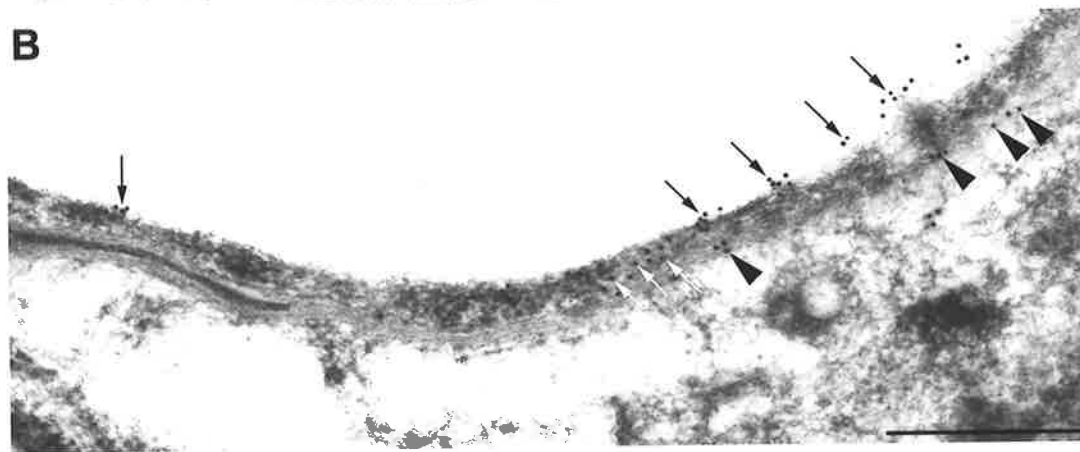
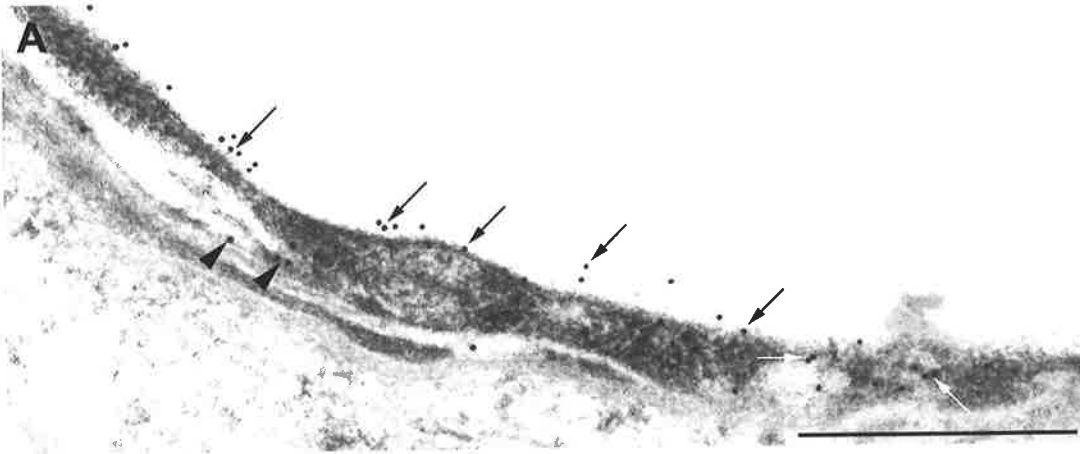


FIG. 4.1.10 (A-D). Electron micrographs of cerebral cortical microvessels from experimental animals, showing the range of changes in EBA expression, at 1h (A-C) and 6h (D) postinjection of 1:400 dilution of *Cl p* epsilon prototoxin, as detected by pre-embedding immunoperoxidase labelling for EBA, with nickel enhancement.

- (A) At 1h after the injection, perivascular space formation (stars) is seen, suggestive of oedema preceding any loss of EBA immunoreactivity at the luminal membrane (small arrows). The reaction product is seen in the full thickness of endothelial cell cytoplasm (between curved arrows) but with unstained vesicles [V].
- (B) At 1h after the injection, an endothelial cell nucleus [N] shows reaction product in the marginal chromatin on the luminal side of the nucleus. The cytoplasm deep to the nucleus is stained but contains clear vesicles [V]. Luminal staining (arrows) is seen.
- (C) At 1h after the injection, a blood vessel shows partial luminal membrane staining (arrows). The luminal membrane at the lower part of the vessel is unlabelled.
- (D) At 6h after the injection, a blood vessel shows absence of EBA labelling, despite lack of perivascular widening or any endothelial cell damage. This indicates that the reduction may precede or follow the oedema formation.

ICJ, intercellular junction.

All micrographs are from grids not counterstained with heavy metals. Bar, 1 μ m.

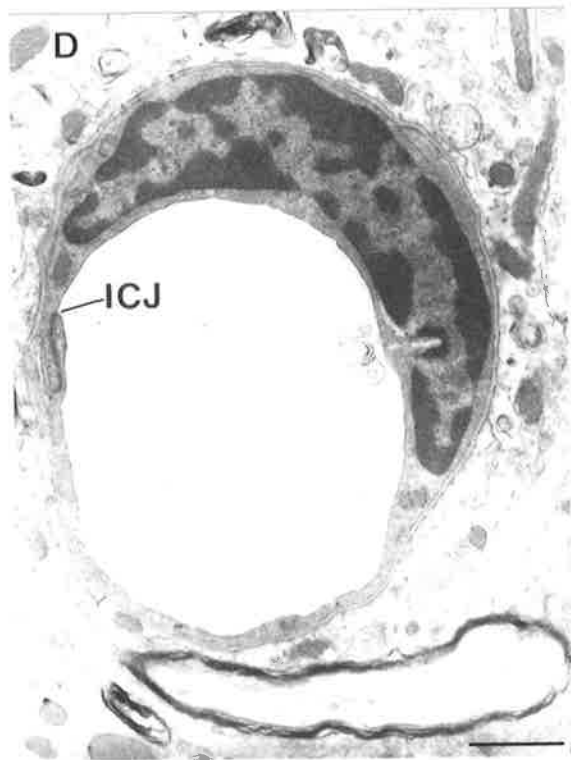
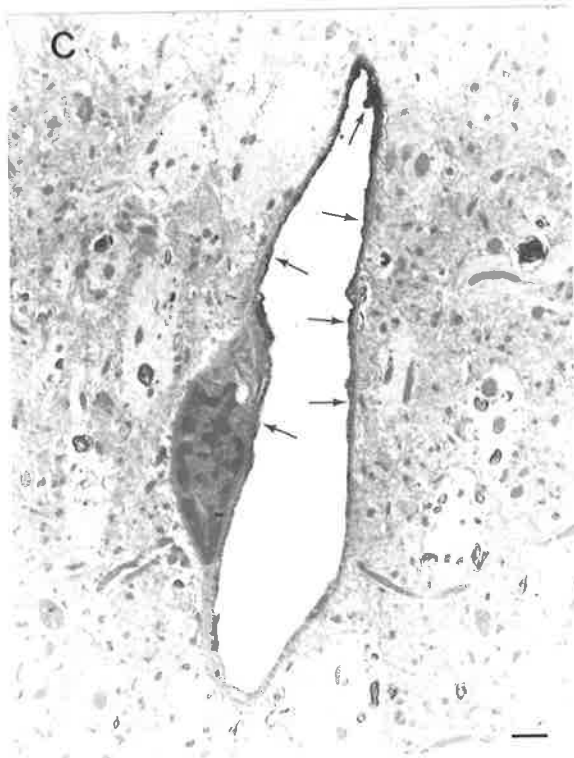
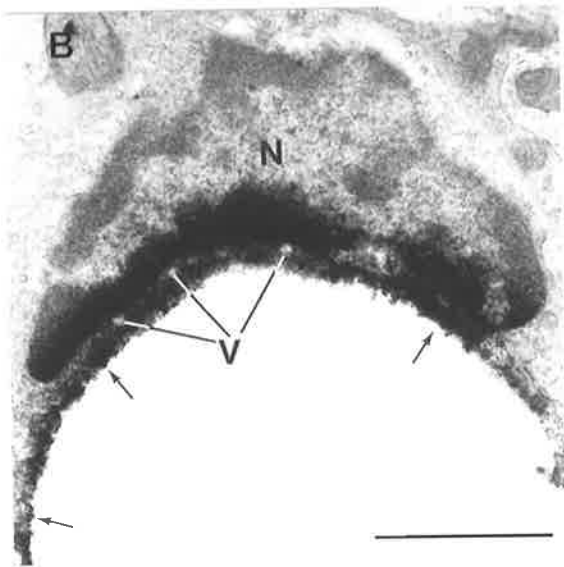
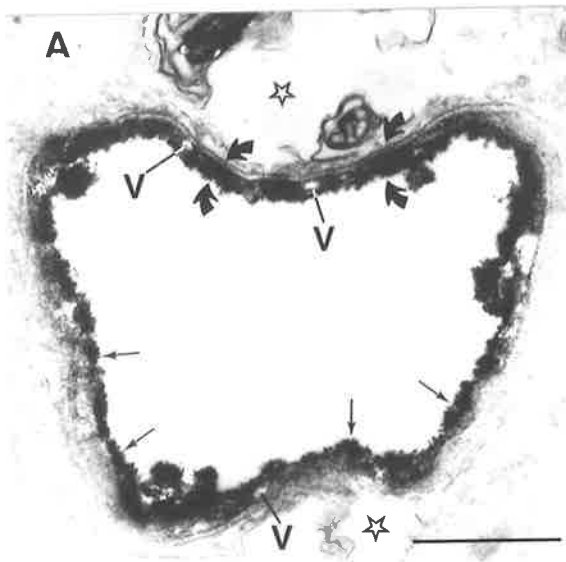


FIG. 4.1.11 (A-D). Electron micrographs of cerebral cortical microvessels from experimental animals, at 1 day (A), 7 days (B), and 14 days (C and D) postinjection of 1:400 dilution of *Clp* epsilon prototoxin, showing pre-embedding immunoperoxidase labelling for EBA, with nickel enhancement.

- (A) Faint reaction product (small arrows) is seen on parts of the luminal circumference.
- (B) No reaction product is seen in the luminal membrane or cytoplasm. The marginal chromatin on the luminal side of the endothelial cell nucleus [N] shows faint reaction product. The perivascular oedema (stars) is distorting the vessel's profile.
- (C) shows complete absence of EBA labelling with minimal perivascular astrocytic oedema (star).
- (D) shows intense reaction product at the luminal membrane (small arrows) and at certain regions of the cytoplasm (between curved arrows) of endothelial cells. The marginal chromatin on the luminal side of the endothelial cell nucleus [N] shows gradation of reaction product. The cytoplasm between the nucleus and the luminal membrane is not stained, indicating that nuclear staining is unlikely to be artifact smearing.

ICJ, luminal end of apparently intact intercellular junctions.

All micrographs are from grids not counterstained with heavy metals. Bar, 1 μm .

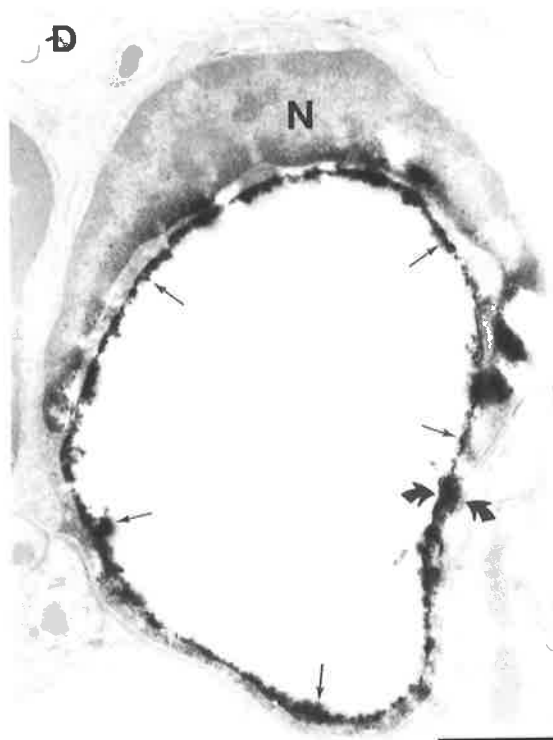
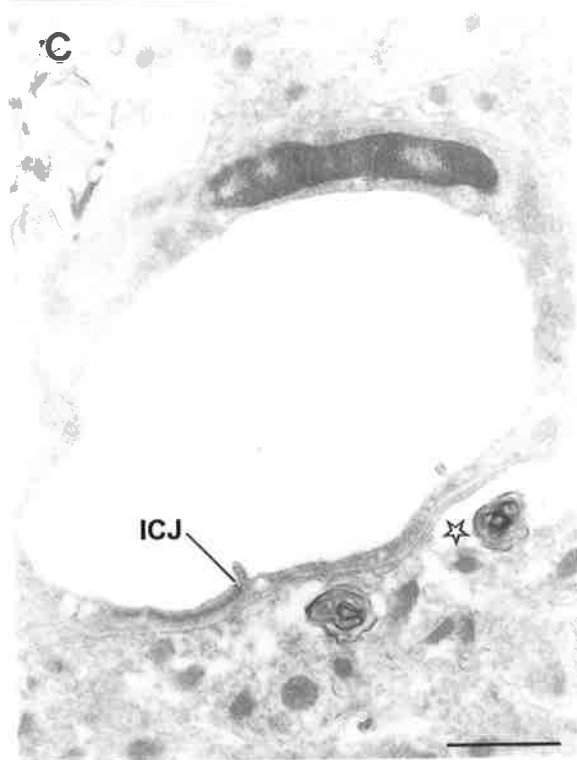
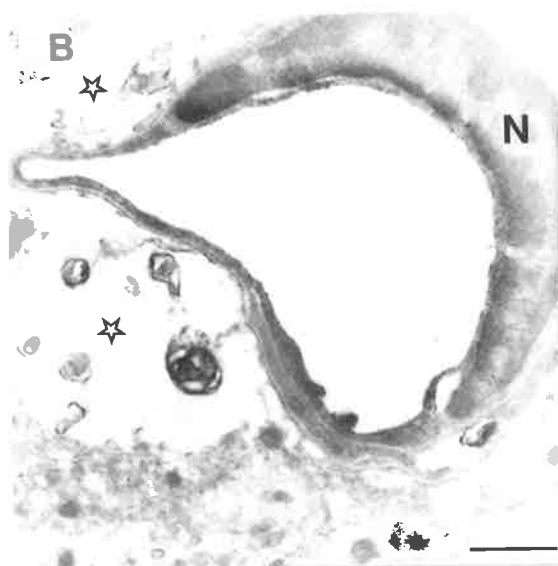
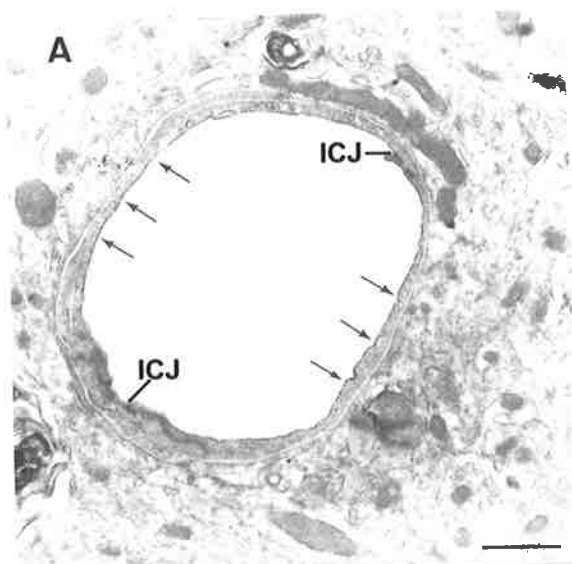
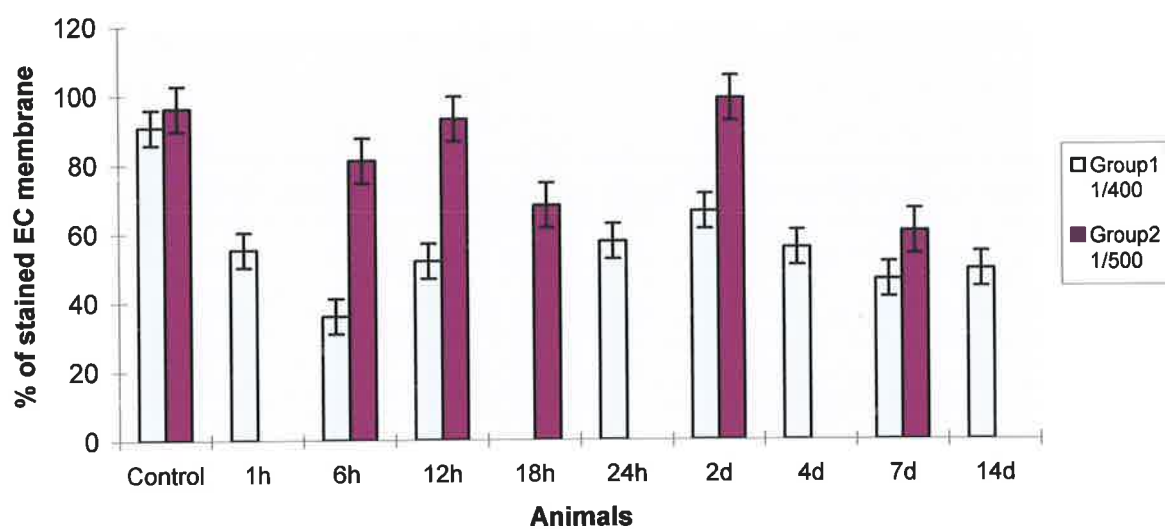


FIG. 4.1.12. A graph showing the quantitative results for the percentage of EC luminal membranes immunoreactive for EBA, in two control animals and in experimental animals injected with 1:400 or 1:500 dilution of epsilon prototoxin and killed at various time intervals ranging from 1h to 14 days postinjection. The histogram bars represent mean values of 16-30 vessels in each time interval. The standard error bars show the error amount for all data points in the two groups. Each experimental group was compared to one control animal, since sections were immunostained at the same time and under identical conditions.

FIG. 4.1.12 Comparison of percentage of length of endothelial cell luminal membrane stained for EBA, in control and experimental animals



4.1.5 GLUT1 expression in prototoxin-induced BBB opening

4.1.5.1 Qualitative analysis of GLUT1 expression

Microvessels in the normal brains showed intense immunostaining for GLUT1 (Figs 4.1.13 and 4.1.14). The peroxidase reaction product appeared to be in the intima at high magnification. The endothelium of pial vessels was also stained. Large blood vessels in the subarachnoid space such as arteries at the base of the brain and in the major fissures did not show immunostaining for GLUT1. Nerve cells such as the Purkinje cells of the cerebellum were not stained. Background staining of the neuropil was minimal. Endothelial cells of blood vessels in the choroid plexus were not stained but epithelial cells of the choroid plexus were distinctly stained, especially at the basal and lateral membranes. No immunoreactivity was observed in the pineal gland vessels.

Experimental materials showed intense immunoreactivity for GLUT1 (Figs 4.1.13 and 4.1.14). Blood vessels in the brain were intensely stained. Qualitatively the density and intensity of blood vessels showing positive staining appeared higher after prototoxin injection compared to controls.

4.1.5.2 Quantitative analysis of GLUT1 expression

Quantitative analysis of GLUT1 immunoreactivity at LM level was carried out in two groups of experimental animals injected with 1:400 and 1:500 dilution of prototoxin and in control animals. In the group injected with 1:500 dilution of prototoxin, the number of GLUT1 immunoreactive blood vessels per mm² in the

frontal cortex increased at 1h after prototoxin injection (398 per mm^2) compared with that of the control animal (297 per mm^2). The density of the GLUT1-immunoreactive blood vessels gradually decreased to near normal level at 7d after the injection (Appendix A.3.1, Fig. 4.1.15). In the cerebellum (Appendix A.3.2, Fig. 4.1.16), the change of GLUT1 immunoreactivity was also observed (Fig. 4.1.16). The density of immunoreactive blood vessels was high at 1h after the injection and showed a trend of reduction towards 7d, where the density of immunoreactive vessels were close to that of the control. However, the animal killed at 24h after the injection had the highest number of reactive vessels. No apparent changes of the density of GLUT1 immunoreactive blood vessels were noted in the inferior colliculus and brainstem in the 1:500 group (Appendix A.3.3, A.3.4) (Fig. 4.1.17, 4.1.18).

In the group injected with 1:400 dilution of prototoxin, although at some time intervals the density of immunoreactive blood vessels appeared higher than that of the control, no obvious trend of changes was observed between the density of GLUT1 immunoreactive blood vessels and time intervals (Appendix A.3.5-8) (Figs 4.1.19-22). Further analysis of five normal animals showed that the standard deviation of the density of immunoreactive blood vessels in the frontal cortex is similar to that of the experimental animals (Appendix A.3.9), indicating that the changes observed in the 1:500 dilution group could be caused by random variation between individual animals. Therefore, the prototoxin does not appear to have an obvious effect on GLUT1 expression in experimental animals.

FIG. 4.1.13 (A-F). Light micrographs of paraffin sections from brains of control (A and B) and experimental animals with survival times of 6h (C), 24h (D), 2d (E) and 7d (F) post-intraperitoneal injection of 1:500 dilution of *Cl p* epsilon prototoxin.

(A and C-F) Blood vessels in the cortex of control and experimental animals are positively stained for GLUT1 (arrows).

(B) Choroidal epithelial cells of a control animal are immunoreactive for GLUT1 (arrows), especially at the basal and lateral membranes.

(C-E) The intensity of GLUT1 immunostaining and the density of blood vessels showing immunoreactivity in the cortices of experimental animals appear to be higher than those in the control animal (A).

(F) At 7d after the injection, the intensity of GLUT1 immunostaining appears similar to that in the control animal (A).

Bar, 50 μ m.

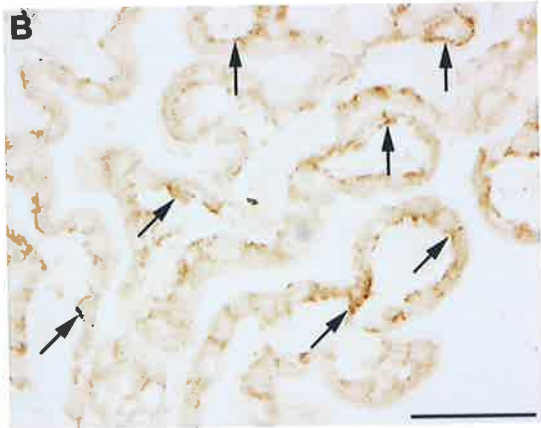
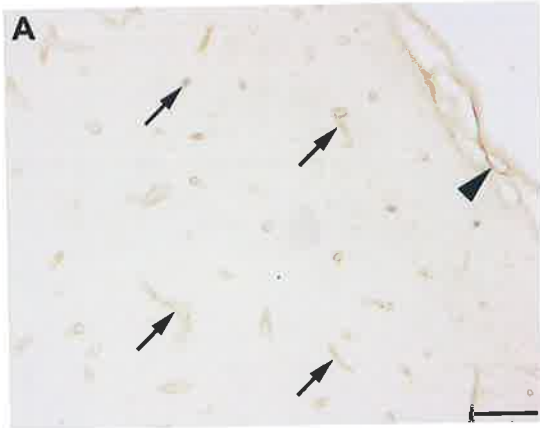


FIG. 4.1.14 (A-F). Light micrographs of paraffin sections from brains of control (A) and experimental animals with survival times of 6h (B), 24h (C), 2d (D), 7d (E) and 14d (F) post-intraperitoneal injection of 1:400 dilution of *Cl p* epsilon prototoxin.

(A-F) Blood vessels in the cortices of control and experimental animals are positively stained for GLUT1 (arrows).

(B-F) In experimental animals, the intensity of GLUT1 immunostaining appears higher and the density of blood vessels showing immunoreactivity appears greater than that in the control animal (A).

Immunoperoxidase, with nickel enhancement. Bar, 50 μ m.

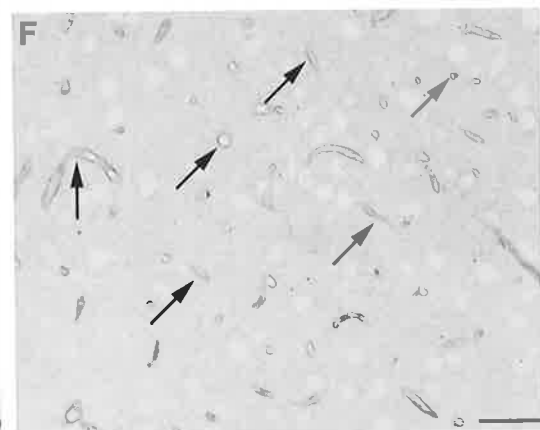
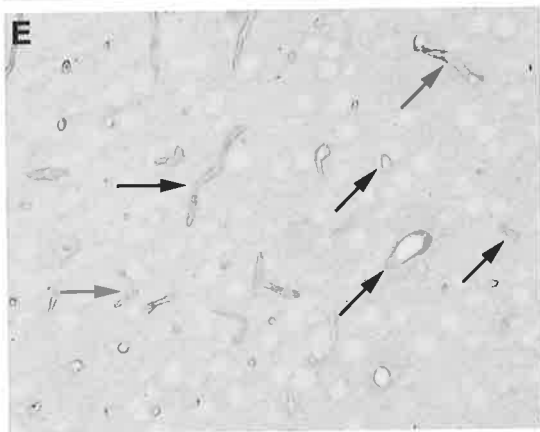
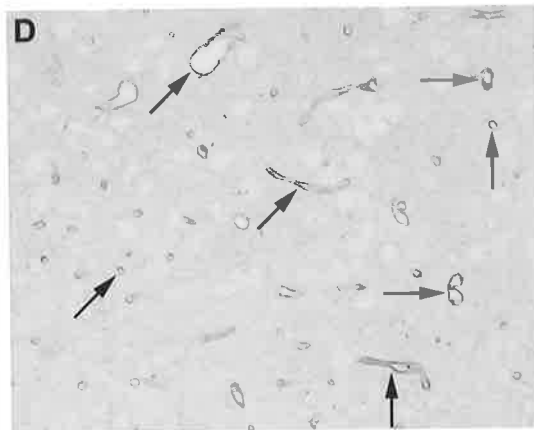
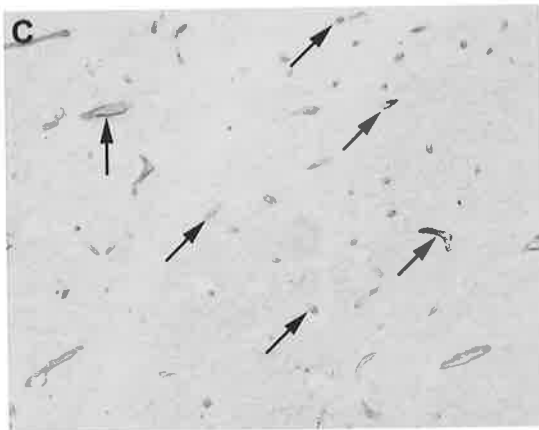
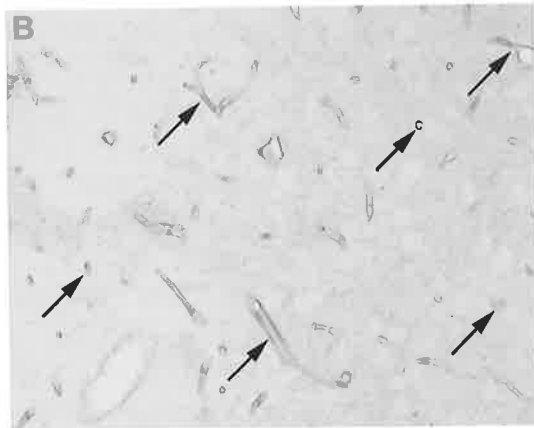
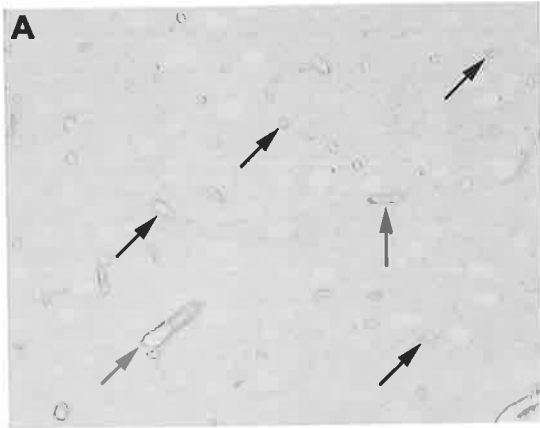


FIG. 4.1.15. A graph showing the number of GLUT1-immunoreactive blood vessels in the frontal cortex in control and experimental animals at 1h, 6h, 9h, 12h, 18h, 24h, 2d, 4d, 7d after the injection of 1:500 dilution of *Cl p* prototoxin. Each data point represents one animal. A regression line is fitted to the data series.

FIG. 4.1.16. A graph showing the number of GLUT1-immunoreactive blood vessels in the cerebellum in control and experimental animals at 1h, 6h, 9h, 12h, 18h, 24h, 2d, 4d, 7d after the injection of 1:500 dilution of *Cl p* prototoxin. Each data point represents one animal. A regression line is fitted to the data series.

FIG. 4.1.15 The number of GLUT1-immunoreactive blood vessels in the frontal cortex in 1:500 prototoxin-induced BBB opening model

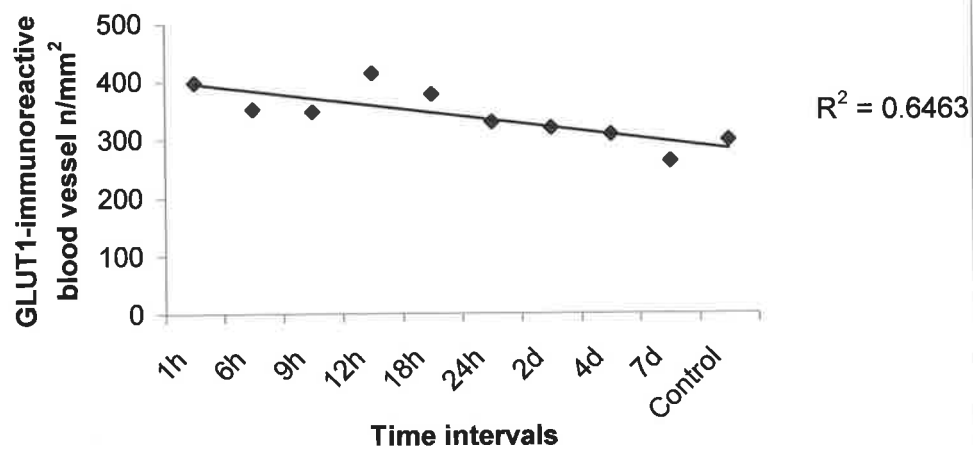


FIG 4.1.16 The number of GLUT1-immunoreactive blood vessels in the cerebellum in 1:500 prototoxin-induced BBB opening model

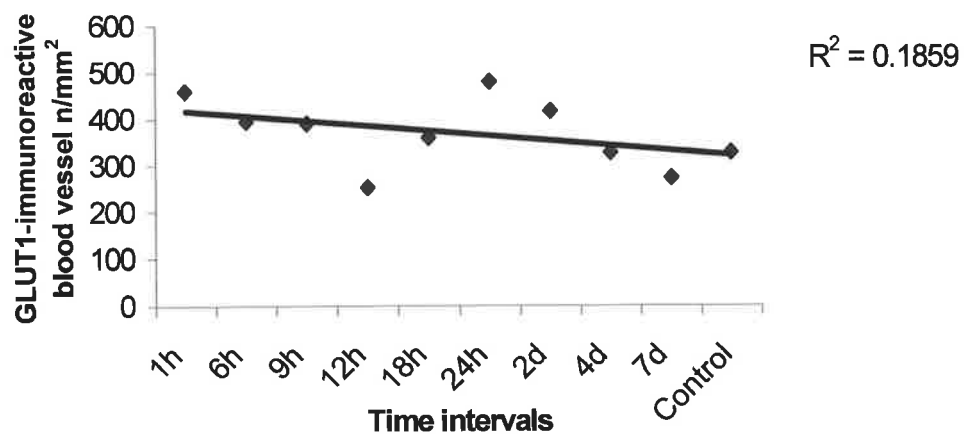


FIG. 4.1.17. A graph showing the number of GLUT1-immunoreactive blood vessels in the inferior colliculus in control and experimental animals at 1h, 6h, 9h, 12h, 18h, 24h, 2d, 4d, 7d after the injection of 1:500 dilution of *Cl p* prototoxin. Each data point represents one animal. A regression line is fitted to the data series.

FIG. 4.1.18. A graph showing the number of GLUT1-immunoreactive blood vessels in the brainstem in control and experimental animals at 1h, 6h, 9h, 12h, 18h, 24h, 2d, 4d, 7d after the injection of 1:500 dilution of *Cl p* prototoxin. Each data point represents one animal. A regression line is fitted to the data series.

FIG. 4.1.17 The number of GLUT1-immunoreactive blood vessels in the inferior colliculus in 1:500 prototoxin-induced BBB opening model

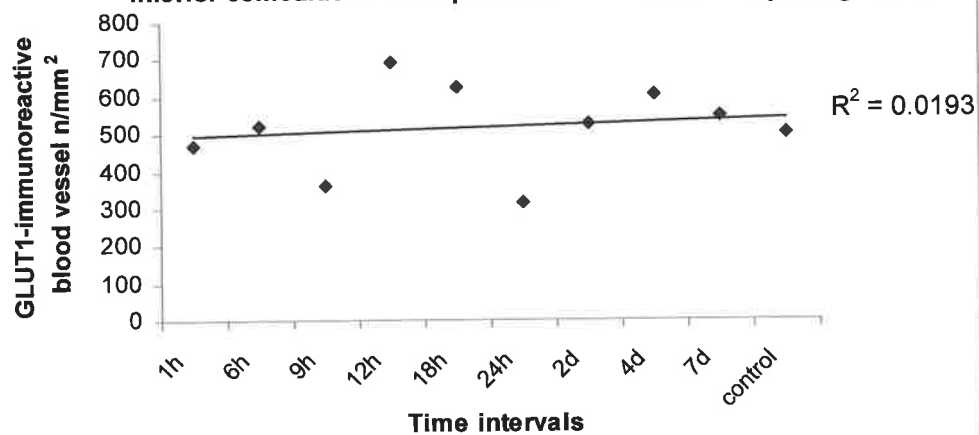


FIG. 4.1.18 The number of GLUT1-immunoreactive blood vessels in the brainstem in 1:500 prototoxin-induced BBB opening model

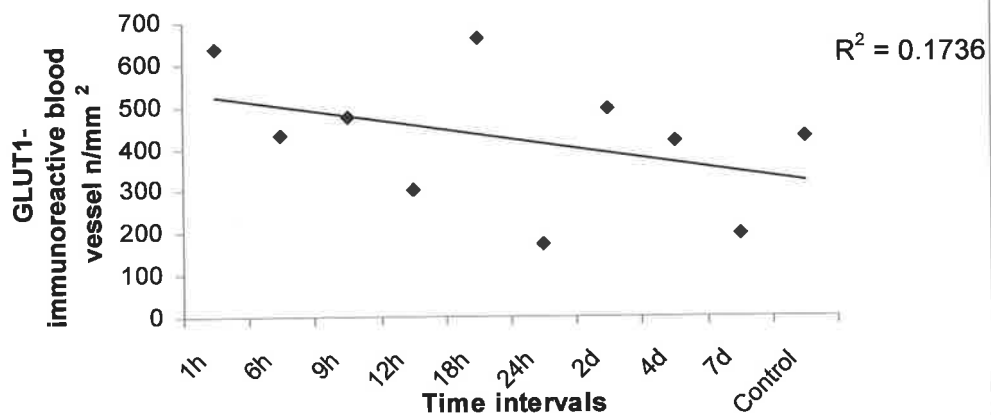


FIG. 4.1.19. A graph showing the number of GLUT1-immunoreactive blood vessels in the frontal cortex in control and experimental animals at 1h, 6h, 12h, 24h, 2d, 4d, 7d, and 14d after the injection of 1:400 dilution of *Cl p* prototoxin. Each data point represents one animal. A regression line is fitted to the data series.

FIG. 4.1.20. A graph showing the number of GLUT1-immunoreactive blood vessels in the cerebellum in control and experimental animals at 1h, 6h, 12h, 24h, 2d, 4d, 7d, and 14d after the injection of 1:400 dilution of *Cl p* prototoxin. Each data point represents one animal. A regression line is fitted to the data series.

Fig 4.1.19 The number of GLUT1-immunoreactive blood vessels in the frontal cortex in 1:400 prototoxin-induced BBB opening model

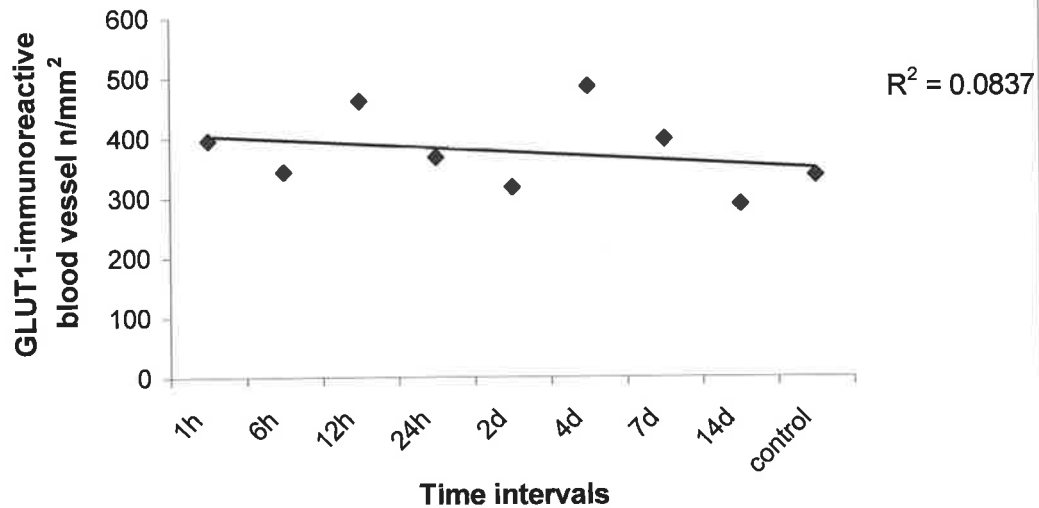


FIG. 4.1.20 The number of GLUT1-immunoreactive blood vessels in the cerebellum in 1:400 prototoxin-induced BBB opening model

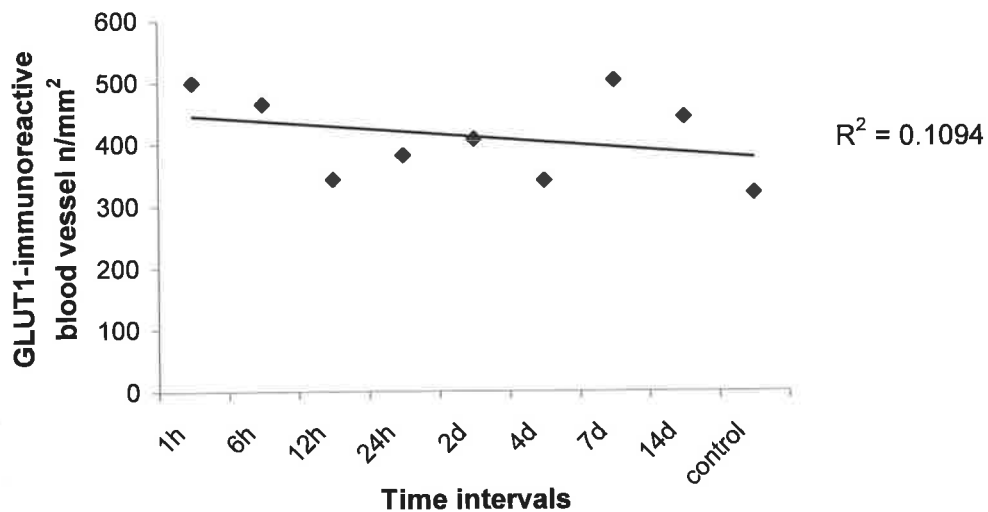


FIG. 4.1.21. A graph showing the number of GLUT1-immunoreactive blood vessels in the inferior colliculus in control and experimental animals at 1h, 6h, 12h, 24h, 2d, 4d, 7d, and 14d after the injection of 1:400 dilution of *Cl p* prototoxin. Each data point represents one animal. A regression line is fitted to the data series.

FIG. 4.1.22. A graph showing the number of GLUT1-immunoreactive blood vessels in the brainstem in control and experimental animals at 1h, 6h, 12h, 24h, 2d, 4d, 7d, and 14d after the injection of 1:400 dilution of *Cl p* prototoxin. Each data point represents one animal. A regression line is fitted to the data series.

Fig 4.1.21

The number of GLUT1-immunoreactive blood vessels in the inferior colliculus in 1:400 prototoxin-induced BBB opening model

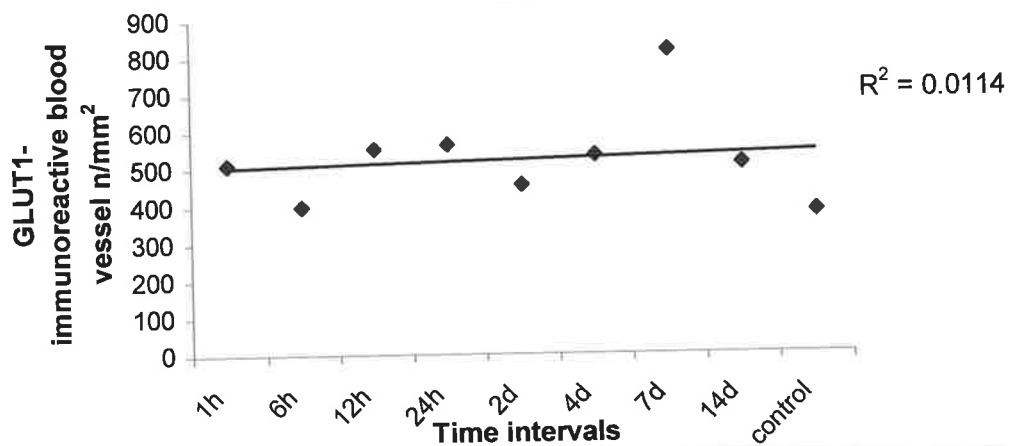
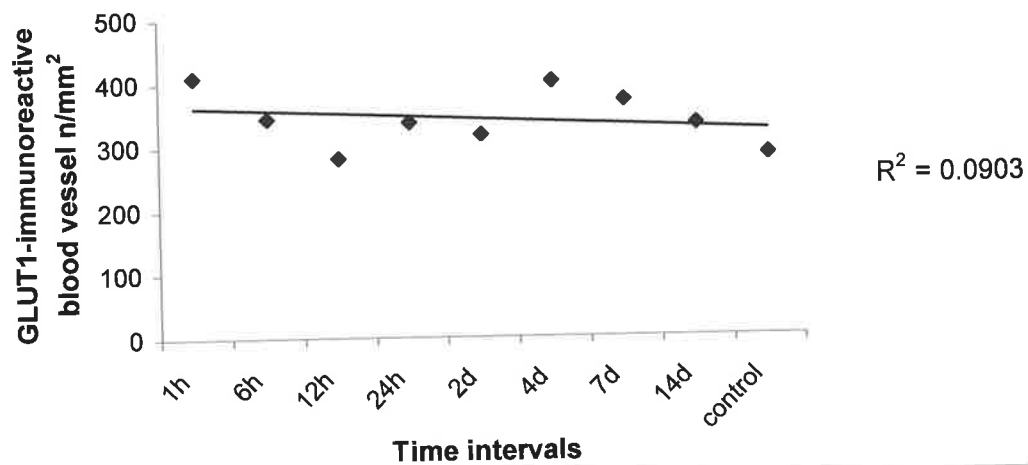


FIG. 4.1.22

The number of GLUT1-immunoreactive blood vessels in the brainstem in 1:400 prototoxin-induced BBB opening model



4.2 Traumatic brain injury model

4.2.1 General conditions of experimental animals

Animals were kept under anaesthesia throughout the experiment and were artificially ventilated via an endotracheal tube. The ventilation rate was kept at 40-50 per minute. The rectal temperature of experimental and sham control animals was maintained at 36.5-37.5 °C. The femoral arterial blood pressure was monitored in some animals. Immediately after the injury, the blood pressure increased from 67 mm Hg to 100 mm Hg. Forty-five minutes after the injury, the blood pressure returned to the basal level recorded in anaesthetised rats.

4.2.2 Gross pathological changes in the brain

The gross pathological changes in the brains of experimental animals did not differ with the survival time after the injury. Hence, the following pathological findings represent experimental animals which survived for 1h, 6h and 12h.

No skull fractures or epidural haemorrhages were observed after the injury. No contusion or focal lesions in the brains were observed. Subarachnoid haemorrhage was observed in the cisterna ambiens, cisterna magna and the subarachnoid space on the cerebral hemisphere in 53% of experimental animals. Intraventricular haemorrhage was also noted in some animals. Subarachnoid and intraventricular haemorrhage in experimental animals were also reported by Marmarou's research group (Foda & Marmarou, 1994). No haemorrhage was present in the brain parenchyma. No haemorrhage was observed in the brains or the subarachnoid space of sham control animals.

4.2.3 Ultrastructure of the BBB in TBI

Normal control and sham control animals showed no modification of the ultrastructure of the BBB (Fig. 4.2.1). The cytoplasm of ECs appeared evenly electron dense. Few vesicles were observed in the EC cytoplasm. Intact tight junctions were observed between ECs.

In experimental animals, one hour after the trauma, increased cytoplasmic vesicles and luminal membrane indentations were observed in ECs (Fig. 4.2.2). Some vesicles communicated with the blood vessel lumen. Some tight junctions showed focal widening or appeared to be connected to a cytoplasmic vesicle. In some sectors of blood vessels, the EC cytoplasm appeared electron lucent and had reduced organelles. Six hours after the injury, the ECs appeared to be more severely altered (Fig. 4.2.3). In addition to the features stated above, large vacuoles were observed in the EC cytoplasm. Swelling of perivascular astrocytic processes was seen in many blood vessels. Twelve hours after the trauma, the ECs appeared to be normalised (Fig. 4.2.4). The cytoplasm of ECs was evenly electron dense. No indentations or excessive vesicles were observed. Tight junctions showed no alterations. However, the swelling of astrocytic processes was more pronounced than at earlier time intervals. The cytoplasm of many astrocytes was electron lucent with dispersed organelles. The perivascular space was also widened and in some blood vessels it distorted the vascular profile.

FIG. 4.2.1 (A-C). Electron micrographs of cerebral cortical microvessels from normal control animals.

(A and B) The extracellular space is compact, cellular elements of neurons and neuroglia are in close apposition. Note the absence of any perivascular spaces.

(C) A higher magnification shows a tight junction (white arrows) between ECs.

N, endothelial cell nucleus.

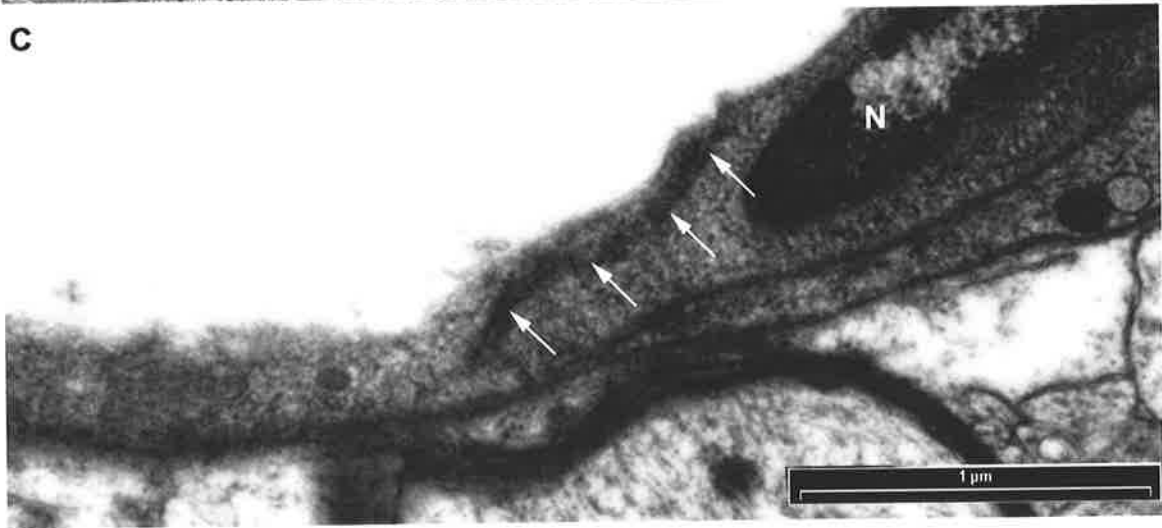
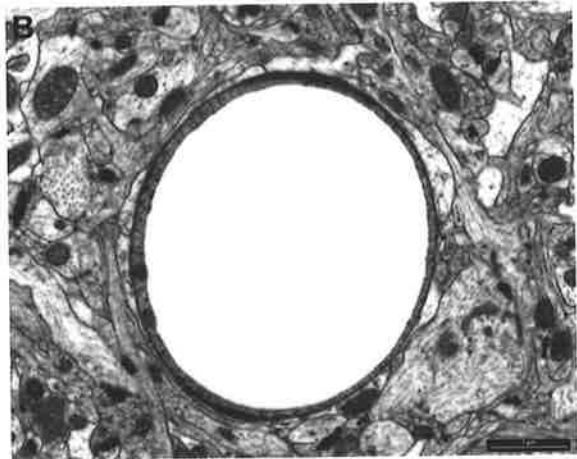
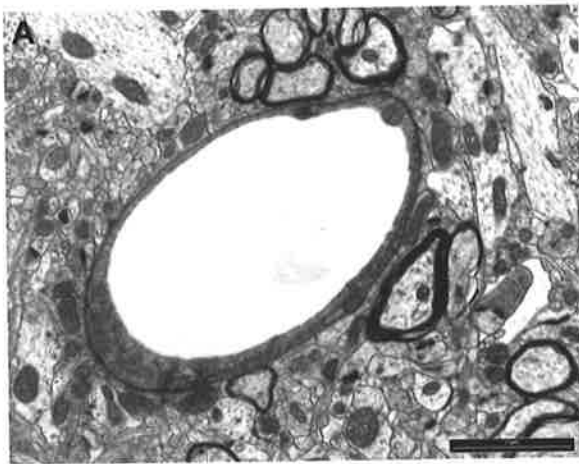


FIG. 4.2.2 (A-G). Electron micrographs showing ultrastructural changes in brain microvessels from experimental animals at 1h after traumatic brain injury.

(A and B) Blood vessels in the brainstem show numerous vesicles in the cytoplasm of ECs (black arrows).

(C) A high magnification shows some vesicles communicate with the lumen or separated from the lumen by a thin membrane (arrows).

(D and E) Cerebral cortical blood vessels show numerous vesicles in the cytoplasm of ECs (black arrows). A large vacuole is seen in the cytoplasm (arrowhead, **D**).

(F and G) High magnifications showing focal widening of tight junctions (white arrows). **G**, shows a large vacuole (black arrow).

ICJ, intercellular junction.

