

Review

Structural pathways for macromolecular and cellular transport across the blood-brain barrier during inflammatory conditions. Review

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Summary. This review presents an overview of the highlights of major concepts involving the anatomical routes for the transport of macromolecules and the transmigration of cellular elements across the blood-brain barrier (BBB) during inflammation. The particular focus will include inflammatory leukocytes, neoplastic cells and pathogenic microorganisms including specific types of viruses, bacteria and yeasts. The experimental animal models presented here have been employed successfully by the authors in several independent experiments during the past twenty-five years for investigations of pathologic alterations of the BBB after a variety of experimentally induced injuries and inflammatory conditions in mammalian and non-mammalian animal species. The initial descriptions of endothelial cell (EC) vesicles or caveolae serving as mini-transporters of fluid substances essentially served as a springboard for many subsequent discoveries during the past half century related to mechanisms of uptake of materials into ECs and whether or not pinocytosis is related to the transport of these materials across EC barriers under normal physiologic conditions and after tissue injury.

In the mid-1970's, the authors of this review independently applied morphologic techniques (transmission electron microscopy-TEM), in conjunction with the plant protein tracer horseradish peroxidase (HRP) to investigate macromolecular transport structures that increased after the brain and spinal cord had been subjected to a variety of injuries. Based on morphologic evidence from these studies of BBB injury, the authors elaborated a unique EC system of modified caveolae that purportedly fused together forming transendothelial cell channels, and later similar EC

profiles defined as vesiculo-canalicular or vesiculo-tubular structures (VTS, Lossinsky, et al., 1999). These EC structures were observed in association with increased BBB permeability of tracers including exogenously injected HRP, normally excluded from the intercellular milieu of the CNS. Subsequent studies of non-BBB-type tumor ECs determined that the EC VTS and other vesicular structures were defined by others as vesiculo-vacuolar organelles (VVOs, Kohn et al., 1992; Dvorak et al., 1996). Collectively, these structures appear to represent a type of anatomical gateway to the CNS likely serving as conduits. However, these CNS conduits become patent only in damaged ECs for the passage of macromolecules, and purportedly for inflammatory and neoplastic cells as well (Lossinsky et al., 1999). In this review, we focus attention on the similarities and differences between caveolae, fused racemic vesicular bundles, endothelial tubules and channels (VTS and the VVOs) that are manifest in normal, non-BBB-type blood vessels, and in the BBB after injury. This review will present evidence that the previous studies by the authors and other researchers established a framework for subsequent transmission (TEM), scanning (SEM) and high-voltage electron microscopic (HVEM) investigations concerning ultrastructural, ultracytochemical and immunultrastructural alterations of the cerebral ECs and the

Abbreviations. AcP: Acid Phosphatase; AP: Alkaline Phosphatase; BBB: Blood-brain barrier; BMVEC: Brain microvessel endothelial cells grown in culture; CNS: Central nervous system; EAE: Experimental allergic encephalomyelitis; EC(s): Endothelial cell(s); HEV: High endothelial cell venules; HVEM: High-voltage electron microscope; ICAM-1: Intracellular adhesion molecule-1 (CD54); LM: Light microscope (y); MS: Multiple Sclerosis; PECAM-1: Platelet endothelial cell adhesion molecule-1 (CD31); SEM: Scanning electron microscope (y); TEM: Transmission electron microscope (y); VCAM-1: Vascular cell adhesion molecule-1 (CD106); VVO: EC vesiculo-vacuolar organelles; VTS: EC vesiculo-tubular system also includes modified caveolae, racemic and fused vesiculo-tubular structures.

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mechanisms of the BBB transport that occurs after CNS injury.

This review is not intended to include all of the many observations that might be included in a general historical overview of the development of the EC channel hypothesis, but it will discuss several of the major contributions. We have attempted to present some of the structural evidence that supports our early contributions and those made by other investigators by highlighting major features of these EC structures that are manifest in the injured BBB. We have focused on currently established concepts and principles related to mechanisms for the transendothelial transport of macromolecules after CNS injury and also offer a critical appraisal of some of this literature. Finally, we describe more recent concepts of transBBB avenues for viruses, including HIV-1, bacterial and mycotic organisms, as well as inflammatory and neoplastic cell adhesion and migration across the injured mammalian BBB. Data from studies of EC-related adhesion molecules, both from the literature and from the author's experimental results and observations made in other laboratories, as well as from personal communications underscore the importance of the adhesion molecules in facilitating the movement of leukocytic, neoplastic cell and human pathogens across the BBB during inflammatory and neoplastic events.

Exciting, ongoing clinical trials are addressing possible therapeutic intervention in neuroinflammatory diseases, including multiple sclerosis, by blocking certain glycoprotein adhesion molecules before cells have the ability to adhere to the ECs and migrate across the BBB. Approaches whereby inflammation may be reduced or arrested using anti-adhesion molecules, by restructuring EC cytoskeletal, filamentous proteins, as well as remodeling cholesterol components of the modified VTS are discussed in the context of developing future therapies for BBB injury and inflammation. Understanding new concepts about the mechanism(s) by which inflammatory cells and a variety of pathogenic microorganisms are transported across the BBB can be expected to advance our understanding of fundamental disease processes. Taken together, the literature and the author's experiences during the past quarter of a century, will hopefully provide new clues related to the mechanisms of transendothelial cell adhesion and emigration across the injured BBB, issues that have been receiving considerable attention in the clinical arena. Learning how to chemically modulate the opening and/or closure of EC VTS and VVO structural pathways, or junctional complexes prior to cellular or microorganism adhesion and breaching the BBB presents challenging new questions in modern medicine. Future studies will be critically important for the development of therapeutic intervention in several human afflictions including traumatic brain and spinal cord injuries, stroke, cancer, multiple sclerosis and conditions where the immune system may be compromised including HIV infection, infantile and adult meningitis.

Key words: Leukocyte migration, Blood-brain barrier, Endothelial cells, Vesiculo-canalicular structures, VTS, Vesiculo-vacuolar organelles, VVOs horseradish peroxidase

Major themes addressed in this review:

1. A brief historical encapsulation of the structure and function of the normal ECs and the normal mammalian BBB.
2. The BBB under pathologic conditions and the development of specialized EC vesicles, caveolae, vesiculo-tubular and racemic structures, EC channels, the VTS, VVOs and EC junctional complexes.
3. Endothelial transport during ontogeny of the BBB.
4. Leukocyte passage across EC barriers in non-BBB-type and BBB-type vessels.
5. Integrins, selectins and adhesion molecules play key roles in regulating leukocytes and tumor cells to chemically sniff, tether and adhere to, and finally penetrate the VTS/VVOs and/or junctional complexes during inflammatory events.
6. Cell culture paradigms based on cellular and molecular biology of the human BBB are powerful adjunct tools to assess how the HIV-1 virus, bacteria and mycotic organisms enter the human CNS.
7. Studies utilizing experimental models of autoimmune disease have contributed to our understanding of the mechanisms of leukocyte recognition of targeted blood vessels.
8. The Cytoskeletal infrastructure plays an important role in modulating the entrance of leukocytes and pathogens into the ECs during inflammatory BBB injury and inflammation.
9. Conclusions and future perspectives.

A brief historical encapsulation of the structure and function of the normal ECs and the normal mammalian BBB

It is generally accepted that the homeostatic environment of the mammalian brain and spinal cord is strictly regulated by morphologic, physiologic and pharmacologic barriers between the nervous tissue and systemic circulation. Considering the morphologic barrier, which is essentially the main focus of this review, the reader is referred to reports by some of the pioneers of the normal and pathological features of the blood-brain barrier (BBB)¹ (Brightman and Reese, 1969; Brightman et al., 1975; Peters et al., 1976; Shivers, 1979a). Ultrastructural studies of the BBB have shown that the restrictiveness of the angioarchitecture of the CNS can be attributed primarily to: 1) tight, belt-like, occluding junctional complexes that fasten together

¹Throughout this review, a distinction between the blood-brain barrier (BBB) and blood-spinal barrier will not be made, and the single abbreviation BBB will refer exclusively to vasculature of the CNS.

adjacent EC membranes (Brightman and Reese, 1969) and 2) a minimal expression of EC vesicular transport activity (Reese and Karnovsky, 1967; Brightman and Reese, 1969; Peters et al., 1976). The BBB normally permits entry of water and ions, small lipophilic molecules and a limited number of nutrients transported via receptor-mediated transcytosis (glucose, some amino acids, heparin and transferrin (Rapoport, 1976; Broadwell et al., 1996; Drews, 1998)). Under normal physiological conditions in the CNS, plasma-borne macromolecules and most cellular elements are restricted from crossing the BBB because it is composed of continuous-type ECs. If one excludes the normally "leaky" circumventricular organs, including the choroid plexus, nucleus infundibularis, the area postrema, pineal gland, neurohypophysis and others, the normal CNS-type blood vessel ECs prevent intravenously injected particulate materials including protein tracers like HRP, ferritin, colloidal carbon ink or gold particles, as well as cellular elements including erythrocytes, leukocytes, platelets, viruses and larger microorganisms from entering the neuropil side of the BBB (Reese and Karnovsky, 1967; Brightman, 1989). Mechanisms controlling the movement of macromolecules into the CNS include non-specific fluid-phase endocytosis and receptor-mediated endocytosis via clathrin-coated membrane invaginations (pits) for certain molecules including insulin, ferritin, glucose and others (Ghitescu et al., 1986; Pino, 1987a; Cornford et al., 1993, 1994; Vorbrodt et al., 1994; Broadwell et al., 1996; Stewart, 2000). BBB-type vessels of the CNS represent a highly selective filtration system which thereby imparts a privileged environment to the CNS.

The concept of dynamic EC vesicles which could form flask shapes with elongated necks was presented by Palade and Burns (1968) who then proposed such vesicles as part of a dynamic system of invaginating endothelial cell vesicles- or pinocytosis². Soon, other investigators employed three-dimensional studies that revealed a pattern of complex interconnecting vesicular and vacuolar networks (Hashimoto et al., 1974 and others), that appeared as grape-like clusters or a series of vesicles fused together to form racemic structures (Bungaard et al., 1979; Frøkjær-Jensen, 1980, 1991; Bundgaard, 1987; Wagner and Chen, 1991). As we will discuss below, single and modified endothelial vesicles appear to exist under normal physiological conditions and seem to form more elaborate network systems after tissue injury, especially during inflammatory conditions of the CNS.

The BBB under pathologic conditions and the development of specialized EC vesicles, caveolae, vesiculo-tubular and racemic structures, EC channels, the VTS, VVOs and EC junctional complexes

The literature over the past forty years has featured an overwhelming number of experimental animal models in which the BBB responds to a variety of

factors that can alter the normal structural and functional behavior of the barrier. It is well known that after the CNS is subjected to an injurious insult, there is a remarkable and immediate change in the physiological and structural features exhibited by the BBB. Using microscopic techniques after injury to the CNS, there is an increase in the permeability of the BBB (Brightman, 1989). If one injects protein tracers into the veins of an animal subjected to cold lesion injury of the brain, the tracer will be observed leaking profusely through the vessel walls around the site of injury into the surrounding extracellular space and into damaged cellular compartments (Klatzo et al., 1967, 1980; Cancilla et al., 1979; Wolman et al., 1981; Vorbrodt et al., 1985). Inflammatory conditions of the BBB include autoimmune conditions, traumatic injury, exposure to various toxic or hypertonic compounds, infections by various microorganisms to name only a few, after which the BBB becomes more permeable to macromolecules such as HRP (Westergaard and Brightman, 1973; Beggs and Waggner, 1976; Westergaard et al., 1976, 1977; Sasaki et al., 1977; Garcia et al., 1978, 1981; Lossinsky et al., 1979, 1981, 1983, 1989a,b, 1995; Petito, 1979; Shivers, 1979a,b, 1980; Houthoff et al., 1982; Petito et al., 1982; Tagami et al., 1983; Wilmes et al., 1983; Wisniewski et al., 1983; Farrell and Shivers, 1984; Shivers et al., 1984a; Simmons et al., 1987; Brightman, 1989; Nag, 1990; Butter et al., 1991; Pluta et al., 1991; Wijsman and Shivers, 1993; Hirano et al., 1994). In many models of CNS injury, the bulk of the earlier literature demonstrated ultrastructurally that the vesicles of the ECs could shuttle carbon, ferritin, lanthanum, and HRP protein tracers across the BBB from the blood facing luminal side of the EC to the abluminal EC surface (Majno, 1965; Farrell and Shivers, 1984) (Figs. 1-3).

Brain microvessel EC junctional complexes have been suggested to serve as an important pathway for transport of macromolecules, fluids and also cellular components across the altered BBB. As mentioned above, several important studies indicated that EC junctional complexes of the CNS are composed of tight junctions or zonulae occludentes (Reese and Karnovsky, 1967; Brightman et al., 1970; Peters et al., 1976; Shivers, 1979a; Farrell and Shivers, 1984; Shivers et al., 1984b; Sedlakova et al., 1999). Under some conditions such as exposure to hypertonic saccharides (Rapoport et al., 1972; Brightman et al., 1973; Rapoport, 1976) and to a variety of disease states in the CNS (Hirano et al., 1994), these junctions have been suggested to open momentarily. The early TEM studies of Brightman et al., (1973) demonstrated HRP reaction product within the junctional complexes that were presumed altered (shrinkage-induced separation) by the hypertonic solution. Our (ASL) own studies with SEM of topographic details of the vascular bed following intra-

²Pinocytosis: The Greek term for drinking of fluids and a depiction of a mechanism by which fluid material can enter cells (Lewis, 1931; Palade, 1953).

