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ULTRASTRUCTURAL STUDIES OF PECAM-1/CD31 EXPRESSION IN THE DEVELOPING MOUSE BLOOD-BRAIN BARRIER WITH THE APPLICATION OF A PRE-EMBEDDING TECHNIQUE

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The aim of this study was to investigate the time of expression of the adhesion molecule platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) in the developing mouse blood-brain barrier (BBB). Ultrastructural studies employing a preembedding technique described herein demonstrate that PECAM-1 is initially expressed on the luminal and abluminal endothelial cell surfaces in the newborn animals. This adhesion molecule expression appears to increase in intensity at 7-10 days *post partum* and then decreases to a weak labelling of the luminal endothelial cell surfaces at two weeks after birth. Our results present immunocytochemical detection of active angiogenesis during early brain development in the mouse. Moreover, because of the important role that adhesion molecules play in immune responses in the central nervous system, upregulation of PECAM-1 prior to structural maturity of the BBB may suggest that the development of an "immune BBB" manifests prior to anatomical closure of the BBB in the mouse.

Adhesion molecules that belong to the immuno-globulin supergene family include intercellular adhesion molecule-1 (ICAM-1/CD54), platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) and several others. These molecules are known to facilitate cell-cell adhesion and transvascular migration of either platelets, leukocytes, inflammatory cells and tumor cells *via* specific attachment molecules called lymphocyte function associated molecule-1 (LFA-1/CD11a) (Marlin, Springer, 1987). During disease states within the CNS, these adhesion molecules are known to up regulate (Sobel et al. 1990). This intense expression of adhesion molecules associated with blood vessels essentially represents an indicator of either homing of leukocytes to high endothelial cells in normal lymphatic tissue (Brown et al. 1993), inflammatory processes (Raine et al. 1990) or angiogenesis (Lossinsky et al. 1995). Thus, other than what is expressed in normal lymphatic endothelia, upregulation of these molecules within the microvasculature is considered as a hallmark for some type of accelerated immune conditions. PECAM-1 has been described as one of the adhesion molecule that is associated with microthrombus formation as well as other

inflammatory responses (Muller et al. 1993; Rosenblum et al. 1993).

Although interactions between inflammatory cells and the endothelial cell plasmalemmal surface *via* adhesion molecule expression have been well characterized in vascular tissue from the adult animal, little is known about the ontogeny of the immune response or when adhesion molecule expression begins in the developing mammalian blood-brain barrier (BBB).

Material and methods

Pregnant C57BL or SJL/J mice were obtained from the animal colony at IBR in New York. The procedures followed in this report conform to the National Institutes if Health's guidelines for animal care and handling. Mice were determined to be pregnant by the first sign of vaginal plugs. Mouse pups were examined at their day 1 (newborn), and on days 3, 5, 7, 10 and 13/14 after birth. Our fixation schedule was similar for human brain biopsy specimens published elsewhere (Lossinsky et al. 1995). Briefly, animals were decapitated and brains were coronally sectioned. Tissue from the parietal cortex was diced into 1 × 2 mm rectangular blocks in
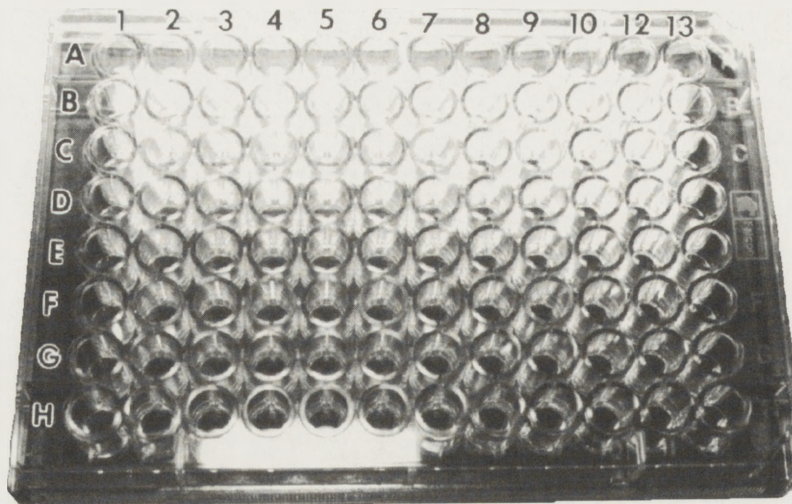
Fig. 1. Microtiter plate illustrates the wells in which the tissue specimens are immunoreacted, washed with buffer, *en bloc* stained with uranyl acetate and postfixed with OsO_4. The plates are covered and enclosed within a larger container with cover that contains water-soaked paper towels. The entire system acts as a hydration chamber to prevent evaporation within the individual wells during overnight incubations. Primary antibodies can also be titrated using this system if optimum antibody concentrations have not been established
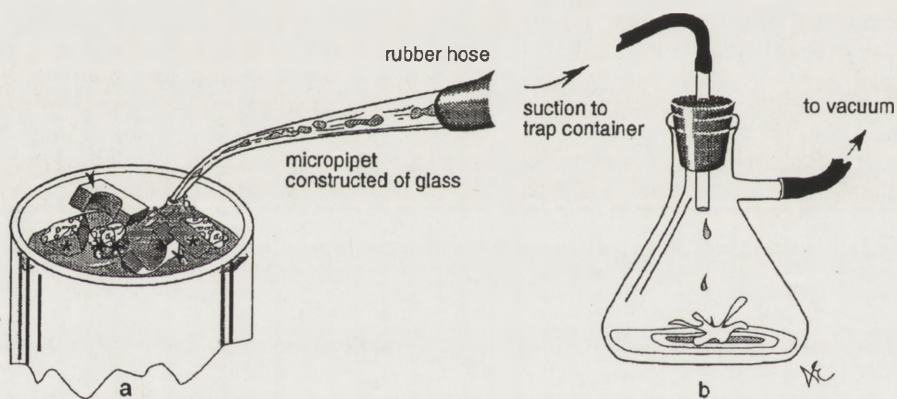