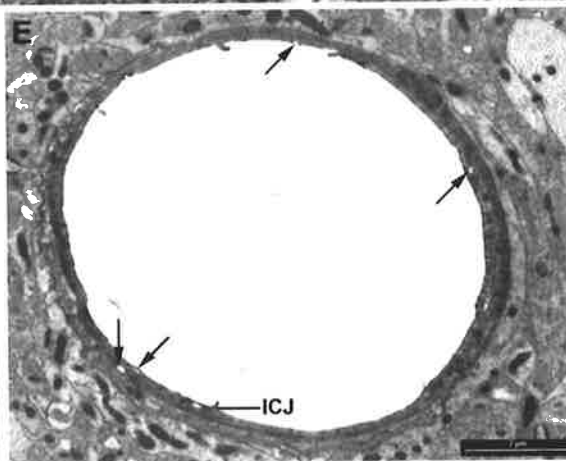
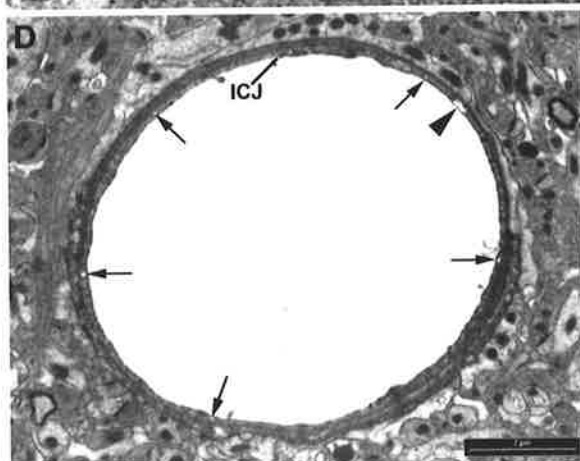
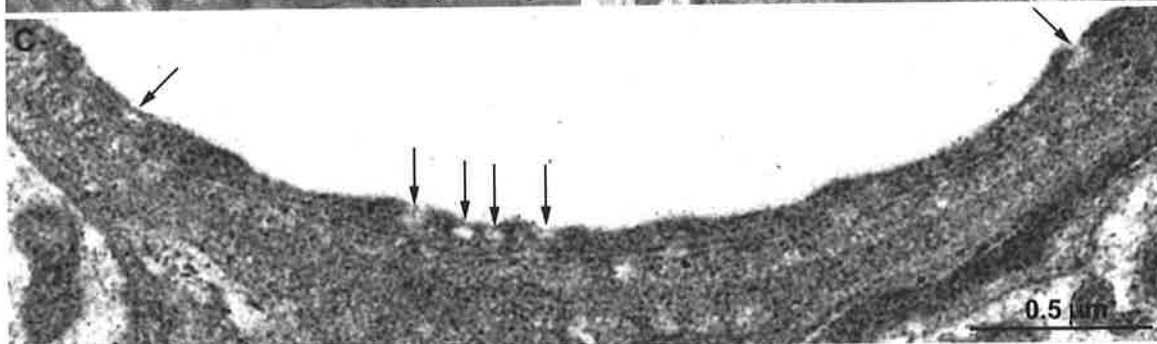
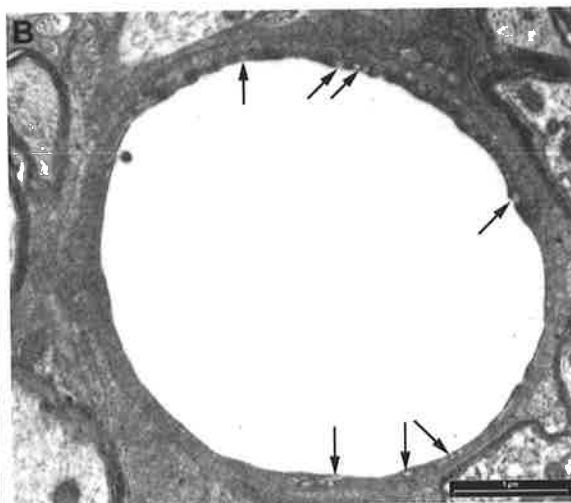
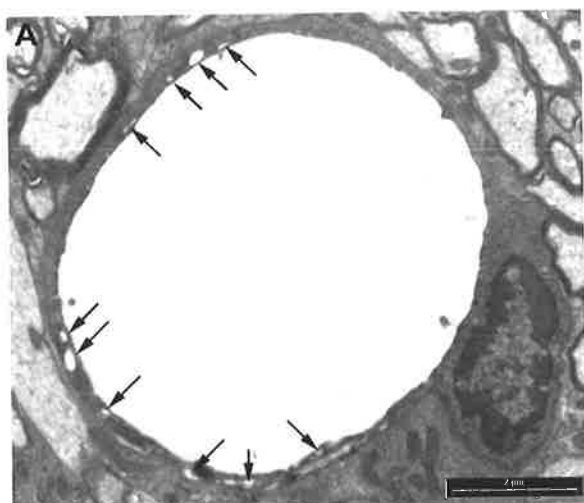


FIG. 4.2.3 (A-D). Electron micrographs showing ultrastructural changes in cerebral cortical microvessels from an experimental animal at 6h after traumatic brain injury.

(A) Numerous indentations on the luminal membrane are seen in a microvessel (arrows).

(B) A large vacuole [V] and numerous indentations (arrows) are observed in ECs. A tight junction is showing focal widening (white arrows).

(C) A higher magnification shows a large vacuole [V] in close proximity to the course of an ICJ, the two ends of which are indicated by arrows.

(D) A higher magnification shows two altered tight junctions (black arrows, luminal and abluminal ends of the junction). Focal widening of the tight junction is observed (white arrows).

ICJ, intercellular junction; N, endothelial cell nucleus.

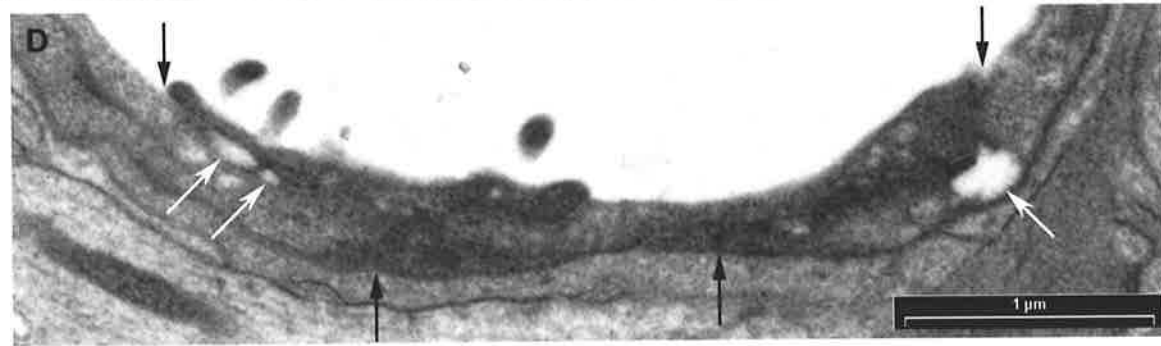
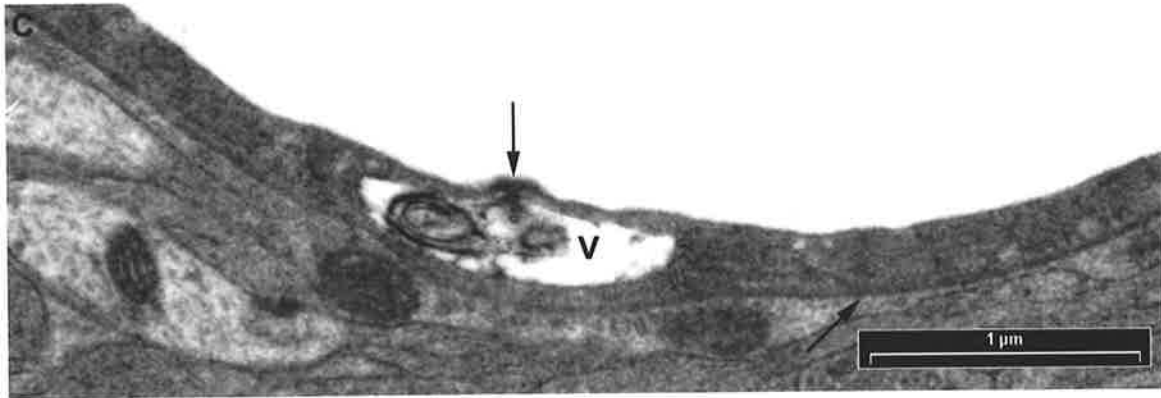
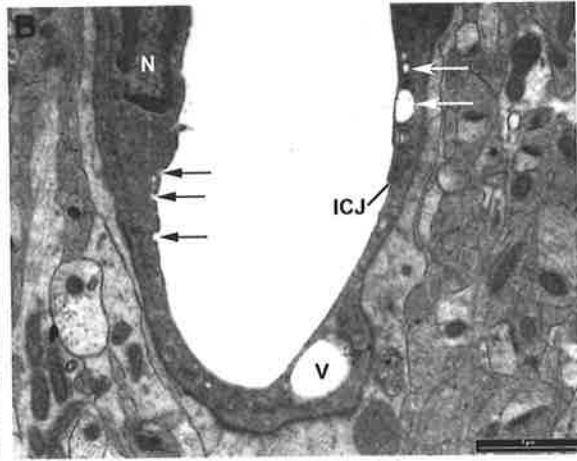
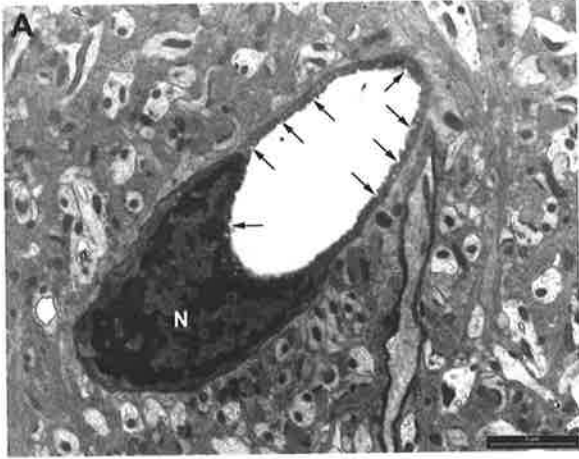
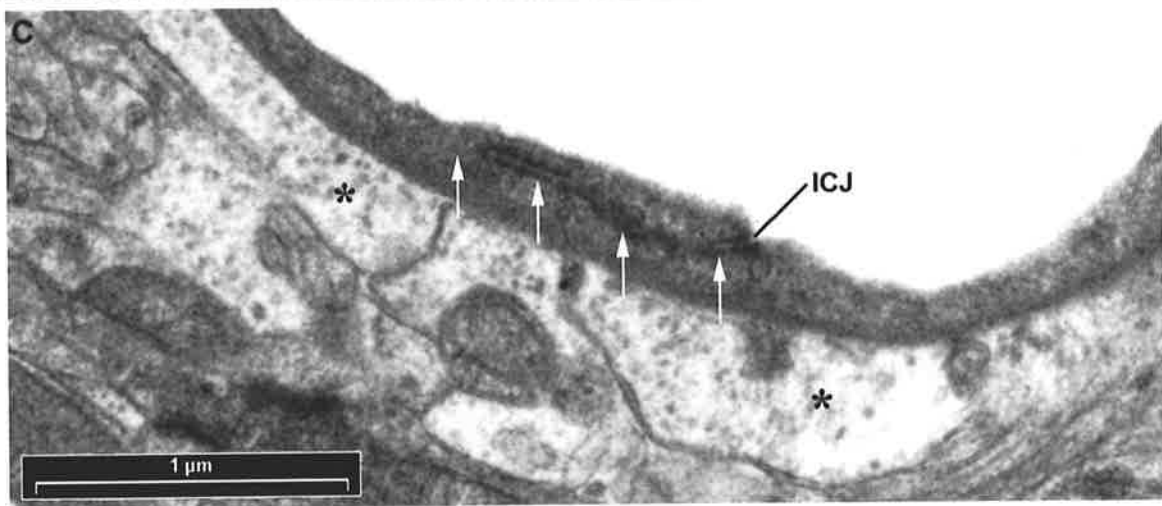
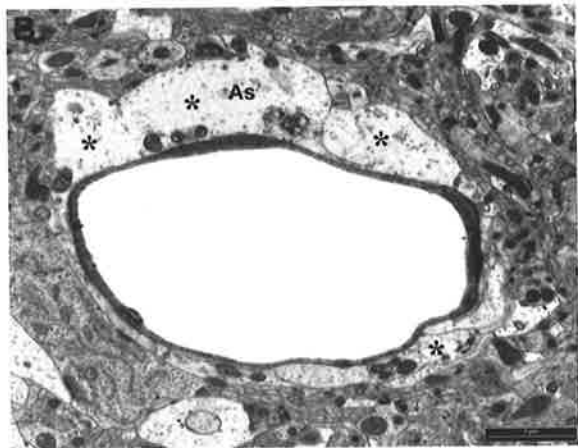
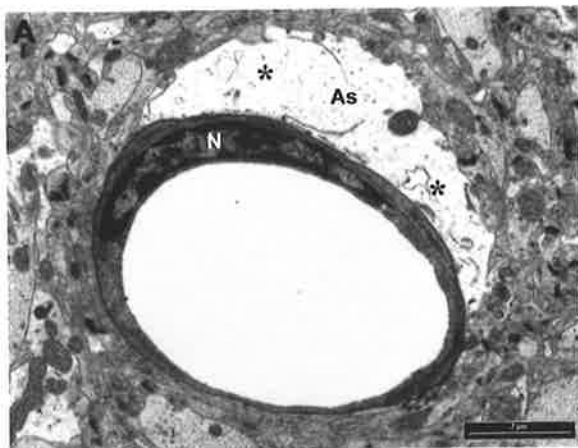


FIG. 4.2.4 (A-C). Electron micrographs showing ultrastructural changes in cerebral cortical microvessels from an experimental animal at 12h after traumatic brain injury.

(A and B) Endothelial cells appear normalised. The swelling of astrocytes is seen (asterisks).

(C) A high magnification shows a structurally intact tight junction (white arrows) between ECs. Astrocytic endfeet are swollen (asterisks).

As, astrocyte; ICJ, intercellular junction; N, endothelial cell nucleus.



4.2.4 Permeability of the BBB in TBI

4.2.4.1 Serum albumin permeability in TBI

Normal and sham control animals showed no albumin in the cerebral cortex, white matter or brainstem (Fig. 4.2.5). Albumin immunoreactivity was only observed in the pineal gland and choroid plexus and to a lesser extent in the periventricular area. In general, the white matter showed faint immunoreactivity not seen in the grey matter.

One hour after the injury (Fig. 4.2.5), some blood vessel walls were highlighted by albumin immunoreactivity, indicating the presence of intramural albumin. Albumin leakage was confined to the immediate vicinity of some blood vessels in the cortex and brainstem. Six hours after the injury (Fig. 4.2.6), albumin leakage in the brain was more widespread than that of 1h. The peroxidase reaction product indicative of albumin was diffusely present in the parenchyma with multiple foci of higher immunoreactivity. Twelve hours after the injury (Fig. 4.2.6), albumin leakage was still present but was less prominent than that of 6h. Similar to the observation seen at 1h, albumin leakage at 12h was confined to the immediate vicinity of some blood vessels in the cortex and brainstem.

EM study of albumin leakage following the trauma showed that albumin leakage was present in the brain at 1h, 6h and 12h after the injury (Fig. 4.2.7). At 1h, the reaction product mainly infiltrated the cytoplasm of brain ECs. Only few vesicles were observed to contain albumin. No obvious infiltration of albumin within tight

junctions was observed. At 6 and 12h the peroxidase reaction product was seen in the perivascular space and the neuropil. In some sections albumin was visualised in the neuropil distant from vascular profile.

4.2.4.2 Lanthanum permeability in TBI

In animals injected with lanthanum chloride, the tracer appeared as an electron dense crystalline deposit in transmission electron microscopy. In the normal and sham control animals, lanthanum deposits were confined to the luminal surface of microvessels in the cortex, corpus callosum and brainstem (Fig. 4.2.8). It labelled luminal membranes at the mouths of intercellular junctions but was not seen within the tight junctions. No lanthanum deposits were noted in the cytoplasm of the ECs or in the brain parenchyma.

Lanthanum leakage in the brain was investigated in experimental animals at 1h and 6h after the injury. One hour after the injury, lanthanum leakage was observed in the cortex, brainstem and corpus callosum (Fig. 4.2.8). Lanthanum deposits were observed in the cytoplasm of ECs as diffuse infiltration. Some vesicles close to the luminal surface of ECs contained lanthanum. Lanthanum was also noted within tight junctions. Lanthanum was seen to fill some altered tight junctions, including the focal widening of these junctions. However, no lanthanum was observed at the perivascular space of microvessels.

Six hours after the injury, lanthanum leakage was still present in the brain (Fig. 4.2.9). As in 1h, lanthanum deposits heavily infiltrated the cytoplasm of brain

ECs. Some vesicles contained lanthanum deposits. Lanthanum was also observed within some tight junctions. In some structurally altered tight junctions, lanthanum filled almost the whole length of the cleft. In some blood vessels, the widened perivascular space contained lanthanum deposits. Lanthanum was also seen in the neuropil among neuronal and glial elements and in some glial processes.

FIG. 4.2.5 (A-F). Light micrographs of sagittal sections of paraffin-embedded brains from control (**A**), 1h sham control (**B**) and experimental animals (**C-F**) at 1h after traumatic brain injury, showing immunoperoxidase staining for serum albumin.

(A and B) In normal control (**A**) and 1h sham control (**B**) animals, no albumin immunoreactivity is observed.

(C-F) At 1h after the injury albumin leakage is seen in the cortex (**C** and **E**) and brainstem (**D** and **F**). Albumin leakage mainly has a perivascular distribution (arrows).

Bar, 50 μm .

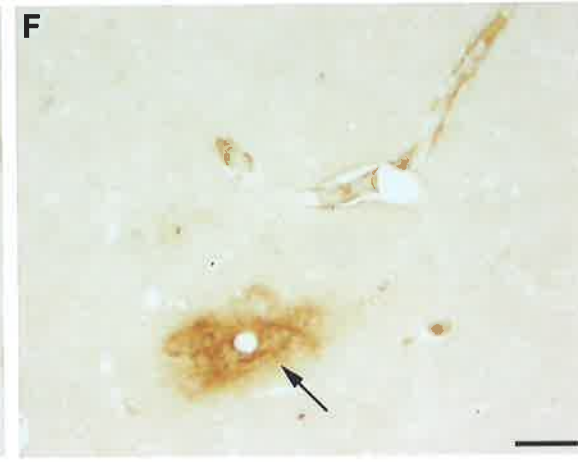
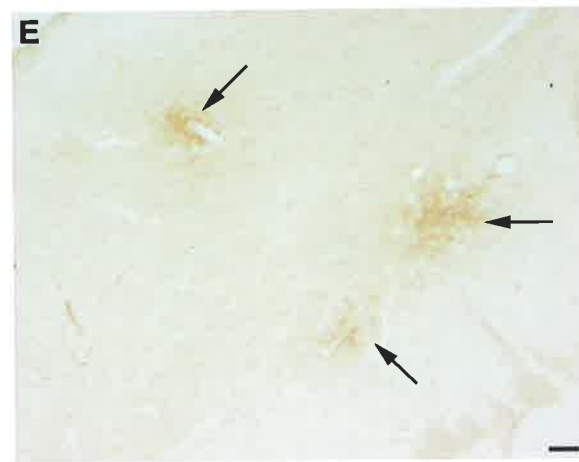
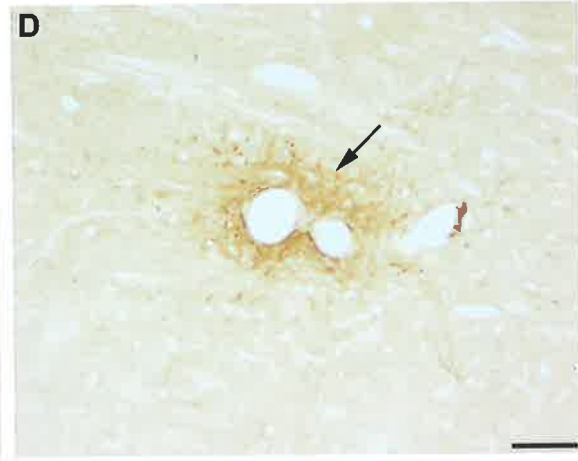
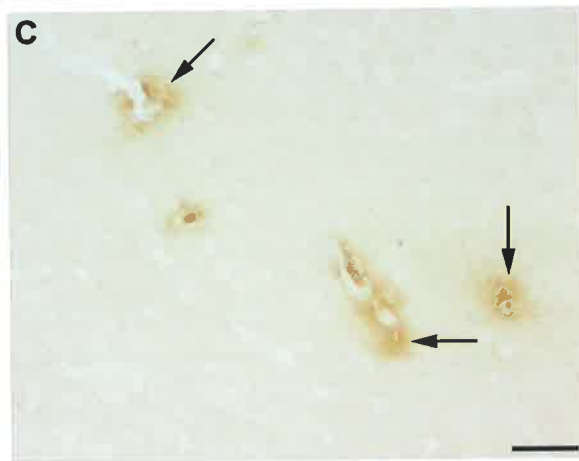
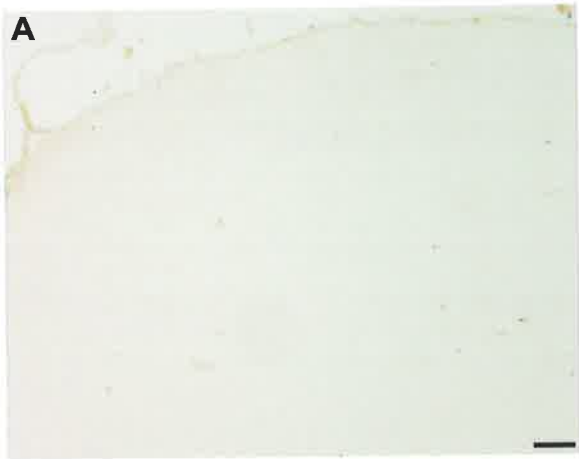


FIG. 4.2.6 (A-F). Light micrographs of paraffin sections of brains from sham controls (6h, **A** and 12h, **B**) and experimental animals with survival times of 6h (**C** and **D**) and 12h (**E** and **F**) after traumatic brain injury. The sections are immunostained for serum albumin.

(A and B) No albumin leakage is seen in brains of sham control animals.

(C and D) Six hours after the injury, albumin immunoreactivity is diffusely present in the parenchyma of the cortex (asterisks, **C**). In the brainstem, perivascular albumin leakage is seen (arrows, **D**).

(E and F) 12 h after the injury, albumin leakage is still present but was less severe compared to that of 6h (**C**). Blood vessel walls in the cortex are highlighted by albumin immunoreactivity with perivascular albumin leakage observed (arrows, **E**). In the brainstem, few foci of peroxidase reaction product is present (arrow, **F**).

Bar, 50 μm .

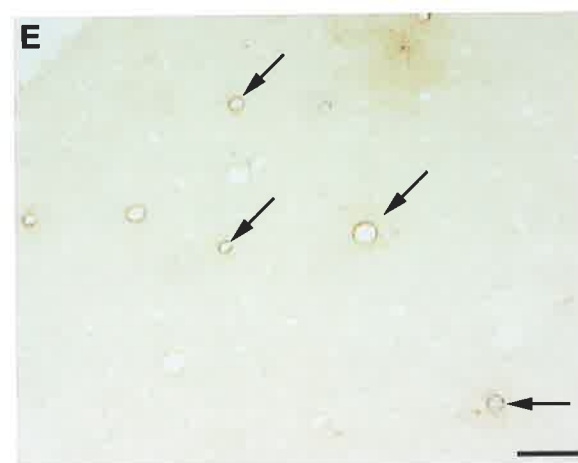
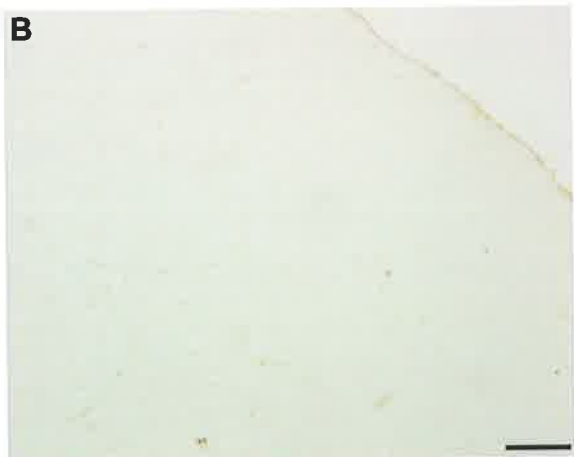


FIG. 4.2.7 (A-E). Electron micrographs of cerebral cortical vessels from normal control **(A)** and experimental animals **(B-E)** after traumatic brain injury, showing pre-embedding immunoperoxidase labelling for endogenous albumin.

(A) No immunoreactivity is seen in the control animal.

(B and C) At 6h after the injury, electron dense deposits, representing albumin labelling, are observed in the cytoplasm of ECs (arrows). Vesicles [V] in the EC are unstained.

(D) At 12h after the injury, albumin infiltration of EC cytoplasm is still present (arrows).

(E) At 12h after the injury, albumin is also present in the brain parenchyma between nerve fibres (arrows).

All micrographs are from grids not counterstained with heavy metals.

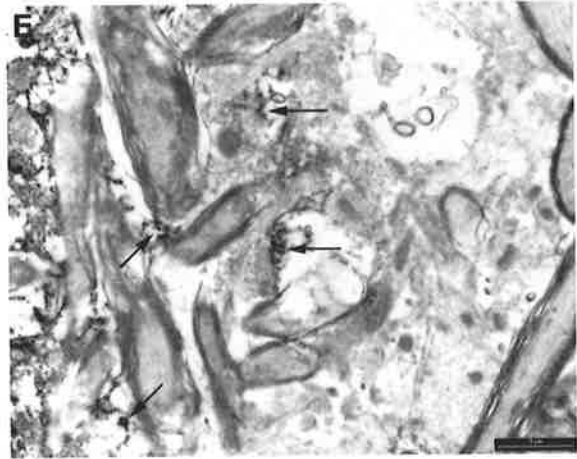
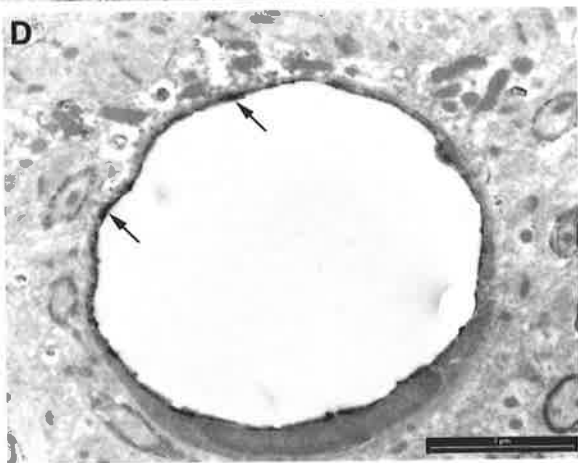
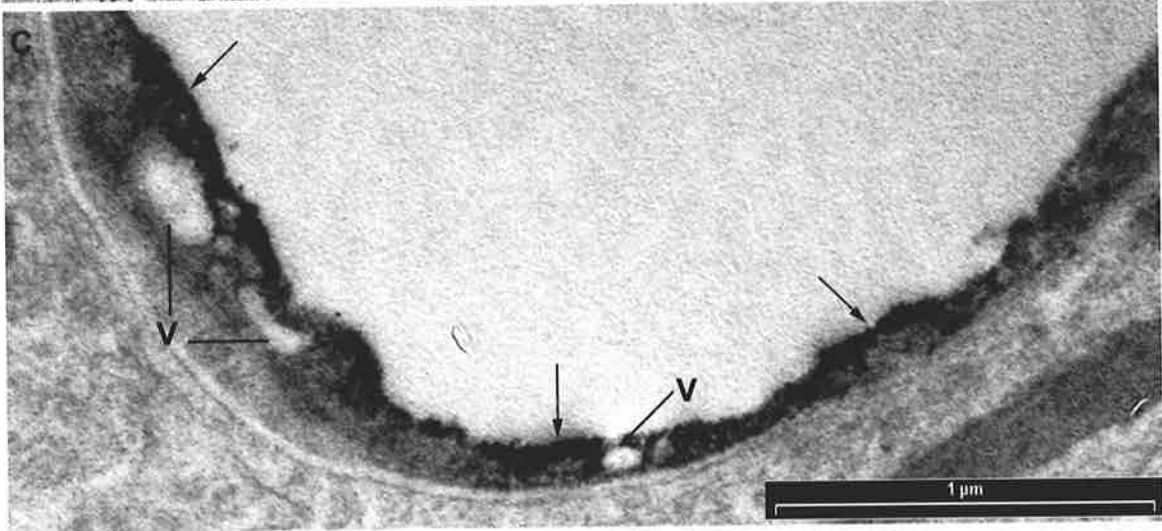
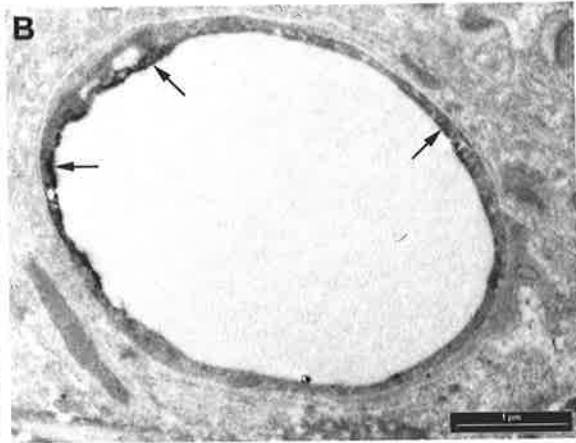
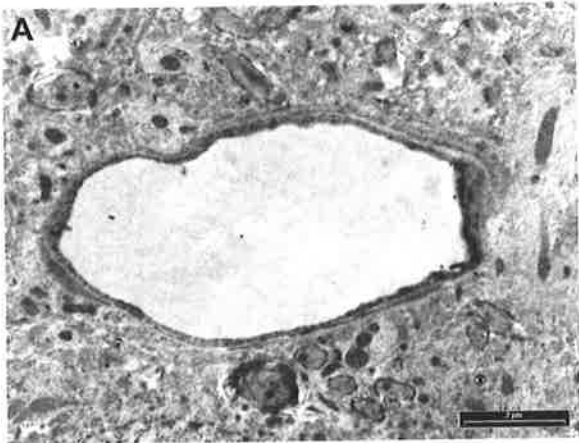


FIG. 4.2.8 (A-E). Electron micrographs of cerebral vessels from a normal control animal (A) and an experimental animal at 1h after traumatic brain injury (B-E), showing lanthanum in the brain.

(A) A cerebral cortical blood vessel from a normal control animal showing lanthanum at the luminal surface (small black arrows) and at the luminal end of a tight junction (large black arrow). No lanthanum is seen within the tight junction (white arrows).

(B and C) Brainstem blood vessels from an experimental animal showing lanthanum at the luminal surface of ECs (small black arrows) and infiltrating altered tight junctions (ICJ, large black arrows).

(D) shows a higher magnification of the rectangle in (B). Lanthanum infiltrates a focally widened tight junction (large arrows). Small arrows indicate lanthanum at the luminal membrane of ECs.

(E) A cerebral cortical vessel from an experimental animal showing lanthanum at the luminal surface (small black arrows) and infiltrating a focally widened tight junction (large arrows).

ICJ, intercellular junction.

All micrographs are from sections lightly stained with uranyl acetate.

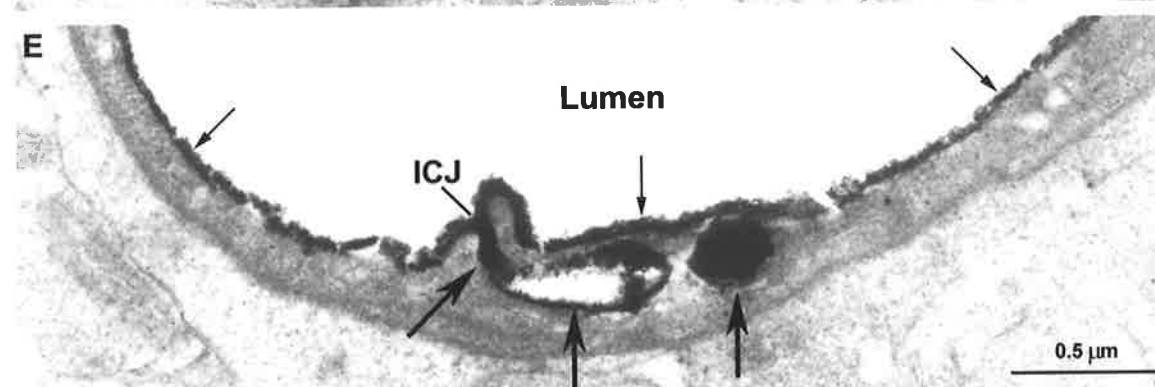
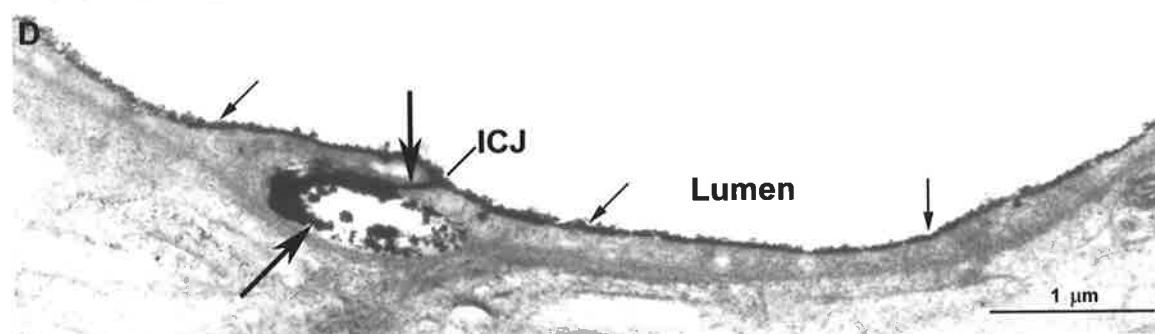
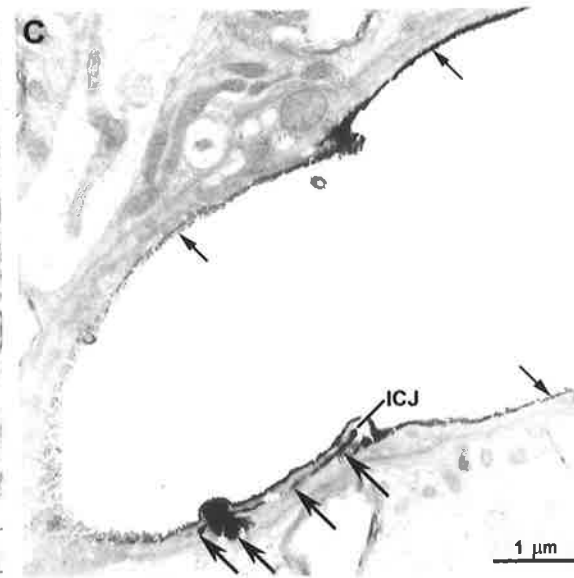
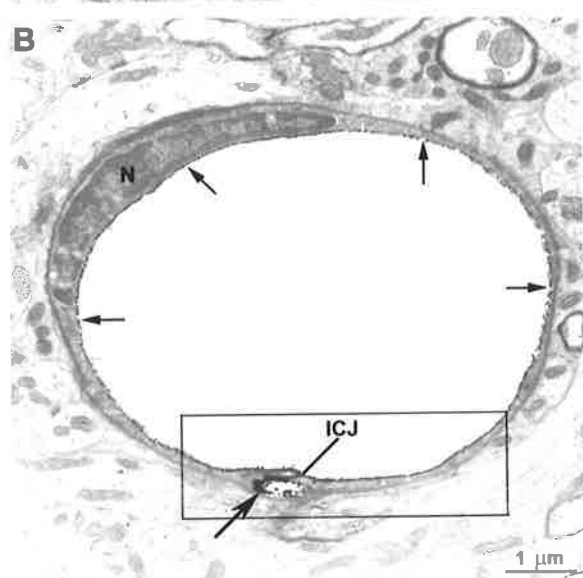
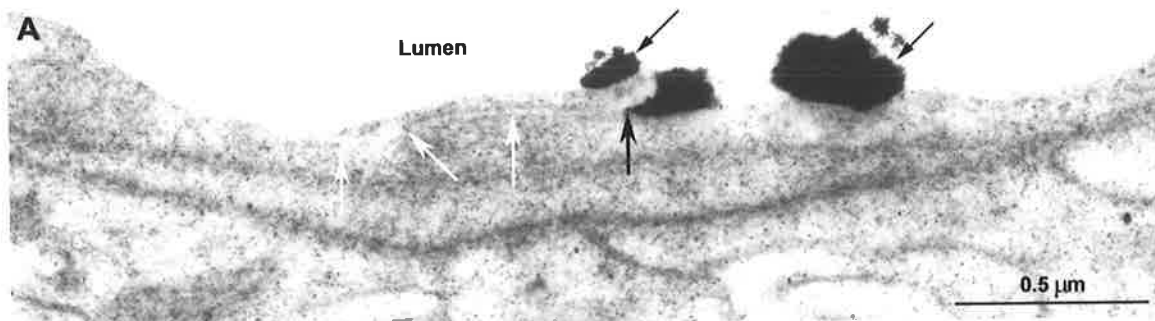
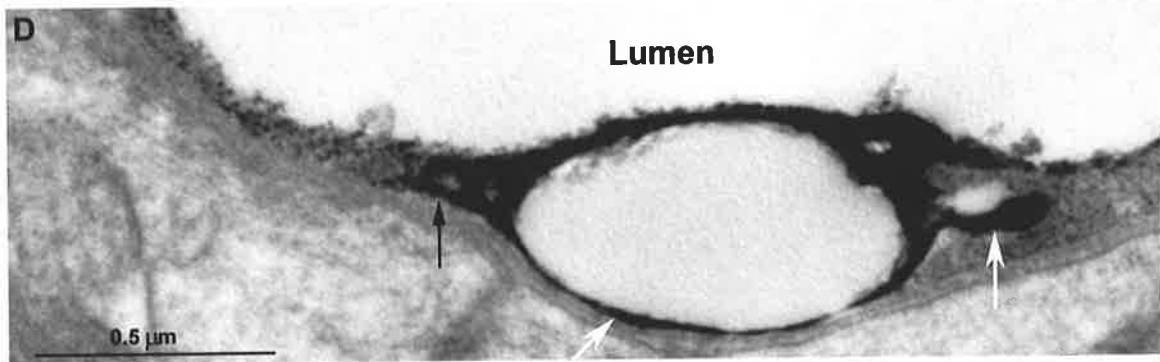
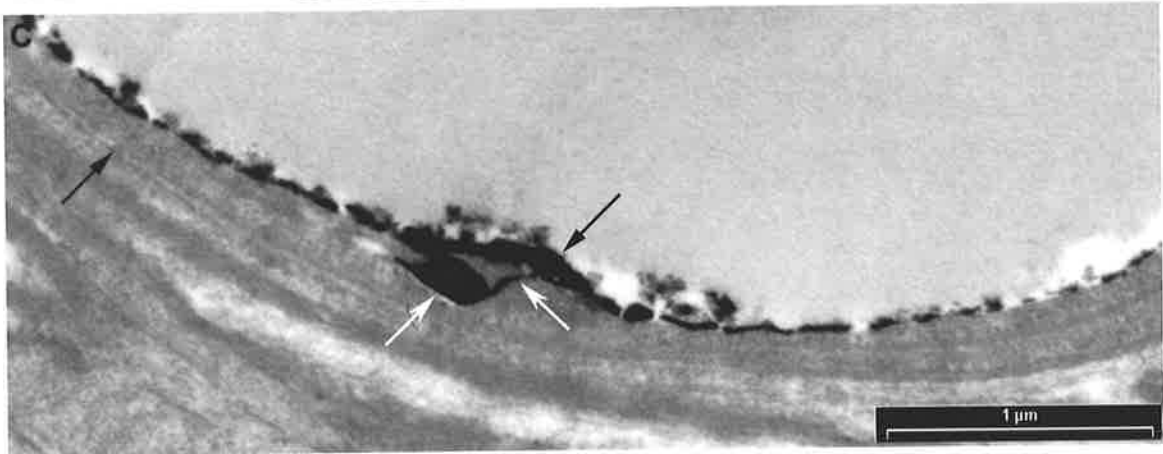
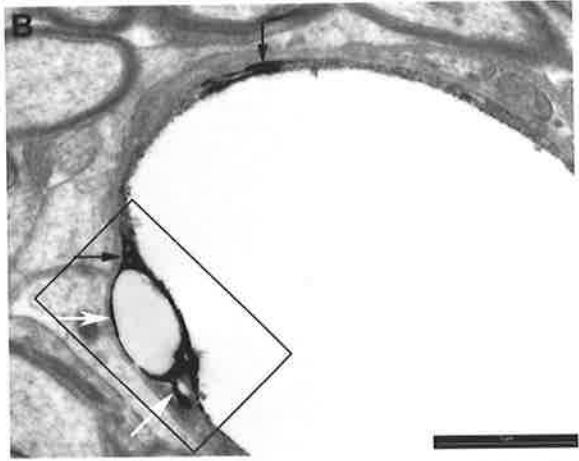
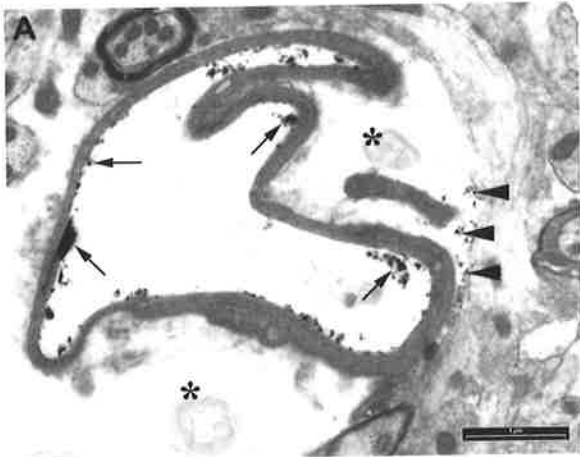


FIG. 4.2.9 (A-D). Electron micrographs of cerebral vessels, in the white matter, from an experimental animal at 6h after traumatic brain injury, showing lanthanum leakage in the brain.

- (A)** Lanthanum is seen at the luminal surface (arrows) and in the perivascular space (arrowheads) of a vessel. The vessel is distorted by the oedema formation, indicated by widening of the perivascular space (asterisks).
- (B)** Lanthanum infiltrates the cytoplasm of ECs (black arrows). Lanthanum is also filling a tight junction, including a focal widening (white arrows).
- (C)** A high magnification showing an altered tight junction (black arrows indicate the luminal and abluminal ends of the tight junction). Lanthanum is seen filling the luminal one third of the junction including a focal widening (white arrows).
- (D)** shows a higher magnification of the rectangle in **(B)**. Lanthanum is seen filling a focally widened tight junction (white arrows) and the adjacent cytoplasm (black arrows).

All micrographs are from sections lightly stained with uranyl acetate.



4.2.5 EBA expression in TBI

LM of Paraffin and Vibratome sections of brains from normal and sham control animals showed strong immunoreactivity for EBA in almost all blood vessels in the cortex, diencephalon, cerebellum and brainstem (Fig. 4.2.10). The staining pattern was similar to that seen in normal Hooded Wistar rats examined in the prototoxin study (section 4.1.4). Occasionally blood vessels in the choroid plexus and pineal gland showed weak immunoreactivity for EBA.

LM of paraffin and Vibratome sections of experimental animals 1h after the injury showed similar pattern of EBA immunoreactivity to that of controls. The majority of blood vessels were positively stained for EBA. However, closer examination revealed reduced EBA immunoreactivity in some blood vessels, where vessel walls were non-reactive or only faintly reactive for EBA staining (Fig. 4.2.10 D). At 6h after the injury, the reduction of EBA immunoreactivity in the brain was apparent by LM. The reduction was most pronounced in the parietal cortex, where many blood vessels were devoid of immunoreactivity for EBA (Fig. 4.2.10 F). At 12h after the injury, considerable recovery of EBA immunoreactivity in the brain was observed (Fig. 4.2.10 H). Most vessels were positively stained. However, there were still some non-reactive blood vessels observed throughout the brain.

EM study showed that in brains of normal and sham control animals, blood vessels were strongly immunoreactive for EBA (Figs 4.2.11 and 4.2.12). Electron

dense peroxidase reaction product indicative of EBA immunostaining was observed along the luminal membrane of ECs and to a lesser extent, in the EC cytoplasm. In brains of normal control animals, EBA staining was present at the luminal membrane of the entire circumference of most blood vessels. Only in a few blood vessels, part of the circumference lacked EBA staining. In brains of sham control animals, the majority of blood vessels showed EBA staining present at the entire circumference of luminal membranes. However, more blood vessels showed sectoral loss of EBA staining compared to those of normal control animals.

One hour after the injury, many blood vessels showed sectoral loss of EBA staining (Fig. 4.2.11). In some blood vessels, no peroxidase reaction product was observed at the luminal membrane or cytoplasm of ECs. Six hours after the injury, the reduction of EBA immunoreactivity in the brain was obvious (Fig. 4.2.12). The majority of cerebral blood vessels were only partially stained or devoid of EBA staining completely. Twelve hours after the injury, the majority of cerebral blood vessels were still only partially stained for EBA (Fig. 4.2.12).

FIG. 4.2.10 (A-H). Light micrographs of paraffin sections showing immunostaining for EBA in normal control (**A** and **B**), sham control (1h, **C**, 6h, **E** and 12h, **G**) and experimental animals after the traumatic brain injury (1h, **D**, 6h, **F** and 12h, **H**).

(**A, C-H**) EBA-immunoreactive vessels (arrows) are seen in various orientations in cerebral cortices.

(**B**) Brain vessels are stained (arrows) in the cerebrum around the choroid plexus but vessels in the choroid plexus are unstained or weakly stained (arrowheads).

(**D, F and H**) Partially- or weakly-labelled vessels (arrowheads) are seen in experimental animals.

Bar, 50 μm .

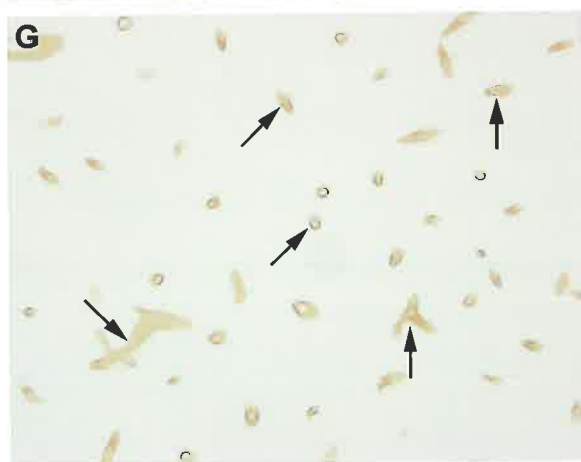
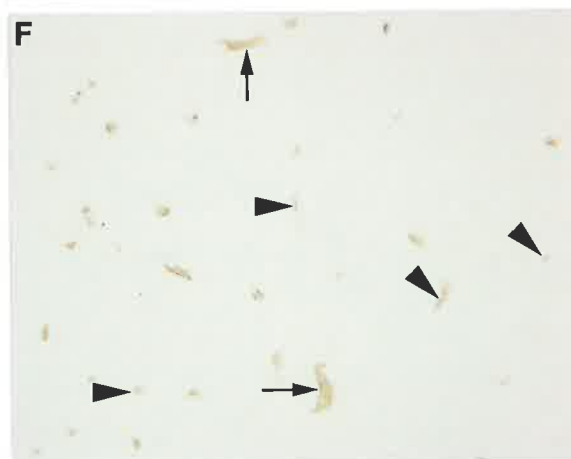
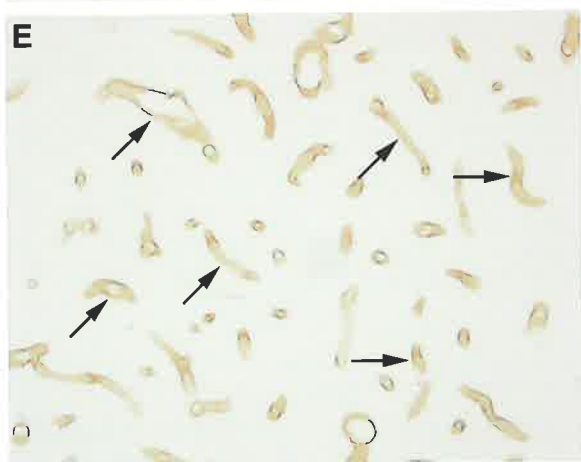
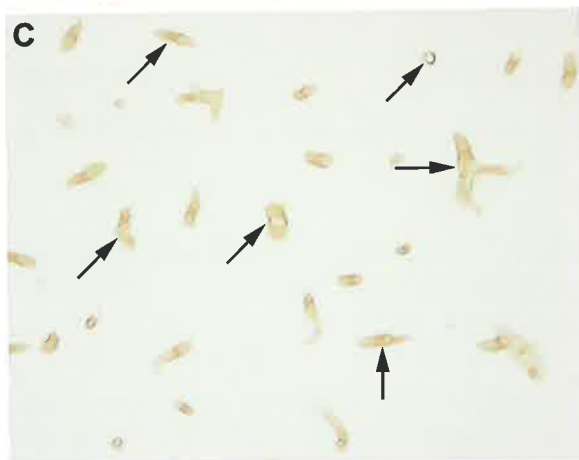
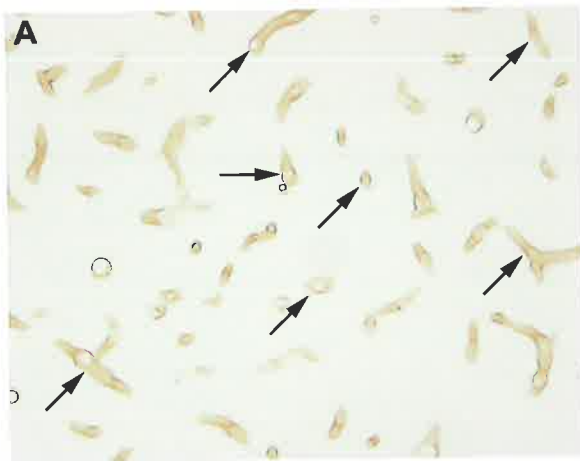


FIG. 4.2.11 (A-E). Electron micrographs of cerebral cortical microvessels from normal control animals (**A-C**), 1h sham control animal (**D**), and an experimental animal at 1h after traumatic brain injury (**E**). The sections show pre-embedding immunoperoxidase labelling for EBA.

(A and B) In normal controls, the labelling appears as electron dense reaction product along the entire length of luminal membrane (small arrows) of ECs. Cytoplasmic vesicles [V] appear unstained (**B**).

(C) Endothelial cells show immunoreactivity in the cytoplasm involving the full thickness between the luminal and abluminal membranes (between large arrows). Cytoplasmic vesicles [V] appear unstained.

(D) A blood vessel from a 1h sham control animal shows strong EBA labelling along the entire length of EC luminal membrane (arrows).

(E) At 1h after the injury, a blood vessel shows partial loss of EBA labelling on the luminal membrane (arrowheads). Cytoplasmic vesicles [V] appear unstained. Widening of the perivascular space (asterisks) indicates the development of oedema at 1h after the trauma.

N, endothelial cell nucleus.

All micrographs are from grids not counterstained with heavy metals.

