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Expanding the definition of the blood–brain barrier to protein

(horseradish peroxidase/acid hydrolases/lysosomes/capillaries/pericytes)

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ABSTRACT **Tight junctions between cerebral endothelial cells and the near absence of pinocytosis and vesicular transport of blood-borne protein into and across these cells are believed to constitute the mammalian blood–brain barrier. In the present investigation evidence is provided to indicate that the capillary endothelium of the mouse brain pinocytoses the enzymatic tracer horseradish peroxidase (EC 1.11.1.17) from cerebral blood under normal conditions. This protein and the internalized endothelial surface membrane associated with it are directed, for the most part, to acid hydrolase-positive lysosomes for degradation. Although peroxidase was never seen in the perivascular clefts, the lysosomes of pericytes were peroxidase-positive. Pericytes are macrophage-like cells located on the abluminal surfaces of cerebral microvasculature; these cells may serve as the first line of defense once the blood–brain barrier is breached. The definition of the blood–brain barrier should be expanded to include consideration of the lysosomal system of organelles in endothelial cells and pericytes.**

Exogenous proteins and serum proteins are excluded from the cerebral extracellular fluid in mammals by the blood–brain barrier. Studies employing the enzymatic tracers horseradish peroxidase (EC 1.11.1.17; hereafter referred to as "peroxidase") and microperoxidase have led to attribution of the blood–brain barrier to two morphological characteristics of cerebral microvascular endothelial cells: circumferential belts of tight junctions between contiguous endothelial cells and the apparent absence of vesicular transport of blood-borne protein from the luminal to the abluminal face of the endothelium (1–4). In these studies, peroxidase-labeled vesicles were particularly rare in capillary endothelial cells, suggesting that, unlike capillaries supplying cardiac and striated muscles (5, 6), those in brain were not actively engaged in pinocytosis to an appreciable degree; however, one of these investigations (4) revealed that in the mouse an insignificant amount of peroxidase did cross segments of *certain* cerebral arterioles to reach the perivascular basement membrane, perhaps by vesicular transport.

This paper will present evidence to suggest that pinocytosis of blood-borne peroxidase does indeed occur normally in cerebral capillaries. Emphasis is placed on pericytes and the lysosomal system within capillary endothelial cells as additional functional components of the blood–brain barrier. The acid hydrolase activities of acid phosphatase (EC 3.1.3.2) (7, 8) and trimetaphosphatase (EC 3.6.1.2) (9) have been used as markers for lysosomes.

MATERIALS AND METHODS

Twenty-six young adult female white mice weighing 25–30 g were used. Eighteen mice received one, two, or three injections of 30 mg of horseradish peroxidase (type VI, Sigma) in 0.25–0.5 ml of 0.9% saline solution per injection into the tail vein. The desired length of the postinjection survival time determined the number of injections each mouse received. Survival times for these mice ranged between 1 and 24 hr. Brains from the remaining eight mice were prepared for normal morphology or acid hydrolase cytochemistry.

Each animal was anesthetized with sodium pentobarbital injected intraperitoneally and perfused through the heart with 100 ml of fixative (pH 7.1–7.2) consisting of 1% formaldehyde, 1.25% glutaraldehyde, and 0.025% calcium chloride in 0.1 M sodium cacodylate buffer (pH 7.35). The brain was removed from the skull, placed in fixative for 1 hr, and then transferred to cacodylate buffer. All brains were cut into 50- to 75-μm thick sections with a Smith–Farquhar TC-2 tissue sectioner (Du Pont). The sections were rinsed overnight in cacodylate buffer at 4°C. Brain sections from the peroxidase-injected mice were incubated for detection of peroxidase activity in a diaminobenzidine/H_2O_2 medium (10). A number of sections were prepared for light microscopic inspection (11). Brain sections from mice not injected with peroxidase were incubated for detection of acid hydrolase activity, as reported previously (12–14), with β-glycerophosphate (7), cytidine 5′-monophosphate (8), and sodium trimetaphosphate (9) as substrates. Some sections from these brains were not incubated for study of enzyme activity and were prepared for normal morphology.

Sections intended for ultrastructural analysis were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 hr. The nonincubated sections were stained *en bloc* with 0.5% aqueous uranyl acetate for 2 hr. All sections were dehydrated in ethanol and embedded in Araldite plastic. Only ultrathin sections cut from the nonincubated material were poststained with 2% uranyl acetate in 50% (vol/vol) ethanol and with Reynolds' lead citrate (15). Capillaries were distinguished from arterioles and venules according to established criteria (16, 17).

RESULTS

In randomly chosen sections of the forebrain and brain stem from mice intravenously injected with peroxidase and fixed at the various survival times, peroxidase reaction product appeared within specific membrane-delimited organelles in capillary endothelial cells. These peroxidase-positive organelles included 40- to 60-nm wide vesicles, vacuoles rimmed with reaction product, and multivesicular bodies and dense bodies, all of which were 0.08–0.5 μm in diameter, and blunt-ended tubules 50–100 nm in diameter and 0.1–0.7 μm in length (Fig. 1). The vesicles and smaller vacuoles, but not multivesicular and dense bodies, increased with the amount of circulating peroxidase and with the duration of the postinjection period. The different classes of peroxidase-positive organelles were scattered throughout the endothelial cell. Some labeled vesicles appeared in close proximity to the abluminal wall of the capillary cells as well as to labeled multivesicular and dense bodies (Fig.

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