Fig. 2. Digrammatic representation of an individual microtiter well to illustrated how this system is used. Using a vacuum system shown, tissue pieces (* and **), as well as incubation solutions, buffer washes and agar particles (arrowheads) can be removed
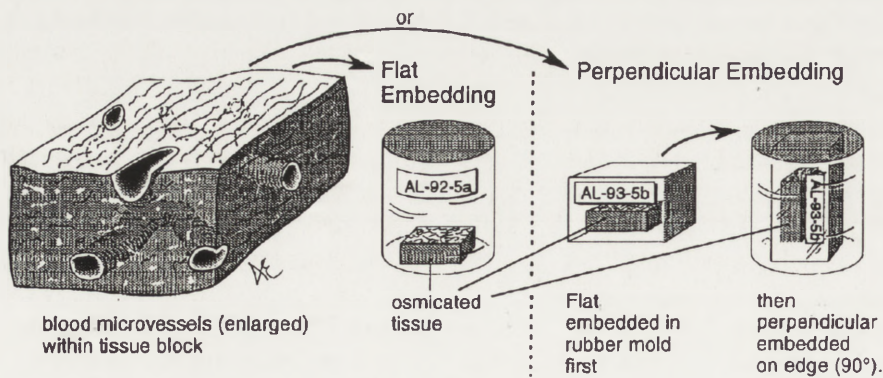
Fig. 3. Diagrammatic view of a rectangular piece of tissue demonstrating the direct exposure that vessel-associated antigens have to the incubation solution. The best tissue preparations resulted when the tissue blocks were embedded perpendicular (on end) to present maximum antibody penetration
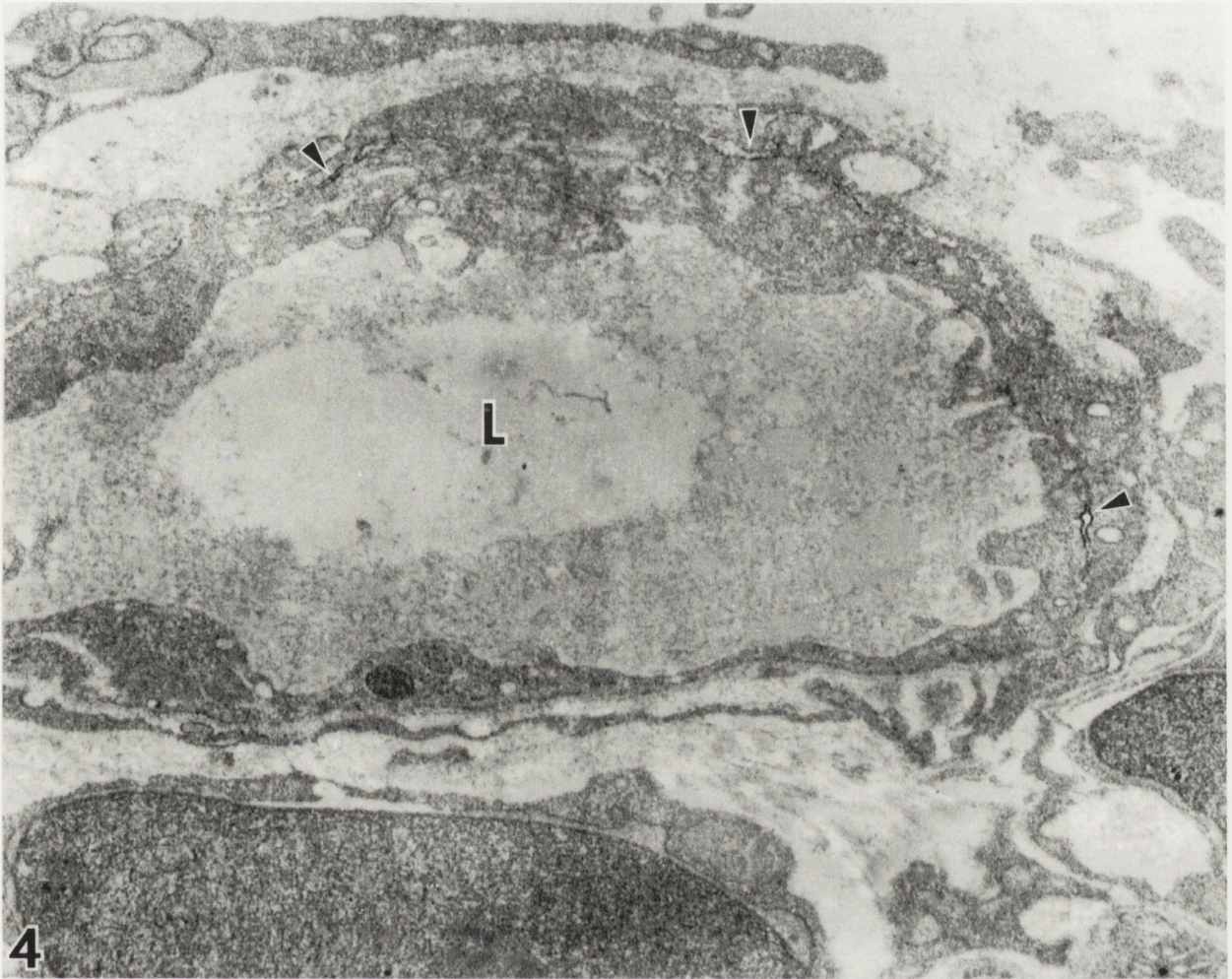
Fig. 4. Newborn mouse brain cortex. Note the weak peroxidase reaction product for PECAM-1 primarily associated with the abluminal vessel front (arrowheads). L = vessel lumen. × 12,000