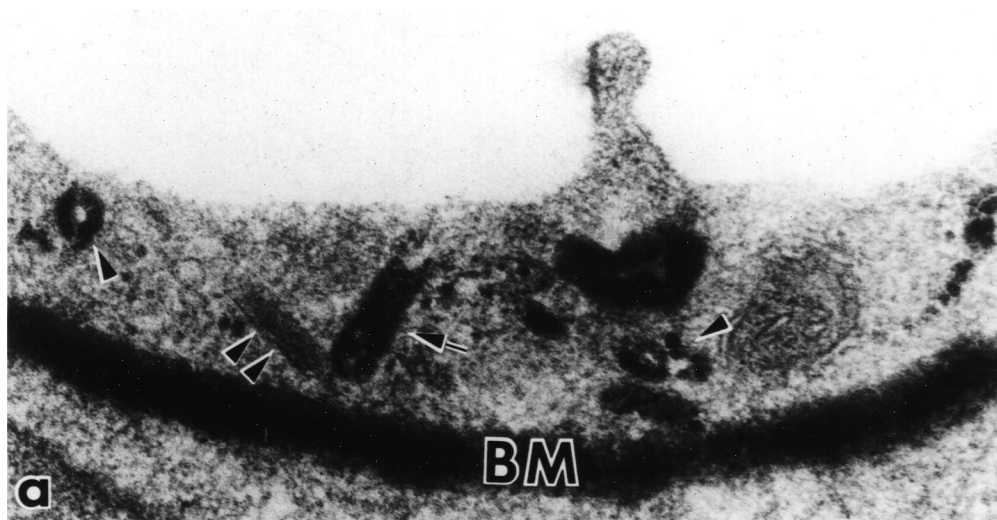
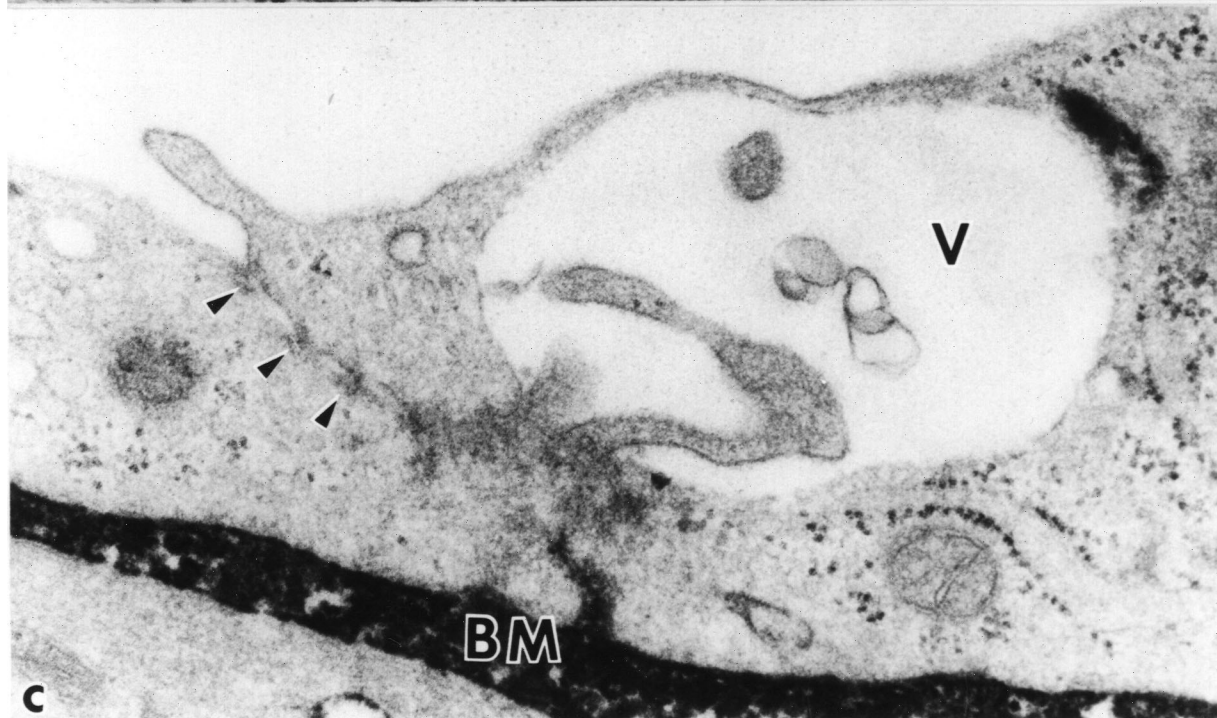
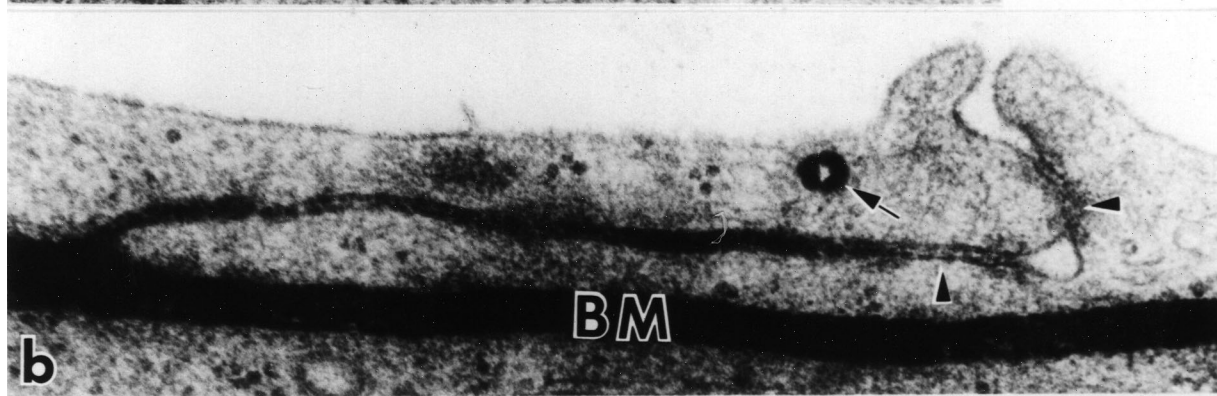


Fig. 1. Adult mouse brain trauma showing increased HRP reaction product (black color) within EC VTS profiles (black arrow), the basement membrane (BM) and within portions of the EC junctional complexes in **b** and **c**. A large parajunctional vacuole (v) is shown in **c**. x 50,000. Reprinted from Lossinsky et al., (1989a), *Acta Neuropathol.* 78, 359-371 with permission from Springer-Verlag Co.



arterial injections of hypertonic saccharides also depicted elongated tubular structures, in addition to patent junctional complexes (Lossinsky et al., 1995a). Later models of CNS injury in which hypertonic solutions were intravenously administered suggested that EC junctional complexes in the brain pulled open due to osmotic shrinkage of the adjacent ECs (Rapoport et al., 1972; Brightman et al., 1973; Rapoport, 1976; Lossinsky et al., 1995a). The junctional perturbation in the BBB is apparently a temporal event that repairs itself after the injury (Rapoport, 1976).

Support for a paracellular pathway for molecular and fluid extravasation across the BBB has not been universal. Notably, all the published reports of investigations of the BBB from both normal and injured central nervous tissue authored by Shivers and his co-workers have provided clear evidence that breaches in brain microvessel endothelial permeability are not a result of disruption of the integrity of interendothelial cell tight junctions (Kenny and Shivers, 1974; Shivers, 1979a,b, 1980; Shivers and Harris, 1984; Shivers et al., 1984a,b, 1987, 1988a; Wijsman and Shivers, 1993; Sedlakova et al., 1999). Instead, strong evidence has been substantiated by these studies for a transcellular conduit system that is likely responsible for extravasation of tracer molecules such as HRP, ferritin etc, in studies of BBB dysfunction (Shivers, 1979a,b, 1980; Shivers and Harris, 1984; Shivers et al., 1984a,b, 1987, 1988a; Wijsman and Shivers, 1993; Sedlakova et al., 1999). Additional support for a vesiculo-tubular route for transendothelial molecular traffic is contained in reports by Hansson et al., 1975; Beggs and Waggener, 1976; Westergaard et al., 1976; Westergaard, 1977; Povlishock et al., 1978; Lossinsky et al., 1979, 1981, 1983; Westergaard, 1980; Coomber and Stewart, 1986;

Lossinsky and Shivers, 2003.

In 1979, one of the authors of this review (ASL) published with others an ultrastructural description of EC tubular structures in ECs from gerbil brains subjected to regional ischemia (Lossinsky et al., 1979). During the same year, the other author of this review (RRS) published similar findings from studies of the effects of hypertonic saccharide solutions on the BBB of the lizard (Shivers, 1979a,b). HRP, like Evans Blue dye chemically links to blood plasma albumin after intravenous injection (Tatton and Crapper, 1970). Because the complex formed by tracer-DAB-osmium reaction product enables TEM visualization of the HRP within numerous vesicles and tubular forms and also within the basement membranes of capillaries and arterioles, it was concluded that the tracer was actively transported across the EC by vesicles fused together to form elongated transendothelial channel-like structures (Lossinsky et al., 1979; Shivers, 1979a,b; Farrell and Shivers, 1984). These elongated complexes of vesicles were later described as endothelial channel-like pores, and were observed in the normal and injured vasculature outside the CNS, as well as in the injured BBB (Hashimoto, 1972; Hashimoto et al., 1974; Simionescu et al., 1975, 1978; Nag et al., 1977; Lossinsky et al., 1979, 1989b; Shivers 1979a,b; Castejón, 1980; Farrell and Shivers, 1984; Shivers and Harris, 1984; Shivers et al., 1984a; Nag, 1990, 2003, Fig. 1).

Although the lizard (*Anolis carolinensis*) and the Mongolian gerbil (*Meriones unguiculatus*) may be viewed as phylogenetically distant (from each other as well as from humans), there are apparent structural similarities in the mechanisms of transporting proteins across CNS microvessels in response to brain injury. These studies demonstrated structurally similar EC

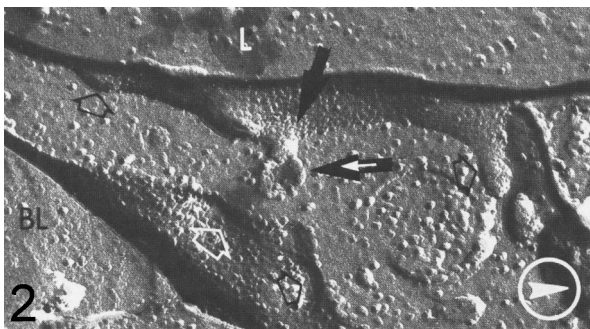


Fig. 2. Complete transendothelial channel is shown in this platinum replica to be continuous with the luminal (black arrows) and abluminal (white on black arrow) surface of the EC. The PF fracture face opening of the channel is seen (white on black arrow), as well as the point at which the channel is continuous with the luminal plasmalemma (black arrow). The abluminal opening of the channel is fluted, indicating that it is the true opening of the channel onto the abluminal surface. Open arrows denote the formation of pinocytotic vesicles. Compare this transendothelial channel with that in Fig. 3. x 80,000. Reprinted from Farrell and Shivers (1984) *Acta Neuropathol.* 63, 179-189 with permission from Springer-Verlag Co.

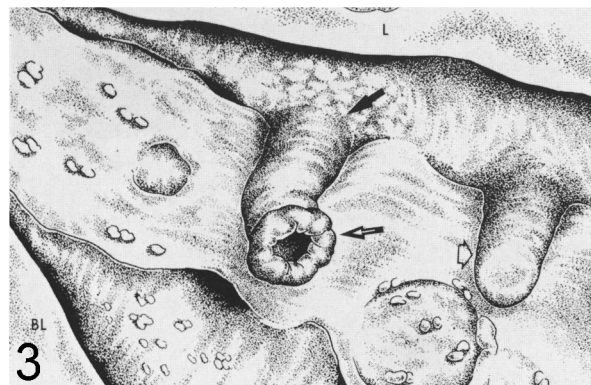


Fig. 3. Artist's view of the complete transendothelial channel illustrated in Fig. 2. The lettering and arrows used here are the same as in Fig. 2 and denote the origin of the channel from the luminal plasmalemma (black arrow) and the abluminal opening (white on black arrow) on the channel on the opposite side of the cell. Open arrow identifies an elongate pinocytotic invagination of the luminal plasmalemma in Figs. 2 and 3. BL: Basal lamina; L: lumen of capillary. Reprinted from Farrell and Shivers (1984). *Acta Neuropathol.* 63, 179-189 with permission from Springer-Verlag Co.

tubular mechanisms and suggested that these EC profiles likely play a key role in the transport of macromolecules during brain injury. No published data exists that is inconsistent with the anatomical similarities between BBB and CNS microvessel endothelial cells of all animal specimens examined to date.

