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Blood-brain barrier permeability in the periventricular areas of the normal mouse brain

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Abstract The main objective of this study was to assess the blood-brain barrier (BBB) permeability in periventricular areas of the normal mouse brain to test the hypothesis that the fragility of the BBB in periventricular areas may play a role in periventricular white matter lesions. Vascular permeability to intravenously injected horseradish peroxidase (HRP) was examined in the periventricular areas of adult mouse brain using light and electron microscopy. Staining for HRP appeared in the periventricular area adjacent to medial side of the lateral ventricle as well as in BBB-free areas, in the lateral septal nucleus, in the medial portion of the hippocampus and in the dorsal portion of the thalamus. In addition, the staining for HRP appeared in ependymal cell layer located near the choroid plexus and was found early after HRP injection in the wall of some vessels located at medial side of the optic tract. Ultrastructural examination of the vessel wall revealed that staining for HRP in the perfusion-fixed mice after circulation of the tracer for 5 min appeared in the perivascular space, in the basal lamina, in several vesicular profiles of the endothelial cell cytoplasm including abluminal pits, in vesicular profiles of perivascular cells and in the adjacent extracellular space. In the mice perfusion-fixed after HRP circulation for 90 min, staining for HRP in the vessels at medial side of the optic tract appeared in the cytoplasm of the perivascular cells, in vesicular structures of the endothelial cell cytoplasm such as plasmalemmal vesicles, endosomes and multivesicular bodies and occasionally in the vascular basal lamina. No clear staining reaction for HRP was found in the periventricular areas adjacent to lateral side of the lateral ventricles. These findings indicate that the BBB in the periventricular area adjacent to medial side of the lateral ventricle near the root of the choroid plexus is not so tight as it is in the cortex or in the lateral periventricular areas, and suggest that the perivascular cells play a scavenger role in the periventricular area as a component of the BBB. In addition, they indicate that blood-borne macromolecules can also invade the areas adjacent to the ventricles such as the lateral septal nucleus, the medial portion of the hippocampus and the dorsal portion of the thalamus.

Key words Blood-brain barrier · Horseradish peroxidase · Periventricular area · Perivascular cells · Virchow-Robin space

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Introduction

The blood-brain barrier (BBB) prevents intravascular macromolecular substances from entering the brain parenchyma [4–6, 27]. In the brain, there are circumventricular organs (BBB-free areas), in which intravascular macromolecules can penetrate the brain parenchyma. Those organs are supplied by permeable capillaries and play an important role in secreting several hormones. It has been also observed that intravascular macromolecular solutes can be transported in some specific BBB segments. It was reported that portions of certain arterioles on the pial surface of the brain, located in the anterior part of the dorsal sagittal fissure, in the cerebellar sulci and in the sulcus between olfactory bulb and cerebral hemisphere, could transfer protein from the blood to the subendothelial basement membrane [36]. Blood-borne peroxidase which had entered the subarachnoid space of the pial surface was reported to move extracellularly through the pia mater into the extracellular

clefts of the underlying neuropil and through the Virchow-Robin spaces for widespread distribution within the perivascular clefts [2, 7, 9]. Tracer injected intraventricularly can penetrate across the ependymal lining into the cerebral parenchyma [5, 6] and invade the brain along the perivascular space [21, 34]. The circumventricular organs such as the median eminence, area postrema and subfornical organ have also been shown to contribute to the distribution of blood-borne macromolecules through the perivascular clefts [3, 7, 8]. In addition, intravascular macromolecules were reported to be infused extracellularly, presumably through leaking vessels present in the circumventricular organs, into other brain areas, such as the corpus callosum [7, 9], the medial portion of the mouse hippocampus [29, 30] and the dorsal portion of the thalamus in the aged mice [30].

Diffuse areas of hypodensity on computed tomography or hyperintensity on T2-weighted magnetic resonance imaging are often encountered in the periventricular or subcortical white matter of the elderly individuals, including patients with ischemic cerebrovascular disease and Alzheimer's disease [14, 16, 26]. The mechanisms whereby the periventricular white matter lesion is rarefied remain unknown. The cause may be chronic cerebral ischemia and/or hypertension [10, 12, 13]. In addition, the BBB was reported to be impaired in the periventricular white matter in the brains of Binswanger's disease patients [1, 22, 28]. Although it is a generally accepted view that the tracer injected intraventricularly can penetrate into the cerebral parenchyma across the ependymal lining [5, 6], the BBB permeability in the periventricular areas has not been well studied. In this study, the BBB permeability to blood-borne macromolecules in the periventricular regions was examined and then the regional differences in the BBB were evaluated.