a fixative containing 2% formalin, 0.1% glutaraldehyde in either 0.1 sodium phosphate or sodium cacodylate buffer at pH 7.3. Immersion fixation continued for 2-3 hrs at 4°C. Some coronal 1 × 2 mm sections were embedded in agar and chopped with tissue sectioner at 50 μm. Tissue pieces were then transferred to a buffer containing 0.1 M sodium cacodylate plus 0.2 M sucrose (S/C buffer) with the addition of 0.01% sodium azide as a preservative.

Immunocubation technique. All immunocytochemical procedures including addition of antibodies, buffer rinses, *en bloc* staining and post-fixation with osmium tetroxide described below were performed in a 96-well microtiter plate (Falcon 3072 Plate, Becton Dickinson & Co., Lincoln Park, NJ, USA, Fig. 1). After overnight blocking with PBS containing 10% goat serum (PBSS) in a refrigerator (4°C) and then quenching aldehyde groups with 0.1 M glycine for 1 hr at 25°C, primary antibody was added to the tissue. Rat anti-PECAM-1 monoclonal antibody (Pharmagen Pharmaceutical Co, San Diego, CA, USA) was used at either 1:50 – 1:100 dilutions

in PBSS overnight at 4°C. All overnight incubations consisted of placing the covered microtiter plate in a hydration chamber to prevent drying. An Eppendorf syringe was used to apply ca. 250 ml of antibody solutions or PBSS rinses to each well containing the tissue and immunoreagent. Tissue pieces were washed numerous times (x8) with PBSS to insure removal of unbound antibodies and/or peroxidase label described below (Fig. 2).