The EC tubular channels were later defined as the vesiculo-tubular system (VTS), since they appeared to form a network of fused vesiculo-tubular structures, especially shown in thicker plastic sections and viewed by high-voltage electron microscopy (Lossinsky et al.,

1979, 1999; Shivers, 1979b, Shivers et al., 1984a; Shivers and Harris, 1984, Figs. 1, 4, 5). The main advantage offered by HVEM to ultrastructural studies of brain microvessel ECs is the ability to see cross sectional views of tissue structures embedded within thick, plastic sections. Since the accelerating voltage in standard TEMs is usually less than 100 kV, penetration of the beam of electrons through the specimen is greatly reduced thereby limiting resolution of the components of the tissue. In order to increase the penetration of electrons through thick specimens and retain a resolution

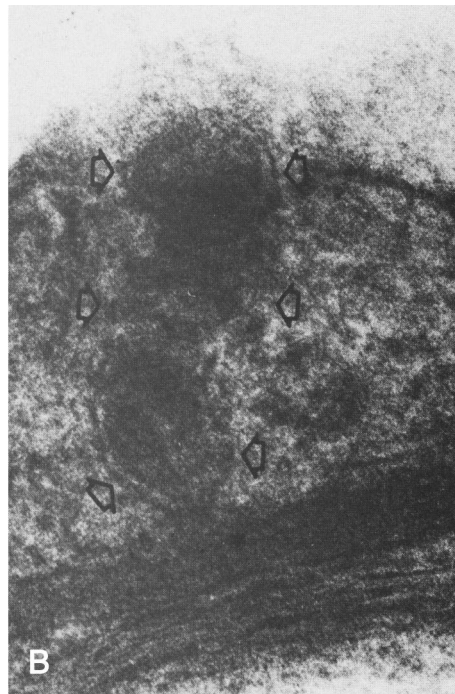
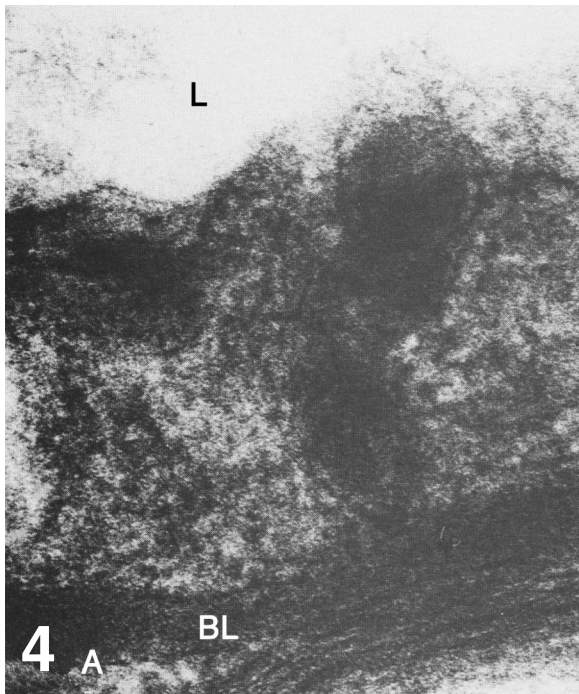


Fig. 4. Stereo HVEM micrographs of a vesiculo-tubular channel. The segmented nature of this conduit is apparent, and also the proximity of its ends to the luminal (L) and abluminal (BL) surfaces of the EC can be seen. This conduit exhibits a feature common to most transendothelial channels, a sigmoid or irregular shape. When this channel is viewed in stereo, it is clear that its course describes an arc (from top to bottom of figure), which proceeds from the surface of the micrograph (or section) to a point deep in the micrograph (section) and back to the surface again (or face of the section). L: lumen; BL: basal lamina. 0.25 μ m section; tilt angl = 14 degrees; x 95,000. Reprinted from Shivers and Harris (1984). *Neuropathol. Appl. Neurobiol.* 10, 343-356, with permission from Blackwell Publishing Co.

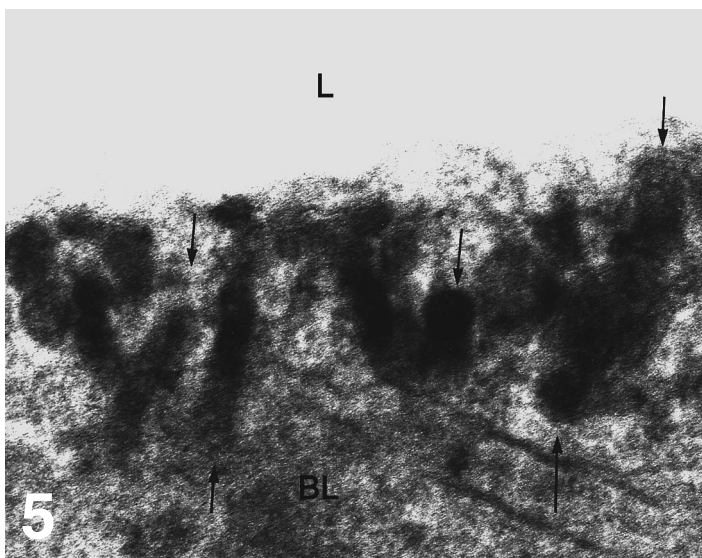


Fig. 5. HVEM image of a rat brain tumor microvessel endothelium containing HRP-filled transendothelial channels (arrows). Note sinuous, ramified profiles of the channels that traverse the endothelial cytoplasm. L: capillary lumen; BL: basal lamina. 0.35 μ m section. x 85,000.

that approximates the theoretical TEM resolution of less than 1 Å (Angstrom), a higher accelerating voltage must be used. The million or 1.2 million volt electron microscopes provide the necessary beam strength and resolving power to render the contents of thick plastic sections of tissue (0.2 to 1-3 µm thick) visible. Advantages of the HVEM include recording of three-dimensional architectural relationships of subcellular structures and even rotational manipulation of the specimen to reveal subtle details of spatial interrelationships. Thick-section specimens can be examined with the HVEM and photographed at various tilt angles to permit examination of stereo pair views of the tissues (Lossinsky and Shivers, 2003). The HVEM has served as an excellent tool for studies of membrane continuity of the EC vesicles, caveolae, EC channels and the VTS (Shivers et al., 1984a; Shivers and Harris, 1984; Wagner and Robinson, 1984; Wagner, 1985; Lossinsky et al., 1989b, 1990, 1999; Lossinsky and Song, 1990; Wagner and Chen, 1991; Wisniewski and Lossinsky, 1991; Lossinsky and Shivers, 2003), and vesiculo-vacuolar organelles (VVOs, Lossinsky et al., 1999), as well as studies of cell-cell interactions during inflammatory conditions of the BBB (Lossinsky and Song, 1990; Lossinsky et al., 1991; Wisniewski and Lossinsky, 1991). The observation of HRP-laden VTS/VVO profiles was confirmed in ECs from rat brain tissue within the penumbra of tumor tissue (Shivers et al., 1984a) and further supported the idea that transcellular pathways likely serve as conduits for the distribution of macromolecules after the CNS is subjected to insult.

Confirmation of the presence of VTS in the injured gerbil and lizard brain microvessel endothelia established a foundation for subsequent investigations concerning the ultrastructural, ultracytochemical and immunocytochemical aspect of BBB transport. Earlier studies of EC tubular structures, the "transendothelial channels", were described in a variety of animal species and experimental conditions (Hirano et al., 1969; Hashimoto, 1972; Hashimoto et al., 1974; Bendayan et al., 1974; Hansson et al., 1975; Nag et al., 1977; Sasaki et al., 1977; Westergaard et al., 1976; Persson et al., 1978; Noguchi et al., 1987; Pino, 1987b; Tripathi and Tripathi, 1985; Shivers et al., 1987; Ogawa et al., 1993; Wijsman and Shivers, 1993; Bendayan and Rasio, 1997; Shivers and Wijsman, 1998). Vesiculo-tubular structures were observed in human brain ECs from patients suffering from stroke (Castejón, 1980), while other reports described this mechanism in other non-BBB-type cells including normal choroid plexus ECs (Van Deurs, 1976) and in alveolar macrophages (Nichols, 1982). Convincing structural transendothelial channels were also observed in skeletal muscle capillaries (Simionescu et al., 1975, 1978). Collectively, these early reports confirmed the existence of a unique endothelial apparatus, verified with the electron microscope, that was present in CNS ECs as a result of a variety of BBB insults (see review of BBB pathophysiology in Shivers

and Wijsman, 1998).

A topic of controversy during the 1980s concerned whether or not the VTS formed continuous conduits that spanned the entire EC thereby linking the luminal with the abluminal surfaces of the microvessel endothelium (Casley-Smith, 1979; Balin et al., 1987; Broadwell, 1989). If this anatomic organization were true, and arranged similar to that demonstrated with the TEM by Simionescu et al. (1975) for non-BBB (skeletal muscle) ECs, then this type of structure could serve as an immediate bi-directional passageway for macromolecules and large amounts of fluids across injured BBB microvessels. The topic of EC "channels" was challenged by Casley-Smith (1979) who suggested they might be an artifact of chemical fixation. Other investigators noted that membrane vesicles could artifactually fuse if only glutaraldehyde fixation was applied and if the tissue remained in buffer in the refrigerator (Hasty and Hay, 1978). Results of experiments to test whether EC vesicle aggregates were a result of fixation artifact demonstrated that the VTS-like profiles were in fact, structures that occurred in situ and not as a post-mortem event (Lossinsky and Wisniewski, 1986). In addition, results of TEM studies on CNS microvessel endothelial cells where the technique of freeze-fracture was used to prepare tissue samples, clearly demonstrated the presence of VTS elements in brain endothelial cytoplasm (Farrell and Shivers, 1984, Figs. 2, 3). Conclusive evidence in support of VTS-like structures as normal residents of brain microvessel ECs can be found in the published results of HVEM and freeze-fracture studies by numerous investigators (Shivers, 1979a,b, 1980; Farrell and Shivers, 1984; Shivers et al., 1984a; Shivers and Harris, 1984; Lossinsky and Song, 1990; Lossinsky et al., 1989b, 1999; Sedlakova et al., 1999) (Figs. 1, 4, 5).

Visualization of transendothelial channels in their entirety, as complete conduits connecting the luminal with the abluminal endothelial surfaces, has been difficult for investigators using thin section TEM techniques. The simplest form of channel is a single cytoplasmic vesicle whose diameter approximates the thickness of the attenuated EC (Shivers et al., 1984a). In such cases, fusion of the vesicle with EC plasma membranes would create an elementary channel. In more complex situations that involve thicker (i.e. numerous diameters larger than a single pinocytotic vesicle), EC linings of brain microvessels, complexes of many cytoplasmic vesicles would be required to form longer conduits to span the endothelium. If the thickness of brain microvessel endothelium, along with the small size of EC cytoplasmic vesicles, are taken into consideration, then it can be assumed a priori, that the conduits which span the endothelium must be serpentine and circuitous in their travel from the luminal to abluminal EC surfaces. The chances of viewing a complete VTS in a single 600 Å thin plastic section (interference color of grey) would be almost nil. Thus, investigators examining specimens in thin plastic sections, would never see an

intact VTS. Evidence presented above on studies of brain EC structures using intermediate voltage electron microscopy and HVEM, provide the necessary proof that complex VTSs do in fact exist (Lossinsky et al., 1979, 1999; Shivers, 1979b; Shivers et al., 1984a; Shivers and Harris, 1984, Figs. 1-5).

The role of the VTS as a transcytotic passageway is supported by cytochemical and immunoultrastructural studies (reviewed by Dermeitzel and Krause, 1991). Cytochemical analyses have investigated the notion that phosphatase enzymes, which are primary components of the lysosome system of cells (Kornfeld and Mellman, 1989), contribute to BBB function after injury (Lossinsky et al., 1983; Vorbrodt et al., 1983; reviewed in Vorbrodt, 1988). One of these enzymes is alkaline phosphatase (AP), an enzyme that is found on the normal luminal EC plasma membrane of capillaries and

arterioles, and to a lesser extent, on venous and post-capillary venules. After brain injury in the mouse, AP was shown to label in addition to the luminal EC plasmalemma, the inner membrane surfaces of the VTS in the injured BBB (Lossinsky et al., 1983; Vorbrodt et al., 1983). These results indicate that the labeled EC VTS were derived from the luminal EC plasma membrane and represent a continuum of the inward invaginations of the luminal EC membrane. Studies of acid phosphatase (AcP) activity in the damaged BBB suggested that this enzyme was associated with the interior aspects of elongated endothelial cytoplasmic profiles including tubular profiles (Lossinsky et al., 1981). These studies suggested that lysosomal enzymes are present in the EC VTS and support the idea that in some situations, vesicles and the VTS may terminate in a cul de sac within the lysosomal system (Broadwell and Salcman, 1981; Broadwell, 1989). In addition, morphologic and histochemical evidence support a connection between the EC VTS and lysosomes (Broadwell and Salcman, 1981; Lossinsky et al., 1981). Whether or not all elements of the EC vesiculo-tubular system come into contact with secondary lysosomes (Broadwell and Salcman, 1981; Lossinsky et al., 1981), with other organelles such as the Golgi complex (Broadwell et al., 1988), or simply connect the luminal EC surface with the abluminal EC surface ultimately opening into the extracellular milieu of the tissue parenchyma (Brightman et al., 1975; Lossinsky et al., 1979, 1989b; Shivers 1979b; Farrell and Shivers, 1984; Shivers and Harris, 1984; Shivers et al., 1984a), or combinations of these, remains to be elucidated.