Materials and methods

For this study 2- to 4-month-old C3H (n = 10) and DBA/2 mice (n = 4) were reared in the animal facilities of the Kagawa Med-

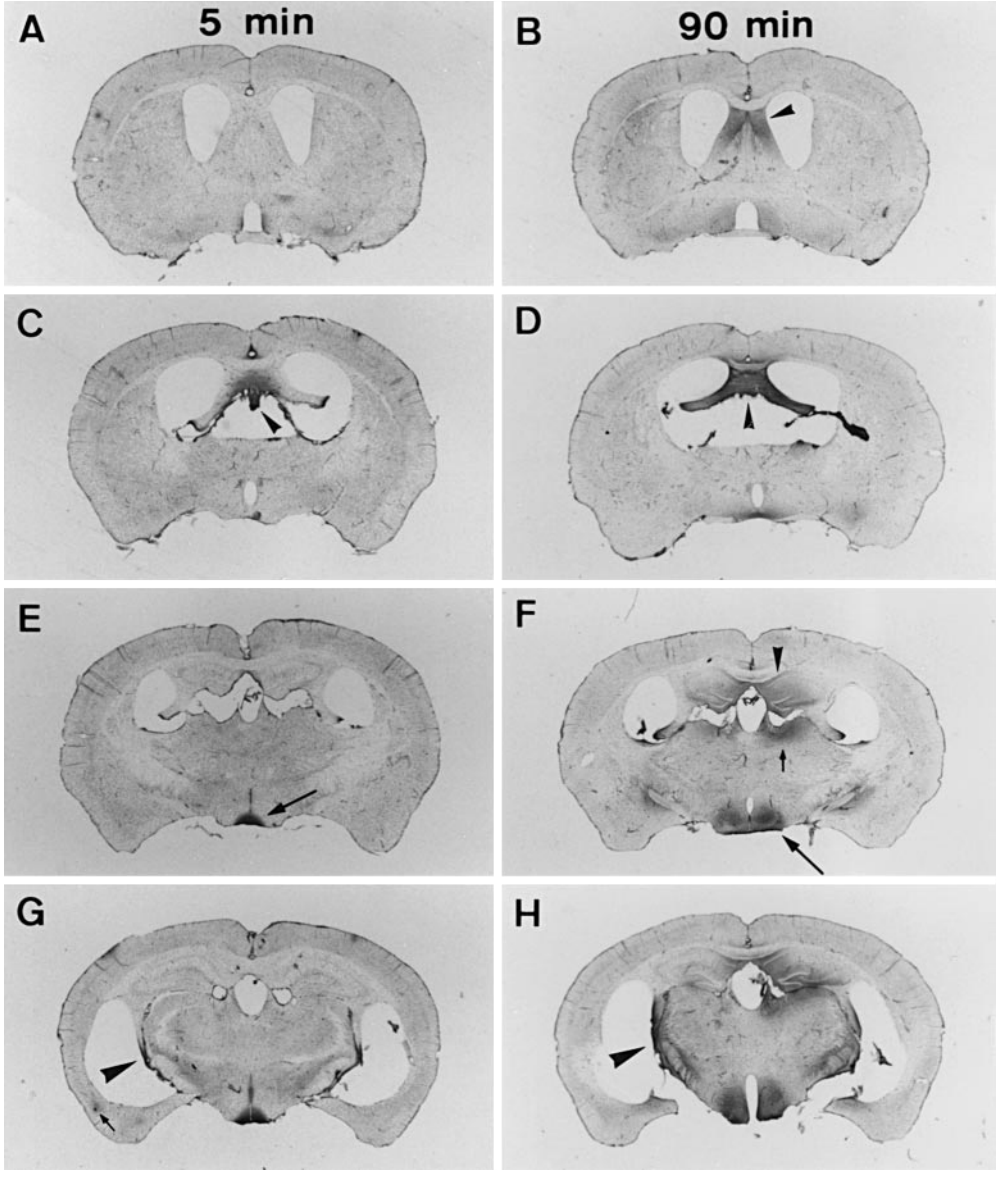


Fig. 1 A–H The staining reaction for HRP with TMB incubation is shown in the sections obtained from perfusion-fixed C3H mice after HRP circulation for 5 (**A**, **C**, **E**, **G**) and 90 (**B**, **D**, **F**, **H**) min. The sections cut through the septum (**A**, **B**) and the hippocampus (**C**–**H**) are shown. In perfusion-fixed mice after HRP circulation for 5 min (**A**, **C**, **E**, **G**), staining for HRP is present in the periventricular area adjacent to medial side of the lateral ventricle (**G**; *arrowhead*), in the hypothalamus around the median eminence (**E**; *arrow*) and in the subfornical organ (**C**; *small arrowhead*). Artifactual staining for HRP (**G**; *small arrow*) is sometimes observed in the perfusion-fixed mice after HRP circulation for 5 min. In the perfusion-fixed mice after HRP circulation for 90 min (**B**, **D**, **F**, **H**), strong staining for HRP appears in the periventricular area (**H**; *arrowhead*), the hypothalamus around the median eminence (**F**; *arrow*), the subfornical organ (**D**; *small arrowhead*), the medial portion of the hippocampus (**F**; *small arrowhead*), the dorsal portion of the thalamus (F; *small arrow*) and the lateral septal nucleus (**B**; *small arrowhead*) (HRP horseradish peroxidase, *TMB* tetramethylbenzidine). **A**–**H** × 6.2