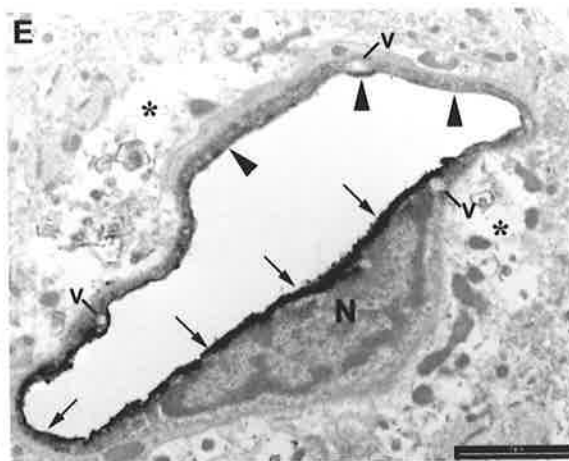
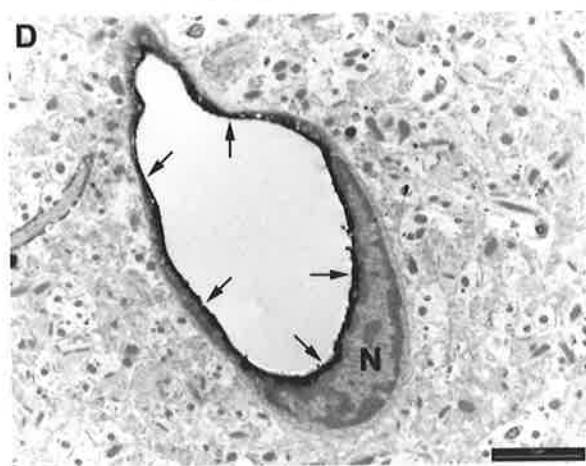
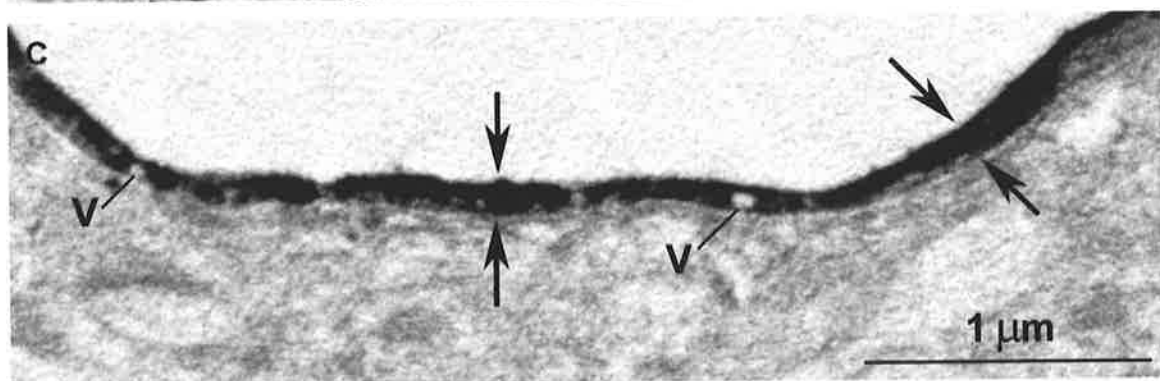
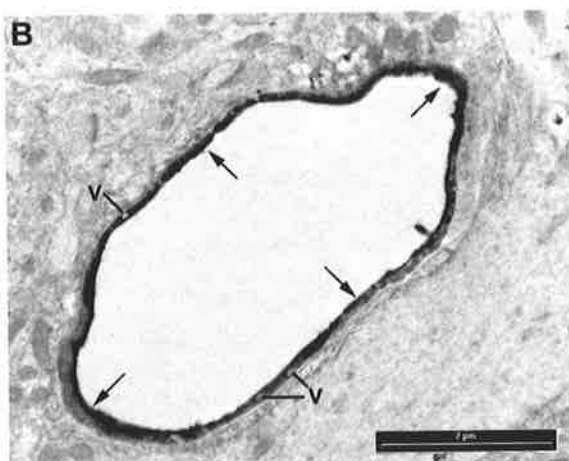
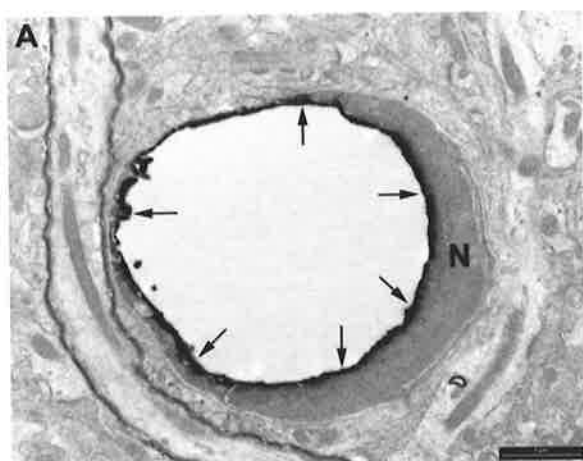
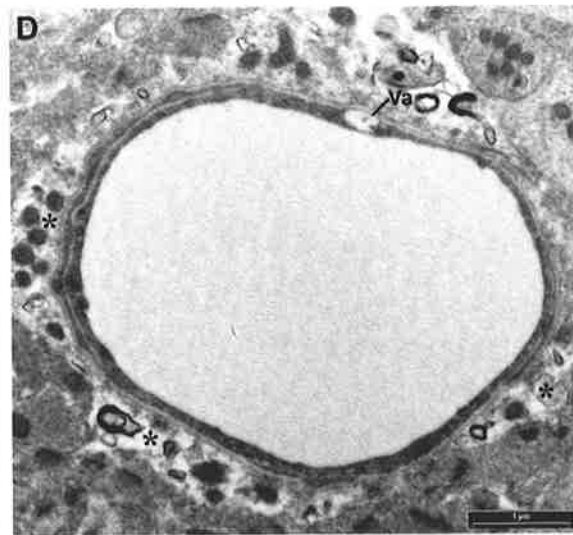
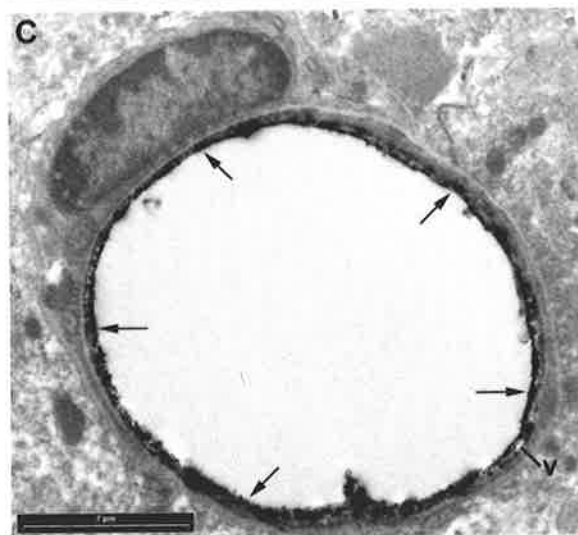
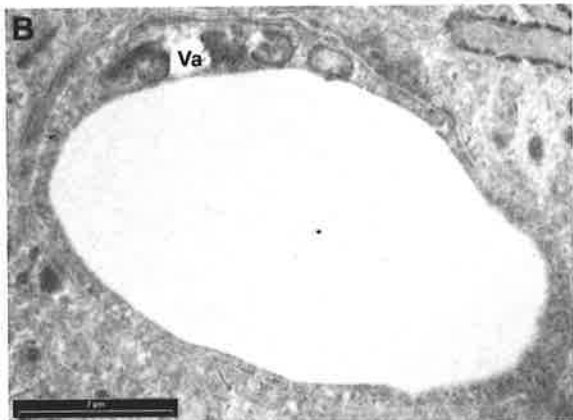
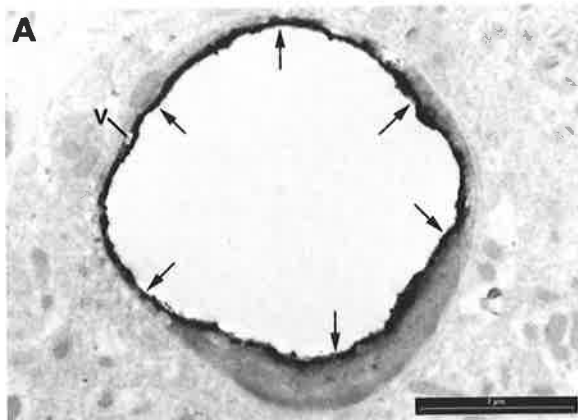


FIG. 4.2.12 (A-D). Electron micrographs of cerebral cortical microvessels from 6h sham control animal (**A**), 12h sham control animal (**C**), and experimental animals at 6h (**B**) and 12h (**D**) after traumatic brain injury. The sections show pre-embedding immunoperoxidase labelling for EBA.

(A and C) Sham control animals show EBA labelling along the entire length of the luminal membrane (arrows). Cytoplasmic vesicles [V] are unstained.

(B and D) At 6h and 12h after the injury, blood vessels show loss of immunoreactivity for EBA. Large vacuoles are present in the cytoplasm [Va]. Wide perivascular space (asterisks) is observed in **D**.

All micrographs are from grids not counterstained with heavy metals.



Quantitative analyses of EBA immunoreactivity in TBI were performed at the LM and EM levels on three experimental groups, 1h, 6h and 12h after injury. Two experimental animals in each group were compared to one sham control animal at each time interval and two normal control animals.

For quantitative analysis of EBA immunoreactivity at the LM level, two areas in the brain were analysed (Table 4.1). Positively labelled blood vessels in the frontal cortex and brainstem were quantified (Appendix A.4). Five areas from each region were photographed and analysed. In the two normal control animals, the mean numbers of immunoreactive blood vessels in the frontal cortex were 406 ± 58 and 430 ± 37 per mm^2 respectively (Fig. 4.2.13). The mean number of EBA-immunoreactive blood vessels in the 1h sham control was 282 ± 54 per mm^2 . One hour after the injury, the number of EBA immunoreactive blood vessels decreased to a mean of 346 ± 82 and 269 ± 21 per mm^2 in the two experimental animals. Six hours after the injury, a further reduction of EBA immunoreactivity was observed (Fig. 4.2.13). The number of positively stained blood vessels was further reduced to a mean of 183 ± 42 and 269 ± 33 per mm^2 , which was apparently less than the normal control and the 6h sham control animals. Twelve hours after the injury, the number of EBA-immunoreactive blood vessels increased compared with 6h, to a mean value of 265 ± 39 and 275 ± 33 per mm^2 , but was still less than the normal control and the 12h sham control animals (Fig. 4.2.13).

All the sham control animals showed no obvious reduction in the number of EBA-immunoreactive blood vessels in the frontal cortex except the one hour sham control animal, which showed a decreased number of EBA-immunoreactive blood vessels when compared with the normal controls.

In the brainstem, the mean numbers of EBA immunoreactive blood vessels of the two normal control animals were 464 ± 43 and 380 ± 47 per mm^2 (images were taken from areas of pontine nuclei) (Fig. 4.2.14). One hour after the injury, the number of EBA immunostained blood vessels decreased to the means of 335 ± 29 and 313 ± 56 per mm^2 (Fig. 4.2.14). Six hours after the injury, the mean number of EBA immunoreactive blood vessels of one experimental animal largely decreased to 273 ± 50 per mm^2 , while another experimental animal studied showed no obvious changes of the EBA immunoreactivity (Fig. 4.2.14). Twelve hours after the trauma, the mean numbers of EBA immunoreactive blood vessels were 282 ± 75 and 363 ± 50 per mm^2 , which were still less than the normal control and the 12h sham control animals (Fig. 4.2.14).

All sham control animals showed no obvious reduction of EBA immunoreactivity in the brainstem except the one hour sham animal, where the mean number of positively stained blood vessels was 310 ± 59 per mm^2 .

Table 4.1. The mean number of EBA-immunoreactive blood vessels in the brain of normal control, sham control and traumatic brain injury (TBI) rats

	Frontal cortex (n/mm ²)	Brainstem (n/mm ²)
1h TBI (B3)	346 ± 82 (240-460)	335 ± 29(288-364)
1h TBI (B4)	269 ± 21 (240-295)	313 ± 56 (254-378)
6h TBI (B16)	183 ± 42 (117-220)	273 ± 50 (227-336)
6h TBI (B17)	269 ± 33 (233-316)	467 ± 73 (384-549)
12h TBI (B32)	265 ± 39 (227-330)	282 ± 75 (185-385)
12h TBI (B48)	275 ± 33 (240-330)	363 ± 50 (282 ± 405)
1h sham (B6)	282 ± 54 (220-343)	310 ± 60 (254-378)
6h sham (B18)	420 ± 22 (391-453)	488 ± 103 (343-604)
12h sham (B49)	385 ± 34 (336-419)	452 ± 114 (261-549)
Normal (B19)	406 ± 58 (343-501)	464 ± 43 (398-508)
Normal (B20)	430 ± 37 (371-467)	380 ± 47 (336-446)

Data shown represent the mean value ± SD (range).

n/mm², number of EBA-immunoreactive blood vessels per mm².

FIG. 4.2.13. A histogram summarising the LM quantitative results of EBA immunoreactive vessels in the frontal cortex. The columns represent the mean number of blood vessels obtained from 2 normal controls, one sham control at 1h, 6h, and 12h and two experimental animals at 1h, 6h, and 12h post TBI. The error bars representing the standard deviation for each group of data are shown.

FIG. 4.2.14. A histogram summarising the LM quantitative results of EBA immunoreactive vessels in the brainstem. The columns represent the mean number of blood vessels obtained from 2 normal controls, one sham control at 1h, 6h, and 12h and two experimental animals at 1h, 6h, and 12h post TBI. The error bars representing the standard deviation for each group of data are shown.

FIG. 4.2.13 The mean number of EBA-immunoreactive blood vessels at the LM level in the frontal cortex in normal control, sham control and TBI rats

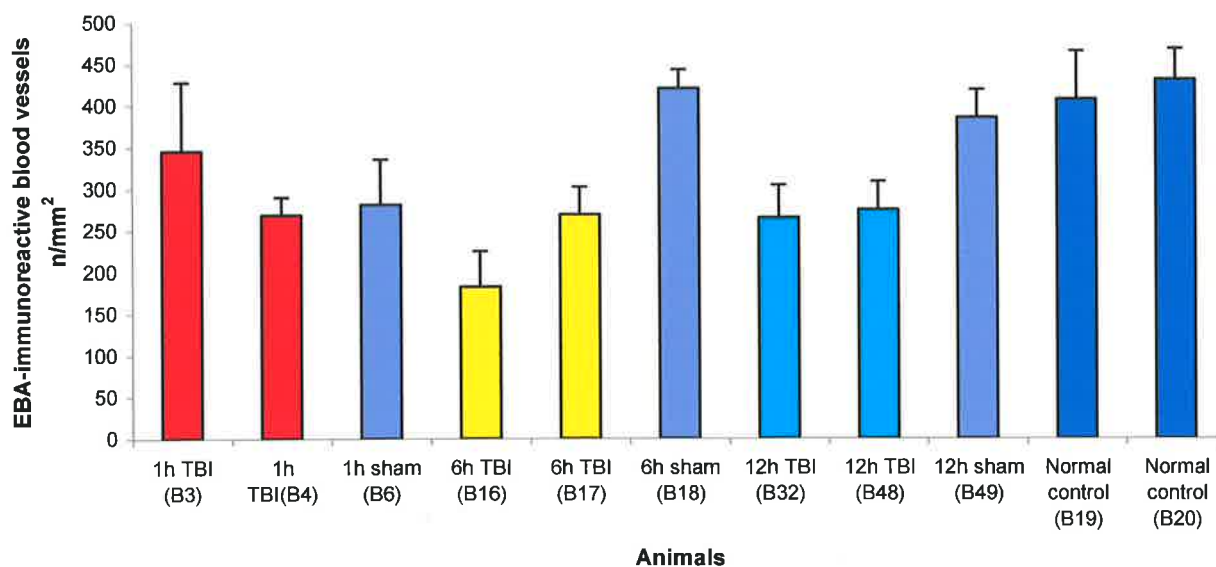
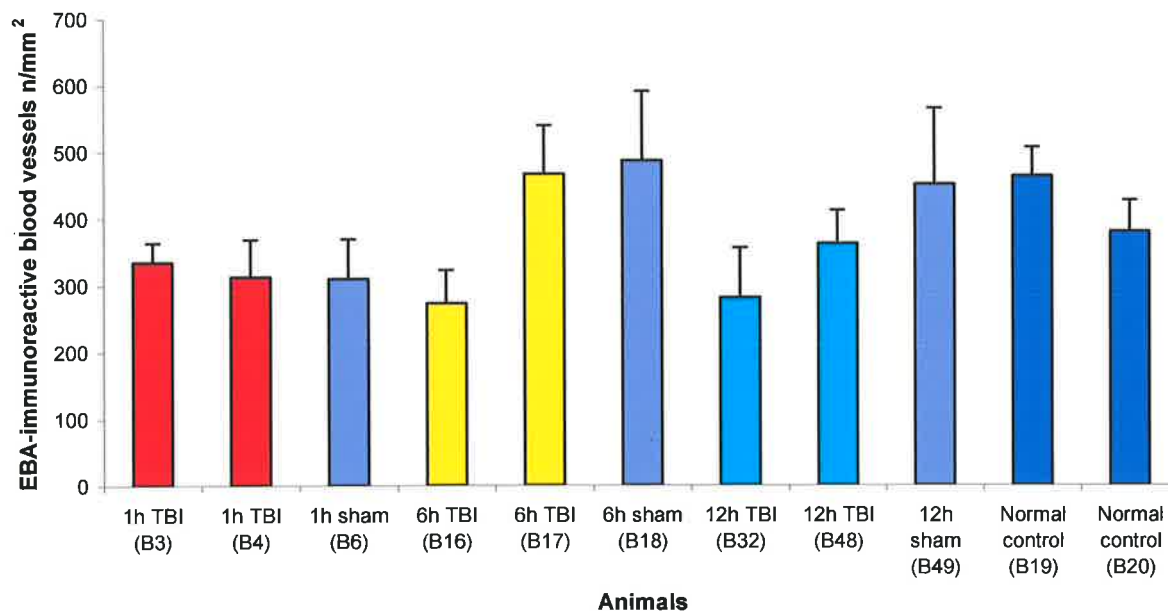


FIG. 4.2.14 The mean number of EBA-immunoreactive blood vessels at the LM level in the brainstem in normal control, sham control and TBI rats



Quantitative assessment of EBA immunoreactivity at the EM level was performed on frontal cortical vessels in two normal controls, one sham control at 1h, 6h and 12h and two experimental animals at 1h, 6h and 12h time intervals. Eighteen and 21 blood vessels in the frontal cortex were examined from the two normal control animals (for raw data see Appendix A.5). The luminal membrane length showing EBA immunoreactivity was measured and the ratio in relation to the total length of luminal membrane for each vessel was calculated. In the normal control animals, most vessels had 100% staining of their luminal membrane. Only occasional vessels were partially stained. The mean values of the percentage of positively stained luminal membrane length of the two normal animals were $92 \pm 20\%$ and $95 \pm 16\%$. In the sham control animals, 22, 22 and 25 blood vessels in the frontal cortex were measured from animals killed at 1h, 6h and 12h respectively (Appendix A.5). Most blood vessels had 100% staining of their luminal membrane. However, compared with the normal animal, more blood vessels in the sham control animals showed sectoral loss of staining and some blood vessels were devoid of EBA staining. In the sham controls, the mean values of the percentage of positively stained luminal membrane lengths were $81 \pm 37\%$ for 1h, $77 \pm 38\%$ for 6h and $83 \pm 22\%$ for 12h.

One hour after the injury, a pronounced reduction of EBA immunoreactivity in cortical blood vessels was observed (Fig. 4.2.15). Many blood vessels showed only partial staining for EBA and some vessels completely lost EBA immunoreactivity. The mean values of the percentage of positively stained luminal membrane lengths were $52 \pm 41\%$ and $45 \pm 38\%$ in the two animals killed

at 1h ($n = 24$ and 22 vessels respectively, see Appendix A.5). Six hours after the injury, more cortical blood vessels showed loss of EBA immunoreactivity (Fig. 4.2.16). The mean values of the percentage of positively stained luminal membrane lengths reduced to $37 \pm 32\%$ and $37 \pm 31\%$ for the two animals killed at 6h ($n = 22$ and 19 vessels respectively, Appendix A.5). Twelve hours after the injury, the reduction of EBA immunoreactivity in cortical microvessels was still evident (Fig. 4.2.17). The mean values of the percentage of immunoreactive luminal membrane lengths were $42 \pm 39\%$ and $54 \pm 27\%$ in the two experimental animals killed at 12h ($n = 23$ and 20 vessels respectively, Appendix A.5).

FIG. 4.2.15. A histogram summarising the EM quantitative results of EBA immunoreactivity in the cortex. The columns represent the mean percentage of EBA labelling of luminal membranes in two normal controls, one sham control at 1h and two experimental animals at 1h post TBI. The error bars representing the standard deviation for each group of data are shown.

FIG. 4.2.16. A histogram summarising the EM quantitative results of EBA immunoreactivity in the cortex. The columns represent the mean percentage of EBA labelling of luminal membranes in two normal controls, one sham control at 6h and two experimental animals at 6h post TBI. The error bars representing the standard deviation for each group of data are shown.

Fig 4.2.15 Mean percentage of EBA labelling of luminal membrane in normal controls, sham control and 1h TBI animals

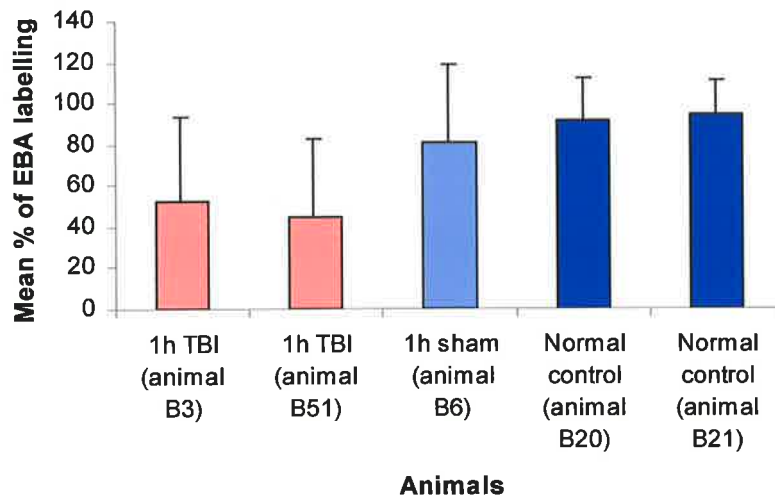


Fig 4.2.16 Mean Percentage of EBA labelling of luminal membrane in normal controls, sham control and 6h TBI animals

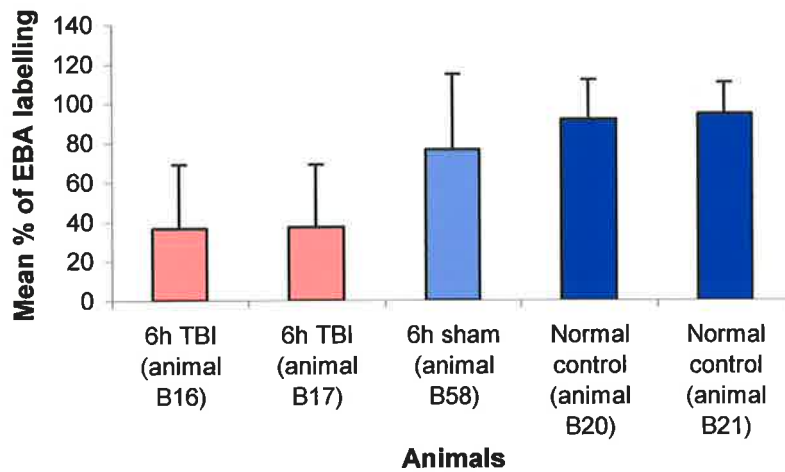
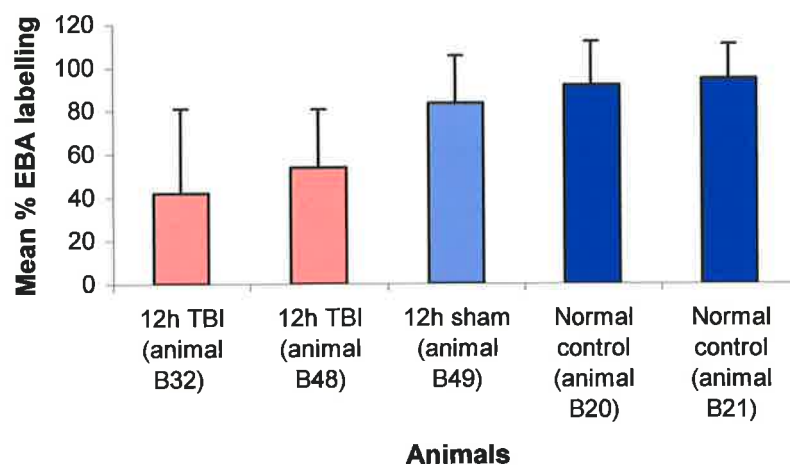


FIG. 4.2.17. A histogram summarising the EM quantitative results of EBA immunoreactivity in the cortex. The columns represent the mean percentage of EBA labelling of luminal membranes in two normal controls, one sham control at 12h and two experimental animals at 12h post TBI. The error bars representing the standard deviation for each group of data are shown.

Fig 4.2.17 Mean percentage of EBA labelling of luminal membranes in normal controls, sham control and 12h TBI animals



4.2.6 GLUT1 expression in TBI

LM of Vibratome and paraffin sections from control animals showed intense and uniform distribution of GLUT1 immunoreactivity in microvessels throughout the brain (Fig. 4.2.18). Blood vessels of various sizes showed positive staining. In the pineal gland and choroid plexus, only few vessels showed faint staining for GLUT1. Choroidal epithelium was immunoreactive for GLUT1. Blood vessels at the pia mater were stained. Sham control animals showed a similar GLUT1 staining to that of normal control animals.

Using electron microscopy, immunoreactivity for GLUT1 appeared as electron dense deposits representing the peroxidase reaction product (Fig. 4.2.19). GLUT1 immunoreactivity was mainly localised to the luminal membrane of ECs. The abluminal membrane was also positively stained, but the staining appeared to be less intense than that of the luminal membrane (Fig. 4.2.19 C, D). The cytoplasm of some ECs was positively stained. Vesicles in the cytoplasm of ECs were unstained (Fig. 4.2.19 D). In normal controls, GLUT1 staining was present at the entire luminal circumference of most cerebral blood vessels. Only occasional blood vessels showed sectoral absence of GLUT1. In sham control animals, more blood vessels showed partial loss of GLUT1 compared with that of normal controls. No GLUT1 immunoreactivity was localised to pericytes or astrocytic endfeet. In the neuropil, neurons and axons were not stained.

Experimental animals showed variations in GLUT1 immunoreactivity in brain vessels. One hour after the injury, LM showed that the majority of microvessels

was positive for GLUT1 staining. However, some blood vessels were faintly stained or lost immunoreactivity completely (Fig. 4.2.18 D). Six hours after the injury, the reduction of GLUT1 immunoreactivity was most pronounced (Fig. 4.2.18 F). In the cortex, more blood vessels were devoid of GLUT1 immunoreactivity or only showed partial staining for GLUT1. At 12h after the injury, GLUT1 immunoreactivity showed obvious recovery as more blood vessels showed staining for GLUT1 (Fig. 4.2.18 H).

By EM (Fig. 4.2.20), 1h after the injury, almost all blood vessels examined showed only partial staining for GLUT1. Some blood vessels showed no immunoreactivity for GLUT1. Six hours after the injury showed maximum loss, the majority of blood vessels showed sectoral loss of GLUT1 and more blood vessels lost immunoreactivity completely. Twelve hours after the injury, unlike LM, the majority of blood vessels still showed partial loss for GLUT1 immunoreactivity.

FIG. 4.2.18 (A-H). Immunolabelling for GLUT1. Light micrographs of paraffin sections from brains of normal control (**A** and **B**), sham controls (1h, **C**; 6h, **E** and 12h, **G**) and experimental animals after traumatic brain injury (1h, **D**; 6h, **F**; and 12h, **H**).

(A and C-F) Blood vessels in the cortices of controls and experimental animals are positively stained for GLUT1 (arrows).

(B) Epithelial cells in the choroid plexus of a control animal are immunoreactive for GLUT1 (arrows).

(D, F and H) Partially- or weakly-stained vessels (arrowheads) are seen in experimental animals. At 1h (**D**) and 6h (**F**) after the injury, large areas in the cortex show loss of GLUT1-immunoreactive blood vessels (asterisks).

Bar, 50 μm .

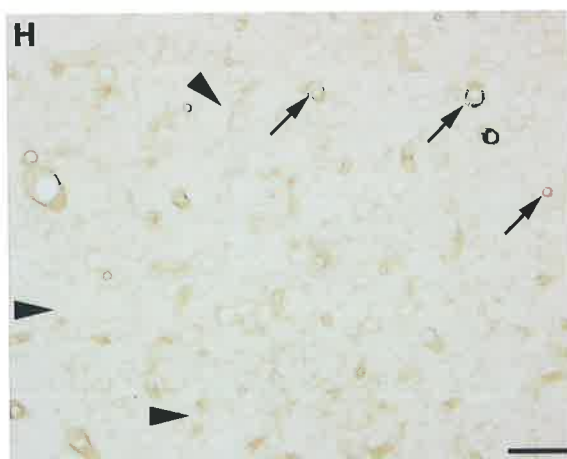
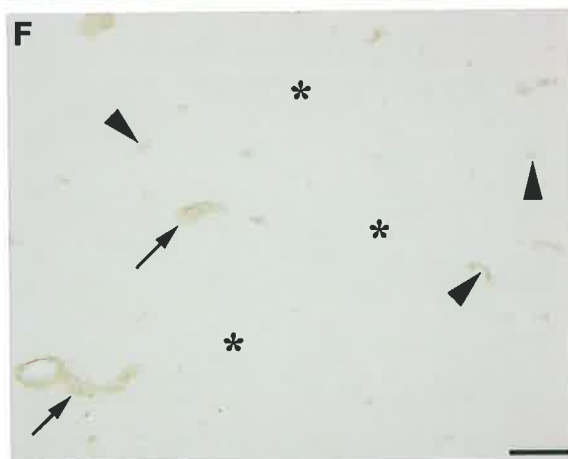
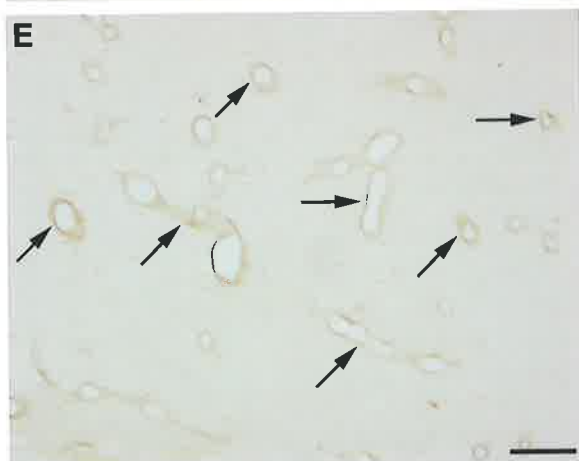
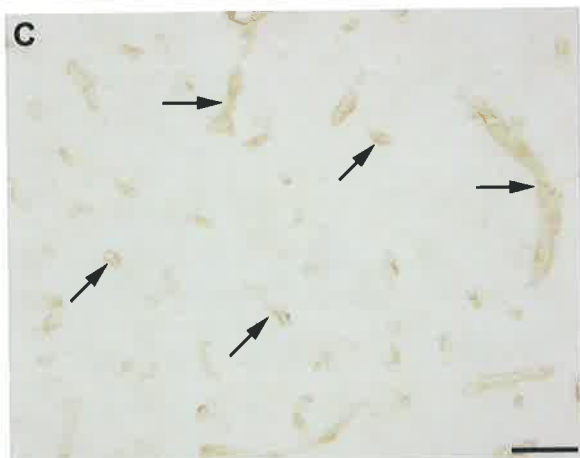
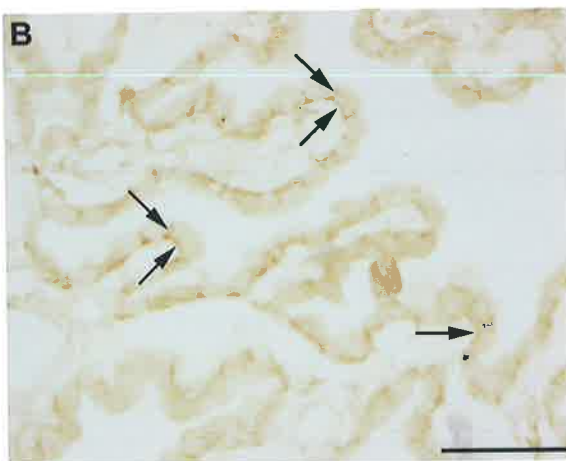


FIG. 4.2.19 (A-D). Electron micrographs of cerebral cortical microvessels from normal control animals, showing pre-embedding immunoperoxidase labelling for GLUT1. The labelling appears as electron dense reaction product along the entire length of the luminal membrane (small black arrows) of ECs. Endothelial cells show immunoreactivity in the cytoplasm involving the full thickness between the luminal and abluminal membranes (between large arrows, **C** and **D**). Abluminal membranes are also stained (white arrows, **C** and **D**). Cytoplasmic vesicles appear unstained [V]. All micrographs are from grids not counterstained with heavy metals.

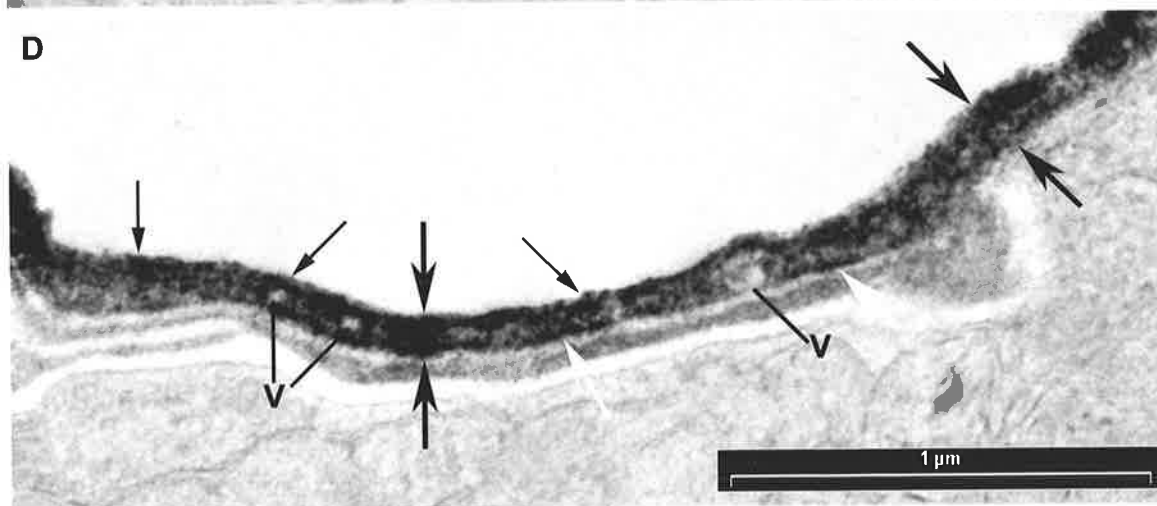
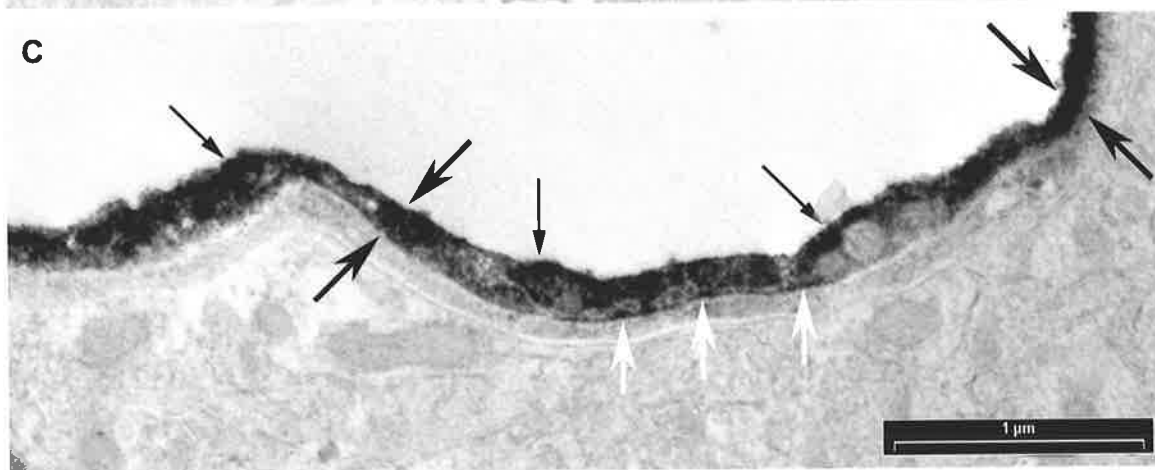
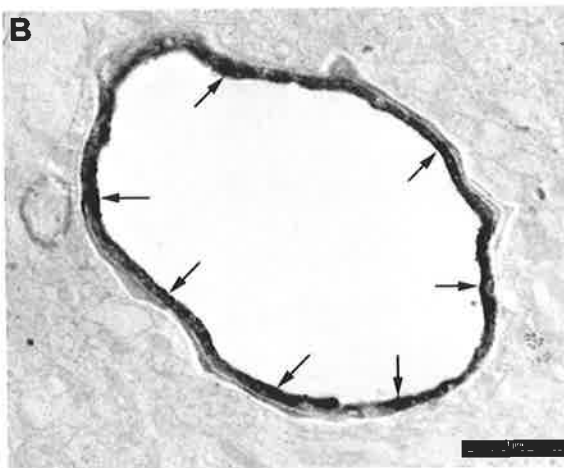
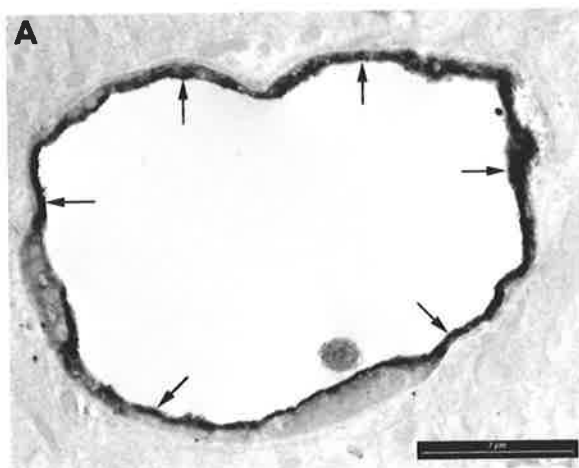


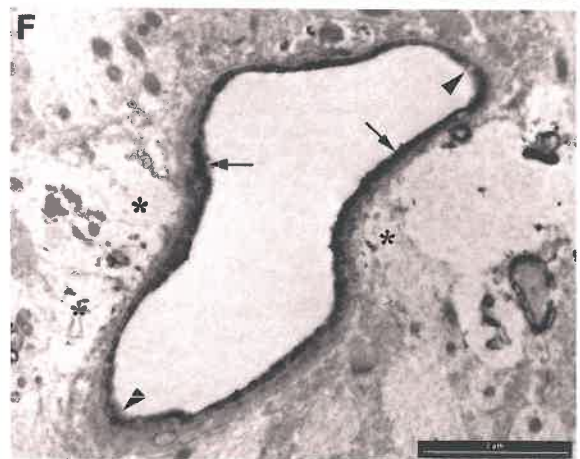
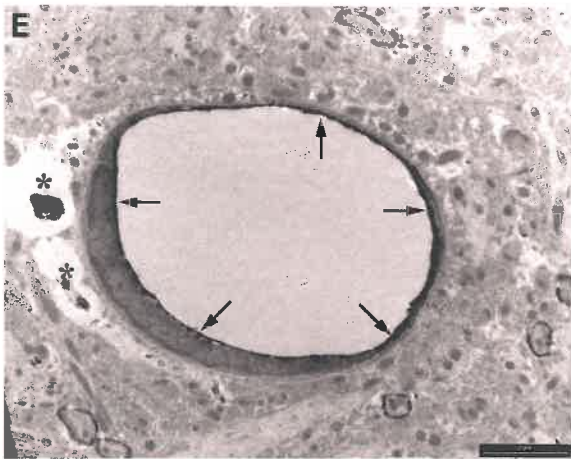
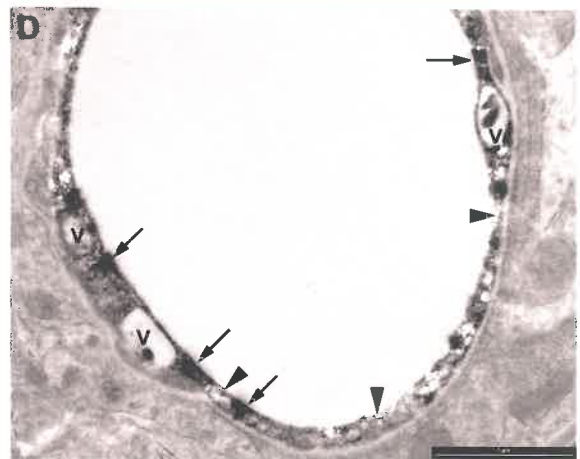
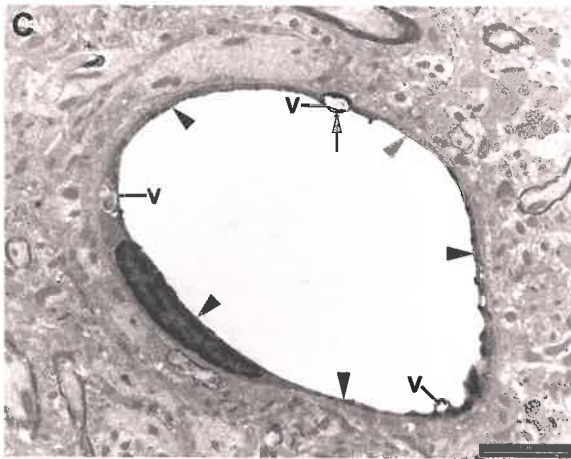
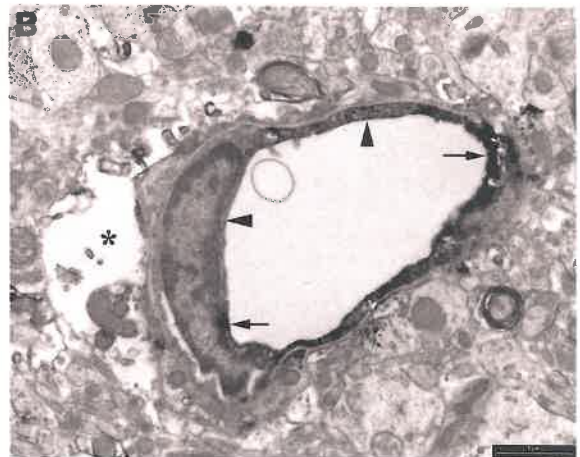
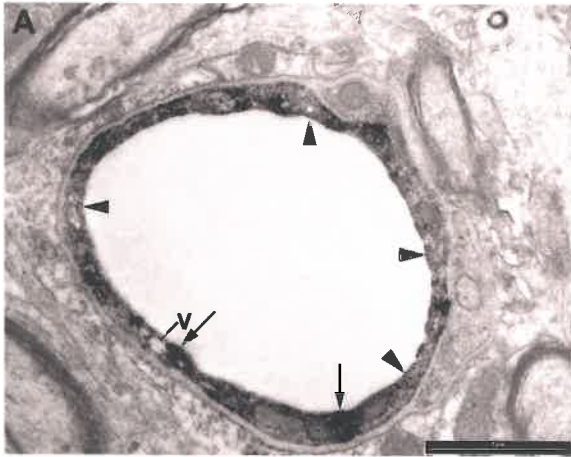
FIG 4.2.20 (A-F). Electron micrographs of cerebral cortical microvessels from experimental animals with survival times of 1h (**A** and **B**), 6h (**C** and **D**) and 12h (**E** and **F**) after traumatic brain injury, showing pre-embedding immunoperoxidase labelling for GLUT1.

(A and B) One hour after the injury, sectoral labelling (arrows) of GLUT1 in ECs is seen. Loss of GLUT1 labelling in ECs is observed (arrowheads). Large vacuoles [V] are present in the cytoplasm of ECs (**A**). Perivascular space is widened (asterisks, **B**), indicating oedema formation.

(C and D) Six hours after the injury, sectoral labelling of GLUT1 in ECs is seen (arrows show stained membrane, arrowheads show unstained membrane). Large vacuoles [V] are seen in the EC cytoplasm.

(E and F) At 12h after the injury, a blood vessel shows GLUT1 labelling at the entire length of ECs (arrows, **E**). Another blood vessel shows partial labelling of ECs (arrows show stained membrane and arrowheads show unstained membrane). Perivascular space is widened (asterisks, **E** and **F**).

All micrographs are from grids not counterstained with heavy metals.



Quantitative analysis of GLUT1 immunoreactive vessels in TBI was carried out at the LM and EM levels on three experimental groups, 1h, 6h and 12h after injury. Two experimental animals in each group were compared with one sham control animal at each time interval and two normal control animals.

For quantitative analysis at the LM level, GLUT1 immunoreactive vessels in the frontal cortex and brainstem were counted (Appendix A.6, Table 4.2). In the frontal cortex of the two normal control brains, the mean numbers of GLUT1 immunoreactive blood vessels were 435 ± 78 and 423 ± 108 per mm^2 (Table 2, Fig. 4.2.21). One hour after the injury, the mean numbers of GLUT1 immunoreactive blood vessels was reduced to 327 ± 33 and 235 ± 13 per mm^2 , which were much less than that in normal controls and slightly less than that of the 1h sham control (Fig. 4.2.21). At 6h after the injury, a pronounced reduction in GLUT1 immunoreactivity was observed, with the mean numbers of GLUT1 immunoreactive vessels decreased to 168 ± 68 and 235 ± 76 per mm^2 . Twelve hours after the trauma, the mean numbers of GLUT1 stained vessels increased slightly to 207 ± 55 and 314 ± 31 per mm^2 , but was still less than that of normal controls and the sham control (Fig. 4.2.21).

The 6h and 12h sham control animals showed a slightly reduced number of GLUT1 immunoreactive vessels in the frontal cortex compared to the normal animals, with the mean values being 382 ± 39 and 387 ± 55 per mm^2 respectively. The sham control animal killed at 1h, however, showed a more obvious reduction

of GLUT1 immunoreactivity in the frontal cortex compared to the normal brains (mean, 316 ± 33 per mm^2).

In the brainstem of normal brains, the mean numbers of GLUT1 immunoreactive blood vessels was 379 ± 21 and 389 ± 33 per mm^2 (Table 4.2, Fig. 4.2.22). At 1h and 6h after the injury, of the two experimental animals studied, one experimental animal showed slightly reduced GLUT1 immunoreactivity in the brainstem (Table 4.2). At 12h after the injury, the reduction of GLUT1 was more obvious, with the mean numbers of GLUT1 immunoreactive blood vessels in the brainstem being 287 ± 39 and 284 ± 33 per mm^2 (Fig. 4.2.22).

Table 4.2. The mean number of GLUT1-immunoreactive blood vessels in the brain of normal controls, sham control and traumatic brain injury (TBI) rats.

	Frontal cortex (n/mm ²)	Brainstem (n/mm ²)
1h TBI (B3)	327 ± 33 (275-357)	530 ± 134 (323-659)
1h TBI (B4)	235 ± 13 (213-247)	328 ± 93 (254-460)
6h TBI (B16)	168 ± 68 (76-233)	255 ± 36 (213-474)
6h TBI (B17)	235 ± 76 (110-323)	401 ± 74 (295-474)
12h TBI (B32)	207 ± 55 (144-288)	287 ± 39 (233-323)
12h TBI (B48)	314 ± 31 (282-350)	284 ± 33 (233-323)
1h sham (B6)	316 ± 33 (275-350)	342 ± 45 (295-391)
6h sham (B18)	382 ± 39 (323-419)	427 ± 86 (364-570)
12h sham (B49)	387 ± 55 (330-453)	472 ± 23 (433-494)
Normal control (B20)	435 ± 78 (378-570)	379 ± 21 (350-398)
Normal control (B21)	423 ± 108 (316-570)	389 ± 33 (343-433)

Data shown represent the mean value ± SD (range).

n/mm², number of GLUT1-immunoreactive blood vessels per mm².

FIG. 4.2.21. A histogram summarising the LM quantitative results of GLUT1 immunoreactive vessels in the frontal cortex. The columns represent the mean number of blood vessels obtained from two normal controls, one sham control at 1h, 6h, and 12h and two experimental animals at 1h, 6h, and 12h post TBI. The error bars representing the standard deviation for each group of data are shown.

FIG. 4.2.22. A histogram summarising the LM quantitative results of GLUT1 immunoreactive vessels in the brainstem. The columns represent the mean number of blood vessels obtained from two normal controls, one sham control at 1h, 6h, and 12h and two experimental animals at 1h, 6h, and 12h post TBI. The error bars representing the standard deviation for each group of data are shown.

FIG. 4.2.21 The mean number of GLUT1-immunoreactive blood vessels in the frontal cortex in normal controls, sham control and TBI rats

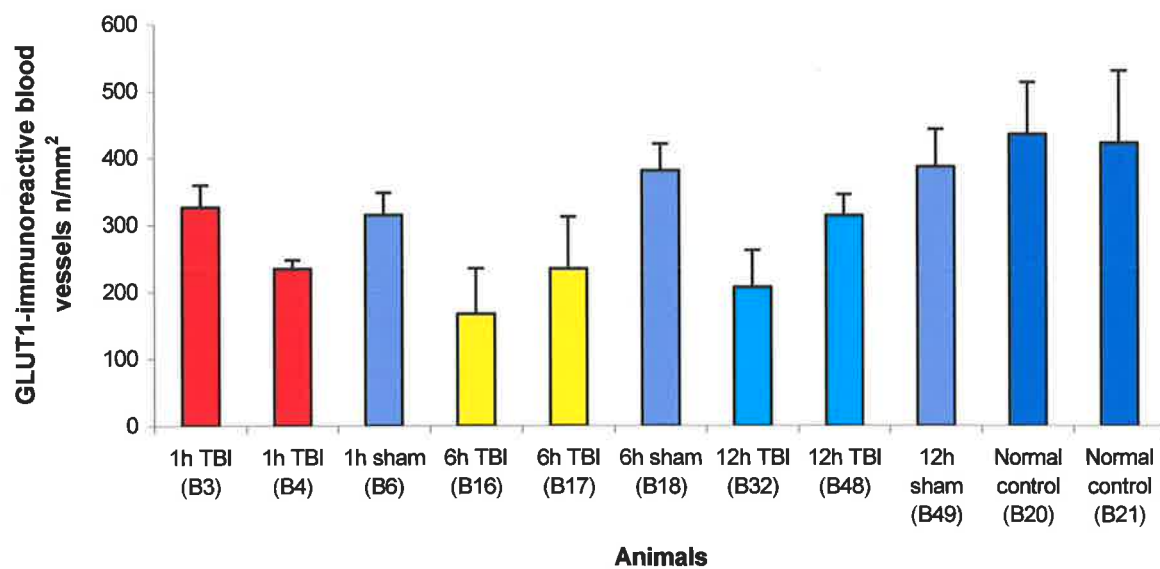
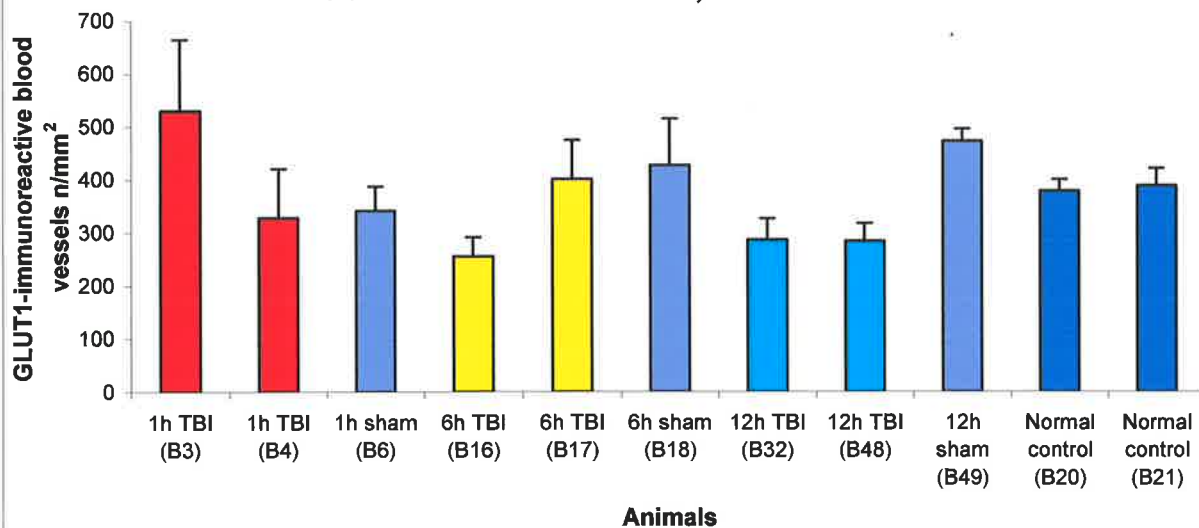


FIG. 4.2.22 The mean number of GLUT1-immunoreactive blood vessels in the brainstem in normal controls, sham control and TBI rats



For quantitative assessment of GLUT1 immunoreactive vessels at the EM level, the EC luminal membrane length showing immunoreactivity was measured in blood vessels in the frontal cortex and the ratio relative to the total length of luminal membrane for each vessel was calculated. The results from all vessels in each animal were pooled and a mean value was obtained for each animal. In the two normal control animals examined in this study, most vessels had 100% staining of their luminal membrane (vessels $n = 16$ and 26 , Appendix A.7, Figs. 4.2.23-25). Only occasional vessels were partially unstained. The mean values of the percentage of positively stained EC membrane length in the two normal animals were $93 \pm 16\%$ and $93\% \pm 15\%$ (Figs. 4.2.23-25). The 1h and 6h sham control animals showed comparable values to the normal control animals, with mean values of $93 \pm 15\%$ and $91 \pm 11\%$ respectively (vessel $n = 20$ and 22 respectively, Appendix A.7, Figs. 4.2.23 and 24). More blood vessels from the brain of the 12h sham control animal showed partial loss for GLUT1 (vessel $n = 22$, Appendix A.7), with the mean value being $83 \pm 18\%$ (Fig. 4.2.25).

