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Brain–blood barrier? Yes and no

(horseradish peroxidase/cerebral endothelium/median eminence/endocytosis/blood–brain barrier)

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ABSTRACT **Ventriculo-cisternal perfusion of horseradish peroxidase (HRP) in the mouse brain has demonstrated that a brain–blood barrier exists at the microvascular endothelium in brain parenchyma but not in the median eminence of the hypothalamus. The brain–blood barrier is similar to the blood–brain barrier in that: tight junctions prevent the movement of protein between endothelial cells, HRP taken into the endothelial cells is directed to lysosomal dense bodies, and, contrary to the literature, a vesicular transendothelial transport of HRP from brain to blood does not occur under normal conditions. The endocytosis of ventricular injected HRP from the abluminal side of the endothelium is demonstrably less than the endocytosis of intravenous injected HRP from the luminal side; hence, the cerebral endothelium expresses a degree of polarity regarding the internalization of its cell surface membrane and extracellular protein. The passage of cerebrospinal fluid-borne or blood-borne HRP between some ependymal cells of the median eminence is not precluded by tight junctions. These patent extracellular channels offer a direct pathway for the exchange of substances between cerebrospinal fluid in the third ventricle and fenestrated capillaries in the median eminence.**

Specific morphological characteristics of cerebral endothelial cells and enzyme activities within these cells define the mammalian blood–brain barrier (1–3). The transcellular transport of large molecular weight, blood-borne substances through the endothelium to the brain extracellular space is believed not to occur under normal conditions. Circumventricular organs (i.e., median eminence, area postrema, etc.) and the choroid plexus possess fenestrated capillaries and lie outside the blood–brain barrier (4, 5); circumferential belts of tight junctions between ependymal cells and between choroid epithelial cells lining the circumventricular organs and choroid plexus, respectively, are considered to provide the physical barrier between the blood and cerebrospinal fluid (CSF) (1, 4, 6). Despite the overwhelming acceptance of a blood–brain barrier, available evidence indicates that the cerebral microvasculature does not manifest a *brain–blood barrier*. The cerebral endothelium is reported to take up protein from the perivascular space and transport it to the luminal surface where the protein is deposited into the blood (7, 8). In this fashion the transendothelial transport of protein from brain to blood could supplement the CSF in the removal of substances from the brain extracellular space.

Evidence obtained with horseradish peroxidase (HRP) as a tracer is presented in this paper to suggest that a brain–blood barrier exists at the level of capillaries and arterioles in the brain but not in the median eminence of the hypothalamus. Open junctions observed between some median eminence ependymal cells permit the bidirectional exchange of substances between the median eminence capillaries and the third ventricle CSF.

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MATERIALS AND METHODS

The microvascular endothelium and the third ventricle median eminence in brains from 43 young adult, female, Swiss mice were analyzed ultrastructurally. Thirty mice were injected with HRP (type VI, Sigma) into one of the lateral cerebral ventricles. Details of the injection procedure have been presented elsewhere (9). Fifteen of the injected mice received 30 μl (2–3 μl/min) of a 30% HRP/saline solution and were sacrificed 3, 6, 12, or 24 hr later. The brainstem, cortex, and hypothalamus from each mouse were saved for processing. Fifteen additional mice were injected similarly with 5 μl (1.25 μl/min) of a 0.2–10% HRP solution and were sacrificed immediately thereafter. Only the hypothalamus from each brain was saved for processing. Twelve mice were injected into the tail vein with 30 mg of HRP dissolved in 0.25 ml of saline; survival times after injection were 30 min or less. The cortex, hypothalamus, and brainstem from these brains were saved for processing. The brain from one noninjected mouse was included as a control to test for endogenous peroxidase activity within the cerebral endothelium. Perfusion-fixation of the animals, preparation of the brains for HRP cytochemistry, and light microscopic and ultrastructural inspection were identical to the descriptions given by Broadwell and Brightman (5, 9, 10).

RESULTS

Cerebral Microvascular Endothelium. Within 3 hr of a cerebral intraventricular injection of 30 μl of a 30% peroxidase solution, the extracellular clefts throughout the forebrain and brainstem were filled with HRP reaction product. Peroxidase remained in the extracellular clefts and bathed the abluminal surface of the cerebral endothelial cells in excess of 24 hr. Tight junctions between endothelial cells prevented the free passage of extracellular HRP to the blood (Fig. 1*A*). Except in portions of the cortex, peroxidase reaction product was not observed coating the luminal surface of endothelial cells. Peroxidase that reached the luminal surface of cortical microvasculature may be attributed to damage caused by the injection cannula passing through the parenchyma to enter the lateral ventricle.