ical University. They were kept under conventional conditions at 24 ± 2 °C, 60 ± 10% humidity and were maintained on a commercial diet and tap water ad libitum. All experiments were performed in accordance with Guidelines for Animal Experiments of the Kagawa Medical University and the National Institutes of Health guide for the care and use of laboratory animals. Native horseradish peroxidase (HRP; type VI, Sigma Chemical Co., St. Louis, Mo.) was dissolved in saline and injected at 0.4 mg/g body weight into the tail vein. At 5 or 90 min after the HRP injection, animals were anesthetized intraperitoneally with sodium pentobarbital, perfused transcardially first with physiological saline at room temperature, then with 2.5% glutaraldehyde (Sigma) and 2% paraformaldehyde

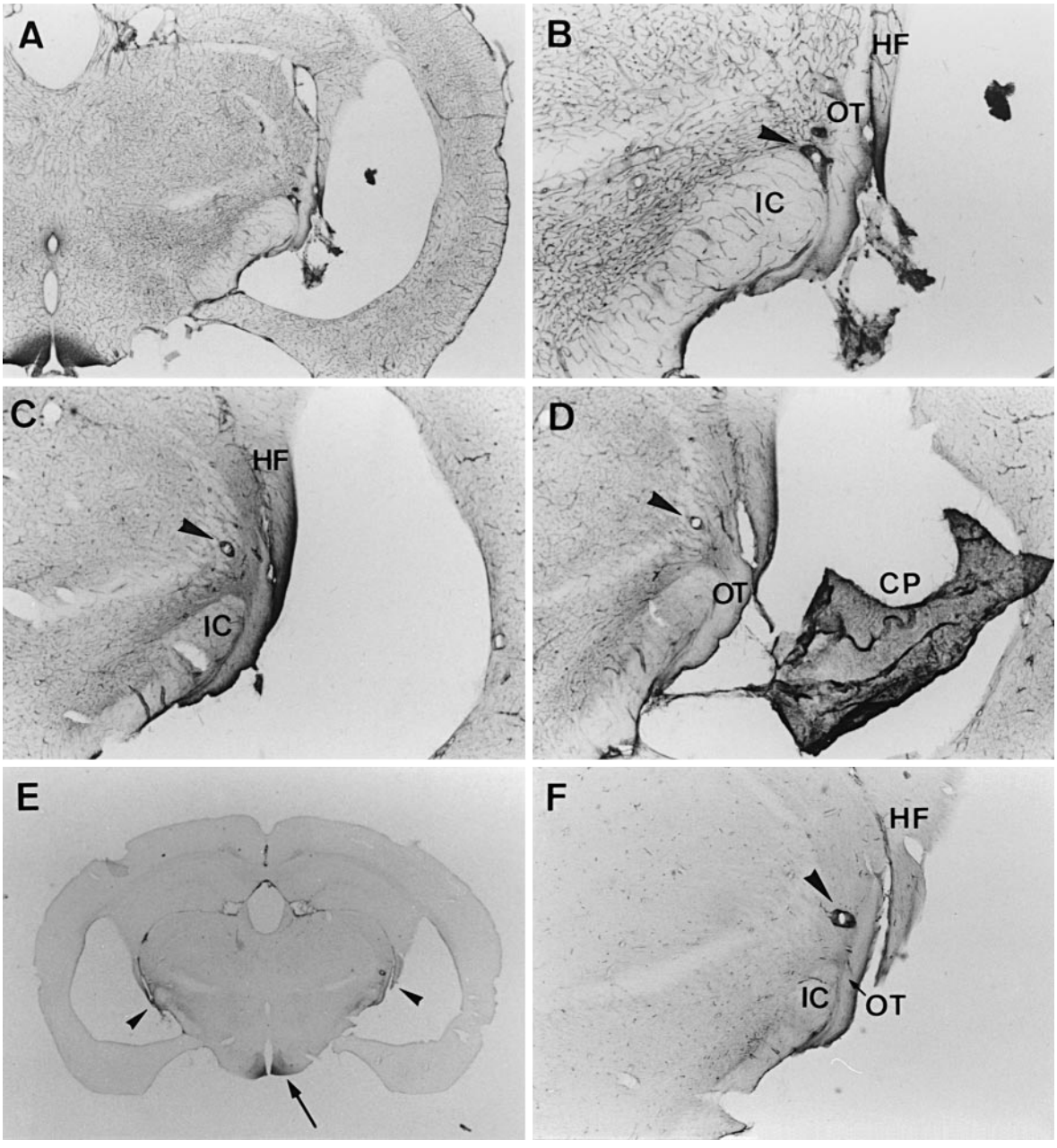


Fig. 2 A–F Light microscopic photographs of staining for HRP with TMB (**A**–**D**) and DAB (**E**, **F**) incubation in the periventricular area are shown in the mice perfusion-fixed after HRP circulation for 5 (**A**, **B**) and 90 (**C**–**F**) min. **A** Localized staining reaction for HRP appears in the periventricular area adjacent to the medial side of the lateral ventricle as well as in the hypothalamus around the median eminence at 5 min after HRP injection. **B** Higher magnification of the staining reaction for HRP in the periventricular area; staining for HRP is present in the hippocampal fimbria (*HF*), ependymal cell layer and the wall of the specific vessel (*arrowhead*) located at medial side of the optic tract (*OT*) or dorsal side of the internal capsule (*IC*). **C**, **D** At 90 min after HRP injection, strong staining reaction for HRP with TMB appears in the hippocampal fimbria and in the periventricular area near the choroid plexus (*CP*) including the vessel (*arrowhead*) located at medial side of the optic tract. **E** In the brain sections which were obtained from the mouse shown in **C**, **D** localized staining for HRP with DAB appears in the hypothalamus around the median eminence (*arrow*) and in the periventricular area (*small arrowheads*), but not in the medial portion of the hippocampus. **F** Higher-power magnification of the staining for HRP with DAB; staining is observed in the vessel wall (*arrowhead*) located at medial side of the optic tract (*DAB* diaminobenzidine). **A** × 18.5; **B** × 45; **C**,**D**,**F** × 28; **E** × 7