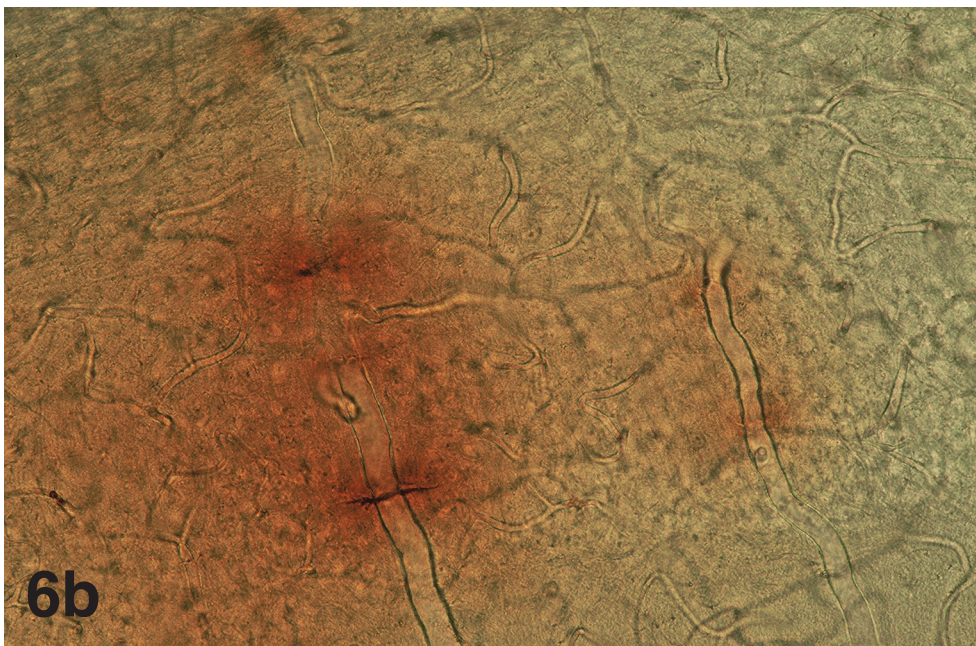
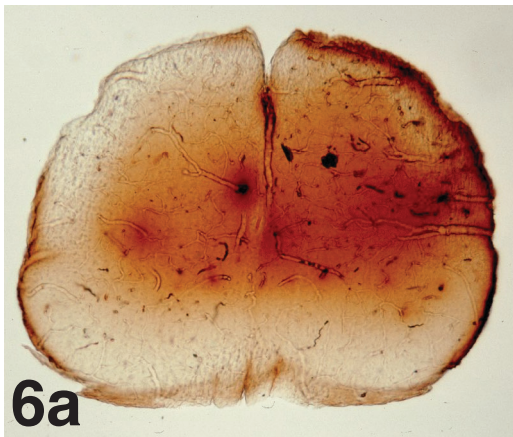


Fig. 6.a. Forty μm Vibratome section of lumbar spinal cord from a 4.5 day neonatal mouse after intraperitoneal injection of HRP solution. Note the increased HRP reaction product surrounding several large and small blood vessels. $\times 40$. **b.** Higher magnification of an area taken from Fig. 6a. Note the HRP leaks are in association with specific vascular regions, either open EC junctional complexes or through the VTS (arrows). $\times 200$

Endothelial transport during ontogeny of the BBB.

The vasculature of the developing BBB in humans has been a topic of great interest in clinical medicine. It is known that striking structural and functional similarities exist in the microanatomy and cell biology of CNS-type blood vessels during brain development and during neoplasia when angiogenesis is an active and ongoing process (Folkman, 1984, 1995; Zagzag et al., 1988; Farrell and Risau, 1994; Damska, 1995; Zagzag, 1995; Nag, 2002). Based on limited ultrastructural data and evaluations of protein concentrations in the cerebral spinal fluid of *post mortem* fetal CNS tissues, the functional and structural BBB in higher mammals including humans, subhuman primates and sheep mature in utero prior to birth (Bradbury, 1979; Saunders and Møllgård, 1984; Møllgård and Saunders, 1986; Møllgård et al., 1988; Johanson, 1989; Saunders and Dziegielowska, 1998). Conversely, the BBB in other, lower mammalian species such as weanling mice and rats forms a structural and functional barrier at ca. 2 weeks postpartum (Lossinsky et al., 1986; Vorbrodt et al., 1986a; Stewart and Hayakawa, 1987).

Earlier experimental studies using mice and rats indicated that intravenous administration of exogenous HRP tracer traverses the barrier via loosely-connected EC junctional complexes in the newborn mouse brains (Lossinsky et al., 1986; Vorbrodt et al., 1986b; Stewart and Hayakawa, 1987, 1994). Some of the tracer was also thought to leak across the ECs via vesicles, modified caveolae and tubular structures (Bradbury, 1979; Lossinsky et al., 1986). This phenomenon appears to last

until approximately 12-14 days postpartum after which the BBB can be considered anatomically mature (Bradbury, 1979; Lossinsky et al., 1986; Vorbrodt et al., 1986a). Similar results in the rat BBB were obtained by Stewart and Hayakawa (1994). We also noted that intraperitoneal administration of HRP tracer demonstrated junctional leakage in blood vessel of the spinal cord at 4.5 days *postpartum* (Lossinsky et al., 1986, Fig. 6). In addition to protein tracer studies, the application of enzyme cytochemistry has contributed to understanding of subtle changes that may occur after injury and during development of the BBB. For example, the cytochemical reaction product for alkaline phosphatase (AP), a phosphatase enzymes thought to play a role in regulating barrier function (Vorbrodt et al., 1983; Vorbrodt, 1988), during development and normal function is known to gradually increase in its localization on capillaries and arterioles, at the approximate time that the BBB becomes structurally mature in the mouse (Goldstein and Harris, 1981; Vorbrodt et al., 1986a). Although one can observe an increase in the appearance of EC VTS-type profiles in the developing BBB during the first week after birth in the mouse BBB (Lossinsky et al., 1986), these structural profiles do not label with AP, a striking hallmark of injured BBB-type vessels. We also know that the luminal EC surface expresses a net negative electrostatic charge (Skutelsky et al., 1975), referred to as "microdomains" of varying charge and density (Simionescu et al., 1981a,b). The BBB during development presents a gradual expression representative of the anionic nature of the EC glycocalyx, composed mainly of acidic glycoproteins (Vorbrodt et al., 1986b, 1990; Vorbrodt, 1989). This pattern of glycoprotein labeling in the developing BBB mimics that of the injured adult BBB in rodents in which one can visualize a patchy distribution of the glycoprotein coating (Lossinsky et al., 1986; Vorbrodt, 1988; Wisniewski and Lossinsky, 1991, Fig. 7). The redistribution of the glycocalyx that one can observe early in the developmental and in the injured BBB permitted cells to come into closer proximity to the endothelial wall, thus, enabling cell-cell contact to occur. During this time, the family of selectins and the adhesion molecules upregulate and establish an important next phase in the process of leukocyte-endothelial interactions, discussed in more detail below. Enzyme cytochemistry of the mammalian BBB has also contributed to the concept of leukocyte and neoplastic cell adhesion that antedated the discovery of adhesion molecules including ICAM-1 and others and is discussed in greater detail in a later section. For more information concerning the ultracytochemistry of the injured BBB, the reader is referred to an excellent monograph devoted to this topic by Vorbrodt (1988).

Mice and rats have served as good experimental models for BBB transport during development using electron dense tracers (reviewed in Bradbury, 1979; Lossinsky et al., 1986; Stewart and Hayakawa 1987; 1994), and the developmental mammalian immune

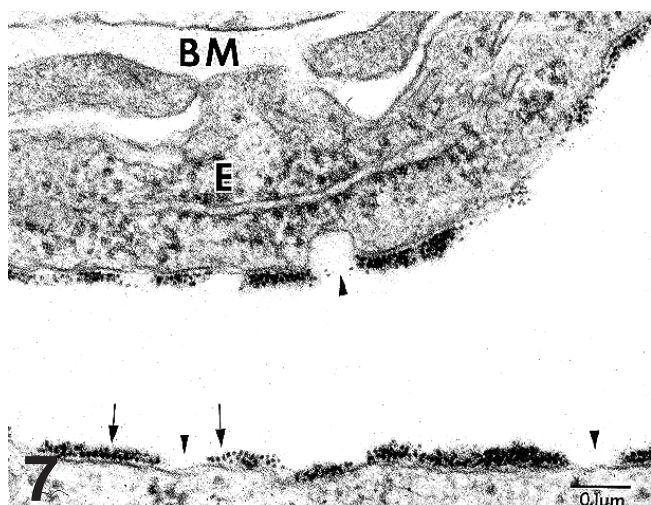


Fig. 7. Four day neonatal mouse brain capillary exposed to *in situ* incubation of cationic ferritin solution after blood washout and prior to vascular fixation with aldehydes. Note the even layer of ferritin particles (arrows) and uneven layer of ferritin particles on the EC plasma membrane. There is a conspicuous absence of ferritin particles near an ostium of an invaginating pit (arrowheads); x 78,000. Reprinted from Lossinsky et al., (1986). *Develop. Neurosci.* 8: 61-75 with permission from S. Karger Co.

system has been characterized. Development of the immature BBB in the fetus and newborn, like that of the immune system of the developmental CNS is thought to proceed at a sluggish pace, an impediment that may have serious complications leading to infection (Lawton and Cooper, 1996). This is important for pathologic and potentially fatal conditions in infants including neonatal meningitis, in which pathogenic microorganisms are more capable of entering an immunologically and structurally insufficient BBB (Davis and Rudd, 1994). Thus, understanding the structural and immunologic maturation processes of the BBB as it develops and matures is of critical importance in human medicine. This topic will be discussed in greater detail in a later section related to recently developed cell culture models that are designed to study the invasion of the human BBB by known pathogenic microorganisms.

Leukocyte passage across EC barriers in non-BBB-type and BBB-type vessels

The question of whether or not an EC VTS system could serve as a transEC route for the emigration of sensitized leukocytes as well as macromolecules during inflammatory or neoplastic conditions of the CNS or in other tissues merits further consideration and is the topic of ongoing research. Current thinking concerning the mechanisms of leukocyte trafficking across the BBB stems from the early classical, descriptive ultrastructural studies of post-capillary venules and veins in several different types of tissues. The studies of leukocyte transport across various blood-tissue barriers under normal and pathologic conditions (Marchesi and Florey, 1960; Marchesi, 1961; Marchesi and Gowans, 1964; Lampert and Carpenter, 1965; Majno, 1965; Lampert, 1967; Bebin, 1968; Schoefl, 1972; Simon, 1979), essentially set the stage for current immunoultrastructural studies of transmigration of leukocytes and neoplastic cells across the BBB. Many reports in the literature, in medical text books and in current diagrams and cartoons in present day immunoreagent catalogs typically present all of the varieties of leukocytes crossing the blood vessels via EC junctional complexes that open and permit their passage. Such dogmatic depiction is presented regardless of the leukocyte subtype and the type of blood-tissue barrier that is being described. Most of the early ultrastructural reports that presented leukocytes penetrating junctional complexes were based on ECs from non-CNS tissues. In both living animals and in culture systems, neutrophils, granulocytes and mononuclear cells appear to traffic primarily between adjacent ECs, i.e., paracellularly through the EC junctional complexes (Marchesi, 1961; Marchesi and Gowans, 1964; Lampert and Carpenter, 1965; Lampert, 1967; Beesley et al., 1979; Shaw, 1980; Pawlowski et al., 1988; Moser et al., 1989; Burns et al., 1997). Granular cells including neutrophils are known to contain hydrolytic enzymes including proteases (Leeson and Leeson, 1970), and it may be reasonable that

released enzymes from these cells could dissolve the tight EC junctions enabling the cells to pass between the adjacent ECs. This attractive concept, however, remains equivocal at present.

Although lymphocytes are known to migrate through the EC junctional complexes of HEVs in lymphatic tissue (Schoefl, 1972), and across ECs in culture (Munroe et al., 1996), other studies favor a transcellular rather than a paracellular route for lymphocyte transmigration across a variety of blood-tissue barriers (Marchesi and Gowans, 1964; Åström 1968; Åström et al., 1968; Lossinsky et al., 1989a), while other researchers were unable to determine the precise transendothelial mechanism for lymphocyte passage (Marchesi and Florey, 1960). Moreover, leukemic cells are also known to traverse EC barriers via a transcellular rather than transjunctional route (Azzarelli et al., 1984, 1985; De Bruyn et al., 1989). At present, the literature indicates that the routes taken by the various types of leukocytes and tumor cells for emigration across ECs are variable depending upon the cell type that is migrating and barrier type that is being breached and whether one refers to either *in vivo* or *in vitro* experimental EC paradigms. Collectively, there is no clear understanding concerning the precise transendothelial route(s) for the various subsets of leukocytes during inflammatory conditions or neoplastic cells in the CNS. A transjunctional pathway may offer the best route with a path of least resistance for leukocytes traversing non-BBB-type vessels such as in lymphatic high ECs, spleen, lymph nodes, hepatic venules and veins. However, there is compelling ultrastructural evidence that this may not be the case for leukocyte transport across BBB-type ECs during CNS injury (Lossinsky et al., 1991). Discussion of the differences between non-BBB-type ECs and BBB-type ECs, thus may have been overlooked in the previous literature and the assumption that all ECs are functionally and structurally the same is not accurate. In fact, convincing evidence has been published to support the tenet that all ECs are not alike, and that individual EC phenotypes are determined by the local tissue environment (DeBault and Cancilla, 1980; Stewart and Wiley, 1981; Tao-Cheng et al., 1986; Arthur et al., 1987; Janzer and Raff, 1987; Shivers et al., 1988b, see also for review; McCurley et al., 1998). Thus, if one critically appraises this literature, one can conclude that uniform agreement does not exist concerning the nature of leukocyte transport across the BBB, the blood-spinal cord barrier, or for that matter in all blood-tissue barriers.

The earlier ultrastructural literature indicates that some types of leukocytes may traverse EC barriers by moving directly through the ECs in a process described as emperipolesis (Åström, 1968; Åström et al., 1968). Emperipolesis occurs during transmigration when the EC embraces and finally engulfs the invading leukocytes as they invade the EC barrier (Azzarelli et al., 1984, 1985; Faustman and Dermietzel, 1985; Lossinsky et al., 1989a; Cross and Raine, 1991). If different subtypes of

leukocytes or neoplastic cells can, in fact, select different transendothelial routes for passage, this raises several important issues regarding the pathophysiology of these various cell types. Evidence from several ultrastructural studies of normal ECs in culture, in autoimmune diseases and in models of experimentally induced tumors of the CNS suggests that there is a unique pore-like region on the EC surface adjacent to the junctional complexes (Marchesi and Florey, 1960; Azzarelli et al., 1984, 1985; De Bruyn et al., 1989; Burns et al., 1997, 2000), i.e., a “parajunctional” route of passage for some leukocytes, as well as certain neoplastic and inflammatory cells. It will be important for researchers to elucidate precisely how the various cell types migrate from the blood side of the vasculature to the brain parenchyma and vice-versa before one can develop specific therapies that may block or reduce the attachment and migration of these cells across the BBB. These kinds of approaches are already being developed in pharmaceutical companies, a topic that will be discussed in greater detail below.