Post-embedding procedures. Tissue samples were incubated in a secondary, biotinylated goat anti-rat IgG (H + L) polyclonal antibody at 1:50 dilution in PBSS (Accurate Chemical & Scientific Co., Westbury, NY, USA) for 1 hour at 25°C. After thorough washing with PBSS (x8), tissue samples were reacted for 30 min with 0.03% H_2O_2 at 25°C to inactive endogenous peroxidase activity. The tissue was then incubated in extravidin-peroxidase label (Sigma Chemical Co., St Louis, MO, USA) at 1:50 dilution in PBSS at 25°C for 1 hour. Tissue samples were then washed with plain PBS × 4. Control incubations included PBSS without
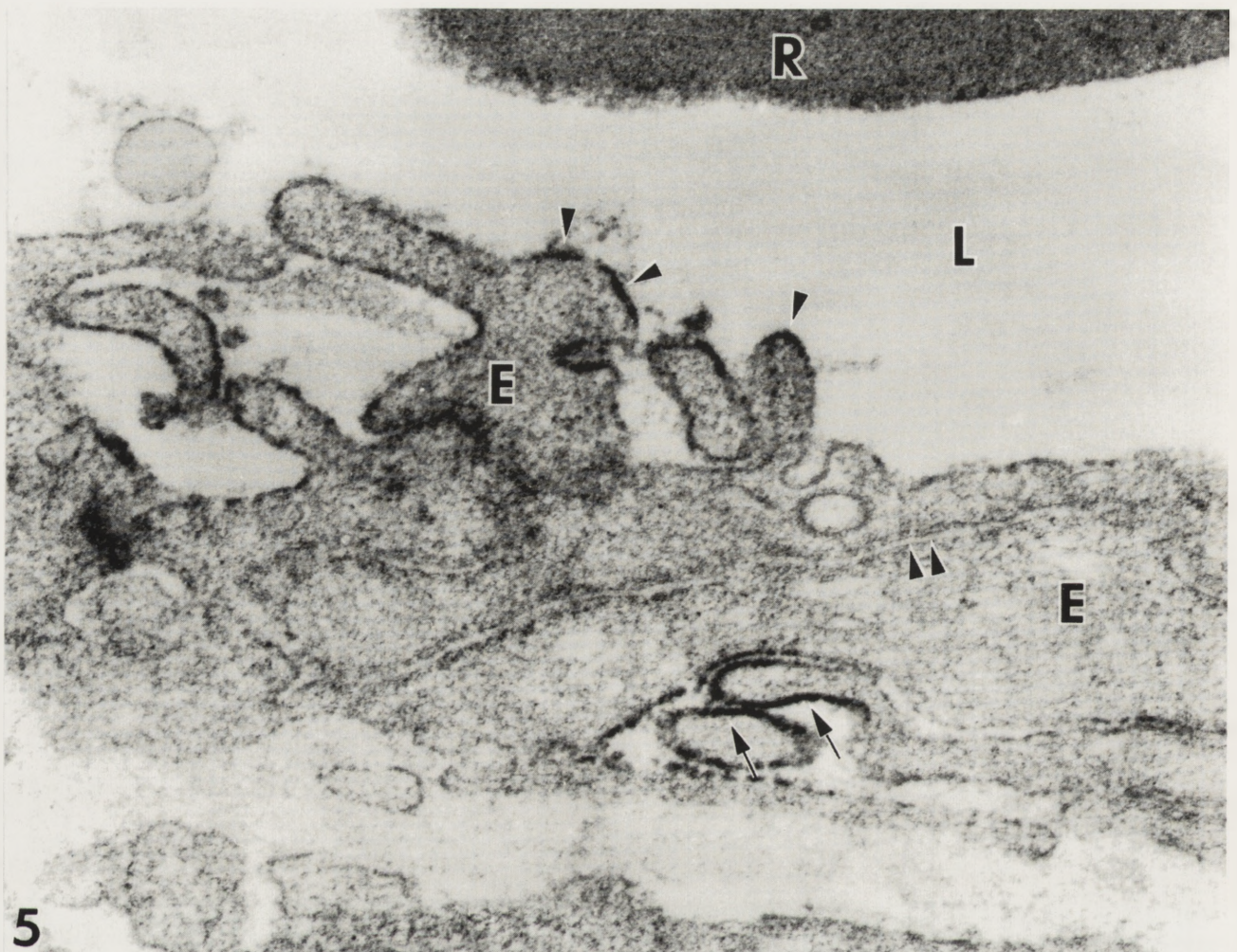
Fig. 5. High magnification of a blood vessel wall from the newborn mouse brain cortex. Note the strong PECAM-1 localization associated with both the luminal (arrowheads) and abluminal (arrows) endothelial cell plasma membranes. The junctional complex in this vessel appears closed (double arrowheads). L = lumen; R = erythrocyte; E = endothelial cell. ×30,000