Randomly selected thin sections through the hypothalamus and brainstem from mice fixed 3–24 hr after injection revealed an extreme paucity to virtual nonexistence of HRP-reactive dense bodies, multivesicular bodies, and vacuoles rimmed with reaction product in longitudinal and cross-sectional views of the microvasculature (Fig. 1 *A*, *C*, and *D*); labeled tubular profiles were never seen. The few peroxidase-positive structures reflected a low degree of endocytic activity or internalization of the abluminal surface membrane in the cerebral endothelia. This low endocytic activity of the abluminal wall was the an-

Abbreviations: HRP, horseradish peroxidase; CSF, cerebrospinal fluid.

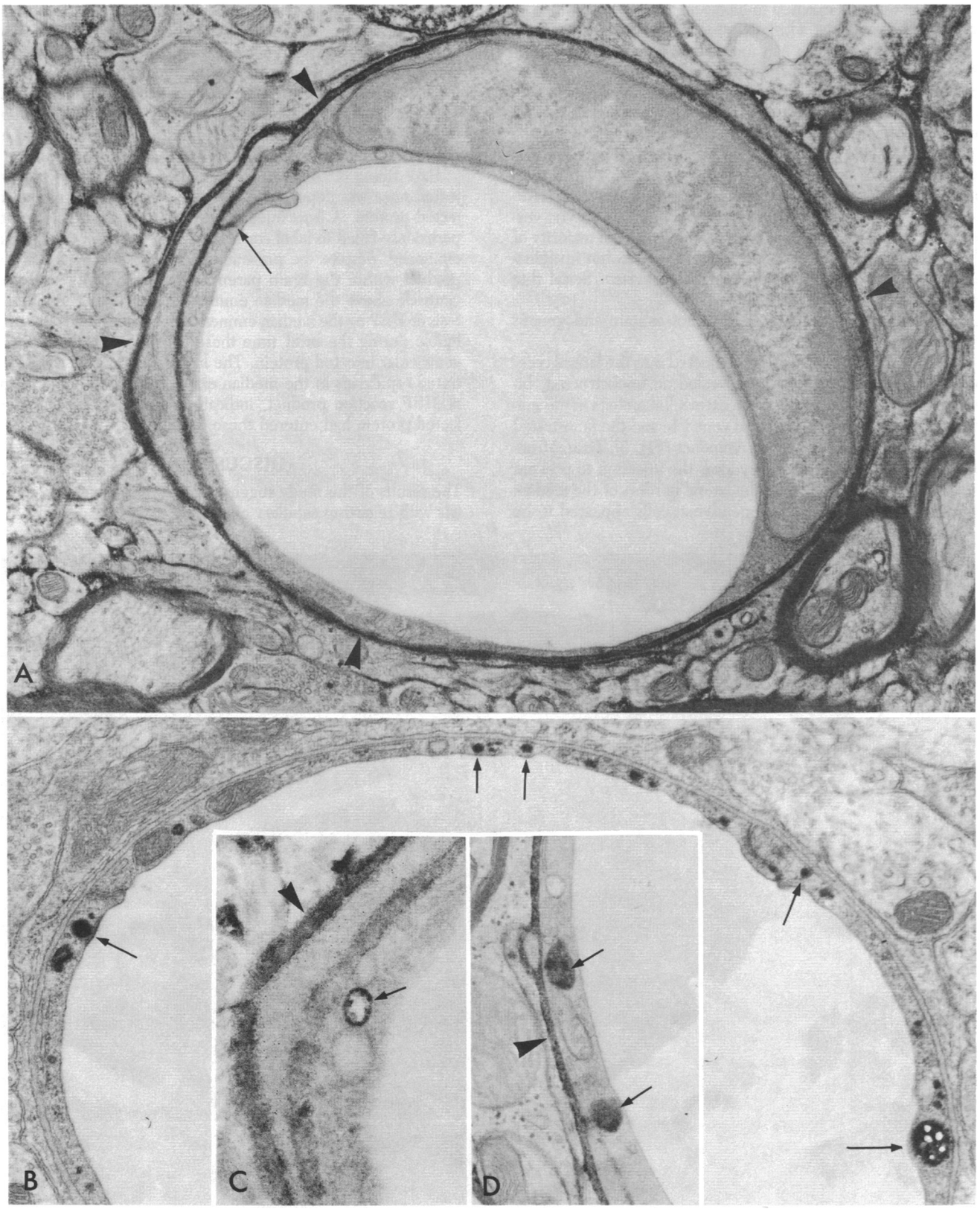

FIG. 1. (*A*, *C*, and *D*) Twelve to twenty-four hours after a cerebral ventricular injection of HRP (30 μl), reaction product remains in the perivascular space (arrowheads) and is prevented from passing between endothelial cells by tight junctions (*A*, arrow). Endothelial cells frequently contain no reactive organelles (*A*) or an extreme paucity of reactive vacuoles (*C*, arrow) and dense bodies (*D*, arrows). (*A*, ×22,500; *C*, ×37,500; *D*, ×30,000.) (*B*) Less than 30 min after intravenous injection of HRP, the cerebral endothelial cells contain many reactive vesicles, dense bodies, and multivesicular bodies (arrows). (×26,700.)

tithesis of an intensive endocytic activity of the luminal wall in cerebral endothelia exposed to blood-borne HRP. Less than 30 min after intravenous administration of peroxidase, cerebral capillaries and arterioles had incorporated the tracer with luminal surface membrane into numerous endocytic vesicles, vacuoles, dense bodies, and multivesicular bodies (Fig. 1*B*).

Many capillaries and arterioles in the intraventricular injected brains contained what appeared to be 40- to 70-nm-wide endocytic vesicles labeled with HRP. These presumptive vesicles were never far removed from the inner face of the abluminal plasmalemma (Fig. 2). Given the near absence of HRP-positive dense bodies and vacuoles with which endocytic vesicles might fuse, the speculation was raised that the majority of the 40- to 70-nm-wide profiles were not vesicles but invaginations or pits in the abluminal endothelial surface. Serial thin sections confirmed this speculation (Fig. 2).