Integrins, selectins and adhesion molecules play key roles in regulating leukocytes and tumor cells as they chemically sniff, tether and adhere to, and finally penetrate the VTS/VVOs and/or junctional complexes during inflammatory events

Some of the earliest observations that leukocytes can target specific types of blood vessels called HEVs were made by Stamper and Woodruff (1976). These investigators studied communications between lymphocytes and HEVs from lymph nodes and established important baseline observations for subsequent research concerning the nature of cell-cell interactions and inflammation. These researchers showed that lymphocytes overlaid onto fixed sections of lymph node tissue sections adhered selectively to the HEV cells and not to any other vascular ECs.

One of the earliest events during an the inflammatory response within any tissue type is the invasion of the tissue by neutrophils from the systemic circulation (Furie et al., 1991). This event is triggered by specific chemical stimuli produced within the tissue that initiates the recruitment of the stimulated leukocytes to the luminal EC surface, adhesion and eventual incursion of leukocytes into the tissue (Paulson, 1992). Once the leukocytes within the vasculature are stimulated by proinflammatory cytokines and chemokines, they are transformed into cells that have a specific mission to leave the circulating blood. In order to cross the BBB, they must first roll along the luminal EC surfaces to establish the initial cell-cell communication. This event may be analogous to a division of infantrymen entering a military theater of operations during battle. To prepare the invading cells to be successful in their mission, the luminal EC surface undergoes an extensive remodeling by producing microvilli and elongated fronds (Povlishock et al., 1980; Kumar et al., 1987; Lossinsky

et al., 1989a; Cross and Raine, 1991; Pluta et al., 1991). Other events occur deeper within the ECs including cytoskeletal protein reorganization will be discussed below. The EC membrane extensions are rich in upregulated selectins (Kaplanski et al., 1994) and adhesion molecules (Wong and Dorovini-Zis, 1995) that facilitate cell-cell adhesion as a type of chemical fly paper. The selectin molecules form an initial, relatively weak attachment as the leukocytes roll along the luminal EC membrane surfaces at specific regions of post-capillary venules and veins (Paulson, 1992), similar to that which was described by Stamper and Woodruff (1976). The leukocytes have the corresponding, integrin molecules on their membrane surfaces that completes the specific chemical linkage between the leukocytes and the EC membrane surfaces. Thus, the invading leukocytes home only to selective regions on the luminal EC surface where they adhere to specific selectin and adhesion molecules that complement corresponding linkage molecules on their exterior membrane surfaces (Bevilacqua, 1993).

The question of why leukocytes and tumor cells recognize only specific EC regions on selected blood vessels, usually post-capillary venules and veins is orchestrated by a sequence of chemical events that occur within the surface membrane components of the EC and the invading leukocytes. Such chemical events have been well characterized during the past twenty years with the discovery of selectins and adhesion molecules that are expressed either constitutively on a variety of normal cells, or upregulated on the membrane surfaces of activated ECs, platelets and leukocytes. These kinds of studies have underscored the important role that membrane-associated glycoproteins play during adhesion and eventual transEC transport of sensitized leukocytes. The reader is referred to several excellent chapters and reviews on this specific topic that includes several of the important selectin molecules including E-L- and P-selectins (Harlin and Liu, 1992; Bevilacqua, 1993; Tedder et al., 1995; Malik and Lo, 1996; Homeister et al., 1998; Kubes and Ward, 2000).

Once the CNS tissue is injured, there is an immediate upregulation of EC immunoglobulin moieties that become available to link specifically to counter receptor integrin molecules on the membrane surfaces of sensitized leukocytes. In EAE and brain trauma, among the most important adhesion molecules include ICAM-1 (CD54), VCAM-1 (CD106), and PECAM-1 (CD31) (Barten and Ruddle, 1994; Cannella et al., 1990; Butcher, 1991; Fabry and Hart, 1993; Brosnan et al., 1995, 1997; Hartung et al., 1995; Tedder, et al., 1995; Luscinskas, 1997; Brosnan and Claudio, 1998). These and other adhesion molecules of the immunoglobulin supergene family serve as important EC homing receptor ligands for their specific integrin receptor molecules on the leukocytes membranes. These Integrins include LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) for lymphocytes, monocytes and neutrophils, and VLA-4 (CD49d/29), expressed on the membrane surfaces of

only lymphocytes and monocytes in response to pro-inflammatory stimuli (Rothlein et al., 1986; Marlin and Springer, 1987; Kuppner et al., 1990; Kishimoto, 1991; Brown et al., 1993; DeLisser et al., 1994; Springer, 1994; Takahashi et al., 1994). The local, affected ECs of veins and post-capillary venules involved in the immune responses become taller, i.e., cuboidal (Simon, 1979; Raine et al., 1990; Cross and Raine, 1991) in preparation for leukocyte contact. These inflamed ECs mimic that

which is observed in normal post-capillary venules of lymph nodes and other lymphatic tissues (Ham and Cormack, 1979). Once the adhesion molecules establish firm chemical attachments to the upregulated ligand receptors on the leukocyte membranes, pseudopodial projections from the leukocytes penetrate and traverse the ECs either through the junctional complexes (Lampert, 1967; Lampert and Carpenter, 1965; Schoefl, 1972), or directly through the EC (Åström 1968; Åström

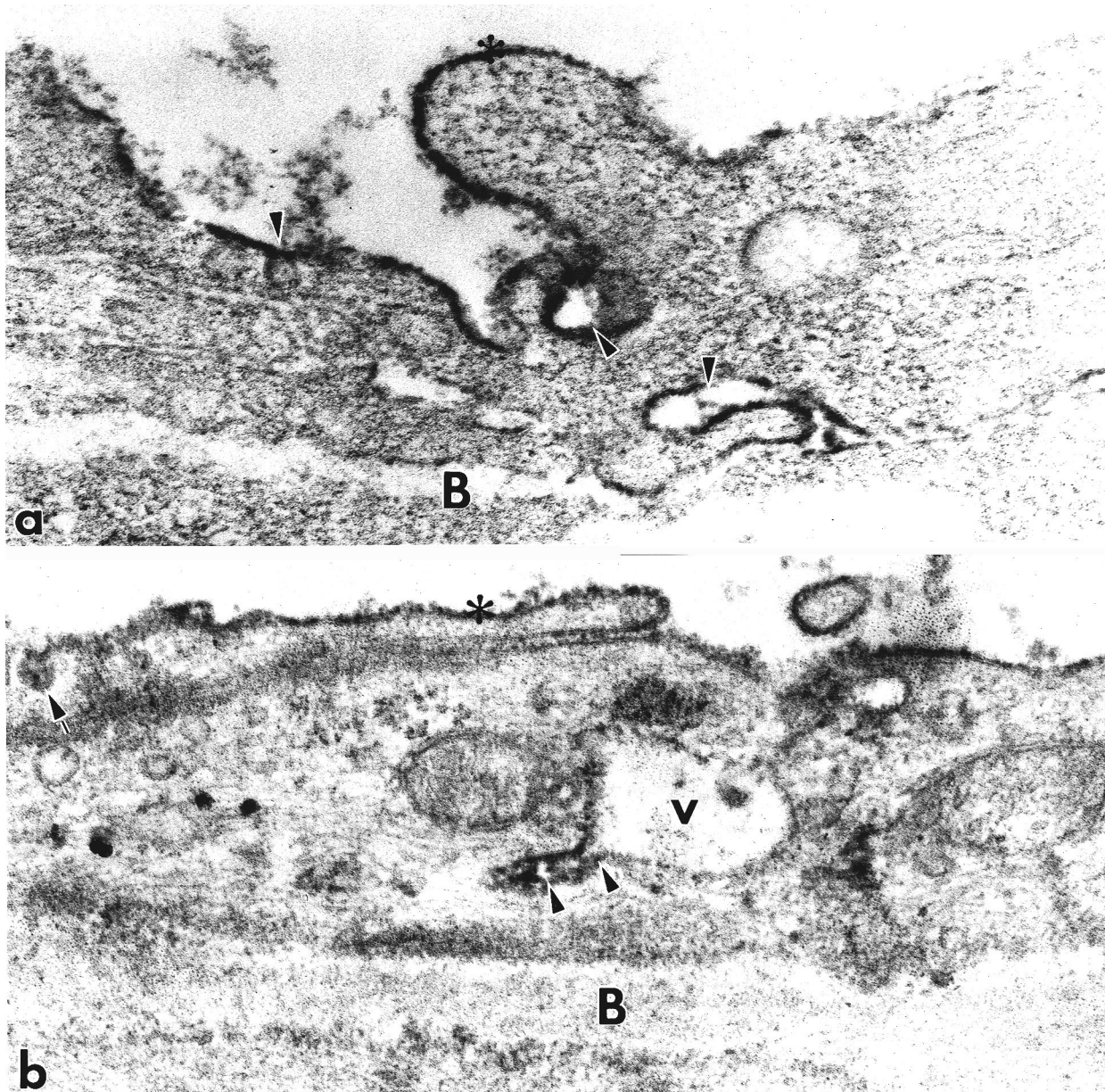


Fig. 8.a. HRP reaction product for PECAM-1 labels the EC surfaces in a newborn mouse. **b.** Human brain angioma biopsy specimen. HRP reaction product for ICAM-1 labels a VTS/VVO structure which is connected to an elongated ICAM-1-positive tubular structure (arrowheads). a, b x 48, 000. B : Base membranes. a: Reprinted from Lossinsky and Wisniewski (1998), *Develop. Neurosci.* 20: 518-524 with permission from Springer-Verlag Co.; b: Reprinted from Lossinsky et al., (1999). *Cell Tissue Res.* 295, 77-88 with permission from Springer-Verlag Co.

et al., 1968). In either situation, the leukocytes eventually emigrate across the blood vessel to the perivascular compartment. What controls each type of adhesion and transport is likely the different types of selectins and other adhesion molecules, and is currently a topic of intensive study.

Based on the literature and anecdotal observations made in several laboratories, it is proposed that lymphocytes and possibly mononuclear and some neoplastic cells enter and pass through transcellular EC pores (Marchesi and Gowans, 1964; Azzarelli et al., 1984, 1985; Faustman and Dermietzel, 1985; Lossinsky and Wisniewski, 1987; Lossinsky et al., 1991), a process also defined as 'intraendothelial channels', dynamic structures formed by the lymphatic ECs under physiological conditions (Azzali, 1988, 1990). It is our contention that these parajunctional pores (Azzarelli et al., 1984, 1985; Tagami et al., 1983) likely represent various conformations of the VTS (Lossinsky et al., 1999), modified vacuoles (Tripathi and Tripathi, 1985), and the VVOs (Kohn et al., 1992; Dvorak et al., 1996; Feng et al., 1999), modulated by the ICAM-1/LFA-1 and VCAM-1/VLA-4 pathways (Lossinsky et al., 1999; Liu et al., 2002). As will be elaborated in greater detail below, cell cultures of human ECs exposed to HIV-1 virus demonstrated ICAM-1 highly expressed within the VTS, ie., modified EC caveolae and racemic structures (Bungaard et al., 1979; Frøkjær-Jensen, 1980, 1991; Wagner, 1985; Bungaard, 1987), suggesting that adhesion molecules may be involved in viral transport through the ECs (Lossinsky et al., 2001; Liu et al., 2002).

In certain circumstances, adhesion molecules including ICAM-1, PECAM-1 and VCAM-1 appear to especially play key roles in facilitating certain cell-cell adhesion and transendothelial migration (Rothlein et al., 1986; Sobel et al., 1990; Lassmann et al., 1991; Harlan and Liu, 1992; Lobb, 1992; Muller et al., 1993; DeLisser et al., 1994; Springer, 1994; Muller, 1995a; Greenwood et al., 2002; Mamdough et al., 2003). At present, however, it remains unclear which specific adhesion molecules upregulate in association with: 1) BBB-type EC vesiculotubular profiles, and 2) tight junctional complexes. For example, upregulation of ICAM-1 and PECAM-1 have been observed within the inner delimiting membrane surfaces of the VTS/VVOs during ontogeny of the BBB in mice during angiogenesis (Lossinsky et al., 1997; Lossinsky and Wisniewski, 1998, Fig. 8a), as well as in ECs from brain tumor biopsies obtained during surgery (Lossinsky et al., 1995b, 1999, Fig. 8b). Conversely, increased expression of PECAM-1 is thought by others to be linked specifically to EC junctional complexes during diapedesis (Mamdough et al., 2003). Nevertheless, these recent studies of adhesion molecules offer novel information related to the ultrastructural similarities and differences of the BBB during ontogeny and in the vasculature of CNS tumors, which share similarities in their angiogenic properties. That these EC VTS/VVOs

may serve as conduits for both macromolecules and cellular components is an intriguing concept to consider in connection to the mechanisms of leukocyte transport across the altered BBB. Additional details concerning the role that the EC VTS/VVOs play in cell-cell interactions are presented below.

There is additional, compelling evidence suggesting that pretreating animals with monoclonal antibodies to various EC adhesion molecules, selectins, leukocyte integrin molecules, CD antigens, major histocompatibility antigens and proinflammatory cytokines can reduce or arrest leukocyte adhesion and transmigration across EC barriers and alter the course of clinical disease. Some of these diseases include autoimmune disease such as EAE, the animal model correlate for human multiple sclerosis (MS), as well as meningitis, stroke and other conditions of the CNS (Furie et al., 1991; Yednock et al., 1992; Archelos et al., 1993; Cannella et al., 1993; Barton and Ruddle, 1994; Bogen et al., 1994; Rosenblum et al., 1994; Weber et al., 1995; Schiffenbauer et al., 1998; Bard et al., 2000). Antibodies developed against $\alpha 4$ integrin molecules (LFA-4/CD49d), a leukocyte surface integrin were shown to reduce clinical relapses of multiple sclerosis (Tubridy et al., 1999; Miller et al., 2003). Similarly, anti-PECAM-1 pretreatment blocks various inflammatory processes (Bogen et al., 1994; McColl et al., 1998; Muller, 1995b; Rosenblum et al., 1994), especially neutrophils and/or monocyte trafficking (Muller, 1995a,b), and similar anti-adhesion molecule therapy reduced neuronal damage after brain ischemia (Bowes et al., 1993). Conversely, anti-adhesion molecule treatment has shown either little or no significant effect on the disease course (Dopp et al., 1994), or it can even augment the disease process (Cannella et al., 1993; Welsh et al., 1993). Thus, manipulating the expression of adhesion molecules or leukocyte integrins is complex and may have variable effects on the disease processes since such treatment can reduce or arrest adhesion and eventual transendothelial migration of stimulated leukocytes.