PECAM-1 antibody or incubating the tissue with an irrelevant antibody (eg. anti-mouse myoglobin) at 1:50 dilution in PBSS.

Tissue samples were post-fixed in 1% OsO_4 in 0.1 M sodium cacodylate buffer for 1 hour at 4°C, rinsed with either S/C buffer of 7.5% sucrose, *en bloc* stained in uranyl acetate, dehydrated in ethanol and embedded in Spurr plastic (Spurr, 1969; Fig. 3). Thick-and thin-sections were cut on a Sorval MT-1 ultramicrotome. Thin-sections were stained with lead and uranyl acetate (material not *en bloc* stained) and examined with a Hitachi 7000 electron microscope.

Results

Peroxidase reaction product for PECAM-1 was observed in the developing blood vessels in newborn mice, primarily noticed on the abluminal endothelial cell surface, but also observed weakly staining the luminal surface (Figs 4, 5). At three, five and seven days, there appeared to be a gradual increase in the intensity of the luminal surface staining of the

peroxidase reaction product (Figs 6, 7). By 13/14 days after birth, a weak reaction product was observed by and large on the luminal surface of the endothelial cells (Fig. 8).

Other ultrastructural observations included endothelial cell junctions that appeared to be widened in the newborn animals (Fig. 4). These junctional complexes appeared to be closed at 13/14 days. Numerous endothelial cell microvilli and fronds were prominent during the first week after birth (Fig. 7). Leukocytes were occasionally observed within the vessel