(Nakarai Tesque) in 0.1 M phosphate buffer, at pH 7.4 and decapitated [29–31]. The brain was removed and immersed in 1.25% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at 4 °C for 5 h. Some mice (n = 4) were anesthetized after the HRP injection and their brains were removed without perfusion and immersion-fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. The brain was then placed in a sucrose-buffer solution consisting of 10% sucrose in 0.1 M phosphate buffer, pH 7.4, at 4 °C for 12–24 h and sectioned coronally on a microslicer into sections 50 μm thick. All sections were collected in 0.1 M phosphate buffer. Some sections were then transferred to an incubation medium composed of 0.01 M acetate buffer (pH 3.3), tetramethylbenzidine (TMB), and hydrogen peroxide, as reported before [25, 29], while other sections were transferred to an incubation medium containing 0.1 M TRIS buffer (pH 7.4), 3,3′-diaminobenzidine (DAB) and hydrogen peroxide. For light microscopic observation, some sections incubated with TMB or DAB were mounted on gelatin-coated glass slides. For electron microscopy, some sections incubated in a medium containing DAB were post-fixed in 1% osmium tetroxide for 1 h, stained en bloc in 1% uranyl acetate, dehydrated in ethanol and embedded in Epon 812 [31]. Sections, 1 μm thick, were taken from each block and stained with 1% toluidine blue. Ultrathin sections were cut, placed on uncoated grids and observed with a JEM-200CX or a JEM-1200EX electron microscope.

Results

Figure 1 shows the staining reaction for HRP with TMB incubation in C3H mice that were perfusion-fixed after HRP circulation for 5 and 90 min. In the perfusion-fixed mice after HRP circulation for 5 min, localized staining for HRP appeared in the periventricular areas including the hippocampal fimbria, ependymal cell layer and the wall of the vessel located at medial side of the optic tract as well as in the subfornical organ and the hypothalamus around the median eminence (Fig. 1 A, C, E, G). Artifactual staining reaction for HRP was sometimes observed in perfusion-fixed mice after HRP circulation for 5 min (Fig. 1 G). In perfusion-fixed mice after HRP circulation for 90 min, staining for HRP appeared in the periventricular areas including the hippocampal fimbria, the ependymal cell layer and the adjoining neuropil at medial side of the lateral ventricle, as well as in the subfornical organ, the hypothalamus around the median eminence, the lateral septal nucleus, the dorsal portion of the thalamus and the medial portion of the hippocampus (Fig. 1 B, D, F, H). In brain samples taken from the perfusion-fixed mice after HRP