In experimental animals, one hour after the trauma, a clear reduction of GLUT1 immunoreactivity was noted (Fig. 4.2.23). The mean values of the percentage of positively stained EC luminal membrane length in the two experimental animals decreased to $44 \pm 27\%$ and $43 \pm 26\%$ (vessel $n = 17$ and 26 respectively, Appendix A.7). Six hours after the injury, a further reduction of GLUT1 immunoreactivity was observed (Fig. 4.2.24). The mean values of the percentage of immunoreactive EC luminal membrane length in the two experimental animals were $18 \pm 31\%$ and $21 \pm 27\%$ (vessel $n = 21$ and 22 respectively, Appendix A.7).

These mean values were apparently less than the mean of the sham control animal and the normal control animals. Twelve hours after the trauma, a limited recovery of GLUT1 immunoreactivity was noted (Fig. 4.2.25). The mean values of the percentage of immunoreactive EC membrane length of the two experimental animals were $48 \pm 27\%$ and $50 \pm 18\%$ (vessel n = 28 and 18 respectively, Appendix A.7). These mean values were still less than the mean of the sham control animal and the normal control animals.

FIG. 4.2.23. A histogram summarising the EM quantitative results of GLUT1 immunoreactivity in the cortex. The columns represent the mean percentage of GLUT1 labelling of luminal membranes in two normal controls, one sham control at 1h and two experimental animals at 1h post TBI. The error bars representing the standard deviation for each group of data are shown.

FIG. 4.2.24. A histogram summarising the EM quantitative results of GLUT1 immunoreactivity in the cortex. The columns represent the mean percentage of GLUT1 labelling of luminal membranes in two normal controls, one sham control at 6h and two experimental animals at 6h post TBI. The error bars representing the standard deviation for each group of data are shown.

Fig 4.2.23 Mean percentage of GLUT1 labelling of luminal membranes in normal controls, sham control and 1h TBI

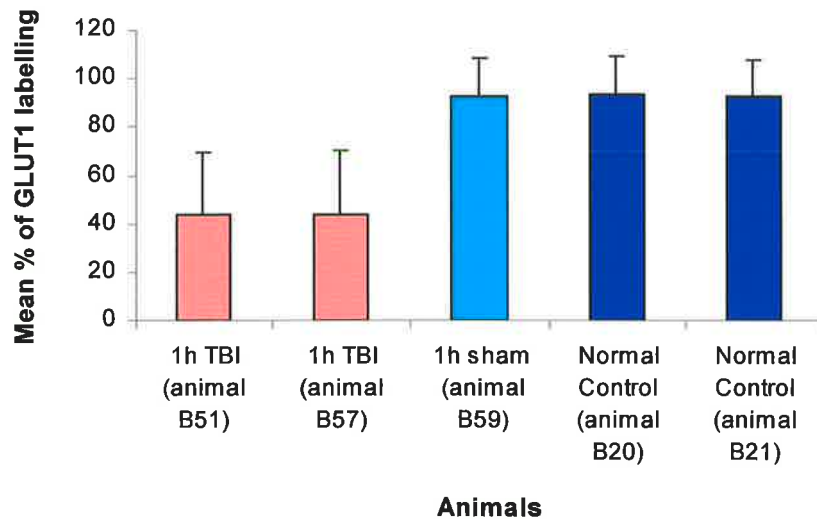


Fig 4.2.24 Mean percentage of GLUT1 labelling of luminal membrane in normal controls, sham control and 6h TBI

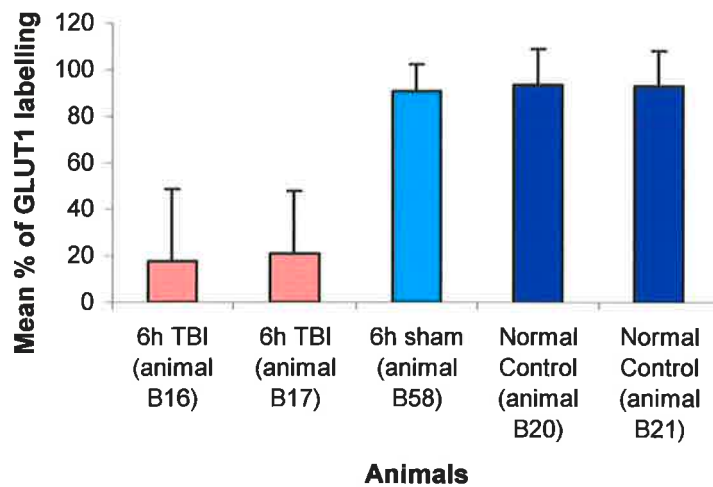
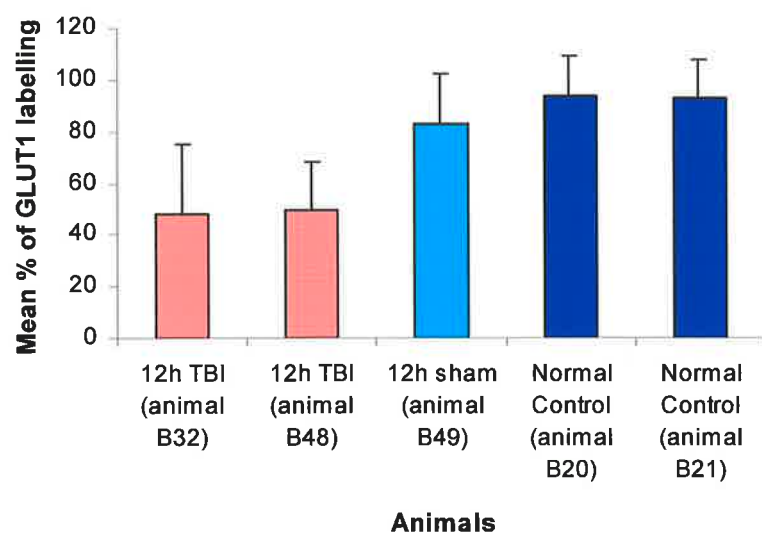


FIG. 4.2.25. A histogram summarising the EM quantitative results of GLUT1 immunoreactivity in the cortex. The columns represent the mean percentage of GLUT1 labelling of luminal membranes in two normal controls, one sham control at 12h and two experimental animals at 12h post TBI. The error bars representing the standard deviation for each group of data are shown.

Fig 4.2.25 Mean percentage of GLUT1 labelling of luminal membrane in normal controls, sham control and 12h TBI



4.3 *In vivo* immunological targeting of EBA

4.3.1 *Permeability of the BBB and BRB*

4.3.1.1 *HRP permeability*

- (1) Brain. None of the control animals injected intravenously with PBS or control antibodies (anti-human cytokeratin, SAL-3, and anti-RECA-1) showed leakage of HRP in the brain. Light microscopy of Vibratome sections showed clear parenchyma throughout the brain (Fig. 4.3.1). Occasional red blood cells seen in the lumina of some vessels showed dark staining due to their content of endogenous peroxidase, indicating the successful development of peroxidase reaction product. Vibratome sections from the liver of control animals injected with HRP showed the tracer in the liver parenchyma, indicating the successful intravenous injection of the tracer and its circulation throughout the body.

On the other hand, experimental animals injected with anti-EBA *in vivo* showed extensive leakage of HRP in the brain, indicating a severely compromised BBB (Fig. 4.3.1). Vibratome sections showed widespread black HRP reaction product in the cerebrum, diencephalon, brainstem and cerebellum. In the white matter, the distribution of the tracer showed a linear pattern, being parallel to nerve fibre tracts (Fig. 4.3.2). In the grey matter the pattern was multifocal with circumferential areas containing HRP, varying in size and superimposed on a diffuse spread of the tracer in the parenchyma (Fig. 4.3.2). The lumina of blood vessels in the brain and liver sinusoids were free of HRP due to flushing of the circulation during

perfusion fixation. HRP was taken up by many neuronal cell bodies and neuronal processes (Fig. 4.3.2). Macrophages occasionally seen in the lumen of blood vessels contained granules of HRP reaction product. Experimental animals anaesthetised with the two different anaesthetic agents, pentobarbitone sodium and urethane, did not show any difference in the distribution of HRP in the brain.

In the transmission electron microscope, HRP appeared as electron dense material (Fig. 4.3.3). Control animals showed no HRP leakage in the brain. No peroxidase reaction product was observed in the cytoplasm of ECs and pericytes, or in the perivascular space and parenchyma. Only very few vesicles in ECs contained HRP. In brains of experimental animals that had 15 min circulation time for HRP, widespread peroxidase reaction product was observed in the parenchyma, especially between nerve cells and fibres. Some axons contained HRP. HRP was also contained in vesicles, multivesicular bodies and dense bodies in EC cytoplasm. HRP also was seen within abluminal part of some tight junctions. However, the luminal ends of tight junctions were full of HRP. The basal lamina of ECs and pericytes was heavily infiltrated with HRP. Many pericytes were diffusely filled with HRP. Peroxidase-positive lysosome-like dense bodies were also observed in pericytes. The perivascular space was increased and contained HRP. In shorter exposure time (7 min) to HRP, the tracer was mainly seen within vesicles of ECs,

but was absent from the pericytes, perivascular space and brain parenchyma.

(2) Retina

Control animals injected with PBS or control antibodies (anti-human cytokeratin, SAL-3), and anti-RECA-1) showed no leakage of HRP in the retina (Fig. 4.3.4). Light microscopy of the wholemount retina showed clear retinal tissue except occasional RBC seen in the lumen of some vessels. In the iris and ciliary body, HRP was taken up by many macrophages. In contrast, experimental animals injected with anti-EBA *in vivo* showed multifocal leakage of HRP in the retina (Fig. 4.3.4). The distribution pattern of the tracer was multifocal with circumferential areas containing HRP, varying in size. The leakage of HRP was predominantly present in regions close to the optic disc.

FIG. 4.3.1 (A-F). Light micrographs of sagittal Vibratome sections from the brains of control and experimental animals stained for peroxidase cytochemistry.

(A-D) Control animals injected intravenously with HRP after the intravenous injection of PBS **(A)**, anti-human cytokeratin **(B)**, SAL-3 **(C)** and anti-RECA-1 **(D)** show no leakage of HRP.

(E) shows a section from the liver of a control animal injected with SAL-3 followed by HRP. Although the brain of this animal did not show any HRP in the parenchyma **(C)** the liver shows HRP reaction product (arrows) indicating the successful administration of HRP into the circulation.

(F) shows a Vibratome section from an experimental animal injected intravenously with anti-EBA followed by HRP. HRP reaction product (arrows) is seen throughout the brain and particularly in the region of projection fibres in the forebrain (asterisks).

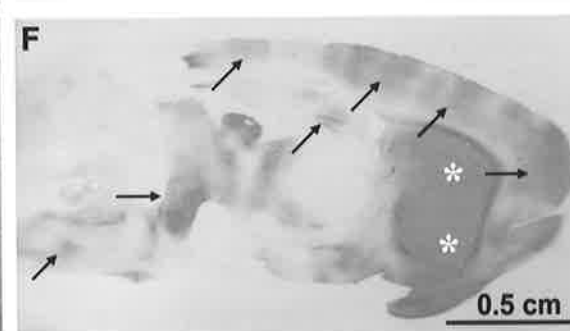
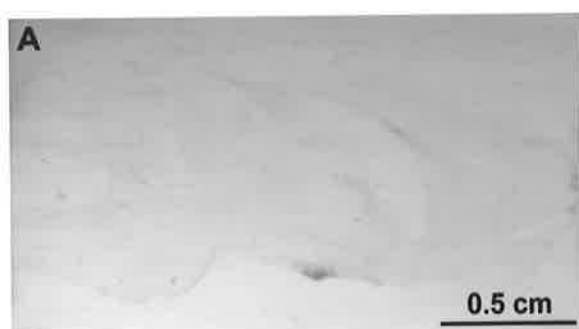


FIG. 4.3.2 (A-D). Higher magnification of sagittal Vibratome sections from the brain of an experimental animal injected intravenously with anti-EBA, followed by HRP. HRP leakage is seen in the brain parenchyma.

(A) In the white matter the distribution of the tracer is linear (asterisks) and runs parallel to nerve fibre tracts.

(B) In the grey matter the distribution of the tracer is circumferential and multifocal (asterisks).

(C and D) Nerve cells (arrows) and nerve fibres (arrowheads) also show HRP reactivity.

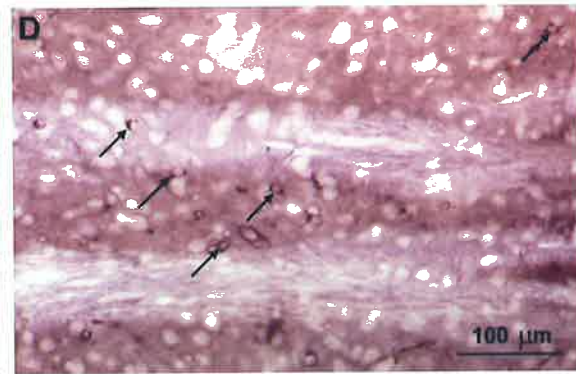
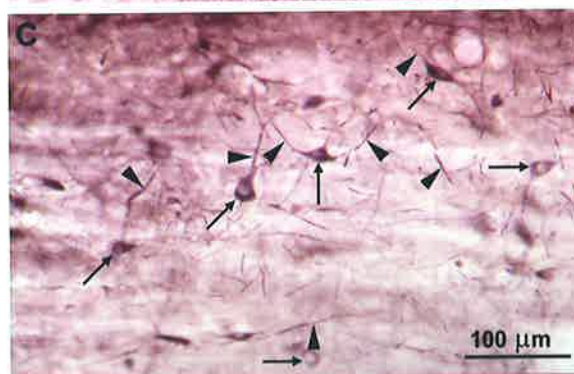
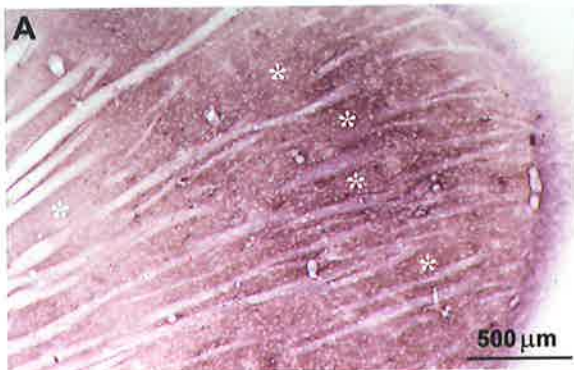


FIG. 4.3.3 (A-F). Electron micrographs of brains from experimental animals injected intravenously with anti-EBA, showing HRP reactivity.

(A and B) Blood vessels show HRP in cytoplasmic vesicles (small arrows) and in the plane of basal lamina (arrowheads) of ECs and pericytes. HRP is present in the parenchyma, between nerve fibres (large arrows).

(C) HRP is seen in cytoplasmic vesicles (small black arrows) and in the plane of basal lamina (arrowheads) of ECs and pericytes. HRP is present in the parenchyma, between nerve fibres (large black arrows). HRP also fills abluminal part of a tight junction (white arrows).

(D) A high magnification showing HRP in cytoplasmic vesicles (arrows).

(E) A blood vessel showing HRP in the plane of basal lamina of ECs and pericytes (arrowheads). A perivascular phagocyte [Ph] contains HRP in the cytoplasm.

(F) HRP is seen in the brain parenchyma, between nerve fibres (arrows)

EC, endothelial cell; Per, pericyte; Ph, phagocyte. Bar, 1 μ m.

All micrographs are from grids not counterstained with heavy metals.

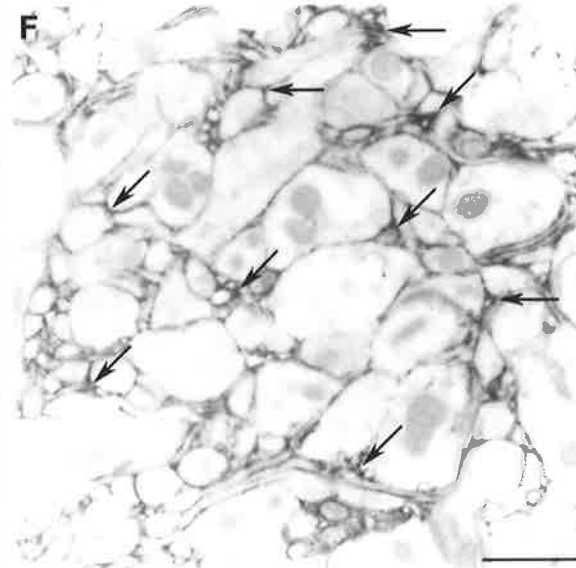
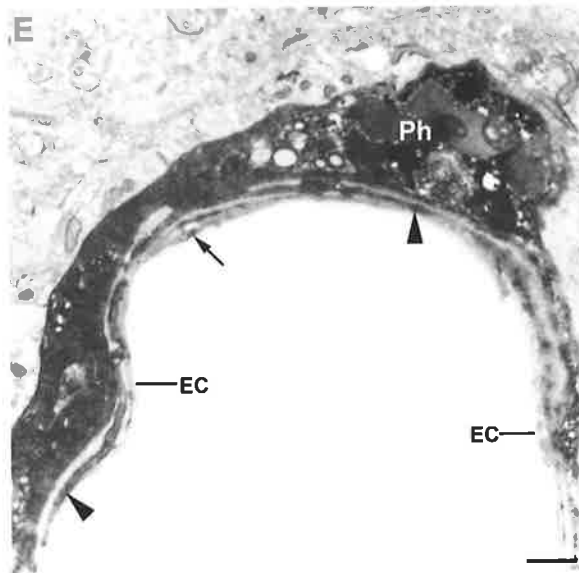
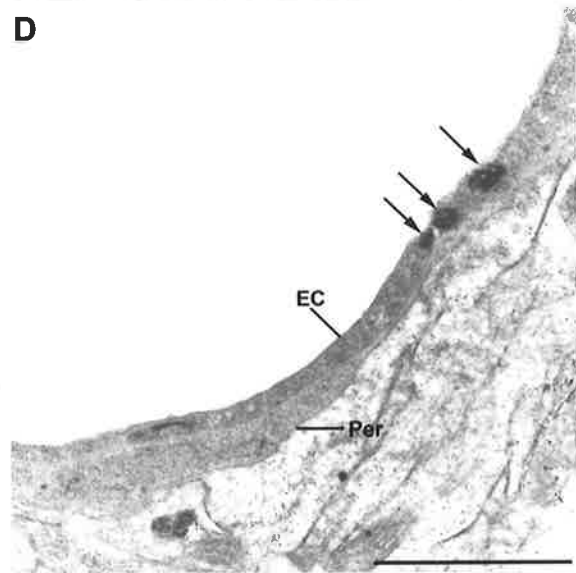
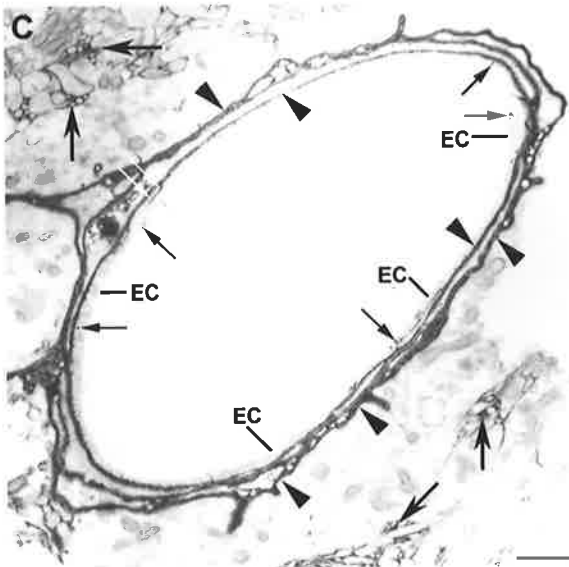
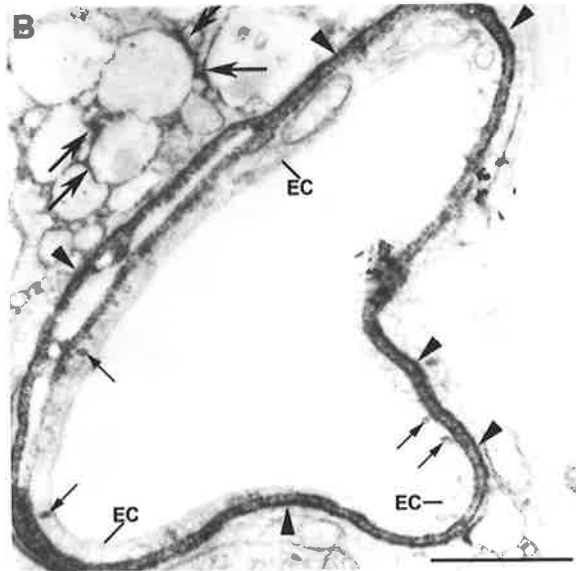
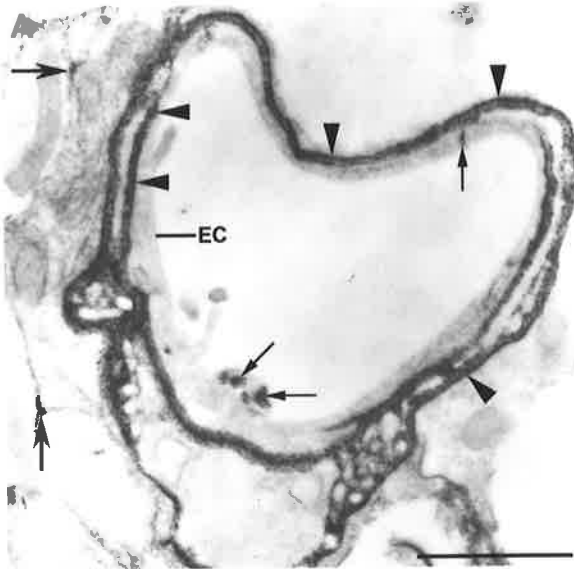
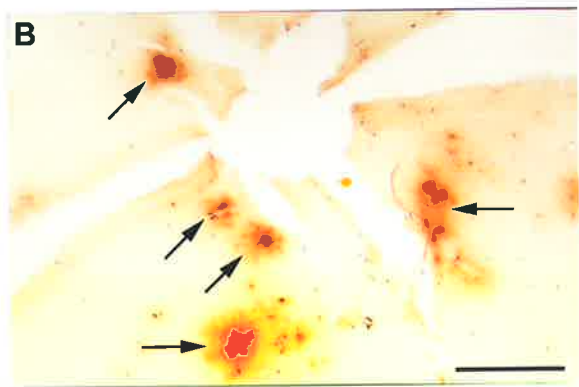


FIG. 4.3.4 (A and B). Light micrographs of wholemount retinas from a control animal injected intravenously with anti-RECA-1 (**A**) and an experimental animal injected with anti-EBA (**B**) stained for HRP cytochemistry.

(**A**) The control animal shows no HRP leakage in the retina.

(**B**) Multifocal HRP leakage is seen in the retina from the experimental animal (arrows).

Bar, 500 μm .



4.3.1.2 Serum albumin permeability

Control animals, which had received an intravenous injection of PBS or control antibodies (anti-human cytokeratin, SAL-3 and anti-RECA-1), showed a faint diffuse immunoreactivity to albumin throughout the grey and white matter of cerebrum, cerebellum and brainstem (Fig. 4.3.5). Some areas in the brain in control animals showed a moderate degree of immunoreactivity to albumin, more than that seen generally in the brain. These areas included the olfactory tract, hippocampus, periventricular zone, rostral diencephalon, superior cerebellar peduncle and multiple foci in the brainstem. The immunoreactivity to albumin was not seen in any of the batches of sections in which the primary antibody to albumin or the primary and secondary antibodies were omitted. Experimental animals injected with anti-EBA, on the other hand, showed widespread and strong immunoreactivity to albumin in the grey and white matter, in paraffin and Vibratome sections (Fig. 4.3.5). The walls of many vessels were highlighted suggesting the presence of albumin intramurally. In the grey matter the distribution of albumin showed circular or oval areas of high immunoreactivity (Fig. 4.3.6). In the white matter the distribution was linear and predominantly followed fibre tracts (Fig. 4.3.6).

Electron microscopy of the brains of experimental animals showed electron dense product indicative of albumin leakage (Fig. 4.3.7). In some ECs, albumin heavily infiltrated the whole thickness of cytoplasm. Most vesicles in the cytoplasm were clear and only occasionally few vesicles contained albumin. Albumin was not

observed conclusively within tight junctions. However, some tight junctions were homogeneously electron dense with loss of definition of the apposed EC membranes, suggesting saturation of the junctions with albumin. Albumin was also observed in the parenchyma, between nerve cells and fibres. In control animals, albumin was only seen along luminal surface of brain blood vessels but not in the cytoplasm or in the parenchyma.

FIG. 4.3.5 (A-F). Immunolabelling for serum albumin. Light micrographs of sagittal Vibratome sections from brains of control animals injected intravenously with PBS (**A**), anti-human cytokeratin (**B**), SAL-3 (**C**) and anti-RECA-1 (**D**). (**E** and **F**) show sections from two experimental animals injected intravenously with anti-EBA.

(A-D) Control animals show no albumin immunoreactivity in the brain parenchyma.

(E and F) Strong albumin immunoreactivity is seen throughout the brain, in the cortex (arrows), brainstem (arrows) and projection tracts of the forebrain (asterisks).

Bar, 0.5 cm.

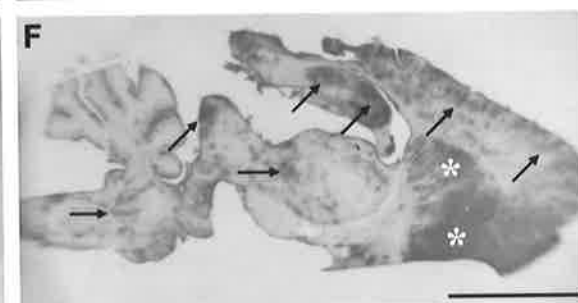
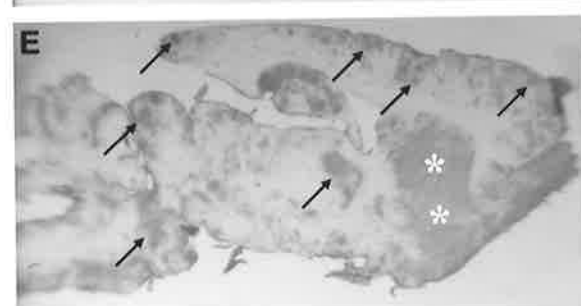
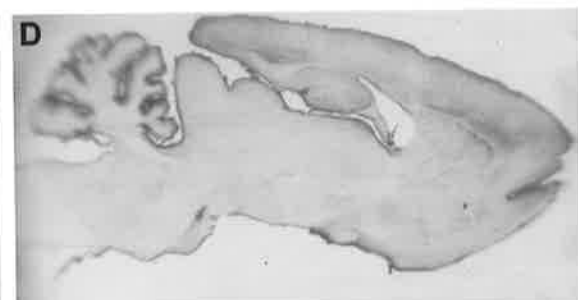
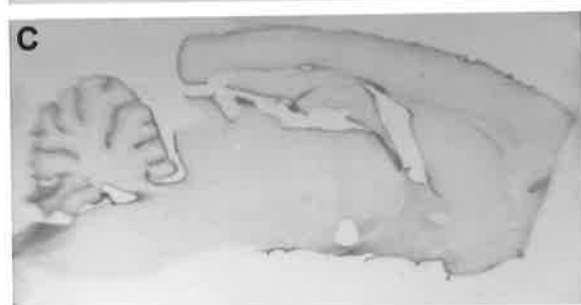
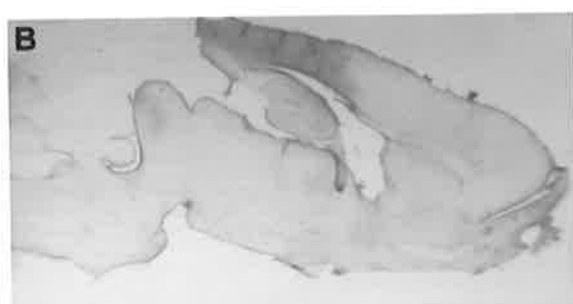


FIG. 4.3.6 (A-D). Light micrographs of Vibratome sections from a brain of an experimental animal injected intravenously with anti-EBA, showing strong immunoreactivity for endogenous albumin in the parenchyma.

(A, C and D) In the grey matter of the cerebral cortex **(A)**, the inferior and superior colliculi **(C)** and the hippocampus **(D)**, the distribution of albumin leakage is multifocal with circumferential areas of spread (asterisks).

(B) In the white matter the distribution is linear (asterisks) and follows the direction of fibre tracts.

SC, superior colliculus. IC, inferior colliculus. Bar, 200 μm .

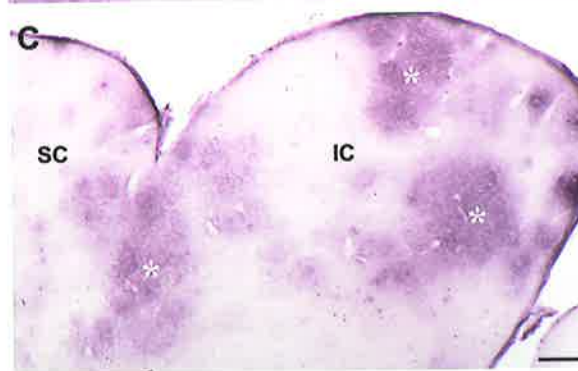
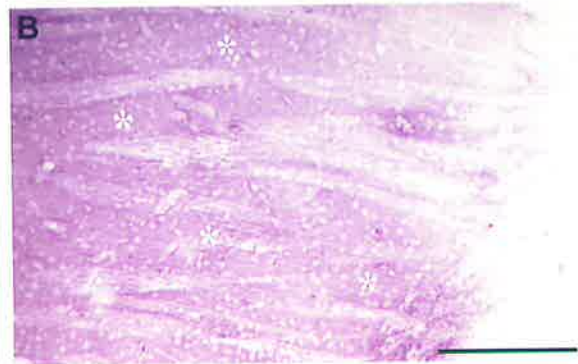
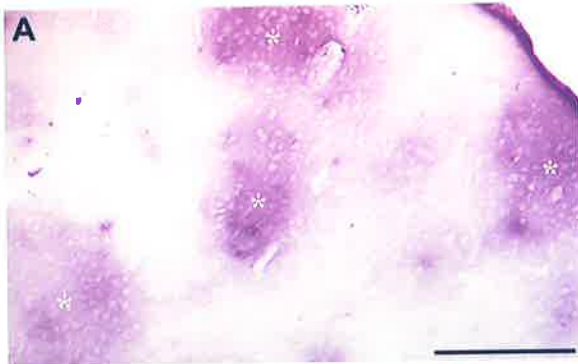


FIG. 4.3.7 (A-E). Electron micrographs of cerebral cortical vessels from experimental animals injected intravenously with anti-EBA, showing pre-embedding immunoperoxidase labelling for serum albumin.

(A) Albumin is seen in the cytoplasm (arrows) of ECs. Vacuoles [Va] are not stained.

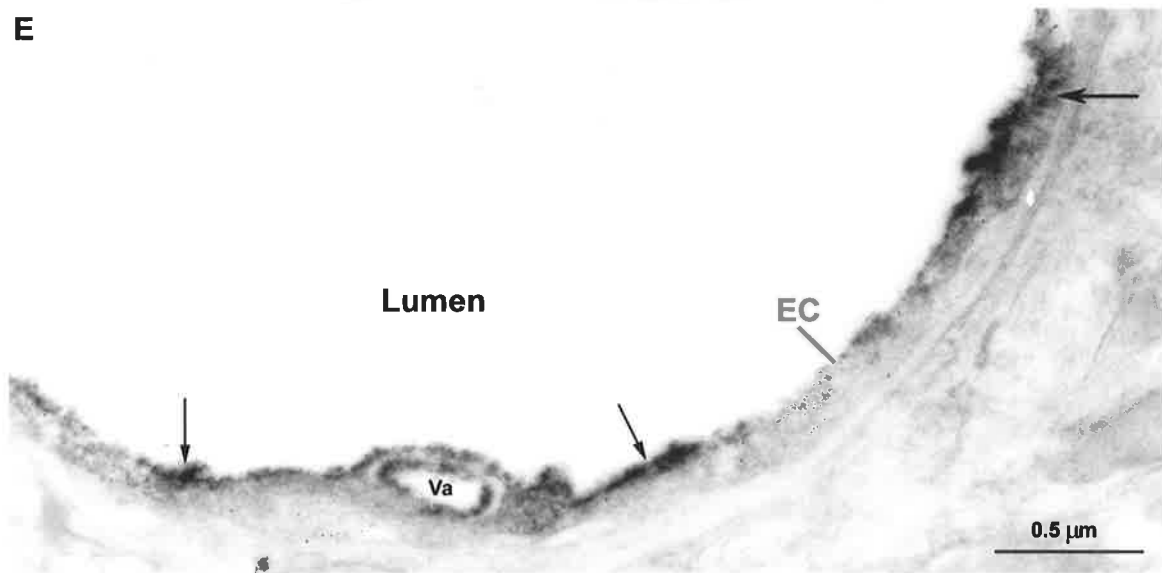
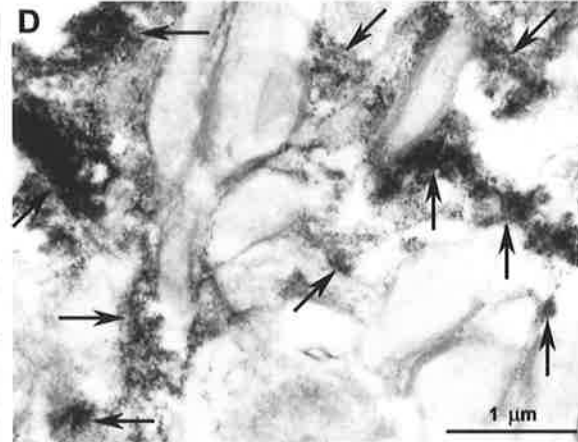
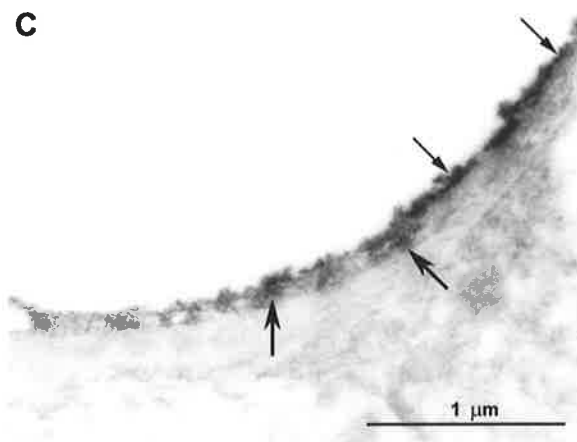
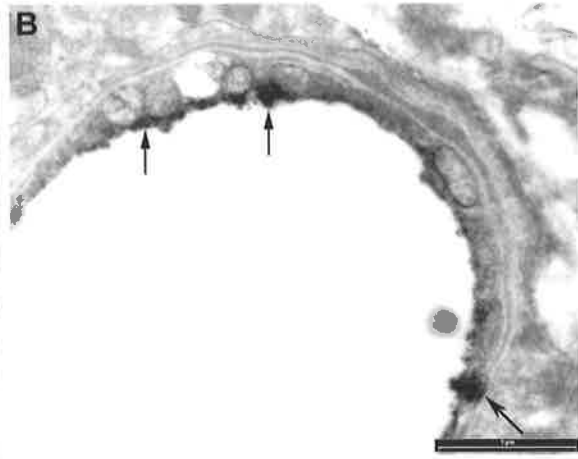
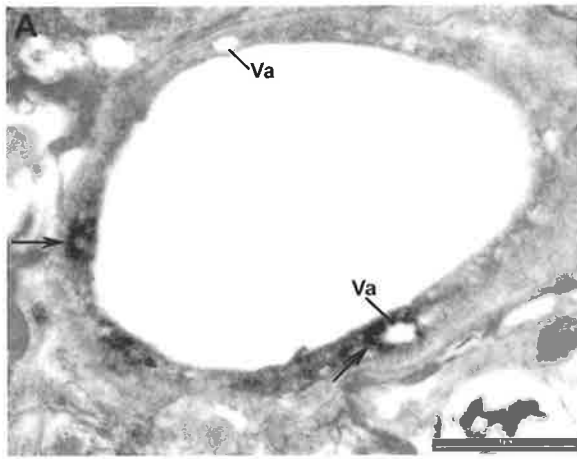
(B and C) Albumin is seen at the luminal surface (small arrows) and in the cytoplasm (large arrow) of ECs.

(D) Albumin is seen in the parenchyma, between nerve fibres (arrows).

(E) A high magnification showing albumin at the luminal surface (small arrows) and in the cytoplasm (large arrow) of an EC. A vacuole [Va] in the cytoplasm contains albumin.

EC, endothelial cell.

All micrographs are from grids not counterstained with heavy metals.



4.3.2 Immunocytochemistry for EBA

(1) Brain

Immunocytochemical staining for EBA was carried out on Vibratome and paraffin sections from brains of experimental and control animals. Control animals (injected intravenously with PBS, anti-human cytokeratin, SAL-3 and anti-RECA-1) showed strong expression of EBA in ECs (Fig. 4.3.8). Blood vessels of various sizes were strongly immunoreactive for EBA in the grey and white matter. Omission of the primary antibody or both primary and secondary antibodies resulted in the loss of all immunoreactivity for EBA.

Experimental animals injected *in vivo* with anti-EBA antibody showed two interesting results. Firstly, Vibratome sections which were not treated with the primary antibody in the staining procedure showed moderate immunoreactivity for EBA in brain ECs in the majority of vessels in the cerebrum, cerebellum, diencephalon and brainstem (Fig. 4.3.8E). In a minority of vessels the staining was either absent or weak. In Vibratome sections which were treated with the primary antibody in the staining procedure the immunoreactivity of brain ECs was much higher than those where the primary antibody was omitted, and almost all vessels were strongly stained for EBA (Fig. 4.3.8F). In sections which were only exposed to DAB no immunoreactivity was seen. The second interesting observation was seen in paraffin sections. Unlike Vibratome sections which were not exposed to the primary antibody, paraffin sections treated in a similar manner (not exposed to the primary antibody, anti-EBA in the staining procedure) did not

show any staining. These sections were obtained from the same animals from which Vibratome sections showed immunoreactivity for EBA despite omission of the primary antibody step.

Pre-embedding electron microscopic study demonstrated the binding of intravenously injected anti-EBA antibody on brain ECs in experimental animals (Fig. 4.3.9). Electron dense peroxidase reaction product was observed on the luminal surface of ECs from sections stained with secondary antibody only. The peroxidase reaction product was also observed in the cytoplasm of ECs. The staining pattern was similar to normal EBA staining observed previously.

(2) Retina

EBA immunocytochemistry was carried out on paraffin sections of the retina and on wholemount retinas. Wholemount retinas from control animals injected with PBS or control antibodies showed normal EBA immunoreactivity in retinal vessels (Fig. 4.3.10). As the wholemount retina is quite thick, about 200 μ m, adequate penetration of antibodies was difficult to achieve. As a result, only blood vessels near the cut edge of the retina were stained for EBA. When the primary or both primary and secondary antibodies were omitted from the immunostaining process, retinas from the control animals showed no immunoreactivity.

Immunocytochemistry for EBA expression in retinas from experimental animals injected with anti-EBA showed similar results to those of the brain (Fig. 4.3.10).

The whole-mount retina showed moderate immunoreactivity for EBA in retinal vessels (near the cut edge) when treated with the secondary antibody only. When treated with primary antibody, anti-EBA, the wholemount retinas showed higher immunoreactivity for EBA than that seen without primary antibody. Stained vessels were again mainly seen near the cut edge of the retina. Paraffin sections showed strong EBA immunoreactivity when treated with primary antibody. When the primary antibody or both primary and secondary antibodies were omitted, the paraffin sections of retina showed no immunoreactivity.

FIG. 4.3.8 (A-F). Sagittal Vibratome sections from the brains of control (**A-D**) and experimental (**E-F**) animals, immunostained for EBA. Figures **A-D** and **F** are from sections stained using the complete protocol of primary and secondary antibodies and tertiary reagents.

(A-D) Control animals, injected intravenously with PBS (**A**), anti-human cytokeratin (**B**), SAL-3 (**C**) and anti-RECA-1 (**D**), show strong immunoreactivity for EBA. Microvessels of various sizes (arrows) are immunoreactive for EBA.

(E) shows a Vibratome section from an experimental animal not treated with the primary anti-EBA in the staining procedure. Brain vessels in this section show moderate immunoreactivity for EBA. This suggests that the anti-EBA injected *in vivo* became bound to ECs and replaced the step of primary antibody in the staining procedure.

(F) A section from an experimental animal injected intravenously with anti-EBA and reacted with anti-EBA primary antibody in the staining procedure shows strong EBA labelling in brain vessels (arrows).

Bar, 200 μm .

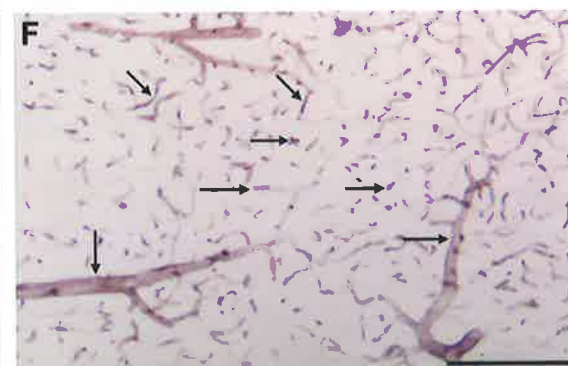
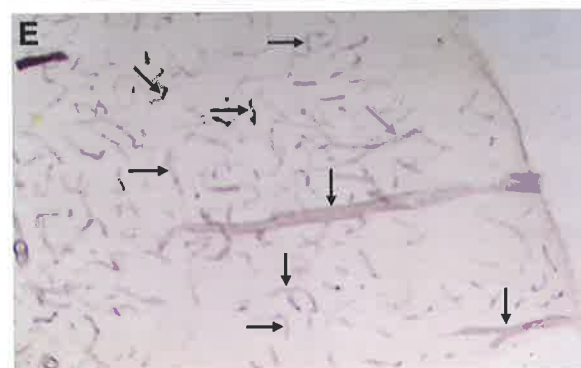
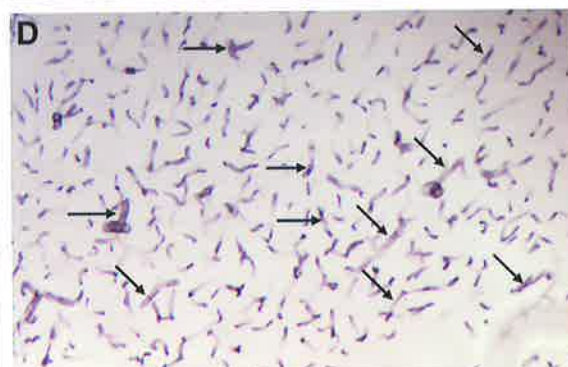
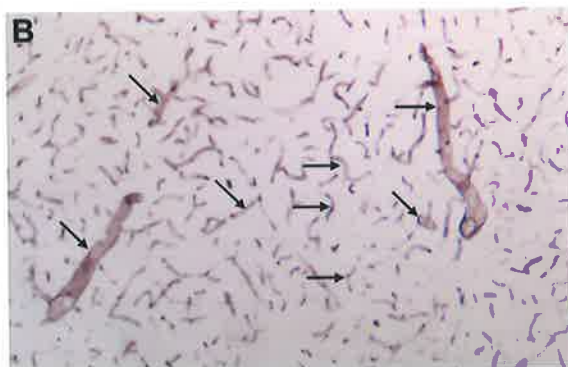
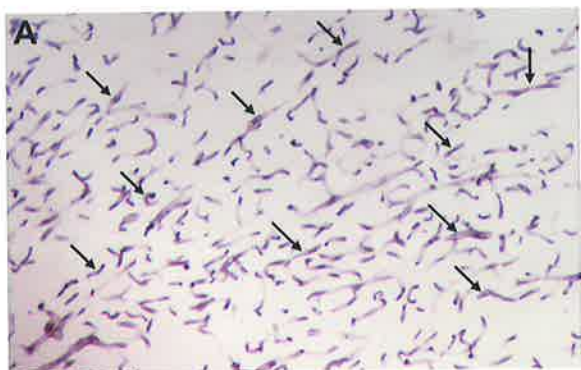


FIG. 4.3.9 (A-E). Electron micrographs of brain blood vessels from control animals injected with SAL-3 (**A**) and anti-RECA-1 (**B**), and an experimental animal injected with anti-EBA (**C-E**), showing pre-embedding immunostaining for EBA.

(A and B) are from sections stained using the complete protocol of primary and secondary antibodies and tertiary reagents. Control animals, injected intravenously with SAL-3 (**A**) and anti-RECA-1 (**B**), show strong immunoreactivity for EBA. EBA labelling is seen at the luminal membrane (small black arrows) and in the cytoplasm (between large black arrows) of ECs. Endothelial cell nuclei also show EBA immunoreactivity at the luminal side of the membrane (white arrows). Vesicles [V] in **B** are not stained.

(C-E) show sections from an experimental animal not treated with the primary anti-EBA in the staining procedure. **(E)** shows a higher magnification of the rectangle in **(C)**. EBA labelling is seen at the luminal membrane (small black arrows, **C-E**) and in the cytoplasm (between large black arrows, **C** and **D**) of ECs. Endothelial cell nuclei also show EBA immunoreactivity at the luminal side of the nucleus (white arrows, **C** and **E**). Vesicles [V] are not stained (**D**).

N, endothelial cell nucleus. All micrographs are from grids not counterstained with heavy metals.

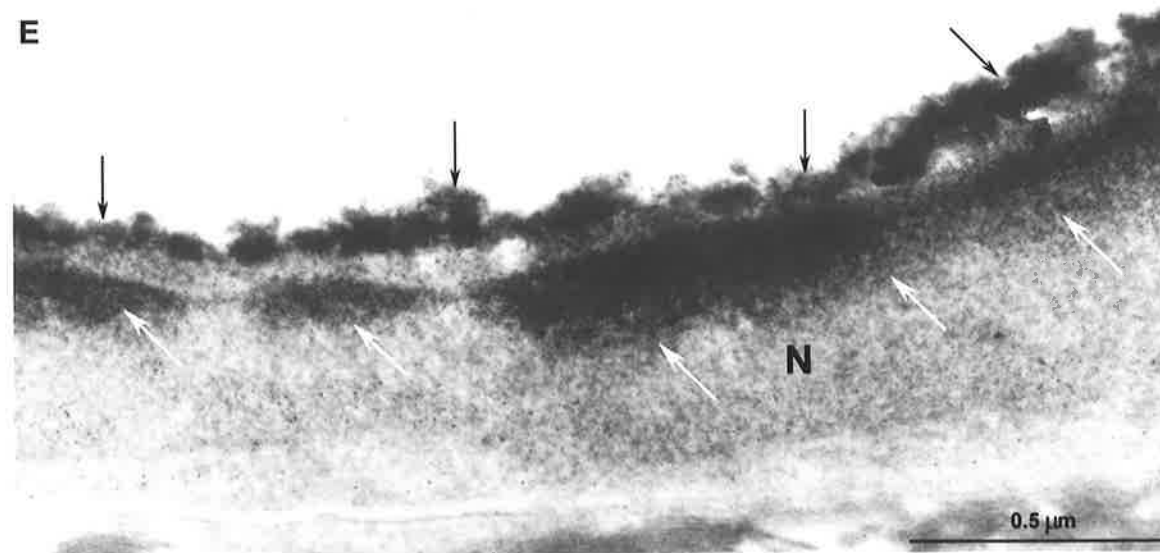
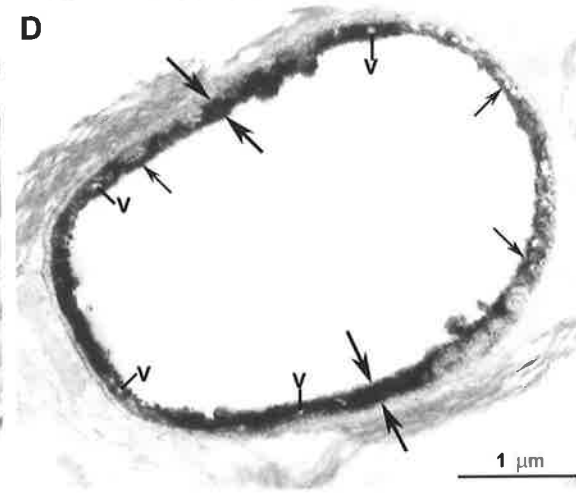
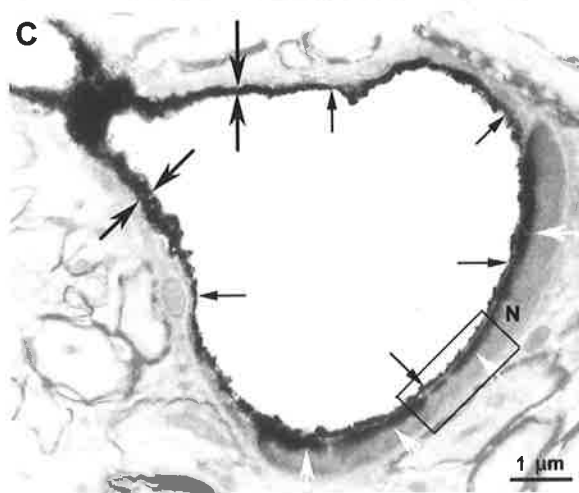
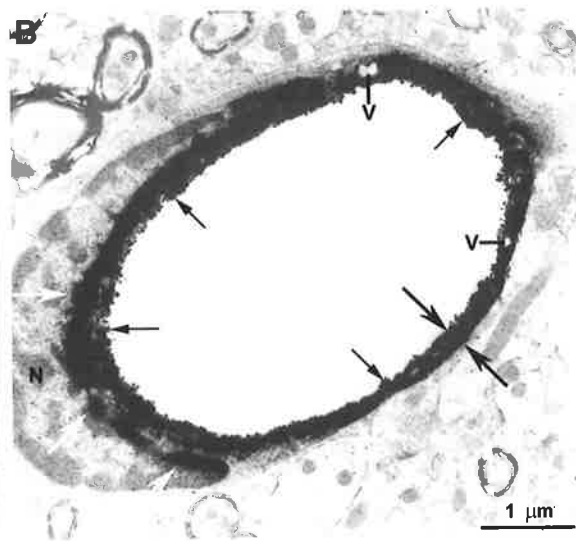
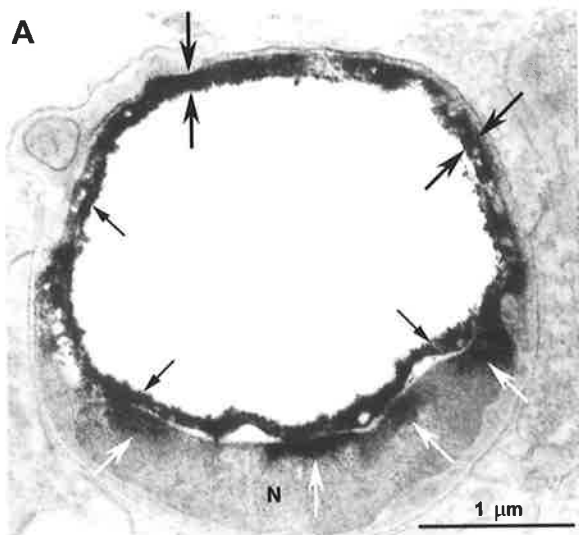
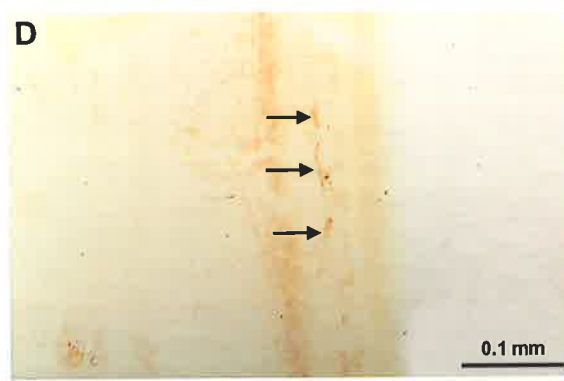
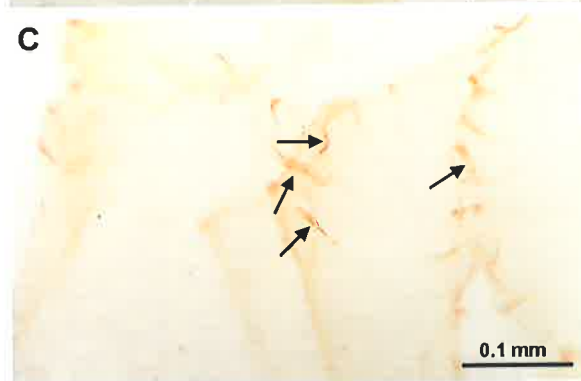
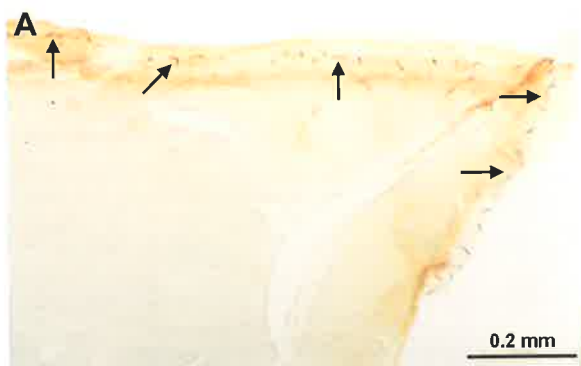


FIG. 4.3.10 (A-D). Light micrographs of wholemount retinas from a control animal injected intravenously with anti-human cytokeratin (**A** and **B**) and an experimental animal injected with anti-EBA (**C** and **D**), showing immunoperoxidase labelling for EBA.