lumina but were not observed attached to or appeared to be in the process of diapedesis (Fig. 6). Endothelial cell vacuoles were also noted in tissue samples that was not well fixed (Fig. 6). Basal lamina in most vessels at all time periods appeared thin or ill-defined. Very little reaction product for PECAM-1 was observed within endothelial cell vesicles or tubular profiles. PECAM-1 reaction product appeared to be associated with both types of cortical blood vessels, especially thin-walled veins, venules, capillaries and arterioles.
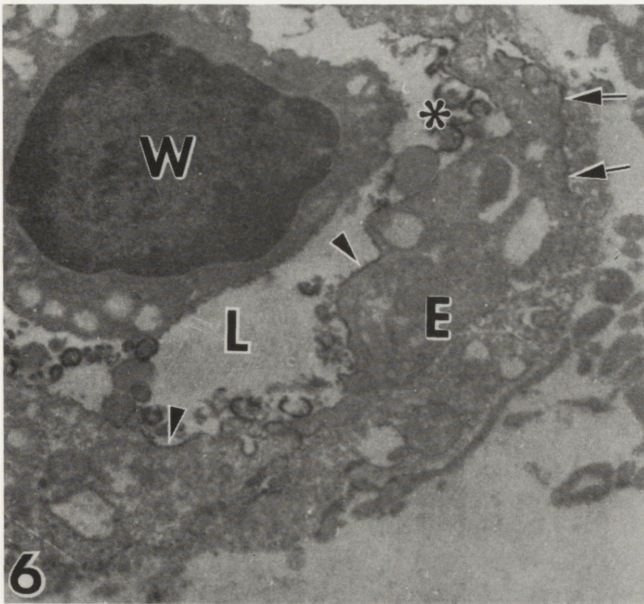
Fig. 6. Day 3, mouse cerebral cortical capillary shows a weak reaction product for PECAM-1 associated with the luminal surface (arrowheads), the abluminal surface (arrow) and with cellular debris (*). Note the leukocyte (W) and swollen cellular organelles in both the leukocyte and endothelial cell, possibly due to improper fixation. E = endothelium; L = vessel lumen. ×6,000
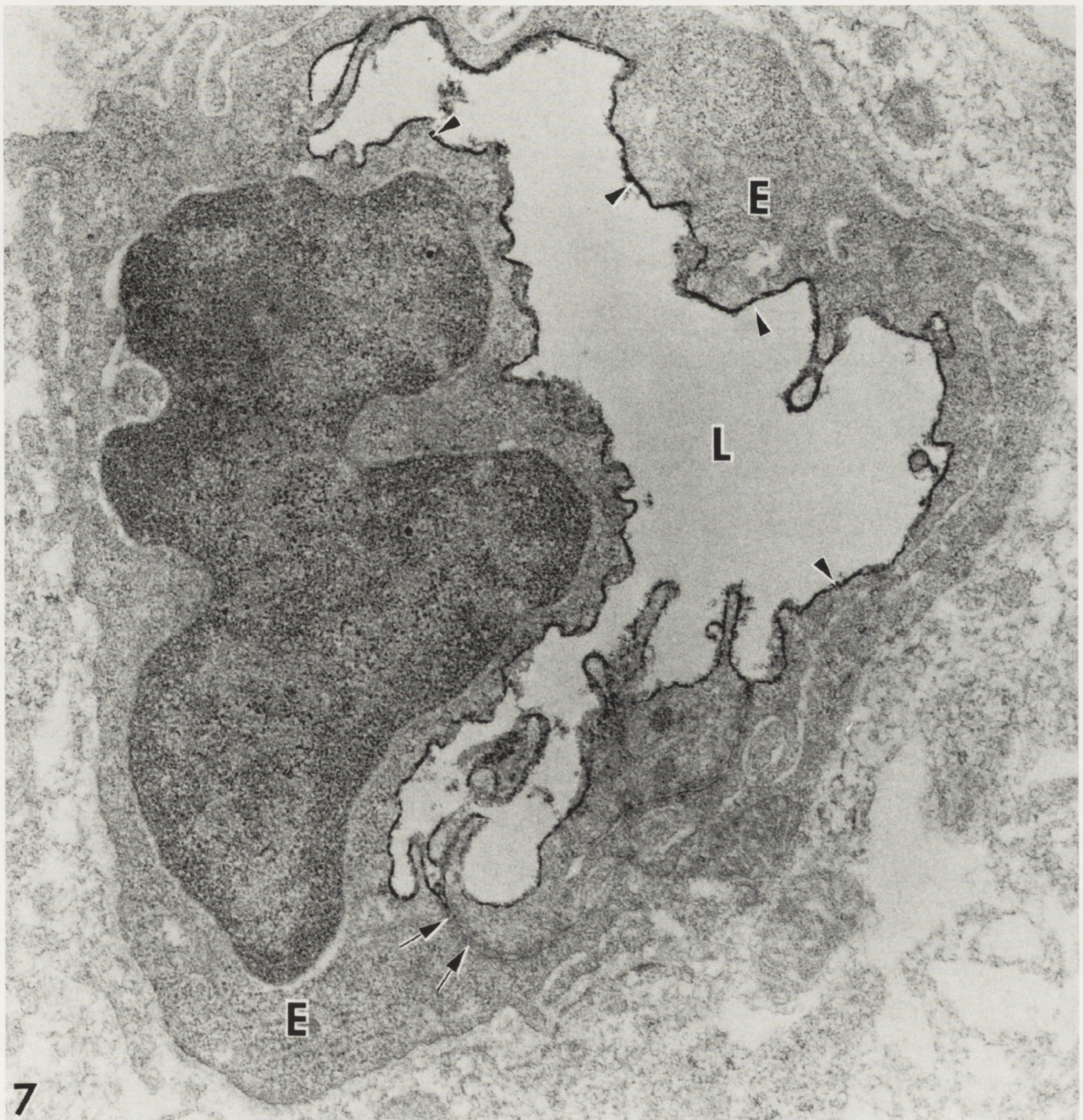
Fig. 7. Day 7, mouse cerebral cortical vessel shows a more intense PECAM-1 reaction product staining the luminal endothelial cell membrane (arrowheads). Note the junctional complex appears to be closed (arrows). L = lumen; E = endothelium. ×13,800
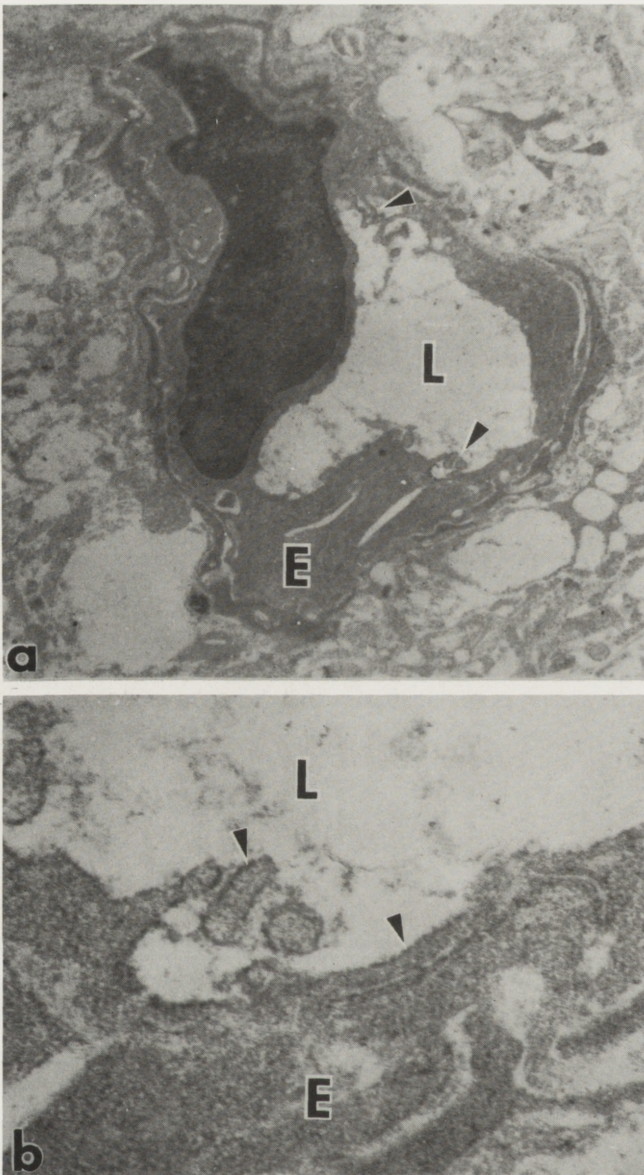
Fig. 8a,b. Day 14, mouse cerebral cortical vessel showing a weak PECAM-1 reaction product staining the luminal endothelial cell membrane (arrowheads). L = lumen; E = endothelium. a ×5,000; b ×17,000