The cerebral endothelial cells did not contain endogenous peroxidase activity.

Median Eminence. Peroxidase injected into the lateral cerebral ventricle or intravenously revealed an unobstructed, bidirectional pathway through some extracellular clefts in the median eminence between the third ventricle and the fenestrated capillaries supplying the median eminence (Fig. 3). Tissues from all mice were fixed immediately after the injection to prevent diffusion of the tracer around the lateral borders of the median eminence (Fig. 3 *A–C*). The ependymal cells appeared to be undamaged. Peroxidase reaction product filled many but not all extracellular clefts between median eminence ependymal cells. Clefts filled with reaction product appeared free of tight junctions between the ependymal cells. In the intravenous injected mice, HRP reaction product traced through the open clefts was evident on the ventricular surface of the median eminence (Fig. 3*D*). Extracellular clefts not completely filled with reaction product in the median eminence from intraventricular and intravenous injected mice were occluded by tight junctions.

HRP filling of the median eminence in the ventricular injected mice was dependent upon the concentration of the injected protein. A 5-μl injection of less than a 1% solution of peroxidase failed to label consistently the whole of the median eminence, despite the presence of a dense band of reaction product within the brain parenchyma surrounding the third ventricle above the median eminence (Fig. 3*B*). The endocytosis of HRP by the median eminence ependymal cells was negligible during the brief time these cells were exposed to the ventricular injected protein. The luminal surface of the fenestrated capillaries in the median eminence exhibited a coating of HRP reaction product, indicating that the ventricular injected protein had entered these capillaries.

DISCUSSION

The results of this study suggest that a brain–blood barrier exists with regard to capillary and arteriole endothelial cells but