It is known that several types of tumor ECs including those from CNS tissues upregulate adhesion molecules (Guarini et al., 1990; Kuppper et al., 1990; Bashir et al., 1992; Kaluza et al., 1994; Lossinsky et al., 1999, Fig. 8b). Two recent studies demonstrated that the inner delimiting membrane surfaces of VTS and VVO labeled with ICAM-1 (Lossinsky et al., 1995b, 1999). These studies of blood vessels from human brain tumor biopsies provided further immunoultrastructural evidence that the VTS/VVO structures may play an active role in cellular trafficking across the altered BBB. This concept was also recently advanced independently by Cordes et al. (1997). These authors suggested that thymic progenitor bone marrow cells may utilize ICAM-1-positive VTS-like profiles in thymic nurse cells. Since leukocyte trafficking is regulated by and large by ICAM-1/LFA-1 and VCAM-1/VLA-4 pathways (Kishimoto, 1991; Kishimoto and Rothlein, 1994), this raises the

possibility that the LFA-1/VLA-4 coated pseudopodia of some types of sensitized immunocytes could link with their ICAM-1/VCAM-1 adhesion molecule-labeled punctate ostia of the VTS/VVOs within the EC as a

mechanism for their transBBB journey. Although speculative at present, the previous immunoultrastructural data presented by Cordes et al. (1997) and Lossinsky et al. (1995b, 1999) are attractive and support

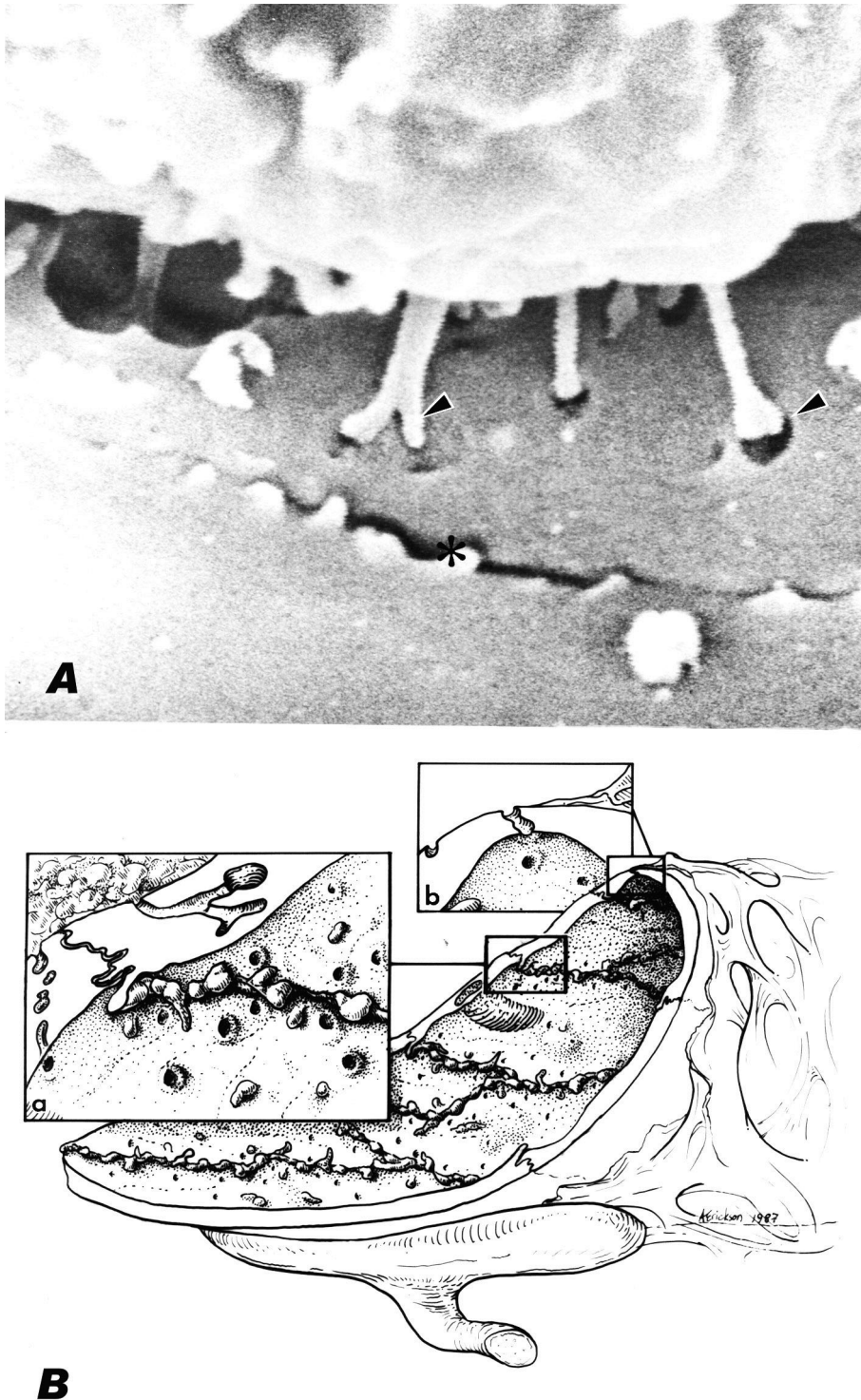


Fig. 9. A. Mouse chronic relapsing EAE. A lymphocyte is shown anchored to the EC surface with fine filopodial extensions (arrowheads) adjacent to the EC junctional complex (asterisk). This attachment is modulated by chemical linkage between the adhesion molecules on the EC surface (ICAM-1, VCAM-1 and others) and integrin molecules (LFA-1, Mac-1, VLA-4 and others) on the leukocyte membranes. x 40,000. **B.** A diagrammatic representation of a cross-sectional view of a post-capillary venule showing a: invaginating vesicles, the junction and a VTS/VVO structures, b: a "transendothelial channel" structure presented in the thinnest portion of the wall of the venule. A: Reprinted from Lossinsky et al., (1991), *Microvasc. Res.* 41, 299-310 with permission from Elsevier Co.; B: Reprinted from Lossinsky et al., (1989b), *Acta Neuropathol.* 77, 480-488 with permission from Springer-Verlag Co.

such a concept. Such an hypothesis, thus, links the VTS and VVOs to the attachment phase and emigration process for some types of sensitized leukocytes to gain entrance into the CNS during inflammatory conditions of the BBB. How the various subsets of leukocytes utilize the VTS and/or VVOs or EC junctions to enter the BBB in the various types of inflammation conditions has not been elucidated at present. Although one may postulate an endothelial infrastructure of VTS- and VVO-like profiles that may function as a transcellular tunnel system that might be manipulated to block the entrance of invading leukocytes using anti-adhesion molecule therapy, additional studies will be necessary to elucidate such an hypothesis.

Additional pieces of evidence support an argument for a VTS/VVO transcellular mechanism may serve as a conduit system and as potential "gateways to the CNS" (Lossinsky and Wisniewski, 1987) for migrating immunocytes or metastatic tumor cells. This is based on the fact that, as we have already mentioned above, the adhesion molecules ICAM-1 and PECAM-1 have been shown to label the inner delimiting membrane surfaces of the VTS and VVOs during brain neovascularization and in CNS tumors (Lossinsky et al., 1995b, 1999; Lossinsky and Wisniewski, 1998), and in other cell types as well (Cordes et al., 1997). If macromolecules can traverse the altered BBB via the VTS, then it is reasonable that leukocytes can negotiate similar pathways. Such a route could merely represent the path of least resistance, as contrasted with enzymatic hydrolysis of the junctions by neutrophils and other cell types (Leeson and Leeson, 1970). This might be the scenario if the invading immunocyte does not possess an adequate proteolytic enzyme system to dissolve open the EC junctions.

The fact that HRP-positive EC tubular structures were observed adjacent to EC junctions in spontaneous hypertensive rats (Tagami et al., 1983) established an intriguing clue that this particular "parajunctional" EC region or "pore" observed by TEM (Garcia et al., 1978; Azzarelli et al., 1984, 1985), may also have unique chemical receptors that can attract sensitized leukocytes to this specific EC region during the initial phase of inflammation. Azzarelli et al. (1984, 1985) demonstrated in a serial thin-section analysis that lymphoma cells actually traversed parajunctional pores during invasion of ECs from leptomeningeal drainage veins. This important observation has been confirmed by others in a cat model of acute meningitis produced by the application of α -bungarotoxin to the leptomeninges (Faustman and Dermietzel, 1985). Previous SEM studies in murine chronic relapsing EAE demonstrated leukocytes, presumably lymphocytes and possibly monocytes inserting pseudopodia into parajunctional openings that appeared to be the VTS and VVOs (Lossinsky and Song, 1990; Lossinsky et al., 1991; Wisniewski and Lossinsky, 1991, Figs. 9, 10). Several *in vitro* SEM studies contributed further structural evidence suggesting that lymphocytes traverse lymph node HEVs

adjacent to EC junctional complexes (Cho and De Bruyn, 1986), and lymphoma tumor cells seek out parajunctional EC regions as they probe the blood vessel surface for their transport across cultured EC barriers (Vlodavsky et al., 1983). Although the parajunctional region on the EC appears to be a very specialized zone for cell-cell contact where probing by the leukocytes and tumor cells may be chemically attracted to adhesion molecules on the luminal EC surfaces, the significance of this EC region remains unclear. Taken altogether, these studies indicate that adhesion and migration pathways of the various leukocyte subsets may be modulated by different adhesion molecules. These kinds of approaches have led to the development of important therapeutic strategies for a number of clinical trials for human diseases. Given that adhesion molecules are being expressed in association with the EC VTS/VVOs, learning how to chemically manipulate the formation of these structures may lead to therapeutic interventions that could open or close the VTS/VVOs at will and either accelerate or arrest the transendothelial trafficking of leukocytes.

Cell culture paradigms based on cellular and molecular biology of the human BBB are powerful adjunct tools to assess how the HIV-1 virus, bacteria and mycotic organisms enter the human CNS

Recent studies have focused on developing cell culture methods to grow purified cultures of brain microvessel ECs (Bowman et al., 1981, 1983, 1990,

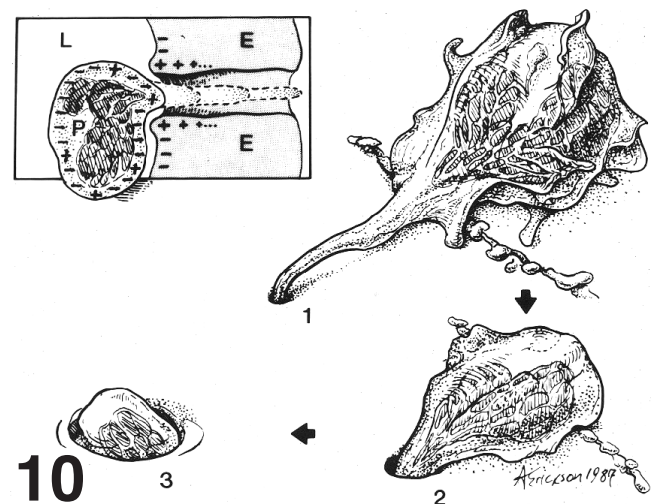


Fig. 10. Top left: This diagram presents an invading pseudopodial projection of a leukocyte capable of inserting into an ostium of the VTS/VVO based on a loss of the glycoprotein domains as net negative electrostatic charge in association with this membrane opening. **Diagram 1-3:** An inflammatory leukocyte probes the EC surface for an ostium. A combination of a loss of glycoprotein domains and upregulation of adhesion molecules facilitates adherence and transmigration. Reprinted from Wisniewski and Lossinsky (1991), Brain Pathol. 1, 89-96 with permission from Brain Pathol.

1991; Arthur et al., 1987; Shivers et al., 1988a,b; Wijsman and Shivers, 1993). Cultivated brain ECs are currently used to study mechanisms of transport of several human pathogenic organisms across the vasculature of the CNS including bacteria, fungi and viruses (Fiala et al., 1997; Persidsky et al., 1997; Persidsky, 1999; Huang et al., 2000; Huang and Jong, 2001; Jong et al., 2001; Chen et al., 2002). Some of these studies have examined human brain ECs grown in culture (BMVEC, Fiala et al., 1997), coronary artery ECs (CAEC, Gujuluva et al., 2001) and cardiomyocytes (Twu et al., 2002) after exposing these cells to the HIV-1, the virus responsible for the production of human AIDS (Fiala et al., 1997, 1998; Liu et al., 2002). The ECs are grown in culture systems on porous membrane filters and represent a cell culture model of the human BBB (Fiala et al., 1997). Using this approach, investigators can manipulate the ECs in culture, either alone or co-cultivated with specific leukocyte subtypes, astrocytes (Clubb and Shivers, 1996; Del Maestro et al., 2001), neurons etc. to study variable aspects of the BBB that cannot be studied in the living experimental animals and in human subjects with assorted viral, bacterial or fungal organisms. Cocaine abuse has been associated with an increased incidence of AIDS infection in humans (Fiala et al., 1998), and it is an important, clinically relevant goal to resolve the issue concerning the precise ultrastructural mechanism(s) by which the HIV-1 virus penetrates the BBB in humans addicted to drugs such as alcohol and cocaine.