Discussion

Results presented herein describe the early appearance of the adhesion molecule PECAM-1/CD31 within the developing microvasculature in the mouse cerebral cortex with the application of a pre-embedding technique. This approach was useful for us since it enables proper exposure of the vessel-associated protein epitopes. The immunocytochemical reactions were performed prior to post-fixation, dehydration and plastic embedding. Although this technique was useful for our PECAM-1 identification, its limits antigenic exposure to surface proteins within the perivascular region (Fig. 3). Antigens that exist deeper within the tissue, such as neuronal and glial proteins usually require post-embedding techniques for detection using water-soluble embedding media and immunoincubation procedures on thin-sections collected on grids (Newman, Hobot, 1989).

We demonstrate that PECAM-1 is already expressed in the brain vasculature at birth in the mouse. This is consistent with the fact that adhesion molecules are known to upregulate during angiogenesis (Polverini, 1996), when blood vessels are rapidly proliferating (Zagzag, 1995). The location of the reaction product for PECAM-1 at both vascular fronts at newborn to five days is similar to results obtained from studies of ICAM-1 localization in human brain tumor biopsies (Lossinsky et al. 1995). This indicates that during angiogenesis, some adhesion molecules are located on either side of the endothelial cells. This may possibly represent an expression of the state of preparedness for platelets or leukocytes intending to attach to and traverse the blood vessels in either direction. After the BBB matures, the primary localization of PECAM-1 in normal resting blood vessels is at the blood facing endothelial surface. Once the BBB matures, platelet or leukocyte adhesion and diapedesis that occurs across the BBB under normal physiological conditions is limited (leukocytes) or non-existent (platelets). Under conditions of new vessel growth, however, inflammatory events or angiogenesis within tumors, increased synthesis of these adhesion molecules facilitates conditions for accelerated cellular adhesion and transendothelial migration of the invading cellular elements.