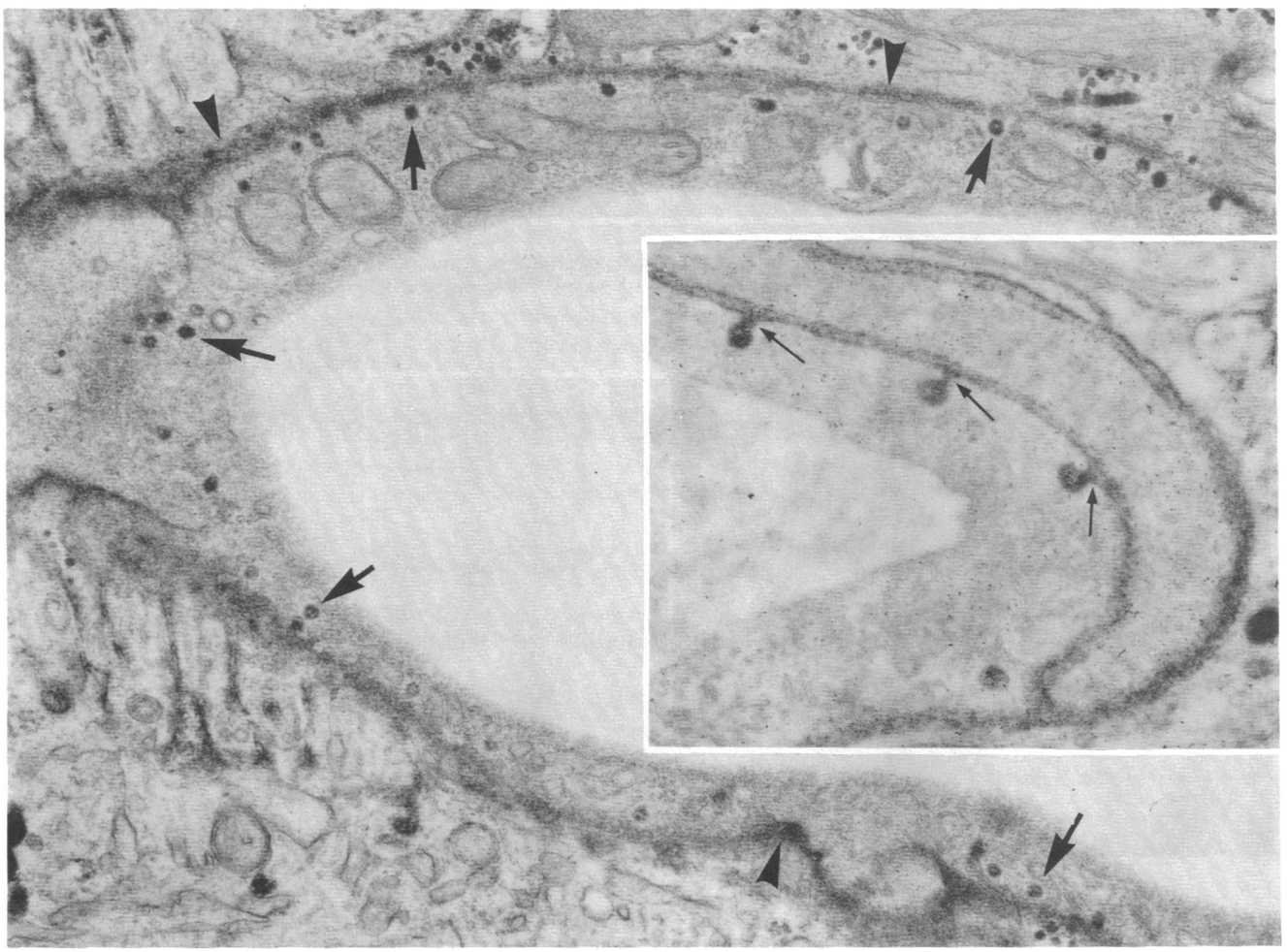

FIG. 2. After a ventricular injection of HRP, reaction product occupies the perivascular clefts (arrowheads) and "vesicular" profiles (arrows) close to the abluminal surface of segments of the cerebral endothelium. (×3,400.) (*Inset*) Serial sections demonstrate that the "vesicular" profiles within the cerebral endothelium are not vesicles but pits (arrows) connected with the abluminal surface. (×73,500.)