Based on the literature, the three major mechanisms for the transport of the HIV-1 across the injured BBB include: a) EC junctional complexes may open briefly allowing transport of free virus via a paracellular route (Fiala et al., 1997); b) free virus may traverse the EC barrier by a transcellular route (Liu et al., 2002); and c) the Trojan Horse mechanism may occur, whereby viral particles first infect leukocytes, primarily lymphocytes and/or mononuclear cells and traverse the BBB by hitching a ride across the BBB (Peluso et al., 1985; Fiala et al., 1997; Huang and Jong, 2001; Liu et al., 2002). The role of selective EC recognition sites such as receptor-mediated mechanisms in viral invasion and human disease remains unclear. It also remains unclear what precise ultrastructural changes occur in human brain ECs exposed to alcohol, cocaine and the HIV-1 virus. However, it is clear that there is an increase in microvilli on the surface of the cultured MBVEC, similar to that which occurs in other types of ECs under injurious conditions (Povlishock et al., 1980; Kumar et al., 1987; Lossinsky et al., 1989a; Cross and Raine, 1991; Pluta et al., 1991; Wisniewski and Lossinsky, 1998). Further, there is an increased expression of ICAM-1 on the inner delimiting membrane surfaces of modified EC caveolae and receme-like vacuoles that appear structurally similar to the VTS (Liu et al., 2002, Fig. 11). The entry of the HIV-1 virus into modified caveolae and movement of the virions across human brain ECs (Lossinsky et al., 2001; Liu et al., 2002), or

cardiac ECs (Gujuluva et al., 2001) grown in culture were blocked with cyclodextrin and nystatin, two chemicals that alter the formation of caveolae by affecting cholesterol chemistry, i.e., by inhibiting lipid raft formation (Liu et al., 2002). Since the VTS/VVOs have been identified in this type of culture system (Liu et al., 2002), one could co-cultivate ECs with HIV-1-infected monocytes to study the ultrastructural changes of the cell-cell interaction that may occur during addition of drugs such as alcohol, cocaine with and without the addition of the HIV-1 virus. Whether or not the HIV-1 virus can enter the EC VTS/VVOs as we have proposed (Liu et al., 2002), or if cocaine may enhance virus endocytosis and monocyte migration across the BBB in cocaine addicts (Fiala et al., 1998) are unresolved questions that will require additional experimentation.

Increased expression of adhesion molecules has been demonstrated on human ECs grown in culture after stimulation with cytokines (Wong and Dorovini-Zis, 1992, 1995). These observations are commensurate with earlier suggestions that the microvilli (fronds) can physically trap and embrace the leukocytes as they approach the ECs (Cross and Raine, 1991). This is another piece of evidence supporting the argument that adhesion molecule-decorated microvilli and fronds may chemically direct leukocyte trafficking as the cells probe the EC surface in preparation for eventual linkage and transBBB emigration. Moreover, CNS EC populations have been observed to exhibit phagocytic properties (Robinson et al., 1991). In addition to uptake of small particles and suspensions of particulate materials, ECs can phagocytize larger particulate materials including colloidal carbon, latex spheres, glass beads, and a host of microorganisms such as viruses, bacteria, and mycotic organisms (Fawcett, 1963; Majno, 1965; Widmann et al., 1972; De Bruyn et al., 1975; 1977; Wantanabe, et al.,

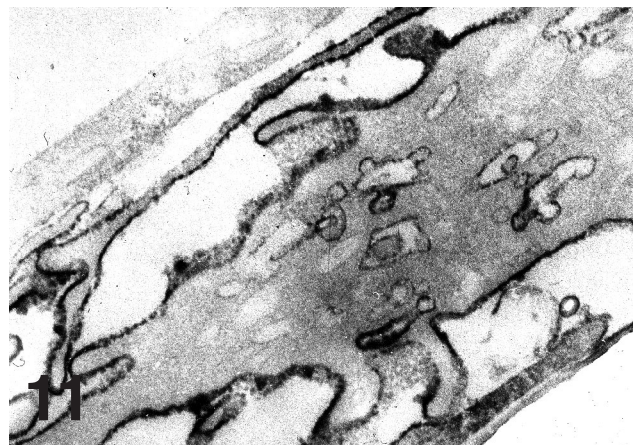


Fig. 11. Three hours after human BMVEC cultures were infected with HIV-1 virus shows upregulated ICAM-1-HRP reaction product (black precipitate) labeling the inner delimiting membrane surfaces of the caveolae (VTS/VVO) and decorates the EC plasmalemmal surface. $\times 40,000$. (See reference by Liu et al., 2002.)

1981; Lossinsky and Wisniewski, 1986; Huang et al., 2000; Huang and Jong, 2001; Jong et al., 2001). Several studies suggest that phagocytosis likely occurs as endothelial leaflets (fronds) embrace the foreign material (Fawcett, 1963). As was pointed out above, emperipolesis (Åström, 1968; Åström et al., 1968) has been observed within the injured BBB only after injury including inflammatory conditions (Cross and Raine, 1991). This phenomenon is certainly observed in hepatic sinusoidal ECs that are highly phagocytic (De Bruyn et al., 1975, 1977). In the CNS, the ECs of the BBB behave similarly (Wantanabe et al., 1981). Endothelial cell leaflets embrace bacterial organisms in cell culture systems, and certain strains of mycotic organisms including yeasts become internalized within large EC vacuolar structures (Huang et al., 2000; Jong et al., 2001). Collectively, these kinds of studies are important for understanding the nature of transmigration of a number of human pathogens and other parasitic organisms and the etiology of meningitis in human adults and infants (Huang and Jong, 2001).

Studies utilizing experimental models of autoimmune disease have contributed to our understanding of the mechanisms of leukocyte recognition of targeted blood vessels

We have already alluded to several important immune events that occur early during an autoimmune response of the CNS such as EAE, characterized by specific hallmarks that include leukocyte rolling and tethering to the luminal EC surfaces of affected veins and post-capillary venules, adhesion and eventual transvascular emigration of the leukocytes across the BBB (Owens et al., 1998). However, similar to other pathologic conditions of the BBB, the precise nature of transbarrier migration of T-cells and mononuclear cells, the leading players in autoimmune diseases has not been clearly delineated at present. Because of the unique characteristics of the BBB, it was previously considered that the CNS under normal conditions was an immunoprivileged organ not permitting cellular elements including leukocytes to traverse the normal barrier. Thus, under normal conditions migration would likely be considered limited to activated T-lymphocytes for immunosurveillance (Hickey et al., 1991; Owens, et al., 1994; Lassmann, 1997; Brosnan and Claudio, 1998; Newman and Wekerle, 1998; Hickey, 2001). An autoimmune reaction will result if lymphocytes (T-cells) have been previously exposed to an antigen such as one of the myelin-associated proteins including myelin basic protein (MBP), a major encephalitogenic component of CNS myelin (Kies et al., 1960; Panitch, 1980). MBP, in conjunction with major histocompatibility molecules (Hickey et al., 1991; Brosnan and Claudio, 1998; Hickey, 2001), plays an important role in producing a reactive autoimmune disease (Brosnan and Claudio, 1998). These antigenic proteins are inaccessible to normal leukocyte immune surveillance because of the

restrictive nature and unique angioarchitecture of the BBB (Hickey et al., 1991; Owens et al., 1994; Newman and Wekerle, 1998). Such an autoimmune response is known to occur in diseases including EAE and MS (Raine et al., 1990; Brosnan and Claudio, 1998). It is thought that entry of T-cells into the CNS depends upon the activation state of these cells and the transmigration across the BBB occurs in a random manner (Hickey et al., 1991). The sensitized T-cells either return to the systemic circulation if no target antigen is recognized, or they initiate an immune response if they recognize a specific target antigen (Brosnan and Claudio, 1998). Thus, as we have already presented, these cells have only two options to move from the blood compartment across the ECs to the neuropil, either paracellularly (i.e., by squeezing through the EC junctions) or by a transcellular pathway (vesicles, modified caveolae, VTS/VVOs).

To reiterate, the factors that control cell-cell adhesion in EAE or in other inflammatory conditions are complex and are thought to be modulated by both soluble pro-inflammatory chemokines, and a specific chemical interaction between membrane-associated leukocyte integrins and EC ligands (Stoolman, 1989; Harlan and Liu, 1992; Lobb, 1992; Fabry and Hart, 1993; Owens et al., 1994; Brosnan et al., 1995, 1997; Eng et al., 1996; Lassmann, 1997; Brosnan and Claudio, 1998; Ransohoff and Tani, 1998). The precise mechanism by which immunogenic myelin-associated proteins are presented from the neuropil to the ECs remains unclear but likely involves perivascular astrocytes, pericytes, microglial cells and macrophages in conjunction with major histocompatibility complex molecules during autoinflammatory events such as EAE and MS (Hickey et al., 1991).

The question of whether or not antigen presentation on ECs may have some modulating effect on CNS inflammation remains open. During inflammatory conditions, the issue concerning how antigenic information is exchanged from/to the invading leukocytes to/from the ECs and other resident perivascular cell types has been the subject of intense investigation. Some investigators focused their attention at the question of encephalitogenic antigen presented to the ECs (Vass et al., 1984; McCarron et al., 1985; Wilcox et al., 1989), while others also studied the contribution made by perivascular pericytes (Pardridge et al., 1989), microglial cells (Hickey and Kimura, 1988), and astrocytes (Traugott et al., 1986; Myers et al., 1993; Tan et al., 1998) as antigen presenting cells. Vass and colleagues (1984) injected MBP into the cerebral spinal fluid of rats. These authors provided immunoultrastructural localization of the MBP-immunolabeled probe within the perivascular basement membranes of blood vessels, within EC caveolae, and on the luminal EC surfaces at regions that mimicked specific binding sites for MBP. These authors, however could not determine if their data represented a physiological expression or their results merely

suggested antigen overload within the system. Nevertheless, this study presented immunoelectron structural evidence that MBP could be presented from the abluminal side of the vessel to the luminal EC aspects. If proteins involved in immune responses of the BBB such as MBP can be presented to the ECs in conjunction with major histocompatibility molecules, this could have important implications concerning the mechanism for antigen identification by sensitized leukocytes as they tether to and probe the luminal EC surfaces in preparation to traverse the BBB. Based on immunoelectron microscopic images presented by Vass et al. (1984), labeling by the MBP-immunogold probe on the luminal plasmalemmal surface suggests a specific receptor-like binding pattern for this protein. Since the

myelin-associated antigens can be presented by the ECs to the T-cells or vice-versa in conjunction with MHC molecules, then the question arises as to how the ECs received these protein complexes. Several interesting questions and issues are raised from these kinds of studies. Are the MBP-laden vesicles presented by Vass et al. (1984) similar structurally and physiologically to the VTS/VVOs? Theoretically, activated T-cells returning to the blood from the parenchyma could deposit MBP-MHC complexes on the inner delimiting membrane surfaces of the VTS/VVOs as they squeeze back through the EC conduits. A portion of this depiction is presented in Fig. 12. Vass et al. (1984) also suggested that minor amounts of MBP at either surface of the EC, once recognized, could initiate a cascade of