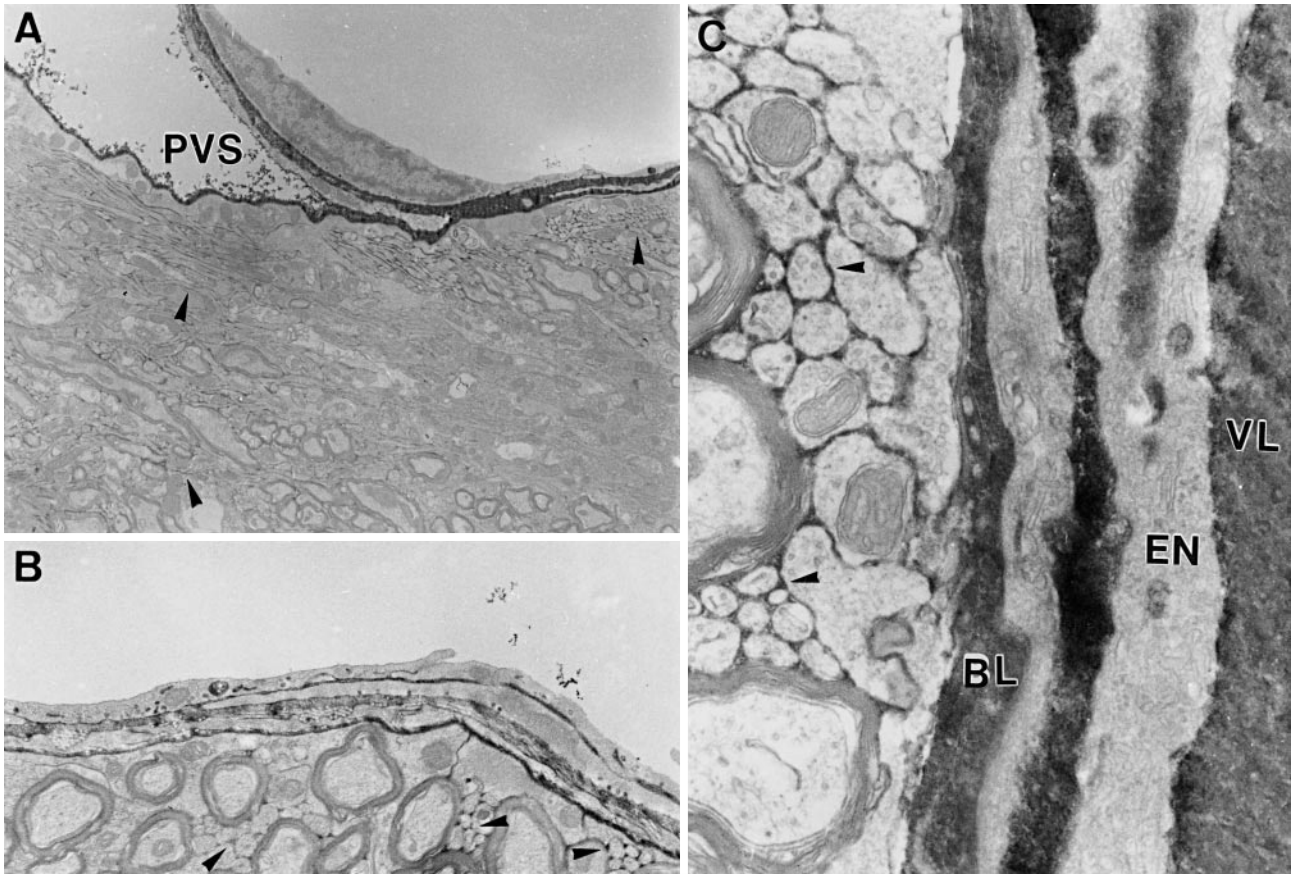


Fig. 3 A–C Electron micrographs of the vessels and their surroundings in the brains of C3H mice perfusion-fixed after HRP circulation for 5 min (**A**, **B**) and in the brain of the mouse immersion-fixed after HRP circulation for 90 min (**C**), showing penetration of HRP into the extracellular space around the vessels located in the periventricular area adjacent to medial side of the optic tract. **A**, **B** Staining for HRP appears in the perivascular space (*PVS*) and the adjoining extracellular space (*small arrowheads*) around the vessel as well as the basal lamina and vesicular structures of the endothelial cell cytoplasm. **C** Staining for HRP is present inside the vessel lumen (*VL*), and in the basal lamina (*BL*), vesicular structures of the cytoplasm of the endothelial cells (*EN*) and the adjoining extracellular space (*small arrowheads*). **A** × 3700; **B** × 10,600; **C** × 37,200)