- (A) A wholemount retina from a control animal injected with anti-human cytokeratin treated with the primary anti-EBA in the staining procedure shows EBA-immunoreactive blood vessels at the edge of the retina (arrows).
- (B) Shows a retina from the same control animal injected with anti-human cytokeratin not treated with the primary anti-EBA in the staining procedure. No EBA immunoreactivity is seen.
- (C) A wholemount retina from an experimental animal treated with the primary anti-EBA in the staining protocol shows EBA-immunoreactive blood vessels at the edge and surface of the retina (arrows)
- (D) Shows that in the experimental animal injected with anti-EBA, moderate EBA immunoreactivity is still seen even when the primary antibody was omitted from the staining procedure (arrows), indicating that the anti-EBA injected in vivo became bound to ECs and replaced the step of primary antibody in the staining procedure.



CHAPTER 5

DISCUSSION

5 Discussion

5.1 *Clostridium perfringens* type D epsilon prototoxin-induced BBB opening

5.1.1 The integrity of the BBB in prototoxin-induced BBB opening

The main findings of this study are firstly, intraperitoneal administration of *Cl P* type D epsilon prototoxin produces a rapid, but mild and reversible opening of the BBB in rats. Secondly, *Cl P* prototoxin produces ultrastructural changes at the BBB. Binding of the prototoxin to the brain was expected, on accounts of previous reports of rapid binding of the prototoxin to mouse brain (Buxton, 1976; Nagahama & Sakurai, 1991). However, the ultrastructural changes in brain ECs and the leakage of endogenous albumin in the brain was not expected. Previous studies have shown that the prototoxin offers a protection to mice from the effects of the toxin (Nagahama & Sakurai, 1991). The prototoxin was shown to inhibit the toxin binding to the brain. Moreover, the prototoxin inhibits the uptake of labelled bovine albumin caused by the toxin in the brain. Formalinised epsilon prototoxin was shown to prevent the extravasation of horseradish peroxidase (HRP) in the brain caused by the toxin for up to 100 min and still have some residual protective effects after 750 min (Buxton, 1976). However, in the current study, some pathological effects of the prototoxin on the BBB were observed. The breakdown of the BBB, demonstrated by the leakage of endogenous albumin and ultrastructural changes of the ECs, were noted as early as 1h and were still present at 14d after the injection of the prototoxin. As previous studies only examined the uptake of albumin in the brain from 10 to 30 min after the injection of the prototoxin (Nagahama & Sakurai, 1991) or employed formalinised prototoxin

(Buxton, 1976), the discrepancy in the findings may be due to different experimental methods.

The mechanism underlying the pathological effects of the prototoxin observed in this study is unclear. Purified prototoxin showed a low lethality, with LD₅₀ being 70,000ng/kg of body weight in mice (Minami *et al.*, 1997). One explanation for the action of the prototoxin may be that the prototoxin was activated within the body. *Clostridium perfringens* type B and D strains also can produce λ -toxin, a thermolysin-like metalloprotease (McDonel, 1986; Minami *et al.*, 1997). Incubation of λ -toxin with the prototoxin at 37°C leads to activation of the prototoxin to the toxin. The λ -toxin activates the prototoxin in a similar manner to trypsin, except that the cleavage occurs between 10th and 11th amino acid residues from the N-terminus of the prototoxin, while trypsin and trypsin plus chymotrypsin induced cleavage occurs between the 13th and 14th amino acid residues (Minami *et al.*, 1997). The prototoxin used in this study was supplied as a partially purified culture supernatant. It is possible that a small amount of λ -toxin was contained in the supernatant. When the supernatant was injected intraperitoneally into the rats, the peritoneum may have provided a suitable environment for the activation of the prototoxin to the toxin.

In this study, endogenous albumin was used as a macromolecular tracer to test the integrity of the BBB. The advantages of using endogenous albumin are that it is one of the normal components of blood and it serves as a carrier protein for many important substances such as fatty acids and hormones (Corvol & Bardin, 1973;

Spector, 1975; Weisiger *et al.*, 1981; Ockner *et al.*, 1983). In the BBB, endothelial cells lining cerebral vessels are joined by tight junctions which seal the intercellular space (Brightman & Reese, 1969). Meanwhile, cerebral ECs have sparse pinocytic vesicles compared with those in most other tissues (Crone, 1986; Saunders *et al.*, 1999a). As a result, the entry of albumin into the brain is highly restricted (Vorbrodt & Dobrogowska, 1994; Balslev *et al.*, 1997). No albumin leakage was detected in the brain of control animals in this study, except in the circumventricular areas, known to lack barrier vessels. In experimental animals, pre-embedding immunoperoxidase labelling for albumin and electron microscopy showed an increased number of vesicles in the cytoplasm of ECs throughout the time course of the experiment and albumin was detected within these vesicles. We thus concluded that vesicular transport of albumin through the cerebral endothelium plays a main role in the albumin leakage observed in this study. This conclusion is in agreement with results from previous studies on albumin transportation in other models of compromised BBB (Vorbrodt *et al.*, 1994; Vorbrodt *et al.*, 1995). Immunogold electron microscopy demonstrated the presence of endogenous albumin in EC cytoplasmic vesicles in protamine sulphate- (Vorbrodt *et al.*, 1995) and hyperosmolar L⁽⁺⁾ arabinose-induced BBB opening (Vorbrodt *et al.*, 1994). The involvement of vesicles in the transendothelial transport of albumin through non-barrier type microvessels has been controversial (Michel, 1992). However, in continuous endothelium, vesicular transport of albumin seems to play an important role in transendothelial albumin transport (Ghitescu *et al.*, 1986; Milici *et al.*, 1987; Michel & Neal, 1999). Under experimental conditions of perfused myocardial capillaries,

vesicular transport of albumin seems to be the major route for transendothelial transport for monomeric albumin and albumin-gold particles (Milici *et al.*, 1987).

The presence of immunolabelling of albumin in the interendothelial junction of ECs was demonstrated by electron microscopy in experimental animals, which suggests the paracellular passageway as an additional route for albumin leakage. Occasionally, focal widening of tight junctions was noted in the ultrastructural study, although the majority of interendothelial junctions retained a normal appearance. The opening of interendothelial tight junctions after both intravascular and extravascular brain injuries was described previously (Brightman & Tao-Cheng, 1993). In cold-induced brain injury, as an extravascular insult, widening of interendothelial junctions is believed to be the basic mechanism for opening of the BBB to macromolecules. On the other hand, in osmotic opening of the BBB, as an intravascular insult, the contribution of modified tight junctions to tracer permeability was questioned (Vorbodt *et al.*, 1993). As only a limited number of junctions were modified and gold labelling for albumin never formed continuous rows linking luminal and abluminal estuaries of the junctions, only small amounts of the leaking tracer were assumed to utilise this route to cross the BBB. In the present study, only a few junctions examined showed modification with the presence of albumin in some junctions. Moreover, we have never been able to find albumin present throughout the length of the junction from the luminal to the abluminal side of the EC. Thus a significant contribution of the paracellular route to albumin leakage in this model is questioned.

Apart from vesicles and modified tight junctions, the EC vacuoles observed in experimental animals could have provided a transcellular pathway for albumin leakage. In a previous study on increased microvascular permeability induced by (a) A23187, a Ca^{2+} ionophore, (b) vascular endothelial growth factor, and (c) histamine, the fusion of vacuoles to both luminal and abluminal surfaces of the ECs was noted (Michel & Neal, 1999). It was suggested that when induced by mediators, the transcellular pathway in microvessels could arise from vacuoles. The fusion of vacuoles with luminal and abluminal membranes could lead to the formation of transendothelial channels, through which exchange might occur. The presence of the large number of EC vacuoles in this study supports this route in the prototoxin model. The exact mechanism of prototoxin induced vacuoles, however is not known.

Neurotoxicity of epsilon toxin has been reported by several authors (Miyamoto *et al.*, 1998; Finnie *et al.*, 1999). Previous studies on neuronal uptake of extravasated endogenous proteins or exogenous horseradish peroxidase suggested two types of neuronal uptake (Tengvar & Olsson, 1982; Tengvar, 1986; Løberg & Torvik, 1991). Immunostaining of both cytoplasm and nuclei was taken to represent damaged neurons while cytoplasmic labelling only, was suggested to indicate intact neurons stained through uptake of proteins by retrograde axonal transport. In the current study of prototoxin injection, experimental animals at 12h and 24h showed immunoreactivity for endogenous albumin in some neurons in the cerebrum and cerebellum. The nuclei of these neurons were devoid of any

staining. The pattern of neuronal immunostaining noted in this study suggests that *Cl P* prototoxin has no neurotoxicity, and that its effect is primarily on endothelial cells of the BBB. Therefore the *Cl P* prototoxin model in the rat appears to be a useful model for the study of primary reversible opening of the BBB in the absence of neuronal cell damage. However, the mechanism underlying the pathological changes observed needs further investigation.

5.1.2 EBA expression in prototoxin-induced BBB opening

The results of the current study showed that EBA immunoreactivity is reduced in cerebral endothelia of experimental animals injected with *Cl p* type D epsilon prototoxin. The reduction of EBA immunoreactivity correlated well with modification of the BBB as indicated by ultrastructural changes of the BBB and serum albumin leakage. As early as 1h after prototoxin injection, some blood vessels partially or completely lost EBA immunoreactivity as observed at LM and EM levels. Quantitative analysis showed decreased EBA expression compared with control animals. In the meantime, ultrastructural changes were observed at 1h. Cerebral ECs showed increased numbers of indentations and vesicles. Immunocytochemistry for serum albumin showed albumin staining in blood vessel walls, indicating intramural albumin. At 1-2d after prototoxin injection, LM showed more blood vessels that had lost EBA immunoreactivity. The most obvious modification of the ultrastructure of ECs also occurred at these time intervals, 1-2d. Large vacuoles were observed in the cytoplasm of ECs and pericytes. Many ECs were swollen, depleted of organelles and had electron lucent cytoplasm. Serum albumin leakage also reached maximum level as the

parenchyma throughout the brain showed albumin with the presence of multiple foci of higher immunoreactivity for albumin in the cerebral cortex, brainstem and corpus callosum. At 7 and 14d after prototoxin injection, quantitative analysis still showed a reduction in EBA expression although at the LM level, the expression of EBA was similar to the control. Ultrastructure of ECs at these time intervals was still showing increased vesicles and EC craters. Animals injected with the higher concentration of the prototoxin showed a faster and stronger response with pronounced reduction in EBA immunoreactivity. The group injected with the lower concentration showed mild and late response, where the maximum effect was seen quantitatively at 7d post-prototoxin injection. The two animals killed at 12h and 2d in this group were not different from the control. This may be due to animal variation, or an inadequate sampling. However, even in this group with a mild response there was a trend of reduction in EBA expression.

The results of the current study support the notion that EBA immunoreactivity correlates well with an intact cerebral endothelium. Almost all cerebral blood vessels of control animals showed 100% staining of their luminal membranes for EBA. No background staining was noted. Neurons and neuropil were not labelled by anti-EBA antibody. Previous studies also found cerebral blood vessels uniformly positive for EBA (Orte *et al.*, 1999; Lin & Ginsberg, 2000). It appears that EBA-immunocytochemistry is highly sensitive and specific to normal cerebral endothelium and provides a reliable index for intact endothelial function. A reduction in EBA expression has also been considered as an indicator of brain

endothelium injury. In stab-wound injury to the brain and EAE, blood vessels in the centre of the lesion lost EBA immunoreactivity while blood vessels in areas peripheral to traumatic and inflammatory lesions were still positive for EBA labelling (Sternberger *et al.*, 1989; Rosenstein *et al.*, 1992). In compression injury to the spinal cord, the reduction of EBA immunoreactivity was most pronounced in the grey matter where structural changes were most severe (Perdiki *et al.*, 1998). In focal cerebral ischaemia, the loss of EBA immunoreactivity was limited to the ischaemic region of the brain while prominent EBA immunoreactivity was observed in the ipsilateral unaffected areas (Lin & Ginsberg, 2000). Apparently, the reduction of EBA immunoreactivity is directly related to cerebral endothelium injury and is accompanied by oedema formation. In this study, ultrastructural changes of cerebral endothelium and brain oedema were observed as early as 1h after prototoxin injection and the cerebral endothelium was not normalised at 14d post-injection. The reduction of EBA immunoreactivity was also recorded from 1h to 14d after prototoxin injection. As indicated by serum albumin leakage and ultrastructural changes of the BBB, the injection of prototoxin induces mild brain oedema. Consistently, in experimental animals, none of the time intervals examined showed complete elimination of EBA immunoreactivity in brain vessels. In previous studies, EBA immunoreactivity had gradually recovered by 3-4 weeks after CNS injury (Sternberger *et al.*, 1989; Rosenstein *et al.*, 1992). However, in this study, although at LM level the expression of EBA was similar to the control by 14d, a clear reduction of EBA immunoreactivity was shown by the quantitative study at the EM level. It is anticipated that with longer time intervals, complete recovery of EBA expression would be seen in this model.

The relationship between the possible effect of the prototoxin and the reduction in EBA expression is not clear. The prototoxin binds to the luminal surface of cerebral EC membranes and is able to transiently protect against the biological effects of the toxin (Buxton, 1976; Nagahama & Sakurai, 1991). It was concluded that the toxin and prototoxin bind to the same sites on cell membranes. However, the nature of binding sites on EC membranes is unknown. Whether the loss of EBA in the current study is a direct effect or an indirect effect of the binding of the prototoxin to ECs membranes is speculative.

In this study vacuolation and swelling of pericytes and astrocytic perivascular processes with depletion of organelles were seen. *In vitro* studies suggest an inductive role for astrocytes on the development of BBB characteristics in ECs with increased production of GGTP and increased complexity of tight junctions between ECs (Maxwell *et al.*, 1987; Tao Cheng *et al.*, 1987). However, the reduction of EBA expression seen in this study may not be due to astrocyte damage. An *in vivo* study on selective astrocytic degeneration, induced by chronic systemic injection of the anti-metabolite 6-aminonicotinamide, showed swelling and organelle degeneration, but there was no alteration in the expression of EBA in brain ECs (Krum, 1996). In the latter experimental model of astrocytic degeneration, the BBB to exogenous and endogenous tracers was not compromised.

In this study, immunoperoxidase staining and immunogold labelling for EBA showed cytoplasmic localisation of EBA. The immunoperoxidase staining involved the full thickness of the cytoplasm between the luminal and the abluminal membranes, or showed a gradient of staining fading away towards the abluminal membrane. Immunogold labelling for EBA showed gold particles located in the cytoplasm of ECs. EBA has been recognised as a membrane protein and specifically localised to luminal EC membranes (Sternberger *et al.*, 1989; Rosenstein *et al.*, 1992). Anti-EBA does not react with the proteins in the endothelial cytosol fraction in immunoblots (Sternberger & Sternberger, 1987). However, cytoplasmic immunoreactivity for EBA was reported in Langerhans' cells in the skin (Sternberger & Sternberger, 1987). The difference between EC cytoplasmic staining in the current study and previous studies may be due to the method used and the penetration ability of antibodies or tertiary reagents. The labelling seen here suggests a cytoplasmic pool whose function may be to replenish the luminal membrane content of EBA. The labelling was not seen in cytoplasmic vesicles, thus an intracellular production pathway involving the Golgi apparatus and vesicular structures is unlikely. The reduced cytoplasmic and luminal membrane labelling seen in experimental animals suggests (a) a reduced EBA production with progressive depletion of the EBA content of the cell, (b) a normal rate of production but with an increased rate of degradation, or (c) some structural alteration in the antigen, that prevented/masked its labelling with the anti-EBA. In this study the loss of cytoplasmic labelling seemed to precede the reduction of luminal membrane labelling, suggesting the first mechanism as an

explanation for the change seen in experimental material. A reduced production may result from EC toxicity affecting the synthesis of EBA.

5.1.3 GLUT1 expression in prototoxin-induced BBB opening

The results of the current study demonstrated that administration of *Cl p* epsilon prototoxin did not alter GLUT1 expression at the BBB. Brain blood vessels of experimental animals examined from 1h to 14d after prototoxin injection maintained intense immunoreactivity for GLUT1. Quantitative analysis revealed no apparent differences between the mean number of positive blood vessels in experimental animals injected with prototoxin and the mean of control animals. As disruption of the BBB has been observed by serum albumin leakage and ultrastructural changes of cerebral endothelium in this model, this finding indicates that unlike EBA, GLUT1 does not correlate with an intact BBB. Such conclusion is consistent with previous studies. GLUT1 is abundant in microvessels of tissues with barrier functions such as the testis and retina (Harik *et al.*, 1990). In the brain, GLUT1 is predominantly expressed by blood vessels with barrier function but is not present in endothelia of the circumventricular organs (Harik *et al.*, 1990; Pardridge *et al.*, 1990). GLUT1 was considered to be a specific marker for cells with occluding junctions (Harik *et al.*, 1990). However, blood vessels of haemangioblastoma, a rare CNS tumour, are highly immunoreactive for GLUT1 (Cornford *et al.*, 1995a). The blood vessels of haemangioblastoma are highly permeable to proteins. In brain cold injury, blood vessels in the injury site and in the perilesional area were permeable to proteins but all maintained GLUT1 immunoreactivity (Cornford *et al.*, 1996). Therefore,

the results of the current study and previous studies suggest that GLUT1 immunoreactivity in the adult brain can not serve as an indicator for intact BBB. However, in the process of brain angiogenesis, regenerated ECs became immunoreactive for GLUT1 by day 5, which coincides with the formation of tight junctions in these cells (Nag, 1996). Developmental up-regulation of GLUT1 immunoreactivity has been observed in rats, rabbits and humans (Devaskar *et al.*, 1991; Cornford *et al.*, 1993; Bertossi *et al.*, 1999). Thus, developmentally the appearance of GLUT1 in the brain endothelium coincides with the formation of the BBB.

Glucose transport at the BBB can be up-regulated or down-regulated by physiological alterations that cause either increased or decreased cerebral glucose metabolism. In normal conditions, local cerebral glucose utilisation is matched with local densities of glucose transporters (Duelli *et al.*, 1998; Duelli *et al.*, 1999). In ischaemic injury to the brain, GLUT1 protein and mRNA are up-regulated (McCall *et al.*, 1996). The depletion of energy supply to the brain in ischaemic conditions compromises the function of mitochondria and increases the demand for glucose. In the current model, that administration of prototoxin did not change the expression of GLUT1 in brain microvessels suggests that the prototoxin did not affect glucose metabolism in the brain. As detected by serum albumin extravasation, injection of prototoxin only induced a rapid, mild and reversible disruption of the BBB. Unlike ischaemic injury which disrupts energy supply to the brain, epsilon prototoxin seems to directly injure brain ECs. It can be assumed that glucose and energy supply to the brain are not dramatically

affected. In support of this speculation, no significant neurotoxicity was observed in experimental animals injected with the prototoxin.

5.1.4 Conclusion

In summary, administration of *Cl p* epsilon prototoxin induces rapid, mild, and reversible opening of the BBB. The pathological effect of the prototoxin appears to be directly on the cerebral endothelium. Ultrastructural changes of cerebral ECs and serum albumin leakage suggest that increased transendothelial transportation and to a lesser extent partial opening of tight junctions are responsible for the brain oedema observed in the current model. EBA immunoreactivity is reduced after the prototoxin injection and the reduction in EBA immunoreactivity correlates with the disruption of the BBB. In agreement with previous studies, EBA immunoreactivity appears to be a sensitive and specific indicator for an intact BBB. However, GLUT1 immunoreactivity is not altered in this model, indicating that GLUT1 expression is not an indicator for injured cerebral endothelium in pathological conditions. It seems that the glucose metabolism of the brain is not affected in the current model.

5.2 Traumatic brain injury (TBI)

5.2.1 The integrity of the BBB in TBI

In this study, serum albumin and lanthanum entered the brain parenchyma, and ultrastructural changes in brain ECs were seen at 1h after TBI. At 6h after the injury, albumin leakage and ultrastructural changes were more severe, indicating that at this time interval EC function at the BBB continued to decline. At 12h after the trauma, albumin leakage was still present but was reduced compared to that seen at 6h and the ultrastructure of most ECs became normalised. We concluded that in this study, the BBB was open to small and large molecules at 1h, the earliest time point examined after the injury. The opening of the BBB was maximum at 6h after the injury. The BBB started to close at some time between 6h and 12h as albumin leakage started to subside but the integrity of the BBB was not restored by 12h postinjury.

The impact acceleration injury model employed in the current study was first introduced by Marmarou *et al.* in 1994. This model induces a closed head injury, without skull fracture, and causes diffuse axonal injury (Povlishock *et al.*, 1997; Stone, J. R. *et al.*, 1999). This model does not cause severe hypertensive surge; thus brain concussion is the single cause of BBB dysfunction in this model (Foda & Marmarou, 1994; Marmarou *et al.*, 1994). In the current investigation, immediately after the injury, the femoral arterial blood pressure of experimental animals increased from the basal level of anaesthetised animals (67 mm Hg) to 100 mm Hg, which was not higher than the normal rat systolic blood pressure (116-145 mm Hg) (d'Uscio *et al.*, 2000). A previous study showed that the

changes of the arterial blood pH, pCO₂ and pO₂ in artificially ventilated experimental animals were not significantly different from those in control animals (Marmarou *et al.*, 1994).

Several studies have investigated the BBB function in this model. Using different tracers and techniques, various observations have been documented. Determined by microvascular changes at the LM and EM levels, it was concluded that brain oedema formation occurs at 6h post injury and appears to reach a maximum after 24 hours in both mild and severe closed head injuries (Foda & Marmarou, 1994). A quantitative study using ¹²⁵I-labelled bovine albumin as a tracer investigated the permeability of the BBB at 2h, 4h and 15h after the injury (van den Brink *et al.*, 1994). The tracer was injected prior to the injury and a cumulative permeability index study was performed 4h and 15h after the injury. The results showed that cumulative radioactive concentration of labelled albumin was apparently more obvious at 4h than at 15h. A “time window study” was also performed at 2h after the injury in which the tracer was injected 30 min before the animal was killed. The BBB was open at 2h after the injury. The authors concluded that the BBB was opened by the impact injury from at least 2 hours and closed within 15 hours (van den Brink *et al.*, 1994). A non-invasive approach to study the BBB is to use magnetic resonance imaging (MRI). By assessing the ability of the BBB to exclude a contrast agent gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA), MW 938 Da, it was found that the BBB was disrupted 4 min after the injury but closed within 30 min (Barzó *et al.*, 1996). Another technique is to use diffusion-weighted MRI to detect the type of oedema based on the measurement

of the apparent diffusion coefficient (ADC). An increase of ADC accompanied by increase of water content of the brain is considered to suggest vasogenic oedema while the reduction of ADC with increased water content of the brain is considered to indicate cytotoxic oedema (Ito *et al.*, 1996; Barzó *et al.*, 1997; Kuroiwa *et al.*, 1999). By using this technique, it was found that the BBB was compromised at the first 60 min after the injury, which led to the formation of vasogenic oedema. However, the ADC began to decrease 40 to 60 min after the injury. Since the water content of the brain continued to increase, it was concluded that cytotoxic oedema was the major contributor to post-traumatic brain swelling (Barzó *et al.*, 1997).

Results from the above previous studies showed varied time courses of the BBB opening and closure following the injury. The duration of the BBB disruption ranged from 30 min to 24 hours after the injury (Foda & Marmarou, 1994; van den Brink *et al.*, 1994; Barzó *et al.*, 1996; Barzó *et al.*, 1997). The present study showed that opening of the BBB following the injury started at least as early as 1h, appeared to reach a maximum level at 6h and then started to close at some time between 6h and 12h. The discrepancy in the reported length of time of the opened BBB may be due to different approaches to the study of BBB permeability and/or the different injury force used. In the present study, the earliest time examined was 1h after the injury. We cannot exclude the possibility that the BBB was opened before the 1h time point. In addition, in the current study, the injury was induced by dropping a 450g metal rod from a height of 1.5 metres, however the other studies used a similar weight but the weight was dropped from a height

of 2 meters. The less force used in this study may have caused less severe but different course of BBB opening.

Brain oedema has been defined into two categories by Klatzo in 1967, vasogenic oedema and cytotoxic oedema (Klatzo, 1967). Vasogenic oedema is characterised by the disruption of the BBB and abnormal leakage of plasma proteins and water into the extracellular space. Cytotoxic oedema is characterised by intracellular swelling without increased permeability of the BBB (Klatzo, 1967). Cytotoxic oedema is mainly manifested by swelling of astrocytes, but also can involve swelling of the myelin sheath of nerve fibres and the dendrites of neurons (Kimmelberg, 1995). Traumatic brain oedema has usually been considered as vasogenic in nature and is characterised by the leakage of blood-borne substances from the vasculature, as a result of direct mechanical injury to the BBB (Marmarou, 1994). Numerous studies have demonstrated the leakage of serum proteins across the BBB following various traumatic brain injury models, such as the fluid percussion injury model (Schmidt & Grady, 1993; van den Brink *et al.*, 1994; Fukuda *et al.*, 1995), cortical impact model (Dhillon *et al.*, 1994; Baskaya *et al.*, 1997a), and impact acceleration model (Foda & Marmarou, 1994; van den Brink *et al.*, 1994; Ito *et al.*, 1996). However, the contribution of cytotoxic oedema to traumatic brain oedema has been suggested more recently. Using the diffusion-weighted MRI, continuation of brain oedema formation was demonstrated despite closure of the BBB. Therefore, it was postulated that cytotoxic oedema plays an important role in traumatic brain oedema. In the present study, increased severity of astrocytic swelling was observed at 12h after

the injury while albumin leakage started to subside, suggesting an initiation of cytotoxic oedema (Kimelberg, 1995). In the current study, astrocytes showed severe swelling, not only in the perivascular endfeet but also in the cell bodies. The cytoplasm of astrocytes was electron lucent with dispersed organelles suggesting cellular swelling. The results of this study support the view that cytotoxic oedema is involved in the formation of brain oedema following traumatic brain injury. However, this study was unable to investigate the development of cytotoxic oedema any further than 12h due to the requirement of the animal ethical approval.

The mechanism underlying the development of cytotoxic oedema is not clear. Cytotoxic oedema is most commonly observed in cerebral ischaemia. In ischaemic insults, the disruption of energy supply leads to ionic pump failure in brain parenchymal cells, mainly astrocytes, resulting in excessive influx of sodium and water and cellular swelling (Klatzo, 1967, 1994). Studies of cerebral blood flow (CBF) in the impact acceleration injury model using T₁-weighted and diffusion weighted MRI showed a temporal decrease of CBF for less than 20 minutes, reaching 60% of the control value, during posttraumatic hypotension, and then increased with the recovery of mean arterial blood pressure (Barzó *et al.*, 1996; Ito *et al.*, 1996). Another quantitative study using ¹⁴C-iodoantipyrine demonstrated uneven increase of CBF in the pontine grey matter, hypothalamus, amygdala, caudate nucleus, and white matter after the trauma (Nawashiro *et al.*, 1994). Furthermore, no energy failure was found in the study of fluid percussion injury model (Unterberg *et al.*, 1988). Therefore, in this particular model, energy

failure may not be severe enough, if it did happen, to cause the prolonged brain oedema observed. In the absence of ischaemia and BBB compromise, another form of oedema, neurotoxic oedema, has been proposed to be responsible for the prolonged oedema process observed in traumatic brain injury (Marmarou, 1994). High levels of excitatory amino acids have been detected in the extracellular space in the brain parenchyma and cerebrospinal fluid, in both experimental animals (Stover *et al.*, 2000) and head injury patients (Bullock *et al.*, 1998; Stover *et al.*, 1999; Yamamoto *et al.*, 1999). Excessive glutamate is known to be able to cause cell death by inducing ionic disequilibrium related to the excessive entry of Na^+ and Ca^{++} (Meldrum, 2000). A recent study showed that traumatic brain injury enhanced glutamate mediated brain damage (Di *et al.*, 1999). It has been reported that glutamate receptor antagonists could reduce regional cerebral oedema after traumatic brain injury (Okiyama *et al.*, 1997). Other factors, such as release of free radicals (Nishio *et al.*, 1997) and impaired potassium homeostasis (D'Ambrosio *et al.*, 1999) have also been suggested to induce neuronal dysfunction and oedema formation. Therefore, it is reasonable to postulate that in the current study, shortly after the trauma, the BBB is disrupted producing predominantly vasogenic oedema. After the closure of the BBB, cytotoxic oedema, possibly induced by excessive neurotoxic substances released in the parenchyma, is initiated.

Lanthanum chloride was used in the current study as a small molecular tracer to investigate the permeability of the BBB at 1h and 6h after the injury. Ionic lanthanum has a MW of 139 Da and ionic radius of 0.4-0.46 nm (Abbott &

Revest, 1991; Brightman & Tao-Cheng, 1993) and is the smallest available EM probe. It has been shown by several studies that ionic lanthanum does not penetrate the normal BBB (Bouldin & Krigman, 1975; Schindelmeyer *et al.*, 1987; Sanovich *et al.*, 1995). Also in the current study ECs excluded lanthanum from entering the brain. Ionic lanthanum is able to displace calcium ions from calcium binding sites (Weiss & Goodman, 1969; Weiss, 1973). To avoid this toxic effect of ionic lanthanum on tissues, lanthanum chloride was perfused into the animals after the tissues were fixed *in situ*. Therefore, the leakage of lanthanum observed in the current study in experimental animals at 1h and 6h after the trauma was not due to any metabolically-dependent process. Although lanthanum deposits were observed in some vesicles, it is not possible that the transfer of lanthanum was through transcytosis since tissues were fixed prior to exposure to lanthanum. Instead, the observation of lanthanum deposits infiltrating the cytoplasm of ECs and its presence in tight junctions indicate that the ionic lanthanum passage is dependent upon its direct diffusion through altered EC membrane and opened tight junctions. Endothelial cell changes and alterations of tight junctions after the trauma were demonstrated in the ultrastructural study. The penetration of lanthanum through ECs was not seen in all blood vessels observed and even in the same blood vessel, lanthanum permeation of ECs varied in different sectors of the vessel. Therefore, it seems that the extent of injury to ECs is not uniform. In summary, according to the observation of lanthanum leakage in the brain after the trauma, at least for small molecules and ions, two pathways may be involved in the opening of the BBB. Molecules in the blood of a comparable size to lanthanum chloride may cross the BBB through the injured

EC membranes or pass through altered tight junctions. Serum albumin infiltrated the cytoplasm of some ECs after the injury. Few vesicles were observed to contain albumin. It seems that serum albumin, as a representative of macromolecules, crossed the BBB mainly through direct diffusion through the endothelium. The involvement of the vesicular system in transporting albumin in the current model may have a subordinate role. Also the intercellular route although was clearly open to small molecules as indicated by lanthanum leakage, it may not be the main pathway for leakage of macromolecules such as albumin.

5.2.2 EBA expression in TBI

The results of the current study demonstrated that EBA immunoreactivity was markedly reduced after the trauma. This is the first study to show a reduction in EBA immunoreactivity in association with and as a sensitive indicator of vascular injury in closed head injury. The reduction correlated well with the time course of BBB breakdown. At 1h after the injury, the tracers employed demonstrated opening of the BBB and the ultrastructural study showed alteration of brain ECs. At this time interval, both LM and EM quantitative data showed a clear reduction of EBA expression. At the LM level, the mean number of EBA-immunoreactive blood vessels in the cortex was reduced to 73% of the mean value of the controls. At the EM level, the percentage of immunostained EC luminal membrane length in the cortex was reduced to a mean of 48%. At 6h after the injury, albumin leakage and changes of ECs became more pronounced. EBA expression at 6h also showed further reduction. At the LM level, the mean number of EBA-immunoreactive blood vessels in the cortex was reduced to 54% of the mean value

of the controls. At the EM level, the percentage of EBA immunostained EC luminal membrane length was decreased to a mean of 37%. At 12h after the injury, albumin leakage was still present in the parenchyma but at a reduced level. Ultrastructurally brain ECs became normalised. EBA expression at this time interval was still reduced compared to controls but slightly increased compared to that of 6h. The mean number of blood vessels showing EBA immunoreactivity was 65% of the mean value of the controls. The mean value of percentage of EBA immunostained EC luminal membrane length was 54%. It appears that the reduction of EBA expression correlates with the severity of the damage to the BBB.

Previous studies have reported a reduction of EBA immunoreactivity following CNS injury, such as EAE (Sternberger *et al.*, 1989), stab-wound injury to the brain (Rosenstein *et al.*, 1992), cerebral infarction (Lin & Ginsberg, 2000; Nishigaya *et al.*, 2000) and spinal cord injury (Perdiki *et al.*, 1998). EBA expression was reduced at the centre of lesions but was retained in the area peripheral to the lesion sites. It appears that the reduction of EBA expression is directly related to the cerebral endothelium injury. In the current study, EBA reduction was observed qualitatively throughout the brain. Two representative regions were selected for quantitative assessment of EBA immunoreactivity: the frontal cortex and brainstem. An apparent reduction of EBA immunoreactivity was found in these two regions. From the qualitative and quantitative results one can conclude that diffuse vascular injury occurred in the current study of closed

head injury model. Previous studies have also demonstrated diffuse axonal injury in this model (Foda & Marmarou, 1994; Stone, J. R. *et al.*, 1999).

Recovery of EBA expression was reported in EAE and stab-wound injury at 3-4 weeks (Sternberger *et al.*, 1989; Rosenstein *et al.*, 1992). In spinal cord injury EBA immunoreactivity is restored at 9 days after the injury (Perdiki *et al.*, 1998). Two weeks after cerebral infarction, EBA expression showed initial recovery in ischaemic areas (Nishigaya *et al.*, 2000). In the current study, EBA expression showed a slight increase at 12h after the injury but was still less than the controls. Longer time intervals are needed to determine if complete recovery of EBA immunoreactivity will occur in this model. At 12h, vasogenic oedema was reduced and EC ultrastructure was largely normalised. The reduction of EBA expression at this time interval indicates that EBA expression is a sensitive indicator of vascular injury, as the recovery of the expression of this protein is lagging behind the reconstitution of the BBB. Such delay may be caused by the protein synthesis process.

At 12h after the trauma, cytotoxic oedema became obvious, as indicated by the severe swelling of astrocytes. On the other hand, the quantitative study did not show any further reduction of EBA immunoreactivity compared to 6h time interval. Therefore, it seems that EBA expression may be an indicator of brain vascular injury but not an indicator of cerebral oedema. Astrocytes have been implicated in the initiation of BBB characteristics in brain ECs (Arthur *et al.*, 1987; Janzer *et al.*, 1993). In the current study, severe swelling of astrocytes was

not accompanied by further reduction of EBA immunoreactivity and indicates that EBA expression is not affected by astrocytic dysfunction. This finding is consistent with a previous study which showed that selective astrocyte damage did not alter the expression of EBA (Krum, 1996). However, the results of this study cannot exclude the possible inductive role of astrocytes on the BBB during development of the brain.

In the current study, quantitative assessment of EBA immunoreactivity was performed at the LM and EM levels. The advantage of LM quantitative analysis is that a relatively large area of tissue can be assessed. However, it cannot assess the staining pattern of individual blood vessels. On the other hand, at the EM level, the expression of EBA in individual blood vessels can be analysed. Sectoral variation of EBA expression of each blood vessel can be assessed. However, the limitation of EM quantitative studies is related to sampling of relatively small areas. Comparing the LM and EM data, EM analysis showed a more severe reduction of EBA immunoreactivity in the cortex. At 1h after the injury, the mean value of the percentage of EBA-immunoreactive EC luminal membrane length was reduced to 52% of the mean value of the controls, while the mean number of EBA-immunoreactive blood vessel at the LM level in the frontal lobe was 73% of the mean value of the controls. At 6h after the trauma, the mean value of the percentage of EBA-immunoreactive EC luminal membrane length was further reduced to 40% of the mean value of the controls, while the mean number of EBA-immunoreactive blood vessels at the LM level was 54% of the mean value of the controls. At 12h after the injury, the mean value of the percentage of EBA-

immunoreactive EC luminal membrane length was 58% of the controls, while the mean number of EBA-immunoreactive blood vessel at the LM level was 65% of the mean value of the controls. The lower value of EBA immunoreactivity obtained at the EM level indicates that sectoral loss of EBA immunoreactivity is more common in this particular model of closed head injury. Therefore, EM analysis seems to be more sensitive to evaluate EBA expression in this model. In the current study, EM quantitative assessment of EBA immunoreactivity complemented and extended the LM quantitative study.

The ultrastructural analysis showed that at all the time intervals studied, EC changes were not severe enough to cause EC degeneration. At 6h after the injury, when the most severe ultrastructural changes were observed, the EC lining the blood vessel wall was complete. Therefore, the reduction in the number of EBA-immunoreactive blood vessels observed after the injury at the LM level is not due to the loss of blood vessels or ECs after the injury but is due to the loss of EBA immunoreactivity.

Sham control animals did not show obvious changes in EBA expression except in the 1h sham control animal when assessed quantitatively at the LM level. The mean number of EBA-immunoreactive blood vessels was reduced in the cortex and brainstem of the 1h sham control animal compared to the normal controls. Such reduction in EBA expression at 1h may be due to individual animal variation or that the sham operation having a transient effect on EBA expression. One may consider that exposure of the skull and mounting of the metal helmet may have

resulted in some as yet unknown haemodynamic, thermal or chemical changes in the vasculature of the brain. Since the reduction in EBA labelling was seen in the brainstem and the cerebral cortex, it is unlikely that any changes are due to local direct effect (of temperature changes or otherwise) being transmitted to cortical vessels.

5.2.3 GLUT1 expression in TBI

The results of the current study showed a pronounced reduction of GLUT1 immunoreactivity after traumatic brain injury. The reduction was observed both qualitatively and quantitatively. At 1h after the injury, the number of GLUT1-immunoreactive blood vessels in the frontal cortex was reduced to 66% of the control. Quantitative EM of the percentage of GLUT1-immunoreactive EC luminal membrane length showed a mean of 47%, which represents 50% of the mean value of the normal controls. At 6h after the injury, a further reduction of GLUT1 expression in the brain was observed by LM and EM. The mean number of GLUT1-immunoreactive blood vessels in the frontal cortex was reduced to 47% of the mean value of the controls. EM analysis showed that the mean value of the percentage of GLUT1-immunoreactive EC luminal membrane length was 19%, which was only 21% of the mean value of the normal controls. Twelve hours after the trauma, a slight increase of GLUT1 immunoreactivity was noted in the cortex at the LM and EM levels compared to 6h time interval although GLUT1 expression was still apparently lower than the controls. The LM quantitative study showed that the mean number of GLUT1-immunoreactive blood vessels in the frontal cortex was 61% of the mean value of the normal

controls. Quantitative assessment at the EM level showed that the mean value of the percentage of GLUT1-immunoreactive EC luminal membrane length was 49%, which was 52% of the mean value of the normal controls.

At 1h, the sham control animal showed reduced GLUT1 expression in the brain. This may be due to individual animal variation or transient effect of artificial ventilation and sham operation on the expression of GLUT1. At 12h, quantitative analysis at the EM level showed reduced GLUT1 expression in the cortex of the sham control compared to the normal controls. This could have resulted from the long anaesthesia. As the experimental animals at this time interval showed a lower GLUT1 immunoreactivity than the sham control animal, it may be concluded that at 12h after the injury, the GLUT1 immunoreactivity is still down regulated.

Despite extensive studies on GLUT1 and traumatic brain injury in isolation, limited information is available on GLUT1 activity in traumatic brain injury. The current study has shown for the first time that GLUT1 activity is down regulated following experimental closed head injury. A study on GLUT1 activity in human brain injury reported that in the central zone of dense erythrocyte infiltration, representing the traumatic focus, capillaries almost totally lost immunoreactivity for GLUT1. However, in the peripheral zone beyond this traumatised zone, the majority of microvessels was highly reactive for GLUT1 (Cornford *et al.*, 1996). Quantitative analysis of GLUT1 immunoreactivity using immunogold staining also showed a high GLUT1 immunoreactivity in that peripheral zone, which was

even higher than that of erythrocytes (Cornford *et al.*, 1996). As it is generally believed that the concentration of GLUT1 in erythrocytes is by far the highest concentration density for any glucose transporter (Mueckler, 1994), it was concluded that GLUT1 activity was increased in this region of the brain in the human cases studied (Cornford *et al.*, 1996). The discrepancy between the previous and the current studies may be caused by several factors. Firstly, different types of traumatic brain injury were studied. The human cases study was carried out on two patients; one had suffered a gun shot injury and the other sustained a motor vehicle accident. In the current study of closed head injury, brains of experimental animals did not show cerebral haemorrhage and contusion. Secondly, as normal human brain material was not available and the GLUT1 activity in the normal human brain is unknown, the GLUT1 activity was compared to that of erythrocytes. Previous studies also found that in human brain resections from other clinical conditions, GLUT1 densities on brain capillary endothelial membranes were higher than on the erythrocyte membranes (Cornford *et al.*, 1994b; Cornford *et al.*, 1995a). Therefore, the density of GLUT1 in cerebral EC membranes may be much higher than in erythrocyte membranes in normal human brain. Thirdly, different quantitative methods were used in the current and previous studies. In the human cases study, focal loss of GLUT1 immunoreactivity was reported. In the present study, a diffuse vascular injury occurred and the loss of GLUT1 is widespread compared to the human cases studied.

In the study of human brain injury, two types of GLUT1-immunoreactive vessels were observed (Cornford *et al.*, 1996). While about 90% of the microvessels showed uniform staining for GLUT1 throughout the entire cross-sectional profile, about 10% of the microvessels showed markedly lower density of GLUT1 labelling in one sector of the capillary profile. This pattern was not observed in normal primate or rodent brains (Cornford *et al.*, 1996; Cornford *et al.*, 1998a). This bimodal GLUT1 distribution has also been observed in brain tissues from patients suffering from seizures (Cornford *et al.*, 1998b). In interictal seizure resections, the distribution of high and low GLUT1 immunoreactive ECs provided three types of GLUT1-immunoreactive microvessels. Type A capillaries display abundant GLUT1 immunoreactivity throughout the entire capillary circumferences on both luminal and abluminal membranes. Type B capillaries have low GLUT1 density in all ECs. Type AB capillaries display both high and low GLUT1 density ECs. In the current study, quantitative analysis at the EM level demonstrated that sectoral absence of GLUT1 immunoreactivity in blood vessels was most common in vessels from experimental materials. Therefore, the findings of the current study indicate that in the rodent brain, bimodal distribution of GLUT1 immunoreactivity in brain ECs is particularly present in response to injury. The differential expression of GLUT1 in contiguous capillary ECs may indicate segmental vascular injury induced by the closed head injury.

Quantitative assessment of GLUT1 at the EM level revealed a more severe reduction than at the LM level. The EM study analysed GLUT1 immunoreactivity of cross sections of each blood vessel, and revealed sectoral

loss of GLUT1 immunoreactivity. Such vessels would have been identified as positively stained in the LM quantitative study. Therefore, the EM analysis appears to be the only suitable method to analyse the bimodal distribution of GLUT1 immunoreactivity in brain EC membranes in traumatic injury.

The current quantitative study of brainstem sections at the LM level showed that GLUT1 immunoreactivity remained unaffected at 1h and 6h after the injury while at these time interval, frontal cortex showed reduced GLUT1 expression. This suggests that glucose uptake in the brainstem is less affected by the injury than in the cortex.

The down-regulation of GLUT1 immunoreactivity observed in this study indicates that the uptake of glucose by brain ECs is reduced in this traumatic brain injury model. This may be due to disturbed production of this protein as a result of vascular injury caused by the mechanical force. Alternatively, the opening of the BBB observed after the injury enabled glucose to diffuse freely into the brain interstitial tissue, which reduced the need for GLUT1 transport activity. However, the latter explanation is less likely, as impairment of cerebral glucose uptake has been reported in experimental traumatic brain injury (Krishnappa *et al.*, 1999) and in severe acute brain trauma in humans (Cruz, 1995). In the experimental study (Krishnappa *et al.*, 1999), cerebral dialysate glucose concentration fell by 50% following severe cortical impact injury while the dialysate lactate concentration significantly increased. Studies on the fluid percussion injury model also reported a significant reduction of the cerebral dialysate glucose and increase of the cerebral dialysate lactate following the injury

(Chen *et al.*, 2000a and b). In human brain trauma (Cruz, 1995), global cerebral glucose extraction was significantly reduced as measured by arteriojugular glucose concentration difference, suggesting impaired cerebral glucose uptake.

The regulation of GLUT1 in the brain is closely related to cerebral glucose metabolism. Studies on neurodegenerative diseases such as Alzheimer's disease and AIDS with late clinical stage of dementia showed that GLUT1 expression is significantly reduced in the brain (Harik, 1992; Simpson *et al.*, 1994; Kovitz & Morgello, 1997). In Alzheimer's disease, down-regulation of both GLUT1 and GLUT3 in the brain in the temporal and parietal cortices was observed (Simpson *et al.*, 1994). Decreased glucose transport in these brain areas in Alzheimer's disease was demonstrated by positron emission tomography (Jagust *et al.*, 1991). In AIDS patients, GLUT1 activity was increased in the brain when no neuropathological changes were observed. However, GLUT1 activity was decreased in brains from patients with HIV encephalitis (Kovitz & Morgello, 1997). The changes of GLUT1 activity in brains in HIV infection seem to parallel the glucose metabolism changes which occur during the course of AIDS. Glucose hypermetabolism was observed at the early stage of AIDS dementia but in the later stage of dementia, glucose metabolism was decreased (Rottenberg *et al.*, 1987).

Metabolic changes have been demonstrated following traumatic brain injury. Impaired ionic homeostasis was documented in several traumatic brain injury models (Hovda *et al.*, 1992; Heath & Vink, 1996; Vink *et al.*, 1996; D'Ambrosio

et al., 1999). The disruption of ionic balance in the brain increases energy demand for the brain to restore the ion gradients. It was reported that local cerebral glucose utilisation increases following traumatic brain injury in the fluid percussion model in rats (Yoshino *et al.*, 1991) and in severe traumatic brain injury in humans (Bergsneider *et al.*, 1997). Therefore, impaired transportation of glucose following traumatic brain injury would further worsen the cellular damage. In the fluid percussion injury model, following the hypermetabolism observed in the early stage of injury, cerebral hypometabolism, indicated by decreased local cerebral metabolic rates for glucose, was observed from 6h after the injury and lasted for as long as 5 days. This indicated that cells were functionally compromised (Yoshino *et al.*, 1991). However, an *in vivo* study showed that changes of blood glucose level failed to affect neural outcome after the trauma (Vink *et al.*, 1997). Infusion of dextrose, or zinc insulin which induced hyperglycaemia or hypoglycaemia respectively in rats following the fluid percussion injury did not affect the brain intracellular pH, free magnesium, cytosolic phosphorylation potential, and neurological outcome (Vink *et al.*, 1997). Another *in vitro* study, on the other hand, showed that higher glucose concentration in the rat hippocampal slice incubation medium improved recovery of neuronal function after cerebral hypoxia (Schurr *et al.*, 1987). The protective effect of glucose was dose dependent. It was suggested that high concentration of glucose in the extracellular fluid increases anaerobic glycolytic production of energy charge to maintain ion gradients across the neuronal membrane. Apparently, the *in vitro* study bypassed the BBB and directly increased the extracellular glucose concentration. The down-regulation of GLUT1 activity in

cerebral EC membranes observed in the current study suggests that increasing the blood glucose concentration may not allow effective increase in the glucose concentration in the extracellular fluid. Recent studies have shown that during traumatic brain injury, lactate may be used as an energy supply. After traumatic brain injury, brain lactate level is significantly increased (McIntosh *et al.*, 1987; Krishnappa *et al.*, 1999; Chen *et al.*, 2000a). Although it was postulated that increased lactate level may contribute to secondary injury to the brain following traumatic brain injury, it has been shown that the level of cerebral lactate was not directly related to the severity of neurological dysfunction (McIntosh *et al.*, 1987). Lactate, traditionally regarded as a bi-product of glycolysis, is now suggested to serve as an energy substrate for the CNS after injury (Schurr *et al.*, 1997; Chen *et al.*, 2000b).

5.2.4 Conclusion

A closed head injury model in rats was employed in this study. Albumin leakage studies carried out here suggest that vasogenic brain oedema is initiated as early as 1h after the injury. Vasogenic oedema appeared to result from the disruption of the BBB. At 12h after the injury, extensive cellular swelling was seen in astrocytes suggesting the development of cytotoxic oedema despite the BBB showing signs of recovery. EBA immunoreactivity, an indicator of vascular integrity, was reduced after the closed head injury. The reduction of EBA expression correlated well with the course of vasogenic oedema. No further reduction of EBA expression occurred when astrocytes showed severe swelling indicating that EBA immunoreactivity is not directly related to astrocyte function.

GLUT1 immunoreactivity was also reduced after the injury, indicating a potential impairment of glucose supply to the brain. Therefore, following traumatic brain injury, multiple factors contribute to the injury to the brain. Apart from the direct mechanical injury to the brain, the disruption of the BBB, subsequent leakage of blood borne toxic substances into the brain interstitium, and energy supply failure may all be responsible for the neuronal dysfunction following traumatic brain injury.