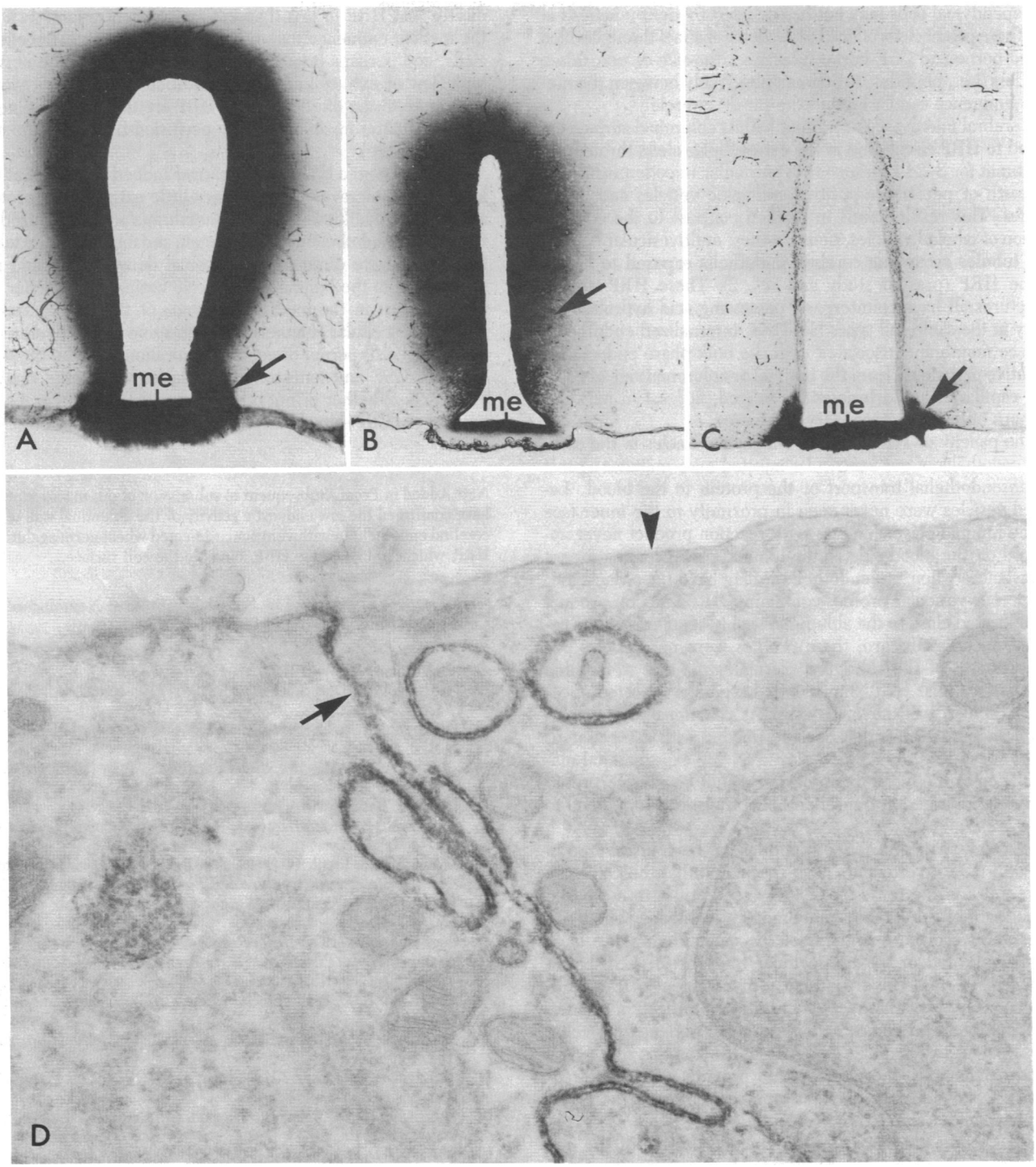

FIG. 3. (*A*) A five-microliter intraventricular injection of 1% HRP immediately followed by fixation of the brain results in HRP reaction product (arrow) filling the median eminence (me) and spreading (through gap junctions between ependymal cells) into the brain parenchyma surrounding the third ventricle. (×130.) (*B*) A similar injection as in *A* but with 0.2% HRP results in the median eminence (me) *not* being filled with reaction product (arrow). (×125.) (*C* and *D*) Intravenous injection of HRP immediately followed by fixation of the brain results in reaction product (arrows) labeling the entire median eminence (*C*, me) and filling many extracellular clefts (*D*, arrow). Reaction product traced through these extracellular clefts also appears on the ventricular surface of the median eminence ependymal cells (*D*, arrowhead). (*C*, ×125; *D*, ×41,250.)

is absent at the level of the median eminence ependymal cells that form the floor of the third ventricle. Endocytosis or internalization of the abluminal surface membrane in the cerebral endothelium is minor compared to that of the luminal surface membrane; therefore, endocytosis in the cerebral microvasculature appears largely vectorial: from the luminal or blood side inward. For this reason, a demonstrable, transendothelial transport of protein from the brain to blood, as advocated by others (7, 8), is unlikely under normal conditions. Our findings also argue against an isolation of the fenestrated capillaries in the median eminence from the third ventricle CSF. Although tight junctions are reported to exist between ependymal cells of the median eminence (4, 6), many of these junctions may not be completely tight or many of the extracellular clefts between

the ependymal cells may not be occluded by tight junctions at all. These patent clefts offer a bidirectional channel through which blood-borne and CSF-borne substances the size of peroxidase and less (i.e., peptides, toxins) can pass freely between the median eminence capillaries and the third ventricle.