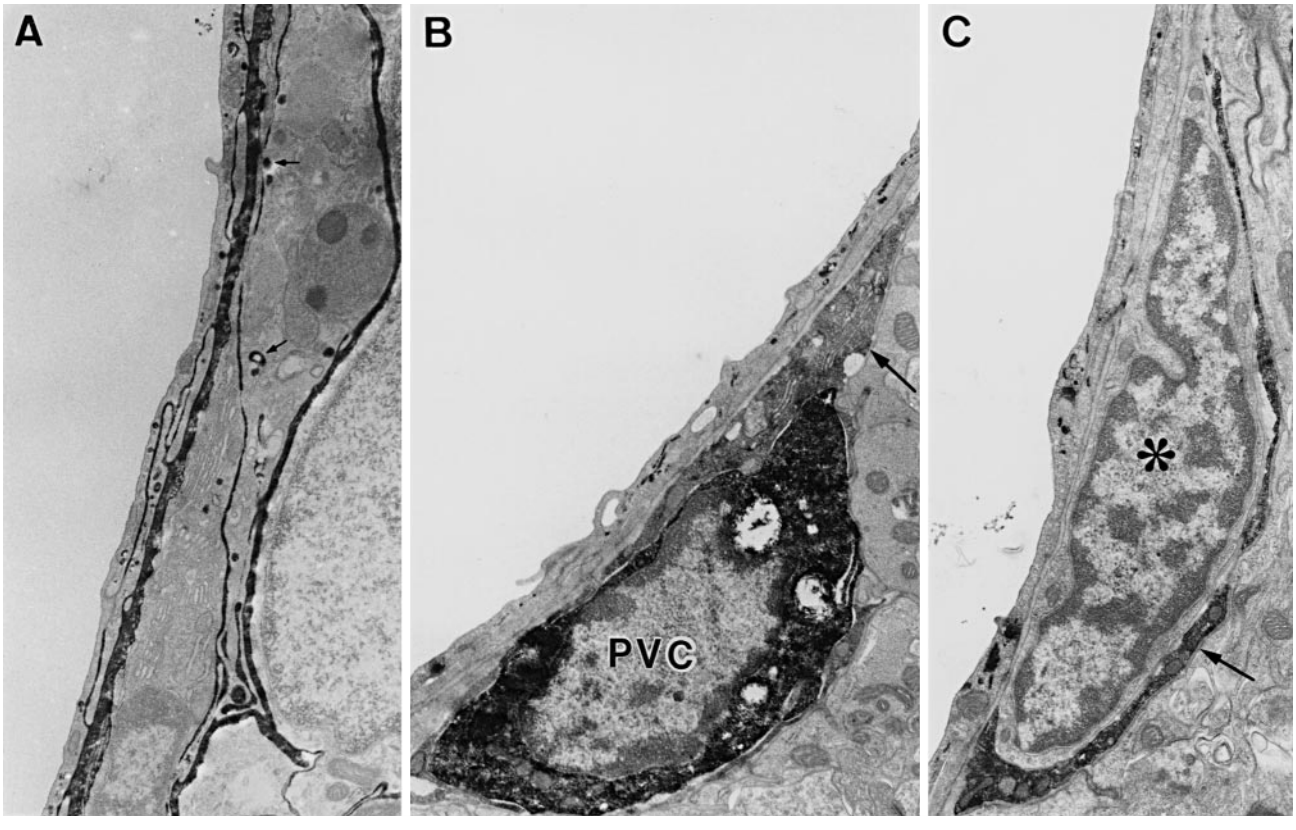
circulation for 5 and 90 min, clear staining for HRP was observed in and around the wall of the specific vessels located at the medial side of the optic tract (Fig. 2 A–D). The choroid plexus was located near the ependymal cell layer showing a positive reaction for HRP (Fig. 2 D). No clear staining for HRP appeared in the periventricular areas adjacent to the lateral side of the lateral ventricles. Localized HRP staining with DAB incubation appeared in the hypothalamus around the median eminence and in the periventricular areas such as the hippocampal fimbria, ependymal cell layer and the wall of the vessels located at medial side of the optic tract, but not in the medial portion of the hippocampus (Fig. 2 E, F).

The vessels located in the periventricular area adjacent to medial side of the lateral ventricle and shown by arrowheads in Fig. 2 B–D, F, as well as their surroundings, were examined by electron microscopy because the vessel wall showed strong staining for HRP in sections obtained from the perfusion-fixed brains after HRP circulation for 5 and 90 min. In perfusion-fixed C3H mice after HRP circulation for 5 min (Fig. 3 A, B), staining for HRP in the vessel wall appeared in the perivascular space, the basal lamina and the adjoining extracellular space. In immersion-fixed C3H mice after HRP circulation for 90 min (Fig. 3 C), HRP staining was present inside the vessel lumen, and in the basal lamina, vesicular structures of the endothelial cell cytoplasm and the adjoining extracellular space. Figure 4 shows the uptake of HRP from the perivascular spaces and basal lamina by the perivascular cells. HRP staining in vesicular profiles of the perivascular cells in perfusion-fixed mice after HRP circulation for 5 min is shown in Fig. 4 A, while that present in the cytoplasm of the perivascular cells in the perfusion-fixed mice after HRP circulation for 90 min is shown in Fig. 4 B, C. These perivascular cells contained dense lysosomal bodies in the cytoplasm. Figure 5 shows uptake of HRP into the endothelial cells. Staining for HRP appeared in several vesicular structures of the endothelial cell cytoplasm in the perfusion-fixed mice after HRP circulation for both 5 and 90 min. Staining for HRP in the former was present mainly in abluminal pits or vesicles and occasionally in endosomes or multivesicular bodies (Fig. 5 A–C), while that in the latter was present in plasmalemmal vesicles, multivesicular bodies and lysosomes rather than in abluminal pits (Fig. 5 D–F).