5.3 *In vivo immunological targeting of EBA*

5.3.1 *The effect of anti-EBA injection on the BBB and BRB*

The most striking finding of this study is that intravenous injection of anti-EBA led to rapid opening of the BBB and BRB, as detected by HRP and endogenous albumin leakage. Numerous studies have shown that EBA immunoreactivity correlates well with an intact cerebral endothelium. The expression of EBA in the brain is at the classic BBB sites and is absent or only weakly present in endothelia of circumventricular organs (Sternberger & Sternberger, 1987). In pathological conditions accompanied with disruption of the BBB, the expression of EBA is reduced. In stab-wound injury to the brain, EAE, compression injury of the spinal cord and cerebral infarction, the BBB breaks down and brain oedema develops. The reduction of EBA expression has been demonstrated in all these experimental models during the acute phases of injury or inflammation (Sternberger *et al.*, 1989; Rosenstein *et al.*, 1992; Perdiki *et al.*, 1998; Lin & Ginsberg, 2000). The re-expression of EBA was also demonstrated in these conditions and the recovery of EBA immunoreactivity correlates with the clearance of brain oedema and reconstitution of the BBB. EBA appears to uphold the BBB integrity in the rat. It has been suggested that EBA expression provides a reliable index for an intact cerebral endothelium. In this study, intravenous injection of anti-EBA appeared to interfere with the function of EBA at the BBB and led to widespread brain oedema. One may hypothesise that the reduction in EBA in previous studies preceded the occurrence of oedema. This hypothesis has a wide implication; that pathological lesions in the rat CNS, which produce opening of the blood-CNS barrier and the occurrence of oedema, are doing so via a final common pathway

that affects EC biology leading to loss of EBA as the primary event. However, such speculation if applicable to the rat, would not apply to other species including humans, who show oedema in similar pathologies. Anti-EBA is specific to the rat, and we found that anti-EBA does not stain vessels in human or sheep brains (unpublished observations). It is tempting to speculate that oedema formation in species other than the rat may involve a reduction in a homologue of EBA or of a corresponding EC protein similar in function to EBA.

The pattern of spread of albumin and HRP in the grey and white matter of brains of experimental animals showed some similarities. In the white matter the tracers showed linear distribution and predominantly followed a path parallel to fibre tracts. The extracellular space in the white matter is relatively simple and follows nerve fibres. It therefore may provide a low resistance pathway for the tracers. In the grey matter the tracer showed a predominantly circumferential spread. The cytoarchitecture of the grey matter is more complex than that of the white matter, with interdigitating neuronal processes and glial cells. Consequently, the extracellular space in the grey matter is sinuous and complex, offering a high resistance pathway for the spread of tracers.

The current pre-embedding immunoperoxidase electron microscopic study has revealed the route of leakage of HRP and serum albumin through the BBB after intravenous injection of anti-EBA antibody. HRP, with a molecular weight of 40 kDa, has been widely used in permeability studies since its introduction as an exogenous tracer for the study of the BBB (Brightman & Reese, 1969). In normal

conditions, a limited amount of HRP is taken up by ECs by fluid-phased endocytosis, internalised and degraded by lysosomes (Broadwell & Banks, 1993). Previous studies on the mode of leakage of HRP through the BBB showed that in pathological conditions, such as heat stress and spinal cord contusion, the tracer traverses the EC barrier principally via increased vesicular transport (Noble & Wrathall, 1989; Wijsman & Shivers, 1993). In this study, HRP was detected in vesicles, multivesicular bodies and dense bodies in the cytoplasm of brain ECs of experimental animals, indicating a transendothelial vesicular pathway for HRP leakage. No peroxidase reaction product was observed within tight junctions. As the expression of EBA is not associated with tight junctions, the intravenous injection of anti-EBA antibody may not disturb the integrity of tight junctions.

Peroxidase reaction product was seen heavily infiltrating the basal laminae of ECs and pericytes. In a study of cold injury to the brain, it was observed that albumin-gold particles were trapped within the basal lamina of brain ECs after the injury (Vorbodt *et al.*, 1993). This observation together with the findings of this study, it can be postulated that the process of passing through the basal lamina may be a time-limiting step in protein leakage during the formation of brain oedema. Many pericytes were diffusely filled with HRP in this model. Lysosome-like dense bodies in pericytes contained HRP, indicating phagocytic activity of pericytes. In cold injury to the brain, HRP was also detected in pericytes (Cancilla *et al.*, 1972). Therefore, the results of this study support the view that pericytes are phagocytic cells in the brain and form a second line of defence at the BBB.

After passing through the BBB, HRP was detected in the brain parenchyma, between nerve cells and fibres. Many axons also contained peroxidase reaction product, confirming the neuronal staining with HRP observed at the LM level. As the enzyme HRP undergoes fluid phase endocytosis, the neuronal uptake depends on the presence of rather high extracellular concentration of the tracer in the vicinity of the neuron (Dumas *et al.*, 1979). Therefore, the neuronal uptake of HRP observed in this study indicates severe HRP leakage after the intravenous injection of anti-EBA antibody. Neuronal uptake of HRP has been described in cold injury to the brain (Tengvar, 1986). HRP was taken up by neuronal processes at the primary focal brain lesion and spread far away by axonal and dendritic transport mechanisms to remote areas which were neuroanatomically connected to the injured region. In this study, as the intravenous injection of anti-EBA induced a diffused brain oedema and the experimental animals were sacrificed shortly after the injection, it is more likely that HRP was directly taken up by neurons and neuronal processes from the interstitial space rather than transported by retro- or antero-grade axonal transport.

Serum albumin, with a molecular weight of 62 kDa, is a natural component of the blood. At the LM level, the leaking of serum albumin in brains of experimental animals was as severe as HRP leakage and the pattern of distribution of albumin leakage was similar to that of HRP. At the EM level, albumin was mainly seen diffusely infiltrating the cytoplasm of ECs. Only occasional vesicles in the ECs were seen to contain albumin. Because the leakage of albumin was rapid and intense after the injection of anti-EBA antibody and only a few vesicles were

observed to contain albumin, one can assume that activation of the transendothelial vesicular transport system was not responsible for the large amount of albumin leaking through the BBB observed. Instead, albumin was seen heavily filling the cytoplasm of ECs, indicating another pathway for albumin leakage, passage of albumin into and through the EC cytoplasm. Passage of macromolecules across the cell cytoplasm can occur in injured or degenerating segments of ECs. Such passage has been observed previously in osmotic opening of the BBB and perfusion of protamine sulphate (Nagy *et al.*, 1983; Vorbrodt *et al.*, 1993). Protamine sulphate perfusion induces BBB disruption, HRP infiltration through the cytoplasm of brain ECs. The action of protamine sulphate, a polycation solution, is due to the reduction in anionic sites on luminal surface of ECs. However, in the current study, diffuse staining of cytoplasm was noted in the albumin study, but not in the HRP study.

HRP leakage in the retina after the injection of anti-EBA antibody was also noted. Embryologically, the retina is an outgrowth of the brain (diencephalon). The blood-retinal barrier consists of retinal capillary ECs supported by retinal pigment epithelial cells. Retinal blood vessels have similar barrier properties as those of the CNS during development and in adulthood (Chan Ling & Stone, 1992). The expression of EBA in retinal blood vessels has been reported (Lawrenson *et al.*, 1995). In the current study, both paraffin sections and wholemount preparations of the retina showed EBA immunoreactivity in retinal blood vessels. The disruption of the BRB after the intravenous injection of anti-EBA antibody

suggests that as in the BBB, EBA plays an important role in maintaining the integrity of the BRB.

HRP leakage observed in the retina after the intravenous injection of anti-EBA antibody showed a different spread pattern from that in the brain. Unlike the widespread leakage of HRP in the brain, HRP leakage in the retina was multifocal with circumferential areas containing HRP. The most affected regions were the areas close to the optic disc. This finding may suggest that segments of retinal blood vessels close to the optic disc are more sensitive to the interference with EBA function or they are generally more vulnerable to injury.

In summary, the study of serum albumin and HRP leakage demonstrated that anti-EBA antibody injection induced a rapid and diffuse vasogenic brain oedema in the rat. The intravenous injection of anti-EBA antibody appears to interfere with the function of EBA at the BBB and leads to the opening of more than one route for macromolecule leakage across the BBB.

5.3.2 The intravenously injected anti-EBA antibody

In the current study experimental animals were injected intravenously with anti-EBA antibody. From these animals Vibratome sections, which were not exposed to the primary antibody in the staining procedure, showed immunoreactivity of brain vessels for EBA, indicating that the systemically injected anti-EBA antibody became localised to ECs, and acted as a substitute for the exposure of tissues to the primary antibody in the staining procedure. Presumably anti-EBA antibody

identified EBA on the EC membranes and formed antigen-antibody complexes. These complexes remained stable for the duration of the experiment, and tolerated 45 min of flushing of the circulation, initially with DPBS then the fixative, using fast and slow perfusion. However, the immunoreactivity for EBA seen in the Vibratome sections was moderate compared with the strong immunoreactivity seen in sections which were treated with anti-EBA antibody as primary antibody in the staining procedure. This may be explained by an insufficient concentration of the anti-EBA injected *in vivo* to saturate all EBA sites in the cerebral vascular bed. The higher staining intensity in the sections exposed to the primary antibody therefore may be due to the labelling of additional EBA sites during the staining procedure. Exposure of the sections to anti-EBA antibody overnight in the staining procedure provides enough time and antibody availability to label a greater number of EBA molecules in brain vessels.

The binding of intravenously injected anti-EBA antibody to the antigen *in situ* was also observed at the EM level. EBA reaction product was observed in blood vessels of brain tissues from experimental animals stained only with secondary antibody and visualised by DAB. The localisation of EBA reaction product in these ECs was similar to normal EBA staining. EBA reaction product was observed along the luminal membrane of ECs. Cytoplasmic staining was also present and some nuclei showed staining at their luminal side. The cytoplasmic staining of EBA observed in these ECs confirmed our earlier finding in the prototoxin study that EBA is not only located at the luminal membrane of ECs but also in the cytoplasm. The cytoplasmic staining of EBA in these ECs may also

indicate that the intravenously injected anti-EBA was internalised by endothelial cells. However, this will not explain the cytoplasmic labelling seen in control animals not injected with anti-EBA.

As stated above, Vibratome sections from experimental animals injected with anti-EBA *in vivo*, showed immunoreactivity for EBA, despite the omission of anti-EBA antibody as the primary antibody in the staining procedure. Unexpectedly, paraffin sections obtained from the other half of the brains from the same animal and subjected to the same staining protocol, did not show any immunoreactivity for EBA. The difference between the results of Vibratome sections and paraffin sections must be related to the method of processing. After the perfusion, brains were removed and immersed in the same fixative used in the perfusion for 3 hours and then transferred to PBS. Vibratome sections were directly cut from these brain tissues without any further processing. Paraffin sections were cut from brains embedded in paraffin, which underwent dehydration and paraffin wax infiltration (at 58°C). As the staining protocols for Vibratome sections and paraffin sections were identical, the lack of immunoreactivity seen in paraffin sections must be due to the heat employed in paraffin embedding. This suggests that the antigen-antibody complexes which were formed *in vivo* were unstable at the temperature (58°C) required for paraffin embedding. Another possibility is that the antibody itself was denatured or altered by the temperature employed.

5.3.3 The significance of control antibodies

The choice of control antibodies in this study was an important consideration. Firstly, anti-human cytokeratin, anti-Salmonella bacterial antigen (SAL-3) and anti-rat EC-specific antigen (anti-RECA-1) are similar to anti-EBA antibody, in that all four are monoclonal mouse antibodies. They are supplied as ascites fluid or tissue culture supernatant. All are IgM molecules, except anti-RECA-1, which is an IgG₁. However, in contrast to anti-EBA, the anti-human cytokeratin and SAL-3 were used to test the effects of circulating foreign antibodies on the BBB. Anti-RECA-1 is a pan-EC marker, and reacts with endothelium in all rat tissues, including the brain (Duijvestijn *et al.*, 1992). However anti-RECA-1 is not a BBB-specific antibody, and was used here to prove that any effect on BBB produced by the injection of anti-EBA antibody is not due to a non-specific effect of antibodies to ECs at large. Experimental animals injected with anti-EBA antibody showed extensive leakage of HRP and albumin. None of the control antibodies or vehicle showed any tracer leakage. Therefore, the results prove that the leakage was neither an allergic response to exposure of the immune system to foreign proteins, nor to the injection of antibodies to ECs in general, but is likely to be due to a specific effect of anti-EBA on the BBB.

The usage of anti-RECA-1 antibody is of great significance in this study. Anti-RECA-1 is strictly specific for rat ECs. It recognises an EC surface molecule as detected by immunofluorescence staining *in vitro* (Duijvestijn *et al.*, 1992). When intravenously injected in rats, anti-RECA-1 antibody was shown to stain all vessel types of the vasculature in the tissues, using a procedure similar to that used in

this study, that is, to stain the sections without adding the primary antibody in the staining procedure (Duijvestijn *et al.*, 1992). Therefore, the intravenous injection of anti-RECA-1 antibody in this study should lead to the formation of RECA-1 antigen-antibody complexes on the ECs. However, no HRP or albumin leakage was observed after the injection of anti-RECA-1 antibody at both LM and EM level. In contrast, anti-EBA antibody injection induced extensive tracer leakage in the brain. This suggests that opening of the BBB in experimental animals was not due to a non-specific cell membrane perturbation as a result of the formation of antigen-antibody complexes or activation of complement system, but is likely to be a specific effect of anti-EBA on the BBB.

The mechanism underlying the disruption of the BBB by intravenous injection of anti-EBA antibody is not known. One can assume that the binding of intravenously injected anti-EBA antibody to the antigen on the ECs disturbs the normal function of EBA and leads to the structural and functional changes of ECs. It has been suggested that EBA is associated with a receptor complex (Sternberger & Sternberger, 1987). The interference with EBA function may disturb cell signalling system or transportation system in ECs. Further studies are needed to clarify the biochemical structure of EBA and its exact function at the BBB.

5.3.4 Conclusion

In vivo administration of antibody to EBA (anti-EBA) resulted in an increased permeability of brain and retinal microvessels to endogenous and exogenous tracers, indicating that interference with EBA function on the BBB and BRB leads

to the opening of both barriers. The intravenously injected anti-EBA became bound to brain and retinal ECs and was detected in Vibratome sections and wholemount retinas by immunocytochemistry after tissue fixation. Intravenous injection of PBS and control antibodies did not alter the integrity of the BBB and BRB, indicating that the effect of intravenous injection of anti-EBA on the BBB and BRB is not due to non-specific antigen-antibody binding on ECs or non-specific allergic reaction to foreign antibodies. The electron microscopic tracer study revealed that intravenous injection of anti-EBA leads to the opening of more than one route for macromolecules to move across the BBB. This study provides direct evidence for the importance of EBA for BBB and BRB integrity in the rat. The mechanisms by which the anti-EBA injected *in vivo* 'neutralised' EBA molecules on ECs, or interfered with the function of EBA, require further investigation.

CHAPTER 6
GENERAL DISCUSSION
AND CONCLUSION

6 General discussion and conclusion

Since the introduction of the concept of the BBB based on the classic dye injection experiment more than 100 years ago, various aspects of the BBB have been extensively studied. We now understand that the BBB consists of a unique endothelium. The cerebral endothelium is non-fenestrated and has a low density of vesicles. Tight junctions seal the interendothelial space. The BBB effectively prevents free exchange between the blood and the brain and is essential for normal neural function. However, despite the enormous amount of knowledge accumulated during decades of research, the BBB is not fully understood. The current study was designed to investigate some aspects of the BBB. Firstly, the integrity of the BBB was studied. Disruption of the BBB leads to the development of vasogenic brain oedema, a common complication of many neurological diseases. The formation of vasogenic oedema can cause further damage to the brain and worsen the outcome of diseases. The mechanism of the development of brain oedema is still unclear. In this study, the ultrastructure of brain ECs and the permeability of the BBB to tracers were examined. Secondly, two BBB markers were studied. Recent studies on the BBB revealed a number of BBB specific proteins, conventionally known as BBB markers. Among these is EBA, a protein selectively expressed by rat brain ECs of barrier vessels. The function of EBA is still unknown. GLUT1, the second marker investigated, is the protein responsible for transporting glucose from the blood into the brain. It is predominantly expressed by ECs of barrier vessels. The changes of GLUT1 in brain injury conditions, especially brain trauma, are largely unknown. In the

current study, the expression of EBA and GLUT1 was investigated at the LM and EM levels.

The current study investigated the BBB in normal and pathological conditions induced by intravascular and extravascular insults. The intravascular insult to the brain was induced by the administration of *Clostridium perfringens* (*Cl p*) type D epsilon prototoxin. *Clostridium perfringens* can cause outbreaks of fatal toxæmia in livestock (McDonel, 1986). It occasionally causes food poisoning and gas gangrene in humans (Rood, 1998; Tallis *et al.*, 1999). *Clostridium perfringens* is also an opportunistic infectious bacteria for patients with AIDS (Caumes *et al.*, 1998). Epsilon toxin produced by *Cl p* type B and D is a potent clostridial toxin. Epsilon prototoxin is an inactive form of the toxin. In the current study, after the injection of the epsilon prototoxin, a rapid, mild and reversible BBB opening occurred. Thus the current study showed for the first time a toxic effect of the epsilon prototoxin on the BBB. Immunocytochemistry for EBA showed that EBA immunoreactivity was significantly reduced in the brain following the injection, and the reduction correlated well with the disruption of the BBB. On the other hand, GLUT1 immunoreactivity remained unchanged after the administration of the prototoxin.

Extravascular insult to the brain was induced by traumatic brain injury. Traumatic brain injury is a common cause of death and disability in young adults in the Western world. In Australia, a report released by the Australian Institute of Health and Welfare in 1999 showed that in 1996 to 1997, 27,437 hospital cases

had a diagnosis of traumatic brain injury (Fortune & Wen, 1999). Among them, young people aged from 15 to 19 were most vulnerable, with the highest rate of 284 per 100,000 population in all age groups. Among the various types of traumatic brain injury, the non-missile head injury, leading to diffuse axonal injury, is the major cause of death and vegetative state in closed head injury patients (Adams *et al.*, 1989; Adams *et al.*, 1991; Gentleman *et al.*, 1995). In the current study, a closed head injury model which induces diffuse axonal injury in rats was employed. The breakdown of the BBB was observed shortly after the injury. The leakage of serum albumin and ionic lanthanum indicated the development of vasogenic brain oedema. Cytotoxic oedema became more obvious later in the time course of the injury, indicated by a severe astrocytic swelling. The reduction of EBA immunoreactivity in the brain was observed. The reduction correlated well with the disruption of the BBB. GLUT1 expression was also down-regulated following the trauma.

The function of EBA at the BBB was further studied using an *in vivo* immunological targeting approach. Intravenous injection of anti-EBA antibody into the rat rapidly opened the BBB and led to a widespread leakage of serum albumin and HRP. The intravenously injected anti-EBA antibody was shown by immunocytochemistry to bind to the antigen on brain ECs. It appears that EBA plays an important role in maintaining the integrity of the BBB.

6.1 The integrity of the BBB

In agreement with previous studies, the present study showed that the BBB in normal conditions is capable of excluding macromolecular tracers such as serum albumin and HRP, and small molecular tracers such as ionic lanthanum. The ionic lanthanum study showed that tight junctions effectively stopped the passage of the tracer. The ultrastructural study revealed that cerebral endothelium is non-fenestrated and has low density of vesicles.

In the current study following injury to the BBB, regardless of the type of insult, increased indentation of the luminal EC membrane and increased vesicles in the cytoplasm of ECs were observed. With further development of the pathological conditions, large vacuoles appeared in ECs. It seems that ultrastructural changes in the luminal membrane of ECs, activation of the vesicular system and the formation of large vacuoles are common responses of brain ECs to injuries. However, the *Cl p* epsilon prototoxin model showed more severe EC injury. At the time intervals which showed the most severe features of BBB disruption, some ECs were denuded and short stretches of the blood vessel wall were formed only by the basal lamina. Subendothelial clefts were observed. Some ECs showed degeneration with depletion of organelles in the cytoplasm. The alteration of tight junctions, on the other hand, was more commonly observed in the traumatic brain injury model.

Accompanying the above ultrastructure changes, leakage of tracers was observed in the two models. Although some common ultrastructure changes were observed

in these two models, EM studies showed that the pathways for tracer leakage were not identical. In the *Cl p* epsilon prototoxin model, the leakage of tracers was mainly through the activated vesicular system and direct diffusion through the damaged endothelium. The rapid leakage of serum albumin following the prototoxin injection may not have been caused mainly by tight junction alteration as only few tight junctions showed focal modification. In the traumatic head injury model, tight junction modification was commonly seen in cerebral blood vessels. Ionic lanthanum heavily infiltrated tight junctions. Tracers were also seen infiltrating the cytoplasm of ECs. However, the involvement of vesicles in transporting the tracers was not evident by EM. Therefore, altered tight junctions and injured ECs were mainly responsible for the leakage of tracers.

In summary, the breakdown of the BBB and the formation of vasogenic brain oedema were observed in two brain injury animal models. However, the mechanism of the development of vasogenic oedema differed in the two models and the nature of the insults to the brain may account for the difference.

6.2 EBA expression and its function

The present study has demonstrated that in normal conditions, EBA is strongly expressed by brain ECs of barrier vessels. EBA is only weakly expressed by few vessels in the choroid plexus and pineal gland, areas known to lack barrier function. These findings are consistent with previous studies. The EM study of EBA expression showed that EBA is localised not only to the luminal EC membranes but also to cytoplasm, which has not been reported previously. The

significance of cytoplasmic localisation of EBA is not clear. It may provide a cytoplasmic pool for protein recruitment.

Previous studies have shown that EBA expression is reduced in some pathological conditions such as EAE, stab-wound injury to the brain, spinal cord trauma and cerebral infarction (Sternberger *et al.*, 1989; Rosenstein *et al.*, 1992; Perdiki *et al.*, 1998; Lin & Ginsberg, 2000; Nishigaya *et al.*, 2000). The reduction of EBA immunoreactivity occurs during the acute phases of the above diseases but recovers in later phases. The localisation of EBA in the brain and its correlation with an intact cerebral endothelium strongly suggest that EBA may uphold the integrity of the BBB. In the current study, EBA reduction was observed qualitatively and quantitatively following *Cl p* type D epsilon prototoxin-induced toxemia and traumatic brain injury at both the LM and EM levels. The reduction of EBA immunoreactivity appeared to correlate with the disruption of the BBB. Although full recovery of EBA immunoreactivity was not observed at the maximum time intervals examined in both models, EBA expression did show signs of recovery along with the clearance of vasogenic oedema and a clear trend towards a full recovery was apparent. It appears that the loss or reduction of EBA immunoreactivity is a sensitive indicator for brain vascular injury.

The correlation between EBA immunoreactivity and an intact cerebral endothelium is well demonstrated by the study of the above two brain injury models. However, whether the reduction of EBA expression observed in the current investigation and previous studies was a cause or consequence of brain

oedema was not known. Therefore, the function of EBA was further investigated by an *in vivo* immunological targeting model. Anti-EBA antibody was intravenously injected into the rat to determine the fate of the injected antibody and its effect on the BBB. Immunocytochemistry showed the intravenously injected anti-EBA became bound to brain ECs. Five to twelve minutes after the injection, widespread leakage of serum albumin and HRP was detected, indicating rapid formation of vasogenic oedema. Intravenous injection of control antibodies (EC non-specific antibodies and a rat EC specific antibody) did not induce disruption of the BBB. The results of the injections of control antibodies ruled out the possibility that the disruption of the BBB observed in experimental animals injected with anti-EBA antibody was non-specific. A non-specific effect could have arisen from the formation of antibody-antigen complexes on EC membrane or an allergic reaction to a foreign antibody. Furthermore, the binding of the intravenously injected anti-EBA to the antigen on ECs was demonstrated by immunocytochemistry. It appears that disturbance of EBA function in brain ECs leads to the opening of the BBB. Therefore, it is reasonable to postulate that the reduction in EBA in previous studies and the current study preceded the occurrence of vasogenic oedema.

In conclusion, the present study showed that EBA expression is a very sensitive indicator of normal brain vascular integrity. EBA seems to be responsible for maintaining the integrity of the BBB. Since a reduction in the expression of EBA was detected in both intravascular and extravascular injury models, it is postulated that in the rat loss of EBA may be the final common pathway in the series of

events of brain vascular injury that lead to the opening of the BBB. Further studies are needed to explore the molecular aspects of EBA and the mechanism of EBA reduction during brain injury.

6.3 GLUT1 expression

GLUT1 is also a BBB marker. It is predominantly expressed by brain ECs of barrier vessels. The function of GLUT1 is to transport glucose from the blood to the brain. Therefore, GLUT1 is closely related to cerebral glucose metabolism. This study showed that in normal conditions, GLUT1 is strongly expressed by blood vessels throughout the brain. In the choroid plexus and pineal gland, only few vessels show weak staining for GLUT1. The epithelium of the choroid plexus, however, expresses GLUT1.

A dissociation between GLUT1 expression and a compromised BBB integrity was demonstrated in the study of *Cl p* type D epsilon prototoxin model. While the breakdown of the BBB was evident, GLUT1 immunoreactivity remained unchanged. Ultrastructurally the prototoxin injection appeared to cause injury to brain ECs but such injury did not significantly affect the expression of GLUT1 in ECs. Therefore, it appears that GLUT1, unlike EBA, is not a vascular injury indicator.

In the current study of traumatic brain injury, a reduction of GLUT1 immunoreactivity was observed. The EM study showed that the reduction of GLUT1 expression was mainly manifested as sectoral loss of GLUT1

immunoreactivity in cerebral blood vessels. The pattern of GLUT1 expression in brain ECs observed in the current investigation is consistent with the bimodal expression of GLUT1 suggested by Cornford *et al.* in the study of human traumatic brain injury and epilepsy (Cornford *et al.*, 1996; Cornford *et al.*, 1998b). The down-regulation of GLUT1 immunoreactivity observed in the traumatic brain injury model suggests that transportation of glucose by brain ECs is reduced. Previous studies have shown that extracellular glucose level in the brain is reduced after traumatic brain injury. Therefore, it can be postulated that cerebral glucose supply is disrupted following brain trauma. As it is well known that glucose is the primary fuel for neurons, the shortage of glucose can cause further damage to the brain. An *in vitro* study showed that increased extracellular glucose level was beneficial for neural function after cerebral hypoxia (Schurr *et al.*, 1987).

In summary, GLUT1 is strongly expressed by brain ECs of barrier blood vessels in normal conditions. GLUT1 expression does not appear to be directly related to the integrity of the BBB. However, GLUT1 is down-regulated in traumatic brain injury model. The mechanism of the reduction of GLUT1 expression is not clear. The down-regulation of GLUT1 may be caused by changes in metabolism following the trauma.

6.4 General conclusion

In the normal state, the BBB is capable of excluding both large and small molecular tracers. EBA and GLUT1 are strongly expressed at the BBB.

Following intravascular and extravascular injuries, the BBB is disrupted and the permeability of tracers is increased. A reduction of EBA immunoreactivity occurs in the two brain injury models employed. The reduction correlates well with the disruption of the BBB, indicating that EBA expression is sensitive to brain vascular injury. *In vivo* immunological targeting of EBA leads to rapid opening of the BBB, suggesting that the disturbance of EBA function affects the integrity of the BBB. GLUT1 expression, on the other hand, is more related to the cerebral metabolism. The intravascular injury to the brain did not affect GLUT1 immunoreactivity suggesting that GLUT1 is not an indicator of vascular injury and the injection of prototoxin does not significantly affect brain glucose metabolism. Following traumatic brain injury, however, GLUT1 is down-regulated. The down-regulation of GLUT1 immunoreactivity may decrease the glucose supply to the brain and induce further damage to the brain.

6.5 Limitations and future studies

The current study investigated the BBB in normal brains and in brains after intravascular and extravascular injuries. Two BBB markers, EBA and GLUT1 were studied qualitatively and quantitatively. Quantitative changes of EBA expression were observed in the two animal models. Quantitative studies showed a trend for the reduction of EBA immunoreactivity at the BBB after both injuries. Both qualitative and quantitative studies revealed a trend for the reduction of GLUT1 immunoreactivity in the brain after traumatic brain injury. However, statistical analysis was not possible due to the small sample size. Therefore, in future studies, more animals should be included in each group to allow statistical

analysis for the changes in EBA and GLUT1. In the *Cl p* epsilon prototoxin induced BBB opening model, EBA immunoreactivity was still reduced at 14 days after the injection. In the traumatic brain injury model, both EBA and GLUT1 immunoreactivity was still reduced at 12h after the injury compared to the controls. Longer time intervals are needed to observe the full recovery of both markers.

The results of this study suggest that EBA is a sensitive indicator of BBB integrity. Disturbance of EBA function will induce rapid disruption of the BBB. However, little is known about the molecular biology of EBA. To understand the relationship between EBA expression and BBB integrity, future studies should be focused on the study of molecular aspects of EBA. The understanding of EBA molecular structure will enable us to study the changes of EBA in pathological conditions at the molecular level. The results of the current study and previous studies have shown a reduction of EBA immunoreactivity in several pathological conditions. It is not clear the reduction of EBA immunoreactivity is caused by increased degradation of EBA protein or decreased gene expression for EBA. More studies are needed to clarify the mechanism of the changes of EBA expression at the molecular level.

Glucose is the essential energy supply for the brain. The activity of GLUT1 is closely related to glucose metabolism. The findings of the current study showed that GLUT1 was down-regulated in the closed head injury model. The reduced immunoreactivity of GLUT1 suggests possible changes of glucose supply to the

brain. Future studies should address the brain glucose metabolism in the closed head injury in relation to the changes of GLUT1 expression. It will be also important to study the status of GLUT3 expression in neurons in this brain injury model to investigate the status of glucose transportation in neurons.

APPENDIX

Appendix

Appendix A.1. Raw data of quantitative assessment of EBA immunoreactivity at the EM level in *Clostridium perfringens* type D epsilon prototoxin-induced BBB opening, 1:400 dilution at time intervals 1h-14d. Percentages of immunostained luminal membranes are shown for consecutive vessels.

Time interval	1h	6h	12h	24h	2d	4d	7d	14d	Normal control
Animal No	V43	V44	V45	V46	V47	V48	V49	V50	V53
Serial vessel No									
1	64.72	69.01	13.28	96.75	95.58	100	100	100	100
2	25.44	36.53	100	0	100	100	68.73	100	100
3	41.98	0	64.38	100	100	68.36	39.14	82.67	100
4	26.26	68.77	35.73	0	100	65.24	69.84	0	0
5	0	24.51	0	26.94	80.68	31.49	0	100	100
6	98.12	100	100	100	100	0	0	0	100
7	100	100	84.59	100	100	65.49	0	27.41	100
8	100	89.04	0	100	85.41	76.21	100	100	92.64
9	100	37.65	100	0	100	100	0	100	100
10	0	0	100	74.32	100	0	85.28	86.92	100
11	84.03	0	0	100	100	58.34	100	63.85	100
12	81.68	0	7.2	0	0	100	0	0	60.83
13	0	0	24.88	100	100	100	86.82	0	100
14	100	0	100	94.23	100	0	0	60.2	100
15	81.6	0	100	100	100	100	0	0	100
16	100	0	0	100	83.72	0	0	0	100
17	0	33.54		100	100	0	0	0	63.12
18	25.03	96.82		0	0	0	37.74	70.86	100
19	88.91	100		0	100	92.37	100	45.77	100
20	0	0		100	100		79.53		100
21	97.29	0		34.91	0		0		
22	0			0	40.09		100		
23				0	0		100		
24				88.4	32.83				
25				23.27	67.68				
26					0				
27					35.4				
28					0				
29					0				
Mean	55.23	35.99	51.88	57.55	66.25	55.66	46.39	49.35	90.83

Appendix A.2. Raw data of quantitative assessment of EBA immunoreactivity at the EM level in CI p type D epsilon prototoxin-induced BBB opening, 1:500 dilution at time intervals 6h-7d. Percentages of immunostained luminal membranes are shown for consecutive vessels.

Time interval	6h	12h	18h	2d	7d	Normal control
Animal No	V10	V12	V15	V17	V18	V11
Serial vessel No						
1	0	100	100	100	100	100
2	100	100	100	100	100	100
3	100	100	100	100	0	100
4	91	100	100	100	60	100
5	100	100	100	100	0	100
6	100	100	100	100	100	100
7	0	100	100	100	0	100
8	100	100	100	100	0	100
9	26	44	92	100	97	100
10	100	74	100	100	100	88
11	100	30	60	100	100	100
12	100	100	100	100	100	100
13	100	100	16	100	100	100
14	100	100	100	100	100	100
15	100	100	0	100	0	100
16	100	100	95	100	100	100
17	0	88	0	100	100	100
18	28	100	0	100	100	100
19	100	100	13	100	0	100
20	100	100	72	100	46	55
21	100	100	100	100	77	100
22	100	100	33	100	100	100
23	100	100	100	100	100	100
24	100	100	73	100	100	100
25		100	31.6	100	8	100
26		83	0	95	0	100
27			100	100	0	100
28			19	75	63	100
29				100	0	48
30				100		
Mean	81.04	93.04	68.02	99	60.38	96.24

Appendix A.3 Raw data of quantitative analysis of GLUT1 immunoreactivity in Cl p epsilon prototoxin-induced BBB opening

Table A.3.1 Raw data of quantitative assessment of GLUT1 expression in the frontal lobe in the 1:500 dilution of prototoxin injection group

Animal Number	Area (mm ²)	Vessel number (Count 1)	n/mm ² (Count 1)	Vessel number (Count 2)	n/mm ² (Count 2)	Mean n/mm ²
V16(1h)	0.20	81	405.00	78	390.00	397.50
V10(6h)	0.19	66	347.37	67	352.63	350.00
V12(9h)	0.22	76	345.45	76	345.45	345.45
V13(12h)	0.21	89	423.81	84	400.00	411.90
V15(18h)	0.20	75	375.00	75	375.00	375.00
V14(24h)	0.19	63	331.58	62	326.32	328.95
V17(2d)	0.22	69	313.64	71	322.73	318.18
V9(4d)	0.22	66	300.00	69	313.64	306.82
V18(7d)	0.22	58	263.64	56	254.55	259.09
V11(control)	0.17	51	300.00	50	294.12	297.06

n/mm², the number of GLUT1 immunoreactive blood vessels per mm²

Table A.3.2 Raw data of quantitative assessment of GLUT1 expression in the cerebellum in the 1:500 dilution of prototoxin injection group

Animal Number	Area (mm ²)	Vessel number (Count 1)	n/mm ² (Count 1)	Vessel number (Count 2)	n/mm ² (Count 2)	Mean n/mm ²
V16(1h)	0.20	90	450.00	94	470.00	460.00
V10(6h)	0.21	83	395.24	83	395.24	395.24
V12(9h)	0.22	88	400.00	84	381.82	390.91
V13(12h)	0.20	50	250.00	51	255.00	252.50
V15(18h)	0.21	75	357.14	76	361.90	359.52
V14(24h)	0.20	96	480.00	96	480.00	480.00
V17(2d)	0.22	92	418.18	91	413.64	415.91
V9(4d)	0.22	73	331.82	71	322.73	327.27
V18(7d)	0.22	59	268.18	61	277.27	272.73
V11(Normal)	0.20	63	315.00	68	340.00	327.50

n/mm², the number of GLUT1 immunoreactive blood vessels per mm²

Table A.3.3 Raw data of quantitative assessment of GLUT1 expression in the inferior colliculus in the 1:500 dilution of prototoxin injection group

Animal Number	Area (mm ²)	Vessel number (Count 1)	n/mm ² (Count 1)	Vessel number (Count 2)	n/mm ² (Count 2)	Mean n/mm ²
V16(1h)	0.21	96	455.11	101	478.81	469.3333
V10(6h)	0.21	112	530.96	108	512.00	521.4815
V12(9h)	0.21	75	355.56	78	369.78	362.6667
V13(12h)	0.21	146	692.15	146	692.15	692.1481
V15(18h)	0.21	135	640.00	129	611.56	625.7778
V14(24h)	0.21	67	317.63	66	312.89	315.2593
V17(2d)	0.21	112	530.96	111	526.22	528.5926
V9(4d)	0.21	128	606.81	127	602.07	604.4444
V18(7d)	0.21	118	559.41	113	535.70	547.5556
V11(control)	0.21	103	488.30	108	512.00	500.1481

n/mm², the number of GLUT1 immunoreactive blood vessels per mm²

Table A.3.4 Raw data of quantitative assessment of GLUT1 expression in the brainstem in the 1:500 dilution of prototoxin injection group

Animal Number	Area (mm ²)	Vessel number (Count 1)	n/mm ² (Count 1)	Vessel number (Count 2)	n/mm ² (Count 2)	Mean n/mm ²
V16(1h)	0.21	133	633.33	134	638.10	635.71
V10(6h)	0.19	80	421.05	84	442.11	431.58
V12(9h)	0.18	87	483.33	84	466.67	475.00
V13(12h)	0.19	57	300.00	58	305.26	302.63
V15(18h)	0.21	138	657.14	140	666.67	661.90
V14(24h)	0.22	37	168.18	38	172.73	170.45
V17(2d)	0.22	109	495.45	109	495.45	495.45
V9(4d)	0.22	88	400.00	96	436.36	418.18
V18(7d)	0.22	40	181.82	46	209.09	195.45
V11(Normal)	0.19	83	436.84	80	421.05	428.95

n/mm², the number of GLUT1 immunoreactive blood vessels per mm²

Table A.3.5 Raw data of quantitative assessment of GLUT1 expression in the frontal lobe in the 1:400 dilution of prototoxin injection group

Animal Number	Area (mm ²)	Vessel number (Count 1)	n/mm ² (Count 1)	Vessel number (Count 2)	n/mm ² (Count 2)	Mean n/mm ²
V43(1h)	0.21	85	402.96	82	388.74	395.85
V44(6h)	0.21	73	346.07	71	336.59	341.33
V45(12h)	0.21	96	455.11	98	464.59	459.85
V46((24h)	0.21	81	384.00	74	350.81	367.41
V47(2d)	0.21	67	317.63	66	312.89	315.26
V48(4d)	0.21	101	478.81	103	488.30	483.56
V49(7d)	0.21	83	393.48	84	398.22	395.85
V50(14d)	0.21	61	289.19	60	284.44	286.81
V54(Normal)	0.21	72	341.33	70	331.85	336.59

n/mm², the number of GLUT1 immunoreactive blood vessels per mm²

Table A.3.6 Raw data of quantitative assessment of GLUT1 expression in the cerebellum in the 1:400 dilution of prototoxin injection group

Animal Number	Area (mm ²)	Vessel number (Count 1)	n/mm ² (Count 1)	Vessel number (Count 2)	n/mm ² (Count 2)	Mean n/mm ²
V43(1h)	0.21	106	504.76	104	495.24	500.00
V44(6h)	0.21	101	478.81	95	450.37	464.59
V45(12h)	0.21	72	342.86	72	342.86	342.86
V46((24h)	0.21	80	379.26	81	384.00	381.63
V47(2d)	0.21	86	407.70	86	407.70	407.70
V48(4d)	0.21	72	341.33	72	341.33	341.33
V49(7d)	0.21	105	497.78	107	507.26	502.52
V50(14d)	0.21	95	450.37	92	436.15	443.26
V54(Normal)	0.20	62	310.00	66	330.00	320.00

Table A.3.7 Raw data of quantitative assessment of GLUT1 expression in the 1:400 dilution of prototoxin injection group in the inferior colliculus

Animal Number	Area (mm ²)	Vessel number (Count 1)	n/mm ² (Count 1)	Vessel number (Count 2)	n/mm ² (Count 2)	Mean n/mm ²
V43(1h)	0.21	107	507.26	109	516.74	512.00
V44(6h)	0.21	83	393.48	85	402.96	398.22
V45(12h)	0.21	117	554.67	116	549.93	552.30
V46((24h)	0.21	118	559.41	119	564.15	561.78
V47(2d)	0.21	96	455.11	95	450.37	452.74
V48(4d)	0.21	111	526.22	114	540.44	533.33
V49(7d)	0.21	169	801.19	174	824.89	813.04
V50(14d)	0.21	107	507.26	108	512.00	509.63
V54(Normal)	0.21	79	374.52	81	384.00	379.26

Table A.3.8 Raw data of quantitative assessment of GLUT1 expression in the brainstem in the 1:400 dilution of prototoxin injection group

Animal Number	Area (mm ²)	Vessel number (Count 1)	n/mm ² (Count 1)	Vessel number (Count 2)	n/mm ² (Count 2)	Mean n/mm ²
V43(1h)	0.21	89	421.93	84	398.22	410.07
V44(6h)	0.21	73	346.07	71	336.59	343.70
V45(12h)	0.21	59	279.70	60	284.44	282.07
V46((24h)	0.21	72	341.33	70	331.85	336.59
V47(2d)	0.21	67	317.63	67	317.63	317.63
V48(4d)	0.21	82	388.74	87	412.44	400.59
V49(7d)	0.21	75	355.56	81	384.00	369.78
V50(14d)	0.21	71	336.59	69	327.11	331.85
V54(Normal)	0.21	60	284.44	60	284.44	284.44

Table A.3.9 Raw data of quantitative assessment of GLUT1 expression in the normal controls in the frontal lobe

Animal Number	Area (mm ²)	Vessel number (Count 1)	n/mm ² (Count 1)	Vessel number (Count 2)	n/mm ² (Count 2)	Mean n/mm ²
V11	0.17	51	300.00	50	294.12	297.06
V51	0.21	81	384.00	80	379.26	381.63
V52	0.21	68	322.37	67	317.63	320.00
V53	0.14	55	381.94	54	375.00	378.47
V54	0.21	72	341.33	70	331.85	336.59

Appendix A.4. Raw data of quantitative assessment of EBA immunoreactivity in traumatic brain injury model at the LM level

Table A.4.1 Raw data of quantitative assessment of EBA expression at the LM level in TBI, 1h (animal B3)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	49	0.145641	336.4437
F-2	35	0.145641	240.3169
F-3	45	0.145641	308.9789
F-4	67	0.145641	460.0353
F-5	56	0.145641	384.5071
Brainstem			
BS-1	50	0.145641	343.3099
BS-2	48	0.145641	329.5775
BS-3	51	0.145641	350.1761
BS-4	53	0.145641	363.9085
BS-5	42	0.145641	288.3803

F, frontal cortex

BS, brainstem

n/mm², the number of EBA-immunoreactive blood vessels per mm²

Table A.4.2 Raw data of quantitative assessment of EBA expression at the LM level in TBI, 1h (animal B4)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	38	0.145641	260.9155
F-2	35	0.145641	240.3169
F-3	41	0.145641	281.5141
F-4	43	0.145641	295.2465
F-5	39	0.145641	267.7817
Brainstem			
BS-1	55	0.145641	377.6409
BS-2	53	0.145641	363.9085
BS-3	44	0.145641	302.1127
BS-4	37	0.145641	254.0493
BS-5	39	0.145641	267.7817

F, frontal cortex

BS, brainstem

n/mm², the number of EBA-immunoreactive blood vessels per mm²

Table A.4.3 Raw data of quantitative assessment of EBA expression at the LM level in 1h sham control (animal B6)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	48	0.145641	329.5775
F-2	40	0.145641	274.6479
F-3	32	0.145641	219.7183
F-4	35	0.145641	240.3169
F-5	50	0.145641	343.3099
Brainstem			
BS-1	55	0.145641	377.6409
BS-2	54	0.145641	370.7747
BS-3	42	0.145641	288.3803
BS-4	37	0.145641	254.0493
BS-5	38	0.145641	260.9155

F, frontal cortex

BS, brainstem

n/mm², the number of EBA-immunoreactive blood vessels per mm²

Table A.4.4 Raw data of quantitative assessment of EBA expression at the LM level in TBI, 6h (animal B16)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	29	0.145641	199.1198
F-2	32	0.145641	219.7183
F-3	31	0.145641	212.8522
F-4	24	0.145641	164.7888
F-5	17	0.145641	116.7254
Brainstem			
BS-1	33	0.145641	226.5845
BS-2	49	0.145641	336.4437
BS-3	46	0.145641	315.8451
BS-4	34	0.145641	233.4507
BS-5	37	0.145641	254.0493

F, frontal cortex

BS, brainstem

n/mm², the number of EBA-immunoreactive blood vessels per mm²

Table A.4.5 Raw data of quantitative assessment of EBA expression at the LM level in TBI, 6h (animal B17)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	46	0.145641	315.8451
F-2	42	0.145641	288.3803
F-3	34	0.145641	233.4507
F-4	38	0.145641	260.9155
F-5	36	0.145641	247.1831
Brainstem			
BS-1	58	0.145641	398.2395
BS-2	70	0.145641	480.6339
BS-3	56	0.145641	384.5071
BS-4	80	0.145641	549.2959
BS-5	76	0.145641	521.8311

F, frontal cortex

BS, brainstem

n/mm², the number of EBA-immunoreactive blood vessels per mm²

Table A.4.7 Raw data of quantitative assessment of EBA expression at the LM level in TBI, 12h (animal B32)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	36	0.145641	247.1831
F-2	39	0.145641	267.7817
F-3	37	0.145641	254.0493
F-4	48	0.145641	329.5775
F-5	33	0.145641	226.5845
Brainstem			
BS-1	42	0.145641	288.3803
BS-2	56	0.145641	384.5071
BS-3	35	0.145641	240.3169
BS-4	45	0.145641	308.9789
BS-5	27	0.145641	185.3874

F, frontal cortex

BS, brainstem

n/mm², the number of EBA-immunoreactive blood vessels per mm²

Table A.4.6 Raw data of quantitative assessment of EBA expression at the LM level in 6h sham control (animal B18)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	57	0.145641	391.3733
F-2	66	0.145641	453.1691
F-3	61	0.145641	418.8381
F-4	62	0.145641	425.7043
F-5	60	0.145641	411.9719
Brainstem			
BS-1	80	0.145641	549.2959
BS-2	88	0.145641	604.2255
BS-3	50	0.145641	343.3099
BS-4	62	0.145641	425.7043
BS-5	75	0.145641	514.9649

F, frontal cortex

BS, brainstem

n/mm², the number of EBA-immunoreactive blood vessels per mm²

Table A.4.8 Raw data of quantitative assessment of EBA expression at the LM level in TBI, 12h (animal B48)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	48	0.145641	329.5775
F-2	38	0.145641	260.9155
F-3	39	0.145641	267.7817
F-4	40	0.145641	274.6479
F-5	35	0.145641	240.3169
Brainstem			
BS-1	55	0.145641	377.6409
BS-2	58	0.145641	398.2395
BS-3	59	0.145641	405.1057
BS-4	51	0.145641	350.1761
BS-5	41	0.145641	281.5141

F, frontal cortex

BS, brainstem

n/mm², the number of EBA-immunoreactive blood vessels per mm²

Table A.4.9 Raw data of quantitative assessment of EBA expression at the LM level in 12h sham control (animal B49)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	49	0.145641	336.4437
F-2	59	0.145641	405.1057
F-3	53	0.145641	363.9085
F-4	58	0.145641	398.2395
F-5	61	0.145641	418.8381
Brainstem			
BS-1	73	0.145641	501.2325
BS-2	74	0.145641	508.0987
BS-3	64	0.145641	439.4367
BS-4	38	0.145641	260.9155
BS-5	80	0.145641	549.2959

F, frontal cortex

BS, brainstem

n/mm², the number of EBA-immunoreactive blood vessels per mm²

Table A.4.10 Raw data of quantitative assessment of EBA expression at the LM level in normal control (animal B19)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	58	0.145641	398.2395
F-2	57	0.145641	391.3733
F-3	50	0.145641	343.3099
F-4	73	0.145641	501.2325
F-5	58	0.145641	398.2395
Brainstem			
BS-1	71	0.145641	487.5001
BS-2	70	0.145641	480.6339
BS-3	74	0.145641	508.0987
BS-4	58	0.145641	398.2395
BS-5	65	0.145641	446.3029

F, frontal cortex

BS, brainstem

n/mm², the number of EBA-immunoreactive blood vessels per mm²

Table A.4.11 Raw data of quantitative assessment of EBA expression at the LM level in normal control (animal B20)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	65	0.145641	446.3029
F-2	54	0.145641	370.7747
F-3	68	0.145641	466.9015
F-4	65	0.145641	446.3029
F-5	61	0.145641	418.8381
Brainstem			
BS-1	60	0.145641	411.9719
BS-2	53	0.145641	363.9085
BS-3	49	0.145641	336.4437
BS-4	50	0.145641	343.3099
BS-5	65	0.145641	446.3029

F, frontal cortex

BS, brainstem

n/mm², the number of EBA-immunoreactive blood vessels per mm²

Table A.4.12 The mean number of EBA-immunoreactive blood vessels at the LM level in the frontal cortex in TBI model

	1h TBI (n/mm ²) (animal B3)	1h TBI (n/mm ²) (animal B4)	6h TBI (n/mm ²) (animal B16)	6h TBI (n/mm ²) (animal B17)	12h TBI (n/mm ²) (animal B32)	12h TBI (n/mm ²) (animal B48)	1h sham (n/mm ²) (animal B6)	6h sham (n/mm ²) (animal B18)	12h sham (n/mm ²) (animal B49)	Normal control (n/mm ²) (animal B19)	Normal control (n/mm ²) (animal B20)
	336.4437	260.9155	199.1198	315.8451	247.1831	329.5775	329.5775	391.3733	336.4437	398.2395	446.3029
	240.3169	240.3169	219.7183	288.3803	267.7817	260.9155	274.6479	453.1691	405.1057	391.3733	370.7747
	308.9789	281.5141	212.8522	233.4507	254.0493	267.7817	219.7183	418.8381	363.9085	343.3099	466.9015
	460.0353	295.2465	164.7888	260.9155	329.5775	274.6479	240.3169	425.7043	398.2395	501.2325	446.3029
	384.5071	267.7817	116.7254	247.1831	226.5845	240.3169	343.3099	411.9719	418.8381	398.2395	418.8381
Mean	346.0564	269.155	182.6409	269.155	265.0353	274.6479	281.5141	420.2113	384.5071	406.4789	429.824

n/mm², the number of EBA-immunoreactive blood vessels per mm²

Table A.4.13 The mean number of EBA-immunoreactive blood vessels at the LM level in the brainstem in TBI model

	1h TBI (n/mm ²) (animal B3)	1h TBI (n/mm ²) (animal B4)	6h TBI (n/mm ²) (animal B16)	6h TBI (n/mm ²) (animal B17)	12h TBI (n/mm ²) (animal B32)	12h TBI (n/mm ²) (animal B48)	1h sham (n/mm ²) (animal B6)	6h sham (n/mm ²) (animal B18)	12h sham (n/mm ²) (animal B49)	Normal control (n/mm ²) (animal B19)	Normal control (n/mm ²) (animal B20)
	343.3099	377.6409	226.5845	398.2395	288.3803	377.6409	377.6409	549.2959	501.2325	487.5001	411.9719
	329.5775	363.9085	336.4437	480.6339	384.5071	398.2395	370.7747	604.2255	508.0987	480.6339	363.9085
	350.1761	302.1127	315.8451	384.5071	240.3169	405.1057	288.3803	343.3099	439.4367	508.0987	336.4437
	363.9085	254.0493	233.4507	549.2959	308.9789	350.1761	254.0493	425.7043	260.9155	398.2395	343.3099
	288.3803	267.7817	254.0493	521.8311	185.3874	281.5141	260.9155	514.9649	549.2959	446.3029	446.3029
Mean	335.0705	313.0986	273.2747	466.9015	281.5141	362.5353	310.3522	487.5001	451.7959	464.155	380.3874

n/mm², the number of EBA-immunoreactive blood vessels per mm²

Appendix A.5 Raw data of quantitative assessment of EBA expression in traumatic brain injury (TBI) model at the EM level. Percentages of immunostained luminal membranes are shown for consecutive vessels.