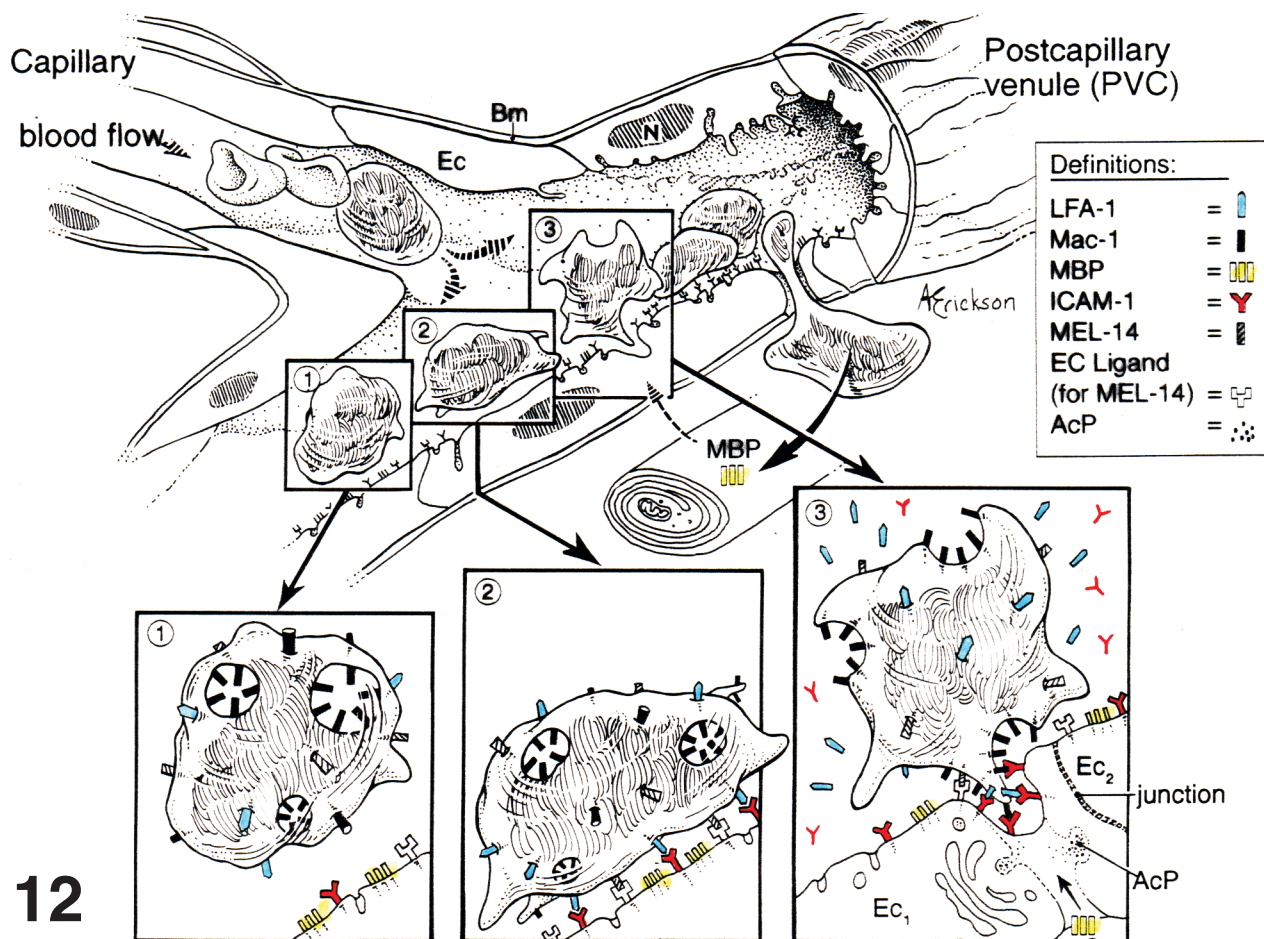


Fig. 12. Diagrammatic composite remodeled with permission by Kishimoto, 1991. This drawing does not present selectins and only depicts some of the important adhesion molecules. In post-capillary venules (top figure), upregulated adhesion molecules play a key role in EC-leukocyte adhesion. The initial step shows blue LFA-1 molecules on the leukocyte (boxes 1, 2) binding to upregulated ICAM-1 molecules (yellow color) on the EC surface. Similarly, VLA-4 binds to VCAM-1 (not shown). This chemical linkage continues within the lining of the invaginating VTS/VVO structure shown (box 3), where LFA-1 and Mac-1 molecules subsequently establish firm binding with ICAM-1 moieties (red color). Later, some of the adhesion molecules shed off and can be identified in the blood plasma. The Mac-1 receptors (black color) later engage the ICAM-1 molecules to further chemically anchor the invading leukocyte to the EC. Note that the binding action is shown to occur at the parajunctional region. According to Vass et al., 1984, yellow-colored myelin-associated proteins shown here as myelin basic protein (BMP) may be presented to and expressed on the luminal EC surface during autoimmune inflammatory conditions.

events that could trigger further liberation of antigens and attraction or homing to the specific vascular region for adhesion of sensitized lymphocytes. Nevertheless, the studies by Vass et al. (1984) and McCarron et al., (1985) and others have focused attention on antigen presentation by the ECs to leukocytes as they tether and adhere to the EC luminal surface T-cells. These kinds of research efforts address important, unresolved issues related to antigen presentation during BBB inflammatory events.

The cytoskeletal infrastructure plays an important role in modulating the entrance of leukocytes and pathogens into the ECs during BBB injury and inflammation

The role that the EC cytoskeleton including microtubular and microfilamentous proteins plays in modulating molecular and cellular transport across the EC has been addressed in recent years. In this context, researchers are currently considering the role that adhesion molecules may contribute in concert with the cytoskeletal infrastructure of the EC during cellular migratory events. EC cytoskeletal proteins have been suggested to be involved with transvascular transport of macromolecules, as well as modulating cell-to-cell adhesion (Nag et al., 1977; Larsson et al., 1979; Shivers et al., 1985; Prakapas, 1990; Takahena et al., 1992; Nag, 1995; Munroe et al., 1996; Yoshida et al., 1996; Lub et al., 1997). There is also considerable literature that suggests that EC cytoskeletal proteins may play a specific role in vesicle shuttling (Shasby et al., 1982; Wolosowich, 1984; Shivers et al., 1985; Joó and Klatzo, 1989; Prakapas, 1990; Gottlieb et al., 1991; Izumi et al., 1991; Travis, 1992; Liu et al., 1993; Nag, 1995). Evidence suggests that remodeling the EC cytoskeleton likely plays an important role in modulating leukocyte trafficking across the endothelial barriers (Lub et al., 1997; Sandig et al., 1997, 1998; Mine et al., 1998; Saito et al., 1998; Wang et al., 2001). Actin filaments accumulate at points on the EC membrane surface where leukocytes make intimate contact (Sandig et al., 1997), and inflammatory leukocytes adhere (Munroe et al., 1996; Yoshida et al., 1996; Peridsky, 1999). Experimental data are available that suggest that alteration of EC actin and microtubular cytoskeletal proteins by drugs such as cytochalasin B or colchicine may alter vascular permeability (Shasby et al., 1982; Steffan et al., 1987; Nag, 1995). These kinds of studies, both earlier and more recent present the argument that during BBB inflammation, chemical rearrangement of the cytoskeletal framework concomitant with restructuring of the EC tubular profiles (i.e., VTS, VVOs, etc) may occur. Such remodeling of the EC infrastructure could possibly block transendothelial translocations of molecules, blood cells and pathogenic microorganisms (Joó and Klatzo, 1989; Lossinsky et al., 1999). Numerous current studies are focused on the cellular and molecular biology of the involvement of the

cytoskeleton with interactions between ECs and other, non-EC types. For example, the molecular biology of actin rearrangement within both host and invading cells during inflammatory episodes is under intensive study. Transcellular migration has received particular attention and it is clear that complex molecular events within and between ECs and invading leukocytes, tumor cells or pathogenic microorganisms must occur before these cellular entities are permitted passage across the BBB. As was already discussed, experimental evidence has revealed that leukocyte transmigration across the EC barrier involves the spatiotemporal regulation of adhesion molecules, chemokines, and cytoskeleton regulators coordinated by sequential integrin activation in consort with the Ras superfamily of small guanosine triphosphatase enzymes (GTPases, Worthylake and Burrage, 2001). These GTPase enzymes include Rho, an enzyme that acts as a molecular switch which controls the signal transduction pathway that links membrane receptors to the organization of the actin cytoskeleton and eventually induces cell adhesion and cell motility (Narumiya, 1996). Another enzyme, Ras, when activated, may lead to the assembly of a meshwork of actin filaments and ultimately to the formation of lamellipodia and membrane ruffling (Hall, 1998). GTPase enzymes may also be important for regulating invasion of the HIV-1 virus (Fiala et al., 1997; Liu et al., 2002) as well as pathogenic bacteria and yeast cells (Huang and Jong, 2001). Another member of the Rho superfamily, Cdc42 has also been shown to induce actin-rich filopodial protrusions (Nobes and Hall, 1992; Ridley et al., 1992; Kozma et al., 1995; Hall, 1998), and is likely linked to the formation of membrane ruffling, lamellipodia and the elongated EC fronds that are expressed during autoimmune conditions of the CNS (Cross and Raine, 1991). Other key molecular players that influence actin cytoskeleton remodeling during EC endocytosis and phagocytosis include cofilin, a major actin-binding and depolymerizing protein in eukaryotic cells that regulates turnover of actin filaments and the formation of their tertiary meshwork structure (Chen et al., 2002). Bierne and colleagues (2001) presented evidence that *Lysteria*-induced phagocytosis is controlled by both activation and deactivation of cofilin, regulated by LIM-kinase, which phosphorylates cofilin and ROCK (the downstream effector of Rho). ROCK, the Rho-associated kinase, has also been implicated in Rho-mediated actin reorganization (Maekawa et al., 1999). Finally, the Rho-GTPase family, as regulators of actin arrangement have been implicated in the mechanism of movement for neutrophils and mononuclear cells (Alblas et al., 2001; Strey et al., 2002).

Mechanisms that facilitate migration of cells and bacteria across blood vessel endothelium have been shown to be complex and, consequently, difficult to characterize. For example, Saito et al. (1998) applied the calcium/calmodulin-dependent myosin light chain kinase (MLCK) inhibitor (ML-9) to human umbilical cord

vessel ECs and observed reduced neutrophil migration in culture. These results suggested that calcium/calmodulin-dependent MLCK plays an important role in regulating transendothelial migration. Chen et al. (2002) have recently studied the effects of nicotine on *E. coli* invasion across the BBB in a culture system of human ECs. These authors noticed that a nicotine-mediated enhancement of actin cytoskeleton rearrangement and EC morphological changes were necessary for *E. coli* invasion of the BBB. Further, they showed that inhibition of phosphatidylinositol 3-kinase (PI3K), an enzyme linked to actin chemistry, abolished the entry of *E. coli* into ECs, an observation that was presumed to be predicated on actin cytoskeleton rearrangement. Collectively, these observations and those discussed previously, suggest that cytoskeleton rearrangement plays a key role in the entry of bacteria into cells and passage of other cells across blood vessels, by exploiting host cell signal transduction pathways which affect cytoskeletal components (Cossart, 2000). Efforts to understand the complex signaling mechanisms that come into play during the invasion of a cell type (foreign to the CNS) or pathogenic organisms across an inflamed BBB are currently being coordinated world-wide.

The kinetics of free viral entry into an in vitro model of the human BBB (Fiala et al., 1997) represents one of the most perplexing problems for molecular neuropathologists today. Evidence obtained by TEM (Liu et al., 2002) has revealed that entry of HIV into brain ECs is not primarily dependent upon clathrin-coated vesicles and also has shown free HIV-1 particles present within EC vacuoles 3 hours post-infection (Liu et al., 2002). The question remains unanswered how the EC cytoskeleton proteins aid in modulation of the formation of caveolae, vacuoles, VTS etc. during uptake of virus particles such as HIV-1. If virus or other pathogenic organisms including fungi (Jong et al., 2001) can traverse the transcellular BBB in vitro, it seems reasonable to assume that leukocytes and tumor cells that follow a transcellular route (in lieu of a paracellular junctional route) may require similar modulations of the cytoskeleton.

Since the cytoskeletal proteins clathrin, actin, dynein and microtubules are involved in physiological endocytosis (Van de Walle et al., 2002), it is thought that some or all of these proteins and protein polymers, may be involved in effecting internalization of pathogens, as well as leukocytes, into ECs of the brain. Dynein and the microtubule network have been suggested to serve as physiological guide cables that facilitate delivery of the HIV viral genome to the nucleus of the cell following entry of the viral particles into the cell. Surely, other pathogenic organisms may utilize a similar cytoskeletal roadway through the cytoplasm of ECs (McDonald et al., 2002). Much remains to be explored at both the molecular and ultrastructural level before the nature of trans-BBB transport of harmful cells and microorganisms through CNS ECs can be elucidated. Details of the role of the cytoskeleton as a modulator of

BBB permeability await further investigations which employ anti-cytoskeletal antibodies to identify which members of the cytoskeleton interact with which parts of the EC VTS/VVOs, and junctional complexes. The question of how cytoskeletal reorganization relates to remodeling of EC caveolae, the VTS and VVOs is an intriguing puzzle that remains unsolved and will be the topic of future studies.

Conclusions and future perspectives

In this review, we have presented a multitude of ultrastructural, ultracytochemical and immunoultrastructural evidence that implicates EC structural profiles defined as the VTS and VVOs, previously purported to represent a mechanism for macromolecular transport. Here we have expanded the definition of these unique EC VTS/VVO structures, originally manifested in the injured BBB as passageways for macromolecules to also represent thoroughfares for transendothelial migration of cellular elements. Although not fully resolved, the EC junctional complexes likely prevent passage of lymphocytes and possibly mononuclear cells, while evidence suggests that neutrophils and possibly mononuclear cells can open (dissolve) the junctional complex. Evidence from the literature and from previous collective studies by the authors of this review support a hypothesis that parajunctional EC conduits are composed of modified caveolae that we and others have defined as the VTS that likely serve as anatomical passageways for the migration of some types of inflammatory and neoplastic cells during a variety of injurious and inflammatory conditions of the BBB. We have also focused attention on several adhesion molecules that play leading roles in the physicochemical attachment of the invading leukocytes to the EC surfaces. It was also suggested that the cell-cell adhesion and linkage to the VTS/VVOs may be analogous to a fighter aircraft jet landing on the deck of an aircraft carrier. The jet can only attach to the carrier deck via a specific cable that forms a specific hook or chemical linkage (Wisniewski and Lossinsky, 1991). Thus, the filopodial projections from the leukocyte are inserted into the punctate EC ostia establishing a firm link for its subsequent cellular journey across the BBB to the CNS.

Remodeling the expression of the adhesion molecules on the surfaces of the ECs or invading leukocytes, ie, their chemical gatekeepers, using antibodies and chemical agents that may alter the EC cytoskeletal infrastructure or membrane lipid chemistry appear to have promise as future strategies for modulating macromolecular transport (e.g. fluid influx into or out of the CNS after stroke, or the cellular trafficking that occurs during autoimmune events of the CNS (e.g. infantile meningitis, cancer, HIV-1 infection, etc.). The recent clinical trials for MS patients using anti-integrin antibodies as a therapeutic treatment may spearhead a new effective strategy for altering the structural nature of cell-cell adhesion within the injured

BBB. These innovative approaches indicate that the entrance of macromolecules and microorganisms into the BBB can be altered experimentally. Such studies may have significant import for the development of future therapeutic intervention related to BBB disturbances in human medicine. Thus, by further elaborating the precise roles that the VTS/VVO and the junctional complexes play in the disease processes, it may be possible to learn how to modulate the opening and closure of these EC structures at will by masking specific EC and/or leukocyte adhesion molecule receptors, by altering the EC cytoskeletal protein infrastructure or a combination of both. Because the nature of leukocytic passage across the BBB remains equivocal at present, it is clear that additional research efforts will be required before the mysteries of cell-cell adhesion and transBBB migration will be fully elucidated. Better understanding of these phenomena can be expected to have profound implications for future therapeutic intervention in human inflammatory and metastatic conditions including traumatic brain and spinal cord injuries (Shivers, 1994), multiple sclerosis, infantile and adult meningitis, AIDS, stroke and cancer.

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