Fig. 4 A–C Electron micrographs of the vessels in the brains of C3H mice perfusion-fixed after HRP circulation for 5 (**A**) and 90 (**B**, **C**) min, showing uptake of HRP by the perivascular cells. **A** Staining for HRP appears in vesicular profiles (*small arrow*) of perivascular cells as well as in the basal lamina and vesicular structures of the endothelial cell cytoplasm. **B** The staining reaction for HRP is strong in the cytoplasm of a perivascular cell (*PVC*), and weak in the cytoplasm of another perivascular cell (*arrow*). These perivascular cells contain dense lysosomal bodies in the cytoplasm. **C** Staining for HRP is present in the cytoplasm of a perivascular cell (*arrow*) but not in the cytoplasm of another cell (*asterisk*), which is located in the perivascular position and does not contain dense lysosomal bodies. **A**, **B** × 9700; **C** × 10,600

Discussion

In this study, the barrier function of the blood microvasculature supplying the periventricular area adjacent to the



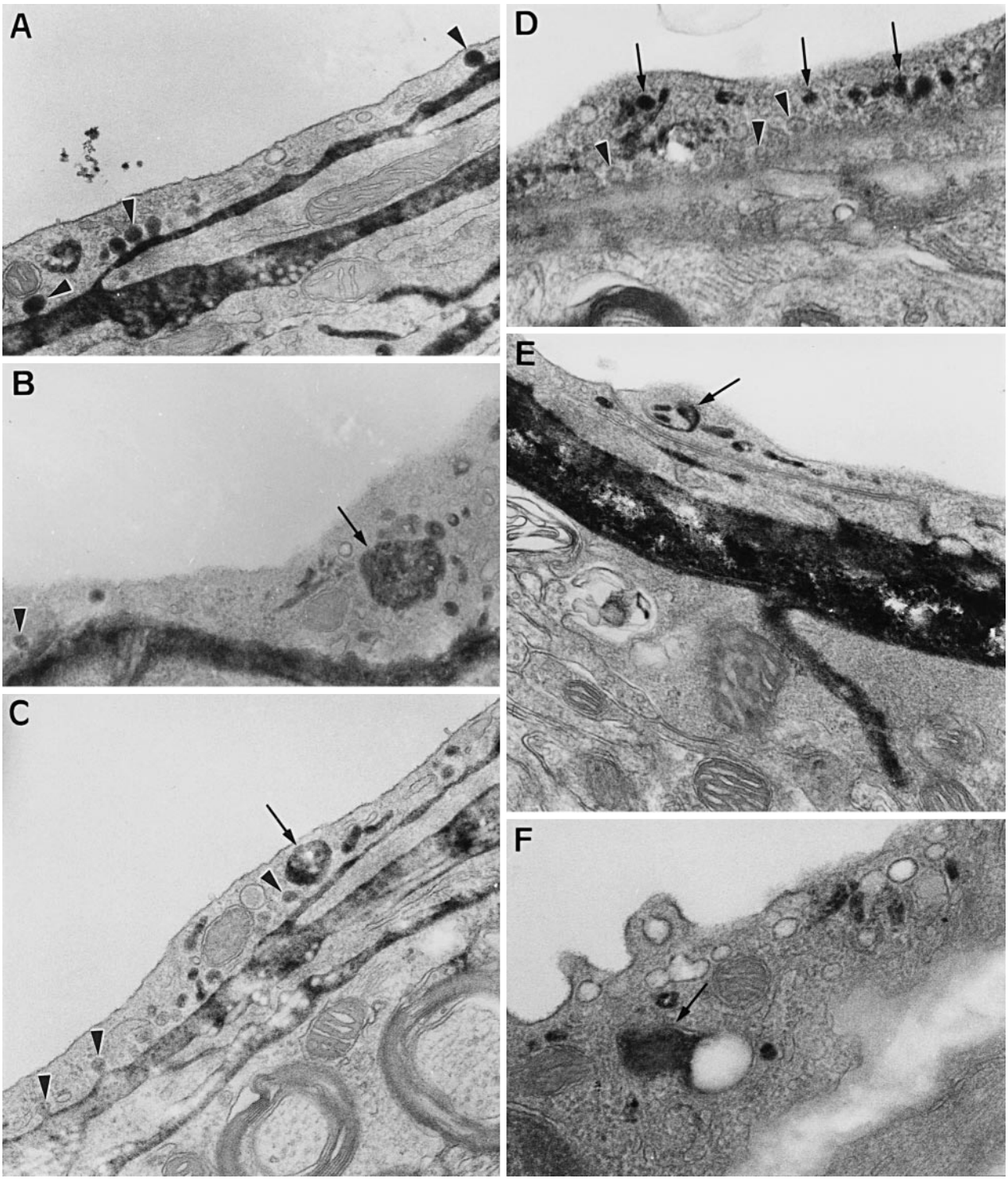


Fig. 5 A–F Electron micrographs of the vessels in the brains of C3H mice perfusion-fixed after HRP circulation for 5 (**A**–**C**) and 90 (**D**–**F**) min, showing uptake of HRP by the endothelial cells. **A**–**C** Staining for HRP appears in the basal lamina, intercellular clefts and several vesicular structures of the endothelial cell cytoplasm, such as abluminal pits (*arrowheads*), plasmalemmal vesicles and endosomes or multivesicular bodies (**B**,**C**; *arrow*). A number of abluminal pits contain HRP-DAB reaction product. **D**–**F** Staining for HRP appears in several vesicular structures of the endothelial cell cytoplasm, such as plasmalemmal vesicles (**D**; *arrow*), multivesicular bodies (**E**; *arrow*) and lysosomes (**F**; *arrow*). Most of abluminal vesicles show negative reaction for HRP (**D**; *small arrowheads*). Staining for HRP is present in the basal lamina of some vessels (**E**) and is not in the basal lamina of others (**F**). The passage of HRP through the glia limitans is restricted (**E**). **A**, **B** × 35,300; **B** × 37,200; **D**, **F** × 47,600; **E** × 32,300