Time interval	1h TBI		1h sham	6h TBI		6h sham	12h TBI		12h sham	Normal control	
Animal No	B3	B51	B6	B16	B17	B58	B32	B48	B49	B20	B21
Serial vessel No											
1	0	100	100	65.16	28.97	100	100	75.01	68.80	100	100
2	35.23	59.73	100	42.09	40.86	100	64.42	57.63	74.62	34.26	100
3	0	72.50	100	2.68	6.62	18.00	100	0	100	100	100
4	100	0	0	0	21.31	100	18.59	100	100	100	100
5	52.45	43.87	0	45.47	3.49	100	100	86.01	100	68.94	100
6	54.86	43.87	100	0	0	0	53.42	77.96	100	100	100
7	45.38	0	32.46	0	52.4	2.63	13.63	35.46	79.27	100	100
8	100	64.67	52.74	48.13	45.19	100	18.31	43.93	53.29	100	100
9	0	0	0	0	11.62	61.28	40.63	100	74.57	100	100
10	0	100	100	0	8.23	62.69	57.25	31.77	57.88	100	100
11	72.02	50.48	100	100	0	100	0	54.46	100	100	100
12	100	80.41	100	24.53	44.19	100	0	0	48.92	100	32.03
13	0	47.03	100	57.67	31.85	40.99	0	48.39	100	100	100
14	38.15	56.99	100	7.20	0	100	22.01	41.36	100	100	100
15	16.61	100	100	58.99	87.75	100	71.60	50.17	23.85	100	100
16	74.52	0	100	62.95	75.57	100	80.66	47.57	53.95	100	100
17	0	17.93	100	20.16	100	100	0	52.37	100	48.03	100
18	45.99	0	100	10.68	89.39	0	0	75.29	100	100	77.97
19	100	0	100	71.03	31.70	100	8.07	48.29	100		100
20	100	100	100	100		100	100	51.27	69.96		100
21	14.76	0	100	51.21		100	0		100		75.90
22	100	48.68	100	37.76		100	29.40		100		
23	100						90.21		78.58		
24	100								100		
25									100		
Mean	52.08	44.83	81.15	36.62	37.33	76.62	42.09	53.85	83.35	91.74	94.57

Appendix A.6. Raw data of quantitative assessment of GLUT1 immunoreactivity in traumatic brain injury model at the LM level

Table A.6.1 Raw data of quantitative assessment of GLUT1 expression at the LM level in TBI, 1h (animal B3)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	51	0.145641	350.1761
F-2	46	0.145641	315.8451
F-3	49	0.145641	336.4437
F-4	40	0.145641	274.6479
F-5	52	0.145641	357.0423
Brainstem			
BS-1	92	0.145641	631.6903
BS-2	96	0.145641	659.155
BS-3	71	0.145641	487.5001
BS-4	47	0.145641	322.7113
BS-5	80	0.145641	549.2959

F, frontal cortex

BS, brainstem

n/mm², the number of GLUT1-immunoreactive blood vessels per mm²

Table A.6.2 Raw data of quantitative assessment of GLUT1 expression at the LM level in TBI, 1h (animal B4)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	35	0.145641	240.3169
F-2	34	0.145641	233.4507
F-3	31	0.145641	212.8522
F-4	35	0.145641	240.3169
F-5	36	0.145641	247.1831
Brainstem			
BS-1	67	0.145641	460.0353
BS-2	37	0.145641	254.0493
BS-3	40	0.145641	274.6479
BS-4	38	0.145641	260.9155
BS-5	57	0.145641	391.3733

F, frontal cortex

BS, brainstem

n/mm², the number of GLUT1-immunoreactive blood vessels per mm²

Table A.6.3 Raw data of quantitative assessment of GLUT1 expression at the LM level in 1h sham control (animal B6)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	42	0.145641	288.3803
F-2	40	0.145641	274.6479
F-3	48	0.145641	329.5775
F-4	51	0.145641	350.1761
F-5	49	0.145641	336.4437
Brainstem			
BS-1	51	0.145641	350.1761
BS-2	55	0.145641	377.6409
BS-3	43	0.145641	295.2465
BS-4	43	0.145641	295.2465
BS-5	57	0.145641	391.3733

F, frontal cortex

BS, brainstem

n/mm², the number of GLUT1-immunoreactive blood vessels per mm²

Table A.6.4 Raw data of quantitative assessment of GLUT1 expression at the LM level in TBI, 6h (animal B16)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	34	0.145641	233.4507
F-2	11	0.145641	75.52818
F-3	19	0.145641	130.4578
F-4	34	0.145641	233.4507
F-5	24	0.145641	164.7888
Brainstem			
BS-1	31	0.145641	212.8522
BS-2	34	0.145641	233.4507
BS-3	39	0.145641	267.7817
BS-4	45	0.145641	308.9789
BS-5	37	0.145641	254.0493

F, frontal cortex

BS, brainstem

n/mm², the number of GLUT1-immunoreactive blood vessels per mm²

Table A.6.5 Raw data of quantitative assessment of GLUT1 expression at the LM level in TBI, 6h (animal B17)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	47	0.145641	322.7113
F-2	34	0.145641	233.4507
F-3	16	0.145641	109.8592
F-4	37	0.145641	254.0493
F-5	37	0.145641	254.0493
Brainstem			
BS-1	54	0.145641	370.7747
BS-2	68	0.145641	466.9015
BS-3	58	0.145641	398.2395
BS-4	43	0.145641	295.2465
BS-5	69	0.145641	473.7677

F, frontal cortex

BS, brainstem

n/mm², the number of GLUT1-immunoreactive blood vessels per mm²

Table A.6.6 Raw data of quantitative assessment of GLUT1 expression at the LM level in 6h sham control (animal B18)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	47	0.145641	322.7113
F-2	61	0.145641	418.8381
F-3	60	0.145641	411.9719
F-4	57	0.145641	391.3733
F-5	53	0.145641	363.9085
Brainstem			
BS-1	66	0.145641	453.1691
BS-2	83	0.145641	569.8945
BS-3	54	0.145641	370.7747
BS-4	55	0.145641	377.6409
BS-5	53	0.145641	363.9085

F, frontal cortex

BS, brainstem

n/mm², the number of GLUT1-immunoreactive blood vessels per mm²

Table A.6.7 Raw data of quantitative assessment of GLUT1 expression at the LM level in TBI, 12h (animal B32)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	21	0.145641	144.1902
F-2	32	0.145641	219.7183
F-3	31	0.145641	212.8522
F-4	42	0.145641	288.3803
F-5	25	0.145641	171.655
Brainstem			
BS-1	38	0.145641	260.9155
BS-2	47	0.145641	322.7113
BS-3	47	0.145641	322.7113
BS-4	43	0.145641	295.2465
BS-5	34	0.145641	233.4507

F, frontal cortex

BS, brainstem

n/mm², the number of GLUT1-immunoreactive blood vessels per mm²

Table A.6.8 Raw data of quantitative assessment of GLUT1 expression at the LM level in TBI, 12h (animal B48)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	51	0.145641	350.1761
F-2	50	0.145641	343.3099
F-3	45	0.145641	308.9789
F-4	41	0.145641	281.5141
F-5	42	0.145641	288.3803
Brainstem			
BS-1	43	0.145641	295.2465
BS-2	47	0.145641	322.7113
BS-3	34	0.145641	233.4507
BS-4	43	0.145641	295.2465
BS-5	40	0.145641	274.6479

F, frontal cortex

BS, brainstem

n/mm², the number of GLUT1-immunoreactive blood vessels per mm²

Table A.6.9 Raw data of quantitative assessment of GLUT1 expression at the LM level in 12h sham control (animal B49)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	48	0.145641	329.5775
F-2	59	0.145641	405.1057
F-3	66	0.145641	453.1691
F-4	48	0.145641	329.5775
F-5	61	0.145641	418.8381
Brainstem			
BS-1	70	0.145641	480.6339
BS-2	63	0.145641	432.5705
BS-3	70	0.145641	480.6339
BS-4	72	0.145641	494.3663
BS-5	69	0.145641	473.7677

F, frontal cortex

BS, brainstem

n/mm², the number of GLUT1-immunoreactive blood vessels per mm²

Table A.6.11 Raw data of quantitative assessment of GLUT1 expression at the LM level in normal control (animal B21)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	47	0.145641	322.7113
F-2	83	0.145641	569.8945
F-3	46	0.145641	315.8451
F-4	70	0.145641	480.6339
F-5	62	0.145641	425.7043
Brainstem			
BS-1	50	0.145641	343.3099
BS-2	58	0.145641	398.2395
BS-3	58	0.145641	398.2395
BS-4	63	0.145641	432.5705
BS-5	54	0.145641	370.7747

F, frontal cortex

BS, brainstem

n/mm², the number of GLUT1-immunoreactive blood vessels per mm²

Table A.6.10 Raw data of quantitative assessment of GLUT1 expression at the LM level in normal control (animal B20)

Brain Area	Vessel Number	Area (mm ²)	n/mm ²
Frontal cortex			
F-1	59	0.145641	405.1057
F-2	57	0.145641	391.3733
F-3	55	0.145641	377.6409
F-4	63	0.145641	432.5705
F-5	83	0.145641	569.8945
Brainstem			
BS-1	51	0.145641	350.1761
BS-2	56	0.145641	384.5071
BS-3	58	0.145641	398.2395
BS-4	58	0.145641	398.2395
BS-5	53	0.145641	363.9085

F, frontal cortex

BS, brainstem

n/mm², the number of GLUT1-immunoreactive blood vessels per mm²

Table A.6.12 The number of GLUT1-immunoreactive blood vessels at the LM level in the frontal cortex in TBI model

	1h TBI (n/mm ²) (animal B3)	1h TBI (n/mm ²) (animal B4)	6h TBI (n/mm ²) (animal B16)	6h TBI (n/mm ²) (animal B17)	12h TBI (n/mm ²) (animal B32)	12h TBI (n/mm ²) (animal B48)	1h sham (n/mm ²) (animal B6)	6hsham (n/mm ²) (animal B18)	12h sham (n/mm ²) (animal B49)	Normal control (n/mm ²) (animal B20)	Normal control (n/mm ²) (animal B21)
	350.1761	240.3169	233.4507	322.7113	144.1902	350.1761	288.3803	322.7113	329.5775	405.1057	322.7113
	315.8451	233.4507	75.52818	233.4507	219.7183	343.3099	274.6479	418.8381	405.1057	391.3733	569.8945
	336.4437	212.8522	130.4578	109.8592	212.8522	308.9789	329.5775	411.9719	453.1691	377.6409	315.8451
	274.6479	240.3169	233.4507	254.0493	288.3803	281.5141	350.1761	391.3733	329.5775	432.5705	480.6339
	357.0423	247.1831	164.7888	254.0493	171.655	288.3803	336.4437	363.9085	418.8381	569.8945	425.7043
Mean	326.831	234.824	167.5352	234.824	207.3592	314.4719	315.8451	381.7606	387.2536	435.317	422.9578

n/mm², the number of GLUT1-immunoreactive blood vessels per mm²

Table A.6.13 The number of GLUT1-immunoreactive blood vessels at the LM level in the brainstem in TBI model

	1h TBI (n/mm ²) (animal B3)	1h TBI (n/mm ²) (animal B4)	6h TBI (n/mm ²) (animal B16)	6h TBI (n/mm ²) (animal B17)	12h TBI (n/mm ²) (animal B32)	12h TBI (n/mm ²) (animal B48)	1h sham (n/mm ²) (animal B6)	6h sham (n/mm ²) (animal B18)	12h sham (n/mm ²) (animal B49)	Normal control (n/mm ²) (animal B20)	Normal control (n/mm ²) (animal B21)
	631.6903	460.0353	212.8522	370.7747	260.9155	295.2465	350.1761	453.1691	480.6339	350.1761	343.3099
	659.155	254.0493	233.4507	466.9015	322.7113	322.7113	377.6409	569.8945	432.5705	384.5071	398.2395
	487.5001	274.6479	267.7817	398.2395	322.7113	233.4507	295.2465	370.7747	480.6339	398.2395	398.2395
	322.7113	260.9155	308.9789	295.2465	295.2465	295.2465	295.2465	377.6409	494.3663	398.2395	432.5705
	549.2959	391.3733	254.0493	473.7677	233.4507	274.6479	391.3733	363.9085	473.7677	363.9085	370.7747
Mean	530.0705	328.2043	255.4226	400.986	287.0071	284.2606	341.9367	427.0775	472.3944	379.0142	388.6268

n/mm², the number of GLUT1-immunoreactive blood vessels per mm²

Appendix A.7 Raw data of quantitative assessment of GLUT1 expression in traumatic brain injury (TBI) model at the EM level.
Percentages of immunostained luminal membranes are shown for consecutive vessels.

Time interval	1h TBI		1h sham	6h TBI		6h sham	12h TBI		12h sham	Normal control	
Animal No	B51	B57	B59	B16	B17	B58	B32	B48	B49	B20	B21
Serial vessel No											
1	87.75	76.02	41.87	0	4.18	92.70	42.10	59.89	33.93	100	100
2	100	46.28	100	0	37.14	100	0	52.27	72.91	100	100
3	62.96	13.74	84.96	23.24	0	83.05	47.69	49.03	82.36	100	100
4	37.25	54.06	96.23	100	14.51	75.59	37.75	69.18	68.86	100	80.35
5	48.42	23.03	100	59.30	0	81.12	52.25	31.84	84.41	100	100
6	40.83	27.71	100	97.31	6.65	100	81.17	49.10	64.43	100	76.89
7	46.91	43.20	82.37	0	34.03	66.08	70.79	65.02	50.79	52.63	100
8	16.60	14.38	100	33.47	0	100	73.47	89.84	100	100	86.79
9	11.68	69.59	100	1.96	34.88	71.59	100	23.14	60.61	100	100
10	74.69	13.34	97.94	0	54.54	91.59	51.79	36.94	73.55	100	51.88
11	64.94	38.25	100	0	27.85	83.12	100	38.64	100	100	100
12	8.77	31.43	100	0	0	100	0	74.01	74.31	100	100
13	17.13	13.51	97.94	4.16	7.91	93.54	42.70	54.91	100	57.48	100
14	41.20	16.45	100	1.69	5.60	100	55.64	20.17	100	100	100
15	30.41	0	100	0	100	68.79	34.08	42.42	100	100	100
16	80.48	54.50	100	0	0	100	59.51	32.56	93.23	82.62	100
17	73.08	26.89	100	8.94	0	93.12	55.95	45.36	100		100
18		14.14	67.24	0	51.89	100	82.98	59.84	100		100
19		43.44	100	29.60	13.26	100	54.09		65.94		68.70
20		57.14		0	0	100	61.23		100		100
21		70.36		9.67	0	92.43	45.25		100		100
22		71.25			64.66	100	0		100		100
23		80.05					41.47				100
24		75.69					0				100
25		78.85					47.83				100
26		77.18					27.98				49.16
27							51.05				
28							25.93				
Mean	43.77	43.48	93.08	17.59	20.78	90.58	47.95	49.68	82.97	93.30	92.84

Clostridium perfringens Prototoxin-Induced Alteration of Endothelial Barrier Antigen (EBA) Immunoreactivity at the Blood–Brain Barrier (BBB)¹

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It has been reported that the severe cerebral edema produced in experimental animals by *Clostridium perfringens* (Cl p) type D ϵ toxin can be prevented by prior treatment with its precursor prototoxin due to competitive binding to endothelial cells (ECs) at the blood–brain barrier (BBB). In this study we investigate the effects of the prototoxin on the BBB, without added toxin. The integrity of the BBB was assessed by its ability to prevent leakage of endogenous albumin. ECs at the BBB were studied by immunocytochemistry for any alteration in the endothelial barrier antigen (EBA), a molecular marker for the intact BBB. Immunocytochemistry showed rapid but mild opening of the BBB to endogenous albumin. Light and electron immunocytochemistry showed qualitative and quantitative reduction in EBA immunoreactivity, with a spectrum of changes at time intervals from 1 h to 14 days post-prototoxin injection. Some vessels with ultrastructural changes and widening of the perivascular space retained EBA immunoreactivity. Many vessels showed partial or complete loss of EBA staining with minimal widening of the perivascular space and edema. Recovery of EBA expression was still incomplete at 14 days postinjection. This is the first report to show endothelial cell damage, mild reversible cerebral edema, and alteration in BBB markers following administration of Cl p prototoxin. This model of mild brain edema may be useful for BBB studies.

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Key Words: endothelial barrier antigen; endothelial cell; blood–brain barrier; *Clostridium perfringens*; prototoxin; immunocytochemistry.

INTRODUCTION

Molecular anatomy of endothelial cells (ECs) at the blood–brain barrier (BBB) has been receiving increasing attention (3, 7–9, 16, 17, 23, 24, 27). The identification of several EC proteins and their detection by immunocytochemistry are progressively broadening our understanding of EC biology. In the rat, an endothelial protein, the endothelial barrier antigen (EBA) has been identified as a marker of CNS barrier vessels (30). The protein is expressed by vessels in the brain (23, 30, 31), spinal cord (24, 30), optic nerve (16), and retina (17) and detected by immunocytochemistry using a monoclonal antibody (anti-EBA) (30). Brain vessels located outside the BBB, such as those in the median eminence, area postrema, and choroid plexus, do not express or only weakly express the protein (25, 30). EBA is not expressed by ECs of peripheral organs and tissues, such as the liver, intestine, heart, skeletal muscles, thymus, and pancreas (30).

In experimental models of BBB alteration, a variation in the expression of EBA occurs in correlation with the state of the disease. Firstly, in acute experimental allergic encephalomyelitis (EAE) in Lewis rats, EBA immunoreactivity is abolished in brain vessels, but is reestablished after recovery from the acute phase (31). Secondly, in stab-wound injury of the brain, EBA immunoreactivity is lacking in directly injured and adjacent vessels, but is restored after 2 weeks (25). Thirdly, in cerebral infarction, EBA expression is reduced by day 1 and is almost lost by day 3. Newly formed vessels, which become identifiable at 3 days, are negative for EBA up to day 7, but show weak EBA expression by 14 days (23). Fourthly, transient reduction in EBA immunoreactivity occurs in spinal cord trauma (24). Edema due to opening of the BBB is a feature of the acute phases of EAE (13), stab-wound injury (25), infarction (1), and spinal cord trauma (10, 24). Thus in the above four etiopathologically different models the reduction of EBA immunoreactivity in

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cerebral vessels appears to be a useful marker for the detection of sites of BBB disruption.

Clostridium perfringens (*Cl p*) is a normal inhabitant of the duodenum of ruminants. It has been associated with gas gangrene and food poisoning in humans and implicated in outbreaks of fatal toxemia in domestic animals (19, 29). These saccharolytic bacteria can proliferate and elaborate large amounts of prototoxin when presented with a diet high in starch. The inactive prototoxin of 311 amino acids (12) becomes activated by the action of trypsin, through cleavage of a small peptide (14 amino acids) from the N-terminus (2). The potent toxin causes increased mucosal permeability facilitating its own absorption (19). Several earlier studies employed *Cl p* epsilon toxin and reported pathological changes in the brain and ECs and an increase in vascular permeability in the brains of mice and sheep (reviewed in 19). Intravenous injection of horseradish peroxidase (HRP) in mice treated with *Cl p* epsilon toxin results in a wide distribution of the tracer in brain parenchyma. In mice, labeled bovine serum albumin accumulates in the brain after toxin injection (21). In the rat, *Cl p* epsilon toxin causes a breakdown in the BBB as detected by immunocytochemistry for serum albumin (11). The toxin binds specifically to the brain of mice as shown by the accumulation of ¹²⁵I-labeled toxin, after its intravenous injection (21). On account of the rapid accumulation of the toxin in the brain, its fast effect and the perivascular edema, it was concluded that the ECs are primary sites of action (4). The toxin is thought to bind to certain receptor sites on ECs (4).

In mice, prior injection of the inactive *Cl p* prototoxin offers transient protection from the effect of the toxin (4, 21), as demonstrated by (a) the length of survival time (21), (b) the reduced binding of labeled toxin to the brain (21), (c) the reduced accumulation in the brain of labeled bovine serum albumin (21), and (d) the lack of HRP leakage in the brain (4). A 90% decrease in ¹²⁵I-labeled toxin in mice brains is detected with prior injection of prototoxin (21). It was suggested that such protection may be due to the prototoxin competing with the toxin at presumed receptor sites, thus averting the action of the toxin on the animals (4, 5, 21). Using rabbit antiserum to formalinized prototoxin and immunocytochemistry it was demonstrated conclusively that *Cl p* prototoxin binds to luminal membranes of brain ECs (5).

Previously mentioned studies employing the inactive prototoxin were principally concerned with the protective effect of the prototoxin and investigated the length of such protection. It was found that the prototoxin offers protection for up to 100 min but was less effective by 750 min (4). It was concluded that the prototoxin may become internalized into ECs freeing the receptor sites for toxin binding (4). It appears that none of the previous studies have investigated the delayed effects of the prototoxin alone on the BBB, without subsequent administration of the toxin. It was of interest therefore to investigate any

delayed effects of the prototoxin on the expression of EBA, which is specifically localized at the luminal membranes of ECs (16, 25, 31). Concurrently we tested the integrity of the BBB, using its ability to exclude albumin from entering the brain parenchyma.

MATERIALS AND METHODS

Animals and Perfusion

Thirty-two adult hooded Wistar rats (10 males, 22 females, 140–220 g) were used. *Cl p* type D ϵ prototoxin was obtained as inactive culture supernatant (Commonwealth Serum Laboratories Ltd., Victoria, Australia). Four milliliters of 1:400 or 1:500 dilution in saline were injected intraperitoneally in experimental animals ($n = 26$). Control animals were either noninjected ($n = 4$) or injected with saline ($n = 2$) and killed after 24 h. Experimental animals were killed at 1 h ($n = 3$), 6 h ($n = 3$), 9 h ($n = 1$), 12 h ($n = 3$), 18 h ($n = 1$), 24 h ($n = 3$), 48 h ($n = 3$), 4 days ($n = 3$), 7 days ($n = 3$), and 14 days ($n = 3$) postinjection. The two animals killed at 9 and 18 h received 1:500 dilution. In all other time intervals, 2 animals received 1:500 dilution and 1 animal received 1:400 dilution. Under pentobarbitone anesthesia the left ventricle was cannulated and the vascular bed washed for 45 s, with Dulbecco's phosphate-buffered saline supplemented with minimum essential medium, gassed with 95% O₂ and 5% CO₂, at room temperature and under constant pressure (90 mm Hg). This was followed by 600 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed and two sagittal blocks, 2 mm thick each, were cut from the right and left hemispheres adjacent to the longitudinal fissure. Fixation was continued by immersion for 3 h in the same fixative followed by buffer wash.

Light and Electron Immunocytochemistry for EBA

One block was embedded in paraffin wax and 7- μ m light microscope (LM) sections were cut and collected on coated slides. The other block was cut into 50- μ m Vibratome sections. The endogenous peroxidase in free floating Vibratome sections and deparaffinized LM sections was blocked, using 0.3% H₂O₂ in double-distilled water for 30 min. Vibratome and LM sections were incubated in 10% normal horse serum (NHS) for 60 min and then incubated, at 4°C overnight, in the primary antibody (anti-EBA, SMI 71, Sternberger Monoclonal Inc., Lutherville, MD) diluted 1:3000 in 1% NHS. Sections were washed in buffer then incubated in a biotinylated horse anti-mouse IgG secondary antibody (Vector Laboratories, Burlingame, CA, diluted 1:200 in 1% NHS) for 30 min (LM sections) or 60 min (Vibratome sections). Following thorough washing in buffer the bound secondary antibody was detected using the avidin-biotin peroxidase method (ABC kit, Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride as chromogen (DAB, 3 mg in 10 ml of 0.1 M phosphate buffer with 0.15

M NaCl, and 30 μ l of 0.3% H_2O_2 for 10 min). Nickel enhancement using the glucose oxidase method (28) was employed in some sections. Negative controls for EBA staining included the omission of the primary antibody or the primary and secondary antibodies or the use of liver tissue. Following immunostaining, LM sections on slides were dehydrated, cleared, and coverslipped. Vibratome sections were prepared for LM and EM examination. For LM, Vibratome sections were mounted on glass slides, air-dried overnight, cleared, and coverslipped. For EM, other Vibratome sections were osmicated, dehydrated, and placed in Taab resin in flat molds. Small rectangular pieces from areas of interest were cut out before or after polymerization and were embedded in resin blocks. Ultrathin sections were examined, mostly without heavy metal staining (uranyl acetate and lead citrate) to enhance the contrast between the peroxidase reaction product and the brain tissue. Sections were viewed in a Philips CM100 electron microscope.

Quantitative and Qualitative Assessment of EBA Immunoreactivity

LM and EM sections were examined for the qualitative assessment of the distribution and intensity of EBA immunoreactivity in brain vessels. Quantitative assessment was performed on two experimental groups of animals, one injected with 1:400 and the other with 1:500 dilution of the prototoxin. Each experimental group was compared to a control animal. Tissues from each group were immunostained at the same time and under the same conditions. A total number of 360 vessels was used for the quantitative study. A range of 16–30 vessels with an average of 24 vessels per animal were photographed randomly from EM sections. An image analysis system (AnalySIS, Soft Image Systems, Germany) was used to quantify the percentage of length of EC luminal membranes immunoreactive for EBA in relation to the total length of luminal circumference. The data for individual vessels were plotted using Microsoft Excel software, and the results from all vessels in each animal were pooled and statistically compared to data from the control animal used in that group, employing Student's *t* test.

Immunostaining for Endogenous Albumin

LM sections on slides were used for the detection of albumin in brain parenchyma employing the avidin–biotin–peroxidase method as above. The primary antibody was goat anti-rat albumin (ICN Pharmaceuticals Inc., Costa Mesa, CA, 1:1000–1:3000), and the secondary antibody was biotinylated rabbit anti-goat IgG, 1:500 (Vector Laboratories). Antibodies were diluted in 1% normal rabbit serum (NRS). Negative controls for albumin staining included the omission of the primary or the primary and secondary antibodies. For positive controls liver tissue was used.

RESULTS

LM Immunocytochemistry for EBA

LM of paraffin and Vibratome sections from normal animals immunostained with anti-EBA showed immunoreactivity in almost all vessels throughout the gray and white matter of the cerebrum, diencephalon, cerebellum, and brainstem. Immunoreactive (IR) microvessels in the brain (Fig. 1A) and pia mater displayed homogeneously dark reaction product. Microvessels were seen in various orientations: transverse, oblique, and longitudinal. Occasional vessels in the choroid plexus and neurohypophysis showed weak staining. The neuropil was clear and no staining of neurons or glial cells was noted. EBA-negative controls, where the primary or the primary and secondary antibodies were omitted, showed no labeling of blood vessels. LM of experimental material obtained from 1 to 18 h postinjection of *Cl p* prototoxin showed immunostained vessels, which in general were similar to those of controls. However, closer examination revealed an increased number of weakly IR or nonreactive vessels (Figs. 1B and 1C). LM of experimental material obtained at 1 and 2 days postinjection of *Cl p* prototoxin showed a pronounced reduction in immunoreactivity of vessels (Figs. 1D and 1E). At 4 and 7 days postinjection many vessels were unstained or weakly stained. At 14 days, IR vessels (Fig. 1F) were not dissimilar to control vessels (Fig. 1A).

EM Immunocytochemistry for EBA

The results obtained from immunoelectron microscopy are shown in Figs. 2–4. The ultrastructure appeared less than optimal for two main reasons. Firstly, glutaraldehyde was omitted from the fixative as it affected the success of immunolabeling. Secondly, ultrathin sections were examined without the conventional heavy metal staining, in order to maximize the contrast between the peroxidase reaction product indicative of the labeling and the brain tissue. EM of control tissue immunostained for EBA showed electron-dense peroxidase reaction product, which was especially apparent in sections unstained with heavy metals due to better contrast (Figs. 2A–2D). The reaction product was essentially located at the luminal membrane of ECs. In the majority of vessels the reaction product was seen along the entire circumference. However, the density of the reaction product was not always uniform in all ECs. A minority of vessels showed sectoral absence of EBA immunoreactivity. The cytoplasm of many ECs showed labeling involving the full thickness of the cytoplasm between the luminal and abluminal membranes or a gradient of labeling fading away toward the abluminal membrane. EC vesicles were not labeled and appeared electron lucent against the darkly stained cytoplasm. Some EC nuclei showed immunoreactivity on the side of the nucleus facing the lumen.

Experimental material showed a spectrum of changes in EBA expression (Figs. 3–5), including ul-

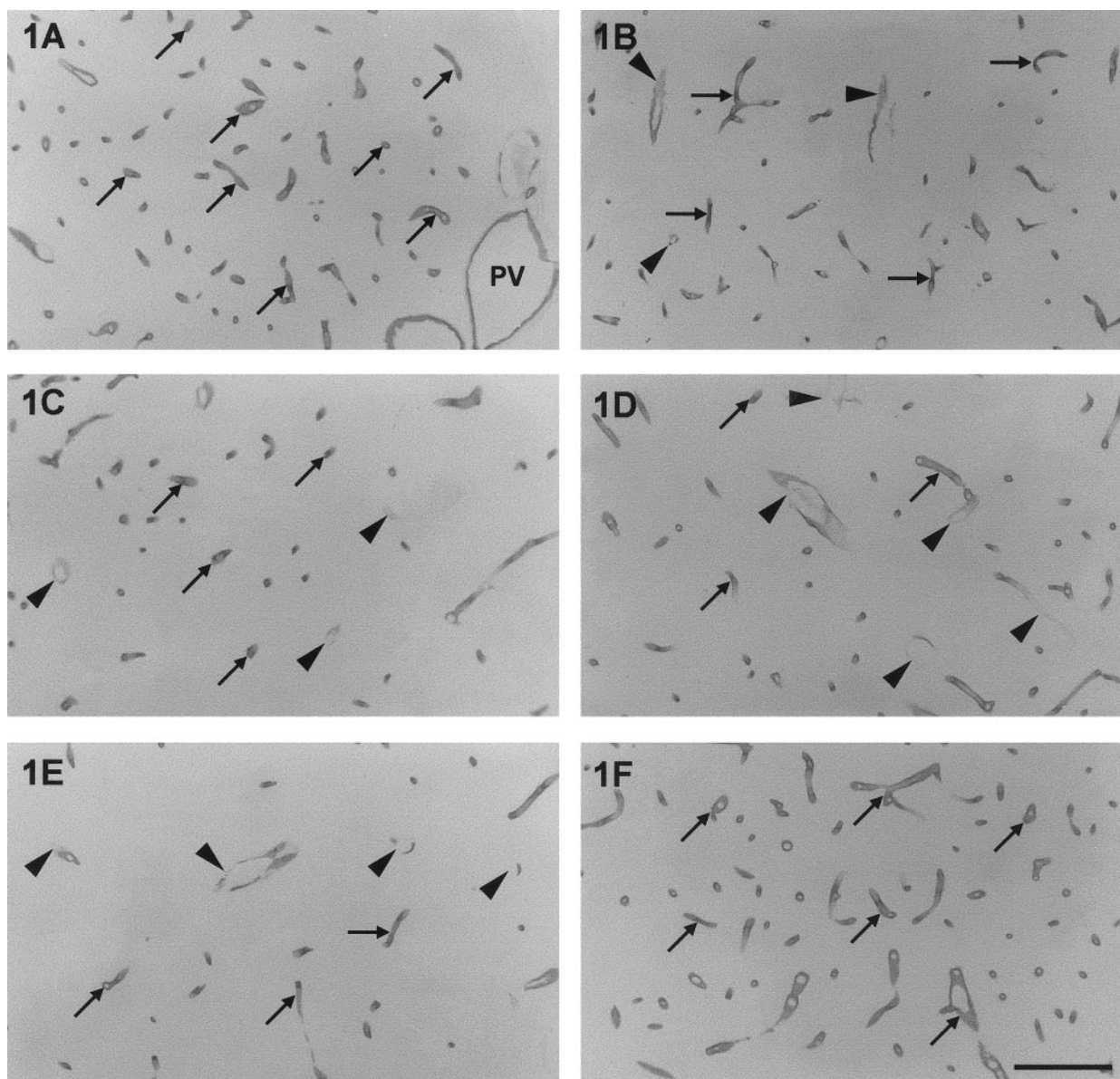


FIG. 1. (A–F) Light micrographs of paraffin sections showing cerebral cortices from control (A) and experimental animals with survival times of 1 h (B), 6 h (C), 24 h (D), 2 days (E), and 14 days (F) post-intraperitoneal injection of *Cl p* prototoxin. EBA-immunoreactive vessels (arrows) are seen in various orientations. Pia mater vessels (PV) on the surface of the cortex (A) are stained. Partially or weakly labeled vessels (arrowheads) are seen in experimental animals (B–E). At 14 days (F) labeling appears similar to the control (A). Immunoperoxidase, with nickel enhancement. Bar, 100 μ m.

trastructural evidence of widening of the perivascular space, suggestive of edema. Vacuolation was often seen in ECs, pericytes, and perivascular astrocytic processes, with apparent reduction in organelles. However, intercellular junctions generally remained intact (Figs. 4A and 4C). Alteration in EBA expression and ultrastructural changes were not synchronized in all vessels of the same section (Fig. 3). In some vessels perivascular widening and EC vacuoles were seen while EBA was still expressed at the luminal membrane of the whole circumference. In many other vessels partial loss of EBA expression in part of the circumference or complete loss of reaction product along the entire circumference was not accompanied by ul-

trastructural changes. At 1 day (Fig. 4A) and 2 days postinjection long stretches of luminal membranes were devoid of the peroxidase reaction product in most vessels. At 4 and 7 days (Fig. 4B) luminal membrane staining was still reduced. At 14 days, with LM examination immunoreactivity of vessels appeared similar to that in controls. However, EM examination showed many vessels with reduced staining (Fig. 4C) among vessels with complete luminal staining (Fig. 4D).

Quantitative Study of EBA Immunoreactivity

Quantitative assessment of EBA immunoreactivity at the EM level was carried out on two groups of

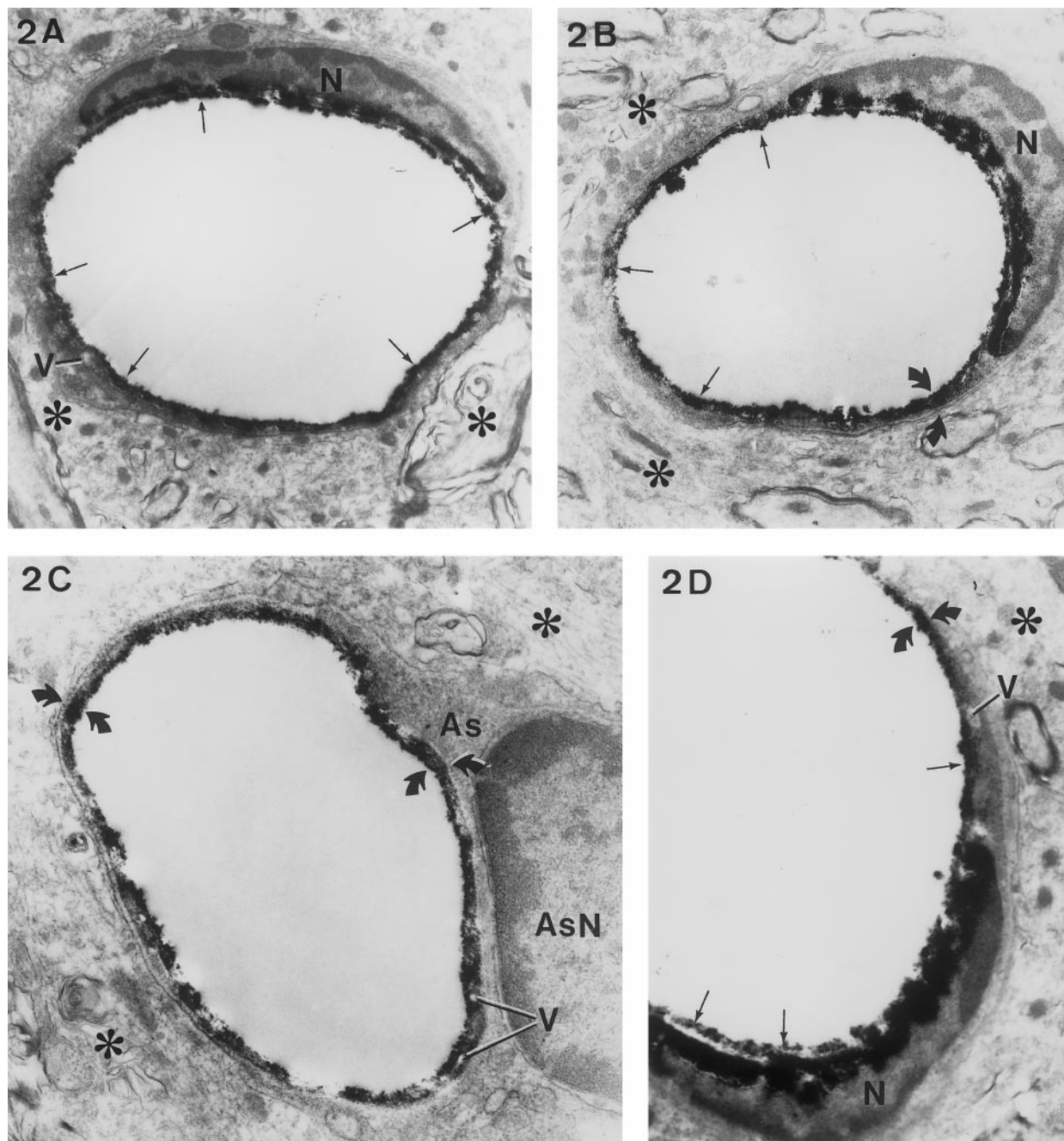


FIG. 2. (A–D) Electron micrographs of cerebral cortical microvessels from control (noninjected) animals, showing preembedding immunoperoxidase labeling for EBA, with nickel enhancement. The labeling appears as electron-dense reaction product along the entire length of luminal membrane (small arrows) of endothelial cells. Endothelial cells show immunoreactivity in the cytoplasm involving the full thickness between the luminal and abluminal membranes (between curved arrows, B–D). The cytoplasmic staining is unlikely to be due to over development of the reaction product or a smearing from the luminal staining since cytoplasmic vesicles (V) appear unstained (A, C, and D). A, B, and D, show endothelial cell nuclei (N) which display immunoreactivity in the marginal chromatin located deep to the nuclear membrane on the luminal side. The astrocyte (As) and its nucleus (AsN) seen in C are not immunoreactive for EBA. Neurons and nerve fibers in the neuropil (asterisks) are nonreactive. All vessels are from grids not stained with heavy metals. A, $\times 9,800$; B, $\times 10,000$; C, $\times 17,000$; D, $\times 13,000$.

experimental animals, injected with 1:400 and 1:500 dilution of the prototoxin, and one control animal in each group. The experimental group which received 1:400 dilution of the prototoxin included one animal killed at 1 h, 6 h, 12 h, 24 h, 2 days, 4 days, 7 days, and

14 days postinjection. The percentage of luminal membrane length showing EBA immunoreactivity was calculated in relation to the total length (circumference) of luminal membrane for each vessel (Fig. 5). In control animals most vessels had 100% staining of their lumi-

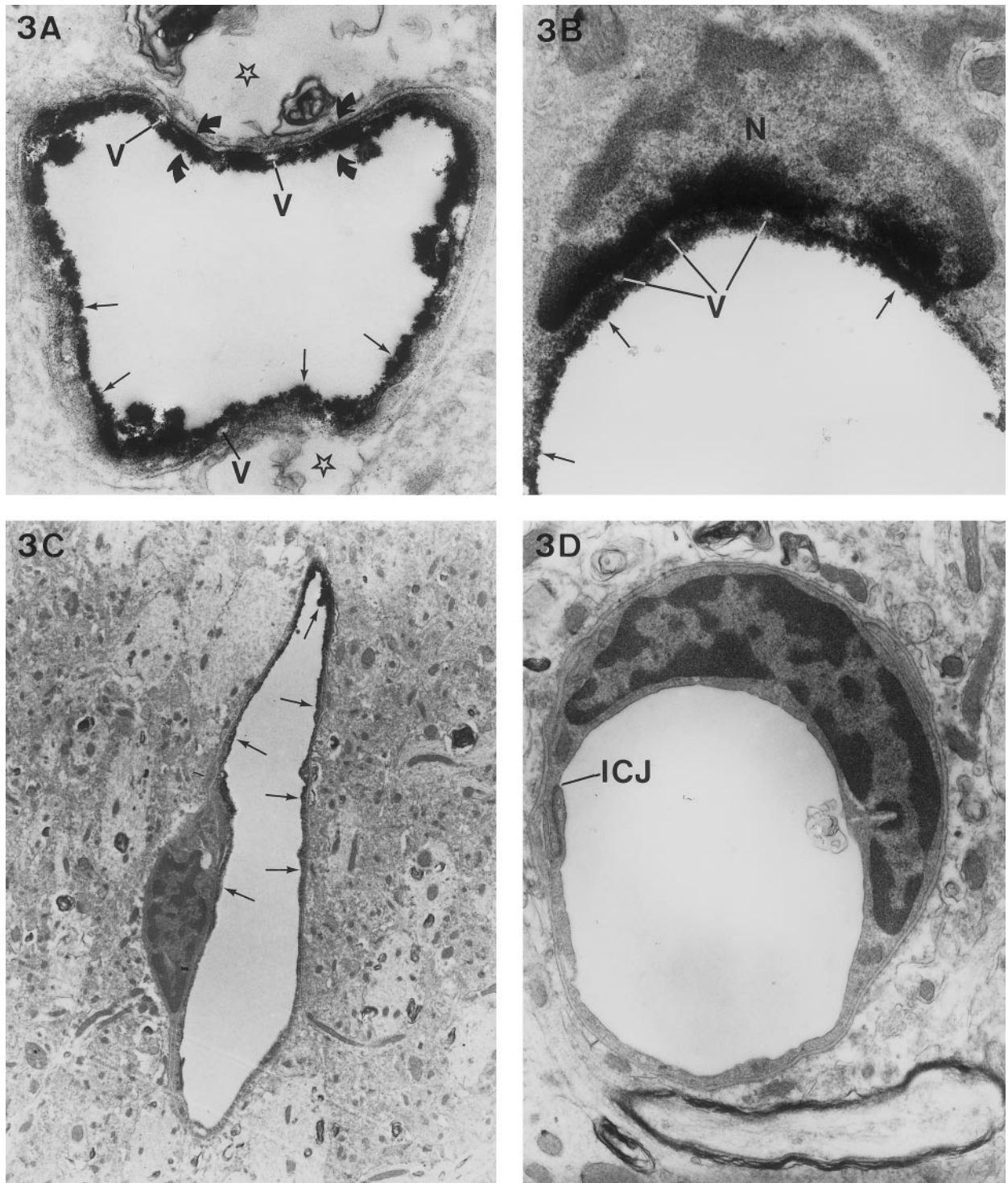


FIG. 3. (A–D) Electron micrographs of cerebral cortical microvessels from experimental animals, showing the range of changes in EBA expression, at 1 h (A–C) and 6 h (D) postinjection of prototoxin, as detected by preembedding immunoperoxidase labeling for EBA, with nickel enhancement. Perivascular space formation (stars) suggestive of edema preceded any loss of EBA immunoreactivity at the luminal membrane (small arrows) in A. In other vessels, luminal membrane staining is partially present as in C (small arrows) or absent as in D, despite lack of perivascular widening or any endothelial cell damage. This indicates that the reduction may precede or follow the edema formation. The reaction product is seen in the full thickness of endothelial cell cytoplasm (between curved arrows, A) but with unstained vesicles (V). The endothelial cell nucleus (N) in B also shows reaction product in the marginal chromatin on the luminal side of the nucleus. The cytoplasm deep to the nucleus is stained but contains clear vesicles (V). ICJ, intercellular junction. All vessels are from grids not stained with heavy metals. A, $\times 22,500$; B, $\times 27,100$; C, $\times 5450$; D, $\times 14,800$.

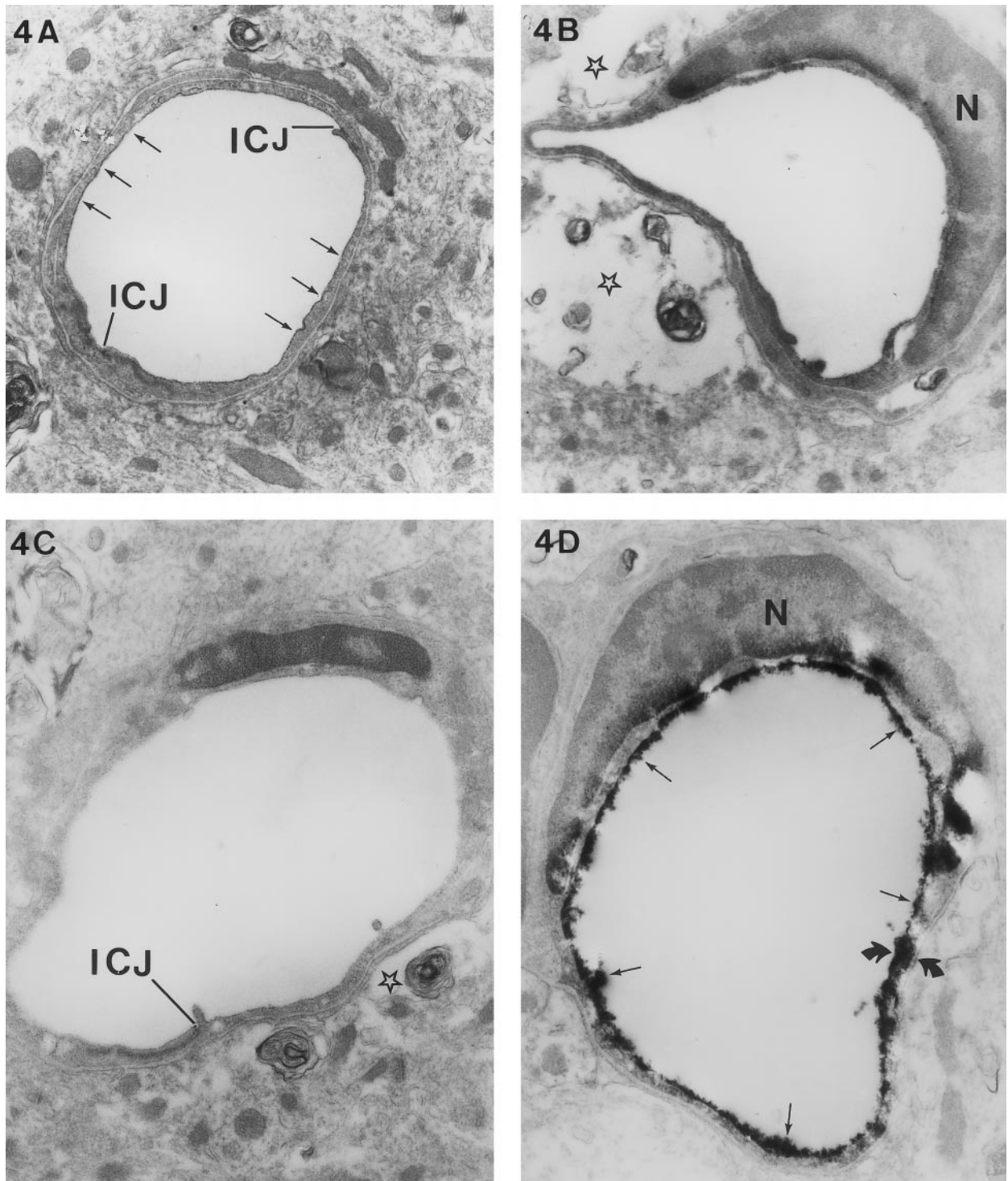


FIG. 4. (A–D) Electron micrographs of cerebral cortical microvessels from experimental animals, at 1 day (A), 7 day (B), and 14 days (C and D) postinjection of prototoxin, showing preembedding immunoperoxidase labeling for EBA, with nickel enhancement. (A) Faint reaction product (small arrows) on parts of the luminal circumference. (B) Absence of reaction product in the luminal membrane and cytoplasm. The marginal chromatin on the luminal side of the endothelial cell nucleus (N) shows faint reaction product. The perivascular edema (stars) is distorting the vessel's profile. (C) Complete absence of EBA labeling with minimal perivascular astrocytic edema (star). (D) Intense reaction product in the luminal membrane (small arrows) and certain regions of the cytoplasm (between curved arrows) of endothelial cells. The marginal chromatin on the luminal side of the endothelial cell nucleus (N) shows gradation of reaction product. The cytoplasm between the nucleus and the luminal membrane here is not stained, indicating that nuclear staining is unlikely to be artifact smearing. All vessels are from grids not stained with heavy metals. ICJ, luminal ends of apparently intact intercellular junctions. A, $\times 12,000$; B, $\times 13,350$; C, $\times 17,500$; D, $\times 19,000$.

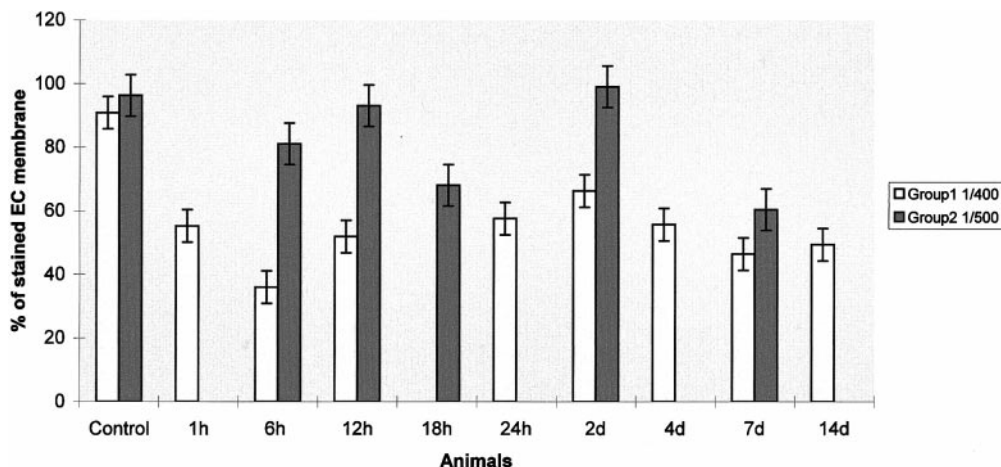


FIG. 5. A graph showing the quantitative results for the percentage of EC luminal membranes immunoreactive for EBA, in two control animals and in experimental animals injected with 1:400 or 1:500 dilution of epsilon prototoxin and killed at various time intervals ranging from 1 h to 14 days postinjection. The histogram bars represent mean values of 16–30 vessels in each time interval. The standard error bars show the error amount for all data points in the two groups. Each experimental group was compared to one control animal, since sections were immunostained at the same time and under identical conditions.

nal membranes. In experimental animals most vessels were either unstained or had values less than 100%. The results from all vessels in each animal were pooled and a mean value was obtained for each animal (Fig. 5). The control animal in this group had a value of 91% (Fig. 5). The percentage of luminal membrane staining in experimental animals ranged from 35 to 66% (Fig. 5). A significant reduction was seen in all experimental animals in this group compared to the control (1 h, $P = 0.002$; 6 h, $P = 0.7^{-5}$; 12 h, $P = 0.002$; 24 h, $P = 0.005$; 2 days, $P = 0.02$; 4 days, $P = 0.003$; 7 days, $P = 0.0002$; and 14 days, $P = 0.0006$). The reduction in EBA expression was more severe in this group compared to the second group, which received 1:500 dilution of the prototoxin.

The second group, which received 1:500 dilution, included one animal at 6 h, 12 h, 18 h, 2 days, and 7 days (Fig. 5). Except in the two experimental animals killed at 12 h and 2 days, all experimental animals showed many vessels with reduced percentage of luminal membrane staining. The control animal in this group showed 96% staining of EC luminal membranes. The percentages of luminal membrane staining were 81, 68, and 60% in experimental animals killed at 6 h, 18 h, and 7 days, respectively. Statistical comparison of these time intervals to the control showed significant reduction (6 h, $P = 0.04$; 18 h, $P = 0.0007$; and 7 days, $P = 0.0001$). The two animals killed at 12 h and 2 days had values of 93 and 99%, respectively, and were not significantly different from the control ($P = 0.4$ and 0.27 , respectively). On the whole the animals injected with 1:500 dilution showed a milder and delayed response to the prototoxin injection.

LM Immunocytochemistry for Albumin

Control rat brain sections showed no albumin immunoreactivity (Fig. 6A) except in the choroid plexus and

neurohypophysis. In general the white matter appeared to have a faint immunoreactivity compared to the gray matter (Fig. 6A). In experimental animals, a dose- and time-dependent albumin leakage was noted. Progressive increase in albumin immunoreactivity was noted from 1 h (Fig. 6B) to 18 h postinjection. At 24 and 48 h, albumin leakage was at its maximum (Fig. 6C). Multiple foci of immunoreactivity were seen in the cerebral cortex and especially in the brainstem and corpus callosum. These were generally the areas with maximal EBA reduction. At 4 and 7 (Fig. 6C) days, albumin leakage was still present but the intensity of immunoreactivity was reduced markedly with only some foci of albumin leakage (Figs. 6D and 6E). At 14 days postinjection (Fig. 6F), the staining was similar to that of control animals, except for occasional foci seen in the cerebrum and brainstem.