Cerebral microvasculature that had its abluminal surface exposed to HRP circulating in the extracellular clefts throughout the brain for 3–24 hr after intraventricular injection exhibited a dearth of peroxidase-positive endocytic vesicles and dense bodies. This result stands in marked contrast to the concentration of labeled vesicles, dense bodies, multivesicular bodies, and tubules occupying cerebral endothelia exposed to blood-borne HRP (present study and ref. 2). These HRP-labeled structures all have counterparts possessing acid hydrolase activity in the same cell types (2). Thus, internalized endothelial surface membrane associated with the bulk-phase endocytosis of native peroxidase from the luminal or abluminal side of cerebral capillaries and arterioles is directed, at least in part and perhaps entirely, to secondary lysosomes.

The paucity of HRP-reactive endocytic vesicles in the cerebral endothelia of our ventricular-injected mice militates against a transendothelial transport of the protein to the blood. Labeled vesicles were never seen in proximity to the inner face of the luminal plasmalemma; HRP reaction product never appeared on the blood side of the luminal plasmalemma except in instances when the endothelium may have been damaged. Numerous vesicular profiles containing HRP reaction product were located close to the abluminal wall in segments of the cerebral microvasculature; these profiles were not vesicles but invaginations of the abluminal plasmalemma that were filled with extracellular HRP. Similar pits have been reported in cerebral capillaries of the hagfish (11). As a cautionary note, abluminal endothelial pits that become flooded with extravasated, *blood-borne* HRP (i.e., in studies involving experimental manipulation of the blood–brain barrier) could be misinterpreted as vesicles engaged in transendothelial transport from blood to brain (12).

In recent years a variety of peptides has been discovered in the CSF (13). Investigators have speculated that the CSF may represent a viable route for delivery of peptides to the portal vasculature of the hypothalamic median eminence. The tanycytes or specialized ependyma of the median eminence have been implicated in the transcellular transport of CSF-borne peptides (14–17). This hypothetical involvement of the tanycytes now appears unattractive with the identification of open junctions between some median eminence ependymal cells. These patent extracellular clefts signal the absence of a CSF–blood barrier and, therefore, would provide for the bidirectional exchange of peptides between the CSF and the blood supplying and draining the median eminence. The presence of peptides within the tanycytes may be a reflection of the fact that these cells act more as phagocytes than as a transport medium (unpublished data).

The failure of earlier investigators to observe open clefts in the median eminence after the ventriculo-cisternal perfusion of peroxidase may be the consequence of a too low volume or concentration (or both) of the injected HRP.[§] Our experience indicates that HRP in less than optimal concentration (<1%) in the median eminence may be washed out of the extracellular clefts, and perhaps into the portal vasculature, by the normal bulk flow of extracellular fluid. Additional factors that would contribute to the dilution of this HRP are the survival time of the animal after injection and the perfusion-fixation of the median eminence.

In conclusion, a brain–blood barrier indeed is present at the level of the microvasculature. Very little extracellular protein and associated abluminal surface membrane are internalized by the cerebral endothelium. What protein and membrane are taken into the cell are directed to lysosomal dense bodies and not transported to the luminal surface. No brain–blood barrier appears to exist in the median eminence of the hypothalamus. Patent extracellular channels between some median eminence ependymal cells permit direct communication between the third ventricle CSF and fenestrated capillaries within the median eminence. Whether or not other circumventricular organs fail to express a CSF–blood barrier has not been determined at this time.

Note Added in Proof. Subsequent to submission of our manuscript, we have confirmed the low endocytic activity of the abluminal wall of the cerebral endothelium with ventricular injected wheat germ agglutinin-HRP, which, unlike native HRP, binds to the cell surface.

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[§] Brightman *et al.* (ref. 4; M. W. Brightman, personal communication) injected 0.01–0.5% HRP into mice. Weindl and Joynt (6) injected 100–200 μl of 1% HRP into adult rabbits.