lateral ventricle was evaluated. Staining for HRP appeared in the hippocampal fimbria, in the ependymal cell layer near the choroid plexus and in the periventricular area adjacent to medial side of the lateral ventricle, as well as in the BBB-free areas, such as the subfornical organ and median eminence, and their surroundings. Strong positive reaction for HRP was conspicuous in the wall of a specific vessel located at medial side of the optic tract. Ultrastructural examination of this vessel revealed that the staining for HRP appeared in and around the vessel wall. There were some differences in the location of HRP-DAB reaction product between the sections obtained from the perfusion-fixed mice after HRP circulation for 5 and 90 min. The staining for HRP in the former appeared mainly in the perivascular space and the basal lamina. On the other hand, after 90 min circulation injected HRP appeared mainly in the cytoplasm of the perivascular cells. In the endothelial cell cytoplasm, staining for HRP at both time intervals was located in several vesicular profiles, such as abluminal pits, plasmalemmal vesicles and endosomes. It is noteworthy, however, that the staining appeared mainly in the abluminal pits in the former and was mainly in the plasmalemmal vesicles in the latter. In addition, endosome-like structures showing positive reaction for HRP were observed for both time intervals. These findings suggest that intravenously injected HRP presumably leaked from permeable vessels supplying BBB-free areas, passed from the ventricle and/or the choroid plexus into the perivascular spaces of the periventricular areas, and ultimately was taken mainly into the cytoplasm of perivascular cells. Part of extravasated HRP was also endocytosed by plasmalemmal vesicles of the endothelial cell cytoplasm [33] and, to a small degree, spread also in the extracellular space. In addition, it passed more extensively into white matter than gray matter areas. Thus, it is conceivable that the perivascular cells play a major role in clearing HRP accumulated in the perivascular space with the endothelial cells playing a minor role.

Some observations in other reports indicate that the blood-borne tracer leaks from blood vessels supplying circumventricular organs and spreads extracellularly from each of the circumventricular organs, such as the subfornical organ, median eminence and area postrema, into the adjacent brain parenchyma [2, 7–9]. We previously reported that blood-borne HRP invaded the medial portion of the hippocampus and the dorsal portion of the thalamus in mice of other strains [29, 30]. In this study, we have shown that in C3H and DBA/2 mice the staining for HRP spread into the lateral septal nucleus and the periventricular area as well as into the medial portion of the hippocampus and the dorsal portion of the thalamus, presumably because they were located near BBB-free areas such as the subfornical organ and choroid plexus. A hypothesized "functional leak" has been proposed at the root of the choroid plexus through which the blood-borne tracer could enter the CSF [5, 6]. This may explain the cause of the escape of blood-borne tracer from vessels supplying the choroid plexus into the ependymal cell layer located near the choroid plexus. Once blood-borne HRP has gained extracellularly entry to the central nervous system from permeable vessels supplying circumventricular organs such as the median eminence, area postrema and subfornical organ and subarachnoid space/pial surface, they are propelled deeper into the central nervous system through the perivascular clefts by the pulsatile activity of arterioles [9]. Consequently, both superficial and deep perivascular phagocytes are able to endocytose the blood-borne proteins. Thus, our findings in the periventricular area are concordant with some observations by other researchers.

The perivascular cells can phagocytose exogenous macromolecules injected into blood vessels or cerebral ventricles [23, 32, 34], express major histocompatibility complex (MHC) class II antigens [15, 20] and have scavenger receptors [24]. It is known that the perivascular spaces are the drainage pathways for interstitial drainage fluid from the brain [18, 19, 35, 37]. In addition, macrophages in the Virchow-Robin space can express MHC class II antigens and are well placed to interact with lymphocytes derived from the blood in initiating and promoting immune response to foreign antigen in the brain [11]. The immunological and neuropathological significance of the Virchow-Robin spaces and the perivascular cells has been pointed out [11, 17]. In this study we have also noted that the perivascular cells in the periventricular area take up blood-borne HRP.

Our findings indicate that the BBB in the periventricular area adjacent to medial side of the lateral ventricle near the choroid plexus is not so tight as it is in the cortex or in the periventricular area adjacent to lateral side of the lateral ventricle, and suggest that the perivascular cells also play a role in scavenger functions in the periventricular areas as a component of the BBB. In addition, they indicate that intravascular macromolecules can also partially invade the septum, hippocampus and thalamus adjacent to the ventricle.

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