DISCUSSION

The most striking finding of this study is the reduction in the expression of EBA immunoreactivity in brain ECs following *in vivo* administration of the inactive *Cl p* epsilon prototoxin. The reduction in EBA immunoreactivity was accompanied by mild opening of the BBB to endogenous albumin. These changes were unexpected since previous reports showed that intravenous injections of the epsilon prototoxin or formalinized prototoxin offer transient protection to experimental animals from the lethal effect of the toxin (4, 21). However, previous studies using the prototoxin did not investigate the effect of the prototoxin alone for longer time intervals; the prototoxin was injected for periods ranging from 3 to 750 min (protection time) before the toxin was injected (4). The prototoxin was protective only up to 100 min, but at 750 min it was not as effective. Thus in previous studies, any mild effect of

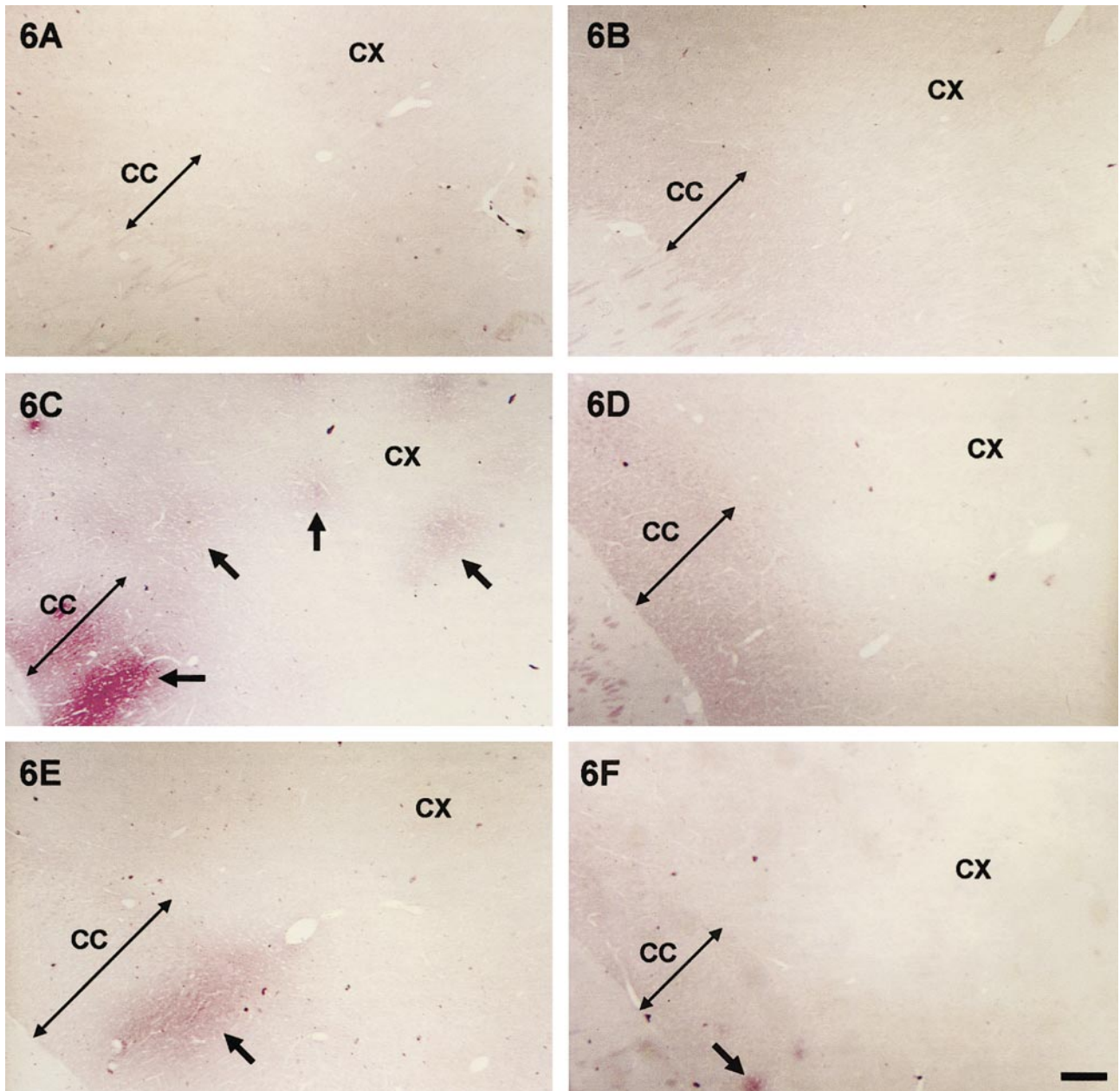


FIG. 6. Light micrographs of sagittal sections of wax-embedded brains from control (A) and experimental animals (B–F) injected with prototoxin. The sections show immunostaining for endogenous albumin with nickel enhancement, but with no counterstaining. In controls (A) no albumin immunoreactivity is seen. At 1 h (B) postinjection slight immunoreactivity is seen especially in the corpus callosum. At 2 days (C) immunoreactivity is visible as multiple foci of peroxidase reaction product in the corpus callosum and the cerebral cortex (arrows). At 4 days (D) and 7 days (E) immunoreactivity is still higher than the control (A) especially in the white matter (arrow). At 14 days (F) postinjection only occasional foci of immunoreactivity are seen (arrow). CC, corpus callosum; CX, cortex. Bar, 200 μ m.

the prototoxin alone would have been overshadowed by the faster and stronger action of the subsequently injected toxin. In the current study, animals were allowed to survive for periods ranging from 1 h to 14 days after the prototoxin injection without injection of the toxin.

The reduction in EBA expression seen qualitatively by light and electron microscopy was confirmed by the quantitative study. Animals injected with the higher concentration of the prototoxin showed a faster and

stronger response with significant reduction in immunoreactivity. The group injected with the lower concentration showed mild and late response, where the maximum effect was seen quantitatively at 7 days post-prototoxin injection. The two animals killed at 12 h and 2 days in this group were not significantly different from the control. This may be due to animal variation or an inadequate sampling. However, even in this group with a mild response there was a trend of reduction in EBA expression. In control animals almost all

vessels showed immunoreactivity for EBA, with a minority of vessels showing absence of immunoreactivity along part of the circumference and only occasional vessels showing complete absence of labeling.

Electron immunocytochemistry employed in the current study demonstrated preferential localization of EBA to luminal EC membranes in control animals, thus supporting previous studies (16, 25, 31). In the current study, experimental animals injected with *Cl p* prototoxin showed a reduction in EBA immunoreactivity. The relationship between the possible effect of the prototoxin and the reduction in EBA expression is not clear. Buxton (5) showed by immunocytochemistry that formalinized epsilon prototoxin of *Cl p* binds to the luminal surface of cerebral ECs in mice. Whether the loss of EBA in the current study is a direct or an indirect effect of the binding of the prototoxin to ECs membranes is speculative.

In this study vacuolation and swelling of pericytes and astrocytic perivascular processes with depletion of organelles were seen. *In vitro* studies suggest an inductive role for astrocytes on the development of BBB characteristics in ECs with increased production of γ -glutamyl transpeptidase (18) and increased complexity of tight junctions between ECs (32). However, the reduction in EBA expression seen in this study may not be due to astrocyte damage. An *in vivo* study on selective astrocytic degeneration, induced by chronic systemic injection of the antimetabolite 6-aminonicotinamide, showed swelling and organelle degeneration, but there was no alteration in the expression of EBA in brain ECs (14). In the latter experimental model of astrocytic degeneration, the BBB to exogenous and endogenous tracers was not compromised (15).

Biochemical studies on the binding of *Cl p* epsilon toxin to rat brain homogenate and synaptosomal membrane fractions indicate a high-affinity binding of the toxin to specific binding sites in brain tissue (22). Pretreatment of synaptosomal membrane fractions with enzymes has been used to study the nature of binding sites (22). The sites appear to be formed of proteins since the binding is reduced by pretreatment with pronase and heat. Also sialic acid residues appear to be important for binding since pretreatment with neuraminidase reduces the binding. Thus the binding sites may be formed of a sialoglycoprotein. Lipase treatment showed that an appropriate lipid environment is important to facilitate the binding (22). The affinity of the toxin to bind to synaptosomal membrane fractions is supported by other studies indicating an effect of the toxin on neurons. The toxin causes the release of acetylcholine from cholinergic nerve endings (26) and causes neuronal damage (11). However, other studies have suggested that the toxin binds to EC membranes (4, 19). The prototoxin also binds to EC membranes (5) and is able to transiently protect against the biological effects of the toxin. It was concluded therefore that the toxin and prototoxin bind to the same sites on cell

membranes (5, 21). However, the nature of binding sites on EC membranes is not known. An investigation employing enzyme pretreatment for the study of binding of the prototoxin/toxin to EC membranes, similar to that employed for toxin binding to synaptosomes (22) is yet to be performed.

Anti-EBA labeling of the cytoplasm of ECs observed in this study in control and experimental animals has not been reported previously. EBA has been recognized as a membrane protein and specifically localized to luminal EC membranes (16, 26, 31). Anti-EBA does not react with the proteins in the endothelial cytosol fraction in immunoblots (30). However, cytoplasmic immunoreactivity for EBA was reported in Langerhans cells in the skin (30). The difference between EC cytoplasmic staining in this study and previous studies may be due to the method used and the penetration ability of antibodies or tertiary reagents. The labeling of EC cytoplasm seen here suggests a cytoplasmic pool whose function may be to replenish luminal membrane content of EBA. The labeling was not seen in cytoplasmic vesicles; thus an intracellular production pathway involving the Golgi apparatus and vesicular structures is unlikely. The reduced cytoplasmic and luminal membrane labeling seen in experimental animals suggest (a) a reduced EBA production with progressive depletion of the EBA content of the cell, (b) a normal rate of production but with an increased rate of degradation, or (c) some structural alteration in the antigen that prevented/masked its labeling with the anti-EBA. In this study the loss of cytoplasmic labeling seemed to precede the reduction of luminal membrane labeling, suggesting the first mechanism as an explanation for the change seen in experimental material. A reduced production may result from EC toxicity affecting the synthesis of EBA.

Most studies employing the administration of ϵ prototoxin *in vivo* have used an intravenous injection route (4, 5, 21). In the current study the prototoxin was injected into the peritoneal cavity. One explanation for the results seen in this study may be that some of the prototoxin has been activated in the body by proteases (2, 20). The epsilon prototoxin was shown by the immunoperoxidase method to bind to the surface of guinea pig peritoneal macrophages *in vitro* and it was concluded that the cell membrane of these cells contains the sites of binding of the prototoxin and the toxin (6). The epsilon toxin was shown to produce ultrastructural changes and death of guinea pig and rabbit peritoneal macrophages in a dose- and time-dependant manner, but no effect was seen on mouse peritoneal macrophages (6). There is no report in the literature on the interaction between the prototoxin and rat peritoneal macrophages; hence we cannot exclude the presence of prototoxin receptor sites on these cells. One may speculate that in the current study the prototoxin became bound to the rat peritoneal macrophages, was internalized, and through lysosomal degradation was

activated, with limited conversion to the toxin. Absorption of the activated toxin via the peritoneum may have produced the transient effect on brain ECs, causing the mild reversible edema and the reduction in EBA expression seen in the current study. The current model may provide a useful approach to the study of BBB markers, formation and clearance of mild brain edema, and the study of therapeutic agents aiming at amelioration of brain edema.

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Research report

Immunological targeting of the endothelial barrier antigen (EBA) in vivo leads to opening of the blood–brain barrier

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Abstract

The role of the endothelial barrier antigen (EBA) in the blood–brain barrier (BBB) of the rat is not fully understood. Pathological conditions which show BBB disruption and leakage of plasma proteins are associated with reduced EBA expression in brain endothelial cells (ECs). However, it is not known if the reduction in EBA is the primary event or is secondary to protein extravasation. We hypothesized that immunological targeting of EBA in vivo would lead to opening of the BBB. To test this hypothesis, a monoclonal antibody (anti-EBA) was intravenously injected in anaesthetized experimental rats. Control animals received intravenous injections of phosphate-buffered saline (PBS) or non-specific antibodies (anti-human cytokeratin, anti-Salmonella bacterial antigen, or anti-pan endothelial cell antigen). Two groups of rats were used, each included experimental and control animals. The first group was used for immunocytochemical detection of EBA in brain ECs and rat albumin in brain parenchyma. In the second group, the permeability of the BBB to horseradish peroxidase (HRP) was tested. Experimental animals, injected with anti-EBA antibody, showed extensive leakage of HRP and albumin in the grey and white matter of the brain. Immunocytochemistry of experimental brains showed that the intravenously injected anti-EBA became bound to ECs and was detected in tissue sections. Control animals did not show leakage of HRP or albumin, and EBA distribution was normal. This study demonstrated for the first time, that immunological ‘neutralisation’ of EBA leads to opening of the BBB, and provided direct evidence for the importance of EBA in maintaining the integrity of the BBB in the rat. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Cellular and molecular biology

Topic: Blood–brain barrier

Keywords: Blood–brain barrier; Endothelial cell; Endothelial barrier antigen; Immunocytochemistry; Albumin; Horseradish peroxidase

1. Introduction

The endothelial barrier antigen (EBA) is a protein selectively expressed by endothelial cells (ECs) of the rat blood–brain barrier (BBB) [22]. EBA is detectable by immunostaining of tissue sections and immunoblotting of

brain homogenate using a monoclonal antibody (anti-EBA) [18,22]. Since it was reported in 1987, the antibody has been employed in several studies to map out the distribution of EBA in normal tissues of the rat. At regions of the brain known to lack BBB, such as the area postrema, pineal gland, median eminence and choroid plexus, the antigen is absent or weakly expressed in some ECs [20,22]. Body organs, such as the intestine, kidney, liver, thyroid and pancreas, which have no blood–tissue barrier, do not express the antigen [22]. EBA is present in vessels of the optic nerve, retina [11,22], iris [12,20] and spinal cord [19,22,23]. The antigen is also expressed by pia mater vessels [3,4,11,20,22], which show several similarities to brain vessels [1]. ECs of the blood–nerve barrier were reported as reactive to anti-EBA [22] or having variable expression of EBA, with some negative and some weakly

Abbreviations: ABC, avidin–biotin complex; BBB, blood–brain barrier; DAB, 3,3'-diaminobenzidine tetrahydrochloride; DPBS, Dulbecco's phosphate-buffered saline; EAE, experimental allergic encephalomyelitis; ECs, endothelial cells; HRP, horseradish peroxidase; mAb, monoclonal antibody; NHS, normal horse serum; NRS, normal rabbit serum; PBS, phosphate-buffered saline; RECA-1, rat endothelial cell antigen-1; SAL-3, Anti-Salmonella bacterial antigen

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positive vessels [12,18]. Thus, it appears that there is a strong body of evidence that EBA is a 'barrier protein' of neural vessels. However, some reported aspects of the expression of EBA do not seem to be in full agreement with the above evidence. Firstly, in the eye, while the optic nerve, retina and iris have 'tight' vessels, which according to the above interpretation, are expected to express EBA, other areas in the eye with permeable vessels show staining for EBA. Examples are vessels of the choriocapillaris and some vessels in the ciliary body [12]. Secondly, anti-EBA stains some ECs in the spleen, and is expressed by some cells in the skin, tentatively identified as Langerhans cells [22]. Thirdly, during development, EBA is detectable only postnatally [3,20,22] although the BBB of the rat becomes impermeable to proteins prenatally [17]. The exact function of EBA is not known. However, from its predominant distribution in barrier vessels, the earlier interpretation of Sternberger and Sternberger [22] of EBA as a 'barrier protein' is corroborated.

To further elucidate the role of EBA in the BBB, several studies looked at changes in its expression in pathological conditions. In experimental allergic encephalomyelitis (EAE) [23], spinal cord trauma [19] and angiogenesis associated with stab-wound injury of the brain and transplantation [20], a reduction in the expression of EBA in brain ECs was reported. The acute phases of the above pathological conditions are accompanied by opening of the blood–CNS barrier and the development of oedema. Leakage of endogenous albumin in the brain was also described in association with neuronal and astroglial damage induced by the local injection of immunotoxins (Thy-1 antibody and the ribosome-inactivating protein saporin). In the latter model, a reduction in EBA expression by ECs was also reported [10]. During recovery of the BBB in the above experimental conditions, clearance of oedema is paralleled by restoration of EBA expression. In experimentally induced diabetes however, there are no significant changes in the expression of EBA [14]. Thus, most studies on pathological conditions have added support to the putative function of EBA in barrier vessels. However, it is not known from the above studies, if the reduction of EBA expression in ECs is a cause or a consequence of opening of the barrier and the development of oedema. It appears that to date, direct evidence for opening of the barrier in response to the loss of EBA is not available.

EBA has been shown by electron-immunocytochemistry to be located at the luminal membranes of ECs [11,12,20,23]. The luminal membranes are the true blood–brain interface and are accessible to factors circulating in the blood. In our search for an experimental model for direct evidence for opening of the barrier in response to the elimination of EBA as a primary event, we hypothesized that systemic injection of anti-EBA would immunologically target the antigen on EC membranes, and we investigated the effect of this injection on the function of

the BBB. We asked the following questions: (a) would the intravenously injected anti-EBA combine with EBA *in vivo*? (b) Can any such antigen–antibody complex be detected by immunocytochemistry after animal perfusion? (c) Would such antigen targeting lead to opening of the BBB? (d) Would any opening of the BBB be specific to the injection of anti-EBA, or would it be a non-specific response to a circulating foreign antibody?

2. Materials and methods

2.1. Animals, intravenous injection of antibodies and tissue preparation

Twenty five Sprague–Dawley adult male rats (350–380 g) were used in two groups. Group 1 ($n=13$) was used for the immunocytochemical detection of EBA and endogenous albumin. Group 2 ($n=12$) was used for HRP permeability study. Each group included experimental and control animals. Animals were anaesthetized (intraperitoneal injection of pentobarbitone sodium, 60 mg/kg, or Urethane, 1.3 g/kg, Sigma, USA) and the femoral vein exposed. Experimental animals received a femoral vein injection of 20 μ l of a monoclonal antibody (mAb) to EBA (anti-EBA, SMI 71, IgM, Sternberger Monoclonals Inc., USA) in 0.4 ml phosphate-buffered saline (PBS). Control animals received femoral vein injections of either an equal volume of the vehicle ($n=5$), or an equal volume of the vehicle containing one of three control antibodies. These included (a) 20 μ l of a mAb to human cytokeratin 8 (clone 35BH11, IgM, Dako Ltd, USA) [9] ($n=5$). (b) 20 μ l of a mAb to Salmonella bacterial antigen (SAL-3, IgM) [16] (gift from Dr L. K. Ashman, Division of Haematology, IMVS, Adelaide) ($n=5$). (c) 20 μ l of mAb to rat EC-specific antigen (anti-RECA-1), clone HIS52, IgG1, Serotec Inc., Oxford, UK) [6] ($n=4$). Antibodies were obtained as mouse ascites fluid (anti-EBA, 10 mg/ml) or culture supernatant (anti-cytokeratin, 511 μ g/ml; SAL-3, 2 mg/ml and anti-RECA-1, 10–50 μ g/ml). The injection times ranged from 5 to 12 min with a further circulation time of 5 min. The left ventricle was cannulated, the right atrium cut, and the circulation flushed with Dulbecco's phosphate-buffered saline (DPBS, pH 7.4), containing 1% minimum essential medium and gassed with 95% O₂: 5% CO₂, for 30 s, followed by 4% paraformaldehyde fixative in 0.1 M phosphate buffer, pH 7.4, under constant pressure of 90 mm Hg. The rate was adjusted to allow a fast flow for the first 300 ml of fixative, followed by 400 ml at a slow rate, with a total perfusion time of 45 min. The brains were removed, immersed for 3 h in the same fixative, then divided in the median plane. One half of the brain was processed in paraffin wax and 7 μ m sections were cut and collected on coated slides. The other half of the brain was cut into 50 μ m sections in the sagittal plane using a Vibratome.

2.2. Group 1: immunocytochemical study for EBA and endogenous albumin

Immunocytochemistry for the detection of EBA in brain vessels was carried out on experimental ($n=3$) and control ($n=10$) animals in group 1. Free-floating Vibratome sections in wells and paraffin sections on slides were washed in PBS, and the endogenous peroxidase activity was blocked using 0.3% H_2O_2 in distilled water for 30 min. Sections were incubated for 1 h in 10% normal horse serum (NHS) before incubation overnight at 4°C in anti-EBA monoclonal primary antibody diluted 1:3000 in 1% NHS. Sections were incubated in a biotinylated rat-adsorbed horse anti-mouse IgG secondary antibody (Vector Lab. USA) diluted 1:500 in 1% NHS. The secondary antibody was detected using the avidin–biotin peroxidase method (ABC kit, Vector Lab.). The peroxidase reaction product was developed, by incubation in 3,3'-diaminobenzidine tetrahydrochloride (DAB) with nickel enhancement in two stages, using the glucose oxidase method [21].

Vibratome and paraffin sections from brains of experimental and control animals in group 1, injected intravenously with antibodies or PBS, were also immunostained for the detection of endogenous albumin in the parenchyma. Immunostaining for albumin was used as a functional assessment of the BBB, and as an indicator for the presence and distribution of vasogenic cerebral oedema [8]. The sections were treated with H_2O_2 (0.3% in distilled water) to block endogenous peroxidase, then incubated for 1 h in 10% normal rabbit serum (NRS), followed by incubation overnight at 4°C in a goat anti-rat albumin primary antibody (ICN Pharmaceuticals Inc., USA). For Vibratome sections dilutions of 1:10,000 to 1:30,000 and for paraffin sections dilutions of 1:2000 to 1:4000 in 1% NRS were used. A biotinylated rabbit anti-goat IgG secondary antibody (Vector Lab.) was applied at a dilution of 1:500 in 1% NRS. The secondary antibody was detected, using the ABC method, and the peroxidase reaction product was developed as above.

In the immunostaining protocol for EBA and endogenous albumin, brain sections (Vibratome and paraffin) in each staining session were divided into three batches. The first batch was treated with the primary and secondary antibodies, ABC and DAB. In batch two the primary antibody was omitted. In batch three both the primary and secondary antibodies were omitted. Batches two and three acted as internal controls for the staining procedure. After staining, Vibratome sections were collected on coated slides, and air-dried overnight. Vibratome and paraffin sections were dehydrated and coverslipped for light microscopy and photography.

2.3. Group 2: HRP permeability study

The second group of rats was used for the HRP permeability study. Experimental ($n=3$) and control ($n=9$)

animals were injected intraperitoneally with an antihistaminic drug to prevent any allergic reaction to HRP (Promethazine, 1 ml of 1% solution in distilled water/kg) [5,13]. The femoral vein was exposed and antibodies or PBS were injected as above. Following the 5 min circulation time of antibodies or PBS, animals were injected with HRP (type II, Sigma, 200 mg/kg, in 0.4 ml of saline) over a period of 5 min. The tracer was allowed to circulate for a further 15 min. Cardiac perfusion was carried out as above. The brain and liver were removed and 50 μ m Vibratome sections were cut. Sections from the brain and liver were treated with DAB to develop the HRP reaction product with nickel enhancement as above. Sections were collected on coated slides, air-dried overnight, dehydrated and mounted with coverslips.

3. Results

3.1. HRP permeability study

None of the control animals in group 2, injected intravenously with PBS or non-specific antibodies (anti-human cytokeratin, SAL-3, or anti-RECA-1), showed leakage of HRP in the brain. Light microscopy of Vibratome sections showed clear parenchyma throughout the brain (Fig. 1A–D). Occasional RBCs seen in the lumina of some vessels showed dark staining due to their content of endogenous peroxidase, indicating the successful development of peroxidase reaction product. Vibratome sections from the liver of control animals injected with HRP showed the tracer in the liver parenchyma (Fig. 1E), indicating the successful intravenous injection of the tracer and its circulation throughout the body. On the other hand, experimental animals injected with anti-EBA *in vivo* showed extensive leakage of HRP in the brain, indicating a severely compromised BBB. Vibratome sections showed widespread black HRP reaction product in the cerebrum, diencephalon, brainstem (Fig. 1F) and cerebellum. In the white matter, the distribution of the tracer showed a linear pattern, being parallel to nerve fibre tracts (Fig. 2A). In the grey matter the pattern was multifocal with circumferential areas containing HRP, varying in size (Fig. 2B) and superimposed on a diffuse spread of the tracer in the parenchyma. The lumina of blood vessels in the brain and liver sinusoids were free of HRP due to flushing of the circulation during perfusion fixation (Figs. 1 and 2). HRP was taken up by many neuronal cell bodies and neuronal processes. Macrophages occasionally seen in the lumina of blood vessels contained granules of HRP reaction product.

3.2. Immunocytochemical study for endogenous albumin and EBA

Control animals which had received an intravenous injection of PBS or non-specific antibodies (anti-human

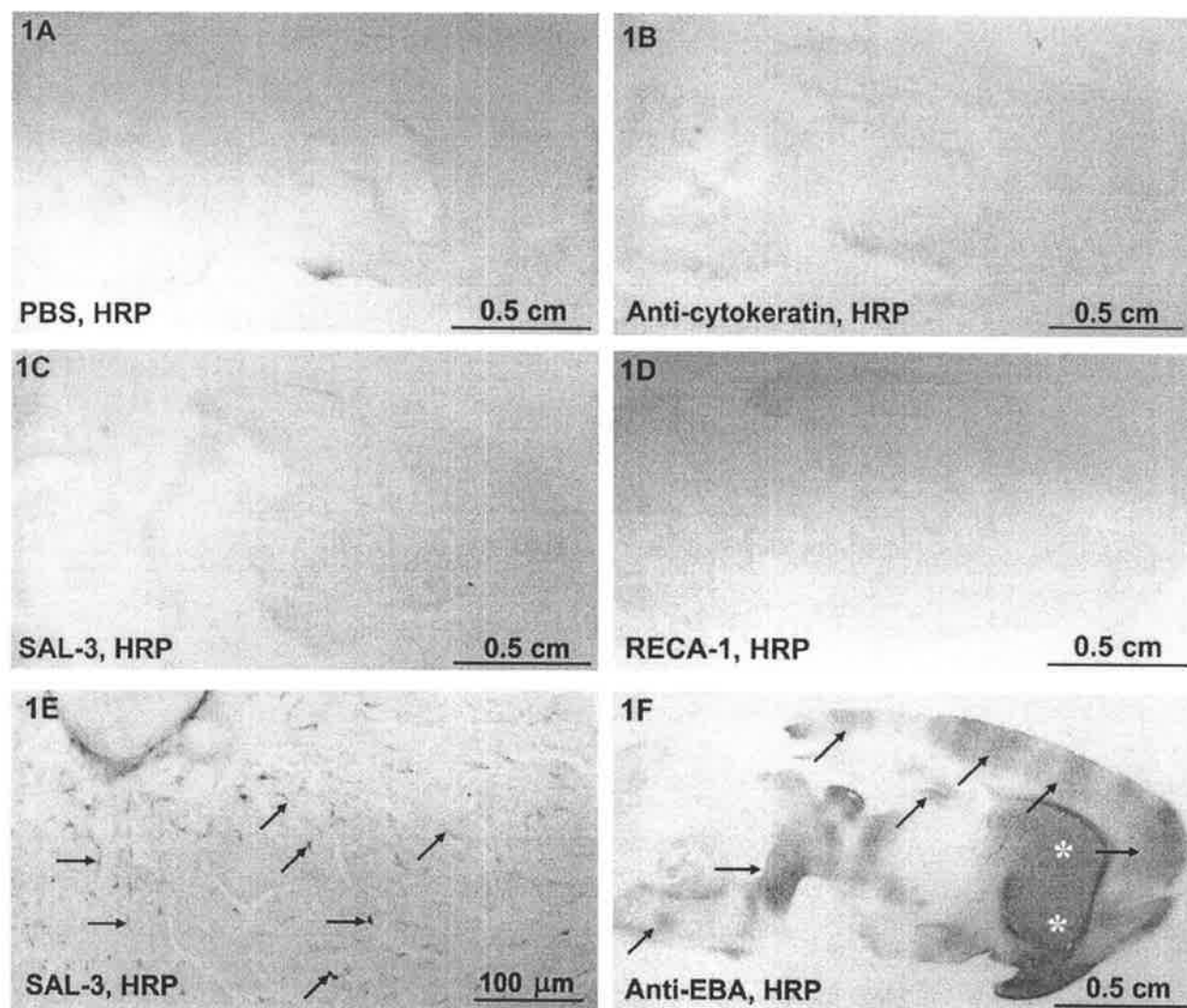


Fig. 1. Light micrographs of sagittal Vibratome sections from the brains of control and experimental animals stained for peroxidase cytochemistry. Control animals injected intravenously with HRP after the intravenous injection of PBS (A), anti-human cytokeratin (B), SAL-3 (C) and RECA-1 (D) show no leakage of HRP in brain parenchyma. (E) shows a Vibratome section of liver from a control animal injected intravenously with SAL-3, followed by HRP. Although the brain of this animal did not show any HRP in the parenchyma (C) the liver shows HRP reaction product (arrows) indicating the successful administration of HRP into the circulation. (F) shows a Vibratome section from an experimental animal injected intravenously with anti-EBA followed by HRP. HRP reaction product (arrows) is seen throughout the brain and particularly in the region of projection fibres in the forebrain (asterisks).

cytokeratin, SAL-3 and anti-RECA-1) showed a faint diffuse immunoreactivity to albumin throughout the grey and white matter of cerebrum, cerebellum and brainstem (Fig. 3A–D). Some areas in the brain in control animals showed a moderate degree of immunoreactivity to albumin, more than that seen generally in the brain. These areas included the olfactory tract, hippocampus, periventricular zone, rostral diencephalon, superior cerebellar peduncle and multiple foci in the brainstem. The immunoreactivity to albumin was not seen in any of the batches of sections in which the primary antibody to albumin or the primary and secondary antibodies were omitted. Experimental animals injected with anti-EBA, on the other

hand, showed widespread and strong immunoreactivity to albumin in the grey and white matter, in paraffin and Vibratome sections (Figs. 3E and F). The walls of many vessels were highlighted suggesting the presence of albumin intramurally. In the grey matter the distribution of albumin showed circular or oval areas of high immunoreactivity (Fig. 4A). In the white matter the distribution was linear and predominantly followed fibre tracts (Fig. 4B).

Immunocytochemical staining for EBA was carried out on Vibratome and paraffin sections from brains of experimental and control animals. Control animals (injected in vivo with PBS, anti-human cytokeratin, SAL-3 and

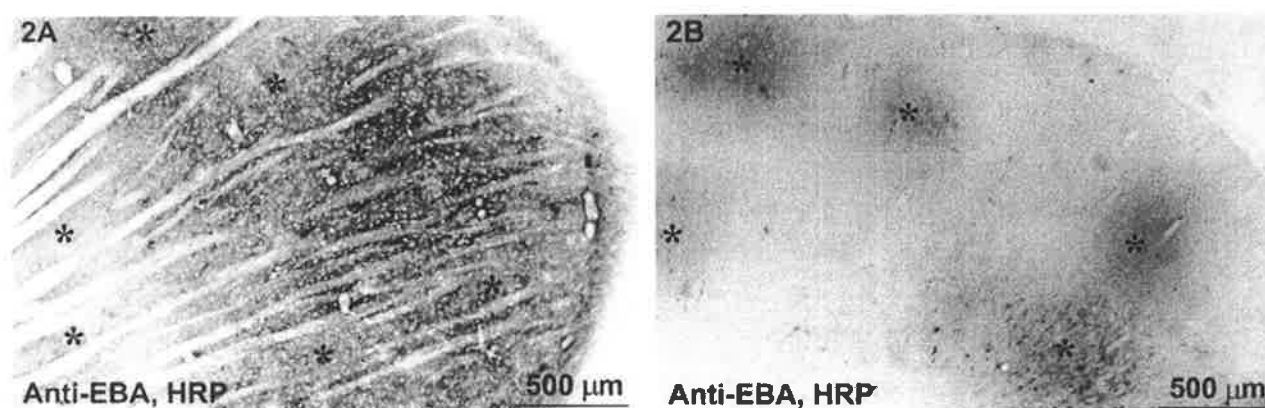


Fig. 2. Higher magnification light micrographs of sagittal Vibratome sections from the brain of an experimental animal injected intravenously with anti-EBA, followed by HRP. HRP is seen in brain parenchyma. In the white matter (A) the distribution of the tracer is linear (asterisks) and runs parallel to nerve fibre tracts. In the grey matter (B) the distribution of the tracers is circumferential and multifocal (asterisks).

anti-RECA-1) showed strong expression of EBA in ECs in batch one exposed to the full staining protocol (Fig. 5A–D). Blood vessels of various sizes were strongly immunoreactive for EBA in the grey and white matter. Omission of the primary antibody (batch two) or primary and secondary antibodies (batch three) resulted in the loss of all immunoreactivity for EBA.

Experimental animals injected *in vivo* with anti-EBA antibody showed two interesting results. Firstly, Vibratome sections in batch two, which were not treated with primary antibody in the staining procedure showed moderate immunoreactivity for EBA in brain ECs in the majority of vessels (Fig. 5E) in the cerebrum, cerebellum, diencephalon and brainstem. In a minority of vessels the staining was either absent or weak (Fig. 5E). In batch one, where Vibratome sections were treated with the primary antibody in the staining procedure the immunoreactivity of brain ECs was much higher than in batch two, and almost all vessels were strongly stained for EBA (Fig. 5F). In batch three of sections, which were not exposed to the primary or the secondary antibodies, no immunoreactivity was seen. The second interesting observation was seen in paraffin sections. Unlike Vibratome sections in batch two, paraffin sections in batch two, which were not exposed to the primary antibody (anti-EBA) in the staining procedure did not show any staining. These sections were obtained from the same animals from which Vibratome sections in batch two showed immunoreactivity for EBA (Fig. 5E).

4. Discussion

Four original findings are reported in this study. Firstly, the *in vivo* administration of antibody to EBA (anti-EBA) resulted in an increased permeability of brain microvessels to endogenous and exogenous tracers. Secondly, the opening of the BBB was not seen in control animals injected with PBS or non-specific antibodies. Thirdly, the

intravenously injected anti-EBA became bound to brain ECs and fourthly, the anti-EBA was detected in Vibratome sections by immunocytochemistry after tissue fixation.

This study showed that immunological targeting of EBA leads to opening of the BBB and vascular leakage of HRP and endogenous albumin. EBA appears to uphold the BBB integrity in the rat. Previous studies on EAE [23] and stab-wound injury of the brain [20] showed opening of the BBB and the formation of cerebral oedema in the acute phases. Extravasation of plasma proteins was also reported in compression injury of the spinal cord [7]. A reduction in the expression of EBA was reported in the above experimental models [19,20,23]. Taking these results with the current findings it is feasible to hypothesize that the reduction in EBA in previous studies preceded the occurrence of oedema. This hypothesis has a wide implication. The implication being that pathological lesions in the rat CNS, which produce opening of the blood–CNS barrier and the occurrence of oedema, are doing so via a ‘final common pathway’ that affects EC biology leading to loss of EBA as the primary event. However, such speculation if applicable to the rat, would not apply to other species including human, which show oedema in similar pathologies. Anti-EBA is specific to the rat [22], and we found that anti-EBA does not stain vessels in human or sheep brains (unpublished observations). It is tempting to speculate that oedema formation in species other than the rat may involve a reduction in a homologue of EBA or of a corresponding EC protein similar in function to EBA.

The patterns of spread of albumin and HRP in the grey and white matter of brains of experimental animals showed some similarities. In the white matter the tracers showed linear distribution and predominantly followed a path parallel to fibre tracts. The extracellular space in the white matter is relatively simple and follows nerve fibres. It therefore may provide a low resistance pathway for the tracers. In the grey matter the tracers showed a predominantly circumferential spread. The cytoarchitecture of

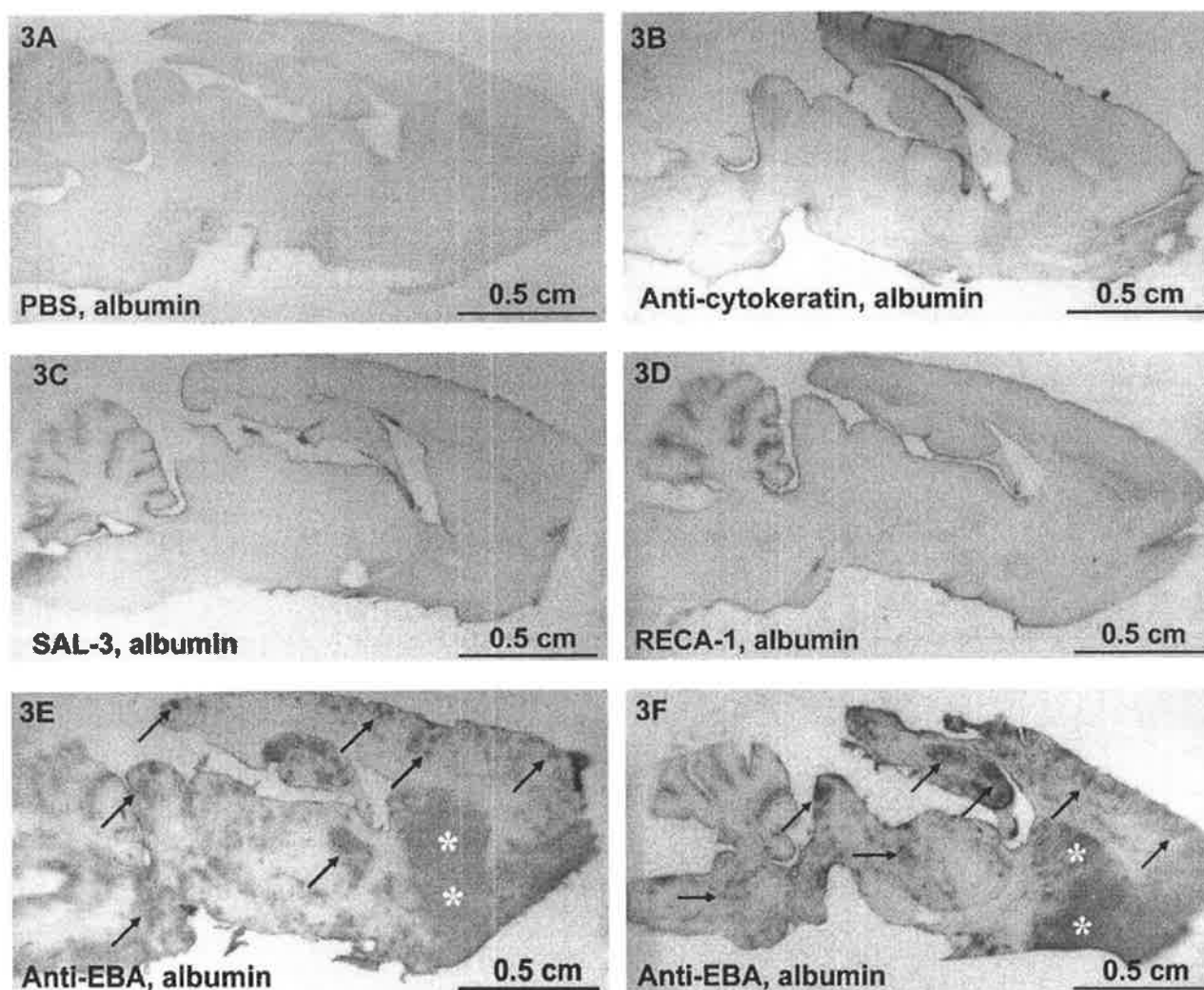


Fig. 3. Light micrographs of sagittal Vibratome sections from brains of control and experimental animals immunostained for endogenous albumin. Faint immunoreactivity for albumin is seen in control animals injected intravenously with PBS (A), anti-human cytokeratin (B), SAL-3 (C) and anti-RECA-1 (D). (E and F) show sections from two experimental animals injected intravenously with anti-EBA. Strong albumin immunoreactivity is seen throughout the brain in the grey matter (arrows) and projection tracts of the forebrain (asterisks).

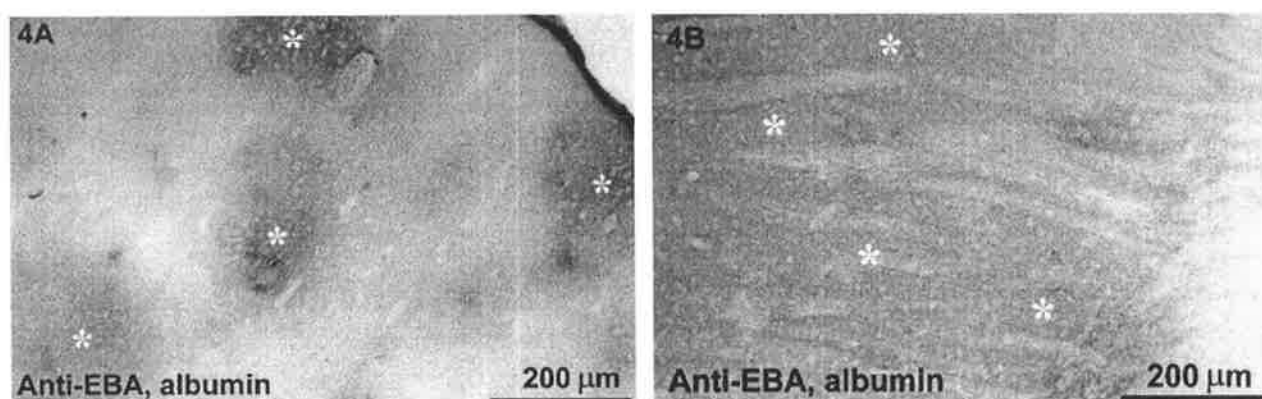


Fig. 4. Higher magnification light micrographs of a Vibratome section from the brain of an experimental animal injected intravenously with anti-EBA, showing strong immunoreactivity for endogenous albumin in the parenchyma. In the grey matter (A) the distribution is multifocal with circumferential areas of spread (asterisks). In the white matter (B) the distribution is linear (asterisks) and follows the direction of fibre tracts.

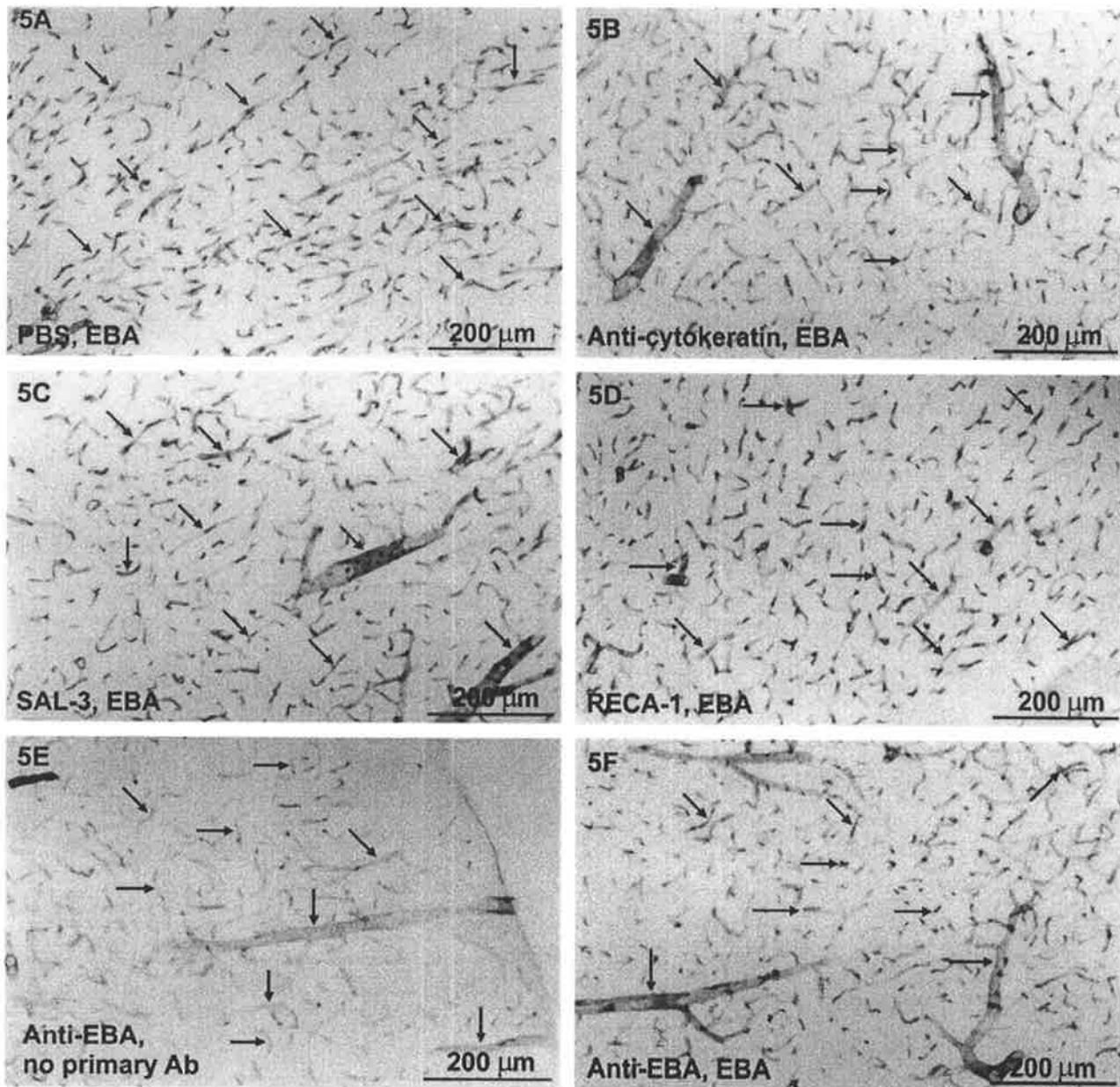


Fig. 5. Sagittal Vibratome sections from the brains of control (A–D) and experimental (E–F) animals, immunostained for EBA. Control animals, injected intravenously with PBS (A), anti-human cytokeratin (B), SAL-3 (C) and anti-RECA-1 (D), show strong immunoreactivity for EBA. Microvessels of various sizes (arrows) are immunoreactive for EBA. The immunoreactivity seen in control animals (A–D) is similar to that seen in the experimental animal (F) injected intravenously with anti-EBA. (E) shows a Vibratome section from an experimental animal not treated with the primary anti-EBA in the staining procedure. Brain vessels in this section show moderate immunoreactivity for EBA. This suggests that the anti-EBA injected in vivo became bound to ECs and replaced the step of primary antibody in the staining procedure.

the grey matter is more complex than that of the white matter, with interdigitating neuronal processes and glial cells. Consequently, the extracellular space in the grey matter is sinuous and complex, offering a high resistance pathway for the spread of tracers. The above interpretation of the spread of tracers applies to routes in the extracellular space, but does not explain the permeability of the tracers through the EC barrier. Although HRP is not a natural

blood constituent, it has been extensively used in permeability studies since its introduction as a tracer for the study of the BBB [2]. Endogenous albumin is a natural component of the plasma and has been used to detect tissue oedema [8,24]. HRP has a molecular weight of 40 kDa, while albumin is a 62 kDa protein. In cryoinjury of the brain, the BBB is compromised and albumin–gold complex passes through opened intercellular junctions

[24]. In osmotic opening of the BBB, albumin–gold complex traverses the ECs via intercellular junctions, EC gaps, the cytoplasm of injured cells and through denuded segments of the lining [24]. Transport of albumin–gold complex via pinocytotic vesicles in this model of osmotic opening is negligible [24]. Previous studies on the mode of leakage of HRP through the BBB showed that the tracer traverses the EC barrier principally via increased vesicular transport, in various models such as heat stress [25] and spinal cord contusion [15]. It appears that the route of leakage of tracers varies according to the pathology affecting the CNS. The current model of anti-EBA injection to ‘neutralize’ EBA in vivo showed opening of the BBB to both tracers. Thus it appears that interfering with the function of EBA leads to opening of one or more routes of leakage of macromolecules. An electron microscopic study on the route of leakage of the above two tracers in this model is being conducted in our laboratory.

In the current study experimental animals were injected intravenously with anti-EBA. Vibratome sections from these animals, which were not exposed to the primary antibody in the staining procedure, showed immunoreactivity of brain vessels for EBA, indicating that the systemically injected anti-EBA became localized to ECs, and acted as a substitute for the exposure of tissues to the primary antibody in the staining procedure. Presumably anti-EBA identified EBA on the EC membranes and formed an ‘antigen–antibody complex’. This complex remained stable for the duration of the experiment, and tolerated 45 min of flushing of the circulation, initially with DPBS then the fixative, using fast and slow perfusion. However, the immunoreactivity for EBA seen in the Vibratome sections was moderate compared with the strong immunoreactivity seen in sections which were treated with anti-EBA as primary antibody in the staining procedure. This may be explained by an insufficient concentration of the anti-EBA injected in vivo to saturate all EBA sites in the cerebral vascular bed. The higher staining intensity in the sections exposed to the primary antibody therefore may be due to the labelling of additional EBA sites during the staining procedure. Exposure of the sections to anti-EBA overnight in the staining procedure provides enough time and antibody availability to label a greater number of EBA molecules in brain vessels.

As stated above, Vibratome sections from experimental animals, injected with anti-EBA in vivo showed immunoreactivity for EBA, despite the omission of anti-EBA as the primary antibody in the staining procedure. Unexpectedly, paraffin sections obtained from the other half of the brains from the same animals and subjected to the same staining protocol, did not show any immunoreactivity for EBA. The difference between the results of Vibratome sections and paraffin sections must be related to the method of processing. Vibratome sections were stained with secondary antibodies and DAB, then dehydrated in alcohols, cleared in HistoClear, and mounted with cover-

slips. On the other hand, brain tissue (which contained the antigen–antibody complex) was processed in paraffin wax before sectioning and immunostaining with the secondary antibody and DAB. Thus the lack of immunoreactivity seen in paraffin sections must be due to the heat employed in paraffin embedding rather than the dehydration and clearing of sections, since the solutions used were identical for paraffin sections and Vibratome sections. This suggests that the antigen–antibody complex which was formed in vivo was unstable at the temperature (58°C) required for paraffin embedding. Another possibility is that the antibody itself was denatured or altered by the temperature employed.

The choice of control antibodies in this study was an important consideration. Firstly, anti-human cytokeratin [9], anti-Salmonella bacterial antigen (SAL-3) [16] and anti-EC-specific antigen (RECA-1) [6] are similar to anti-EBA, in that all four are monoclonal mouse antibodies. They are supplied as ascites fluid or tissue culture supernatant. All are IgM molecules, except anti-RECA-1, which is an IgG1. However, in contrast to anti-EBA, the anti-human cytokeratin and SAL-3 are not EC-specific antibodies. Therefore, these two control antibodies were used to test the effect of circulating foreign antibodies on the BBB. Anti-RECA-1 is a pan-EC marker [6], and reacts with all ECs of the rat. Thus anti-RECA-1 is not a BBB-specific antibody, and was used here to prove that any effect on BBB produced by the injection of anti-EBA is not due to a non-specific effect of antibodies to ECs at large. Experimental animals injected with anti-EBA showed extensive leakage of HRP. None of the control antibodies showed any HRP leakage. Therefore, the results prove that the leakage was neither an allergic response to exposure of the immune system to foreign proteins, nor to the injection of antibodies to ECs in general, but is likely to be due to a specific effect of anti-EBA on the BBB.

Anti-RECA-1 recognises an EC surface molecule [6]. Intravenous injection of anti-RECA-1 in rats leads to its localization to ECs, presumably due to the formation of antigen–antibody complex [6]. Anti-EBA labels luminal membranes of ECs of brain vessels [20,23]. Since EC luminal membranes are the true blood–brain interface, we anticipated that when anti-EBA is administered into the circulation in experimental animals it would have access to EBA on EC membranes. Experimental animals injected with anti-EBA showed opening of the BBB to endogenous and exogenous tracers. However, control animals injected with anti-RECA-1 did not show any BBB opening. This proves that opening of the barrier in experimental animals was not due to cell membrane ‘perturbation’ as a result of the formation of antigen–antibody complex, but is likely to be a specific effect of anti-EBA on the BBB. It has been suggested that EBA is a receptor protein, different from other EC proteins such as factor VIII and transferrin receptor [22]. The mechanisms by which the anti-EBA injected in vivo ‘neutralized’ EBA molecules on ECs,

interfered with the function of EBA, and possible signalling mechanisms require further investigation.

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