Our results are also consistent with previous studies of the development of the BBB where it has been shown that the structural maturity of the BBB does not occur until ca. two weeks *post partum* in the mouse (Vorbrodt et al. 1986; Lossinsky et al. 1986; Stewart et al. 1987). The timing of this event varies in different mammalian species (Bradbury, 1979). Previous studies concerning vasculogenesis and angiogenesis in early mouse brain development (Bradbury 1979; Vorbrodt et al. 1990) point attention to the fact that during early brain maturation, the appearance of certain glycoproteins within the actively growing brain vasculature often precedes structural maturity of the barrier. In the developing vasculature of the mouse brain, it is known that a variety of BBB-associated protein enzymes are, in fact, expressed at different times during brain development. BBB-associated enzymes including y-glutamyl transpeptidase was shown to be present at embryonic day 15 in the brain and increased rapidly in *post natal* rat brains up to 30 days (Sessa, Perez 1975). Alkaline phosphatase, and butyryl cholinesterase were shown to be present as early as embryonic day 17 in the mouse brain (Risau et al. 1986) and both enzymes are expressed fully in the mature BBB (Risau et al. 1986; Vorbrodt et al.

1986). We report evidence in this report that PE-CAM-1 appears associated with the vasculature during early brain development and then decreases in intensity with structural maturity of the barrier. This inverse correlation appears to suggest that the above-mentioned enzymes are related to the modulation of BBB function (Vorbrodt, 1988), while adhesion molecules such as PECAM-1 and others are strongly expressed during angiogenesis and then diminish to a weakly expressed marker of the luminal endothelial cell plasma membrane. During inflammatory processes, these adhesion molecules are synthesized and are directed to the luminal endothelial front to serve as modulators of inflammatory processes.

Because of the important role that PECAM-1 plays in cell-cell interactions and the immune response, data presented here appear to indicate that an "immune BBB" precedes the development of a "structural BBB". This contention is consistent with the observed increased numbers of endothelial microvilli and fronds (Figs. 5, 7), noted to be associated with inflammatory vascular conditions of the CNS (Cross et al. 1990; Lossinsky et al. 1991; Wiśniewski, Lossinsky 1991). Whether or not increased cell-cell adhesion occurs during the period of BBB development does not seem to be the case according to our observations, but this question remains unclear at present. Additional research with other adhesion molecules will be necessary in developing brain material in order to answer questions concerning the nature of the ontogeny of the anatomical and immune mammalian BBB.

Badania ultrastrukturalne ekspresji PECAM-1/CD31 rozwijającej się bariery krew-mózg u myszy, z zastosowaniem techniki przedzatopieniowej

Streszczenie

Przedmiotem pracy było zbadanie ekspresji cząsteczki adhezyjnej PECAM-1/CD31 w rozwijającej się barierze krew-mózg u myszy. Badania ultrastrukturalne wykazały, że PECAM-1 początkowo u noworodka ujawnia się na obu (zewnętrznej i wewnętrznej) powierzchniach komórki śródbłonka. Intensywność przylegania cząsteczki wzrasta siódmego-dziesiątego dnia po porodzie, a następnie w około 2 tygodnie po porodzie zmniejsza się do słabej aktywności od strony światła naczynia. Przedstawione wyniki pozwalają na immunocytochemiczne udokumentowanie aktywnej angiogenezy we wczesnym rozwoju mózgu myszy. Ponadto, ponieważ adhezyjne cząsteczki odgrywają rolę w odpowiedzi immunologicznej ośrodkowego układu nerwowego, wykształcenie ekspresji PECAM-1 przed osiągnięciem strukturalnej dojrzałości bariery sugeruje, że rozwój "immunologicznej bariery" występuje wcześniej niż jej zamknięcie anatomiczne u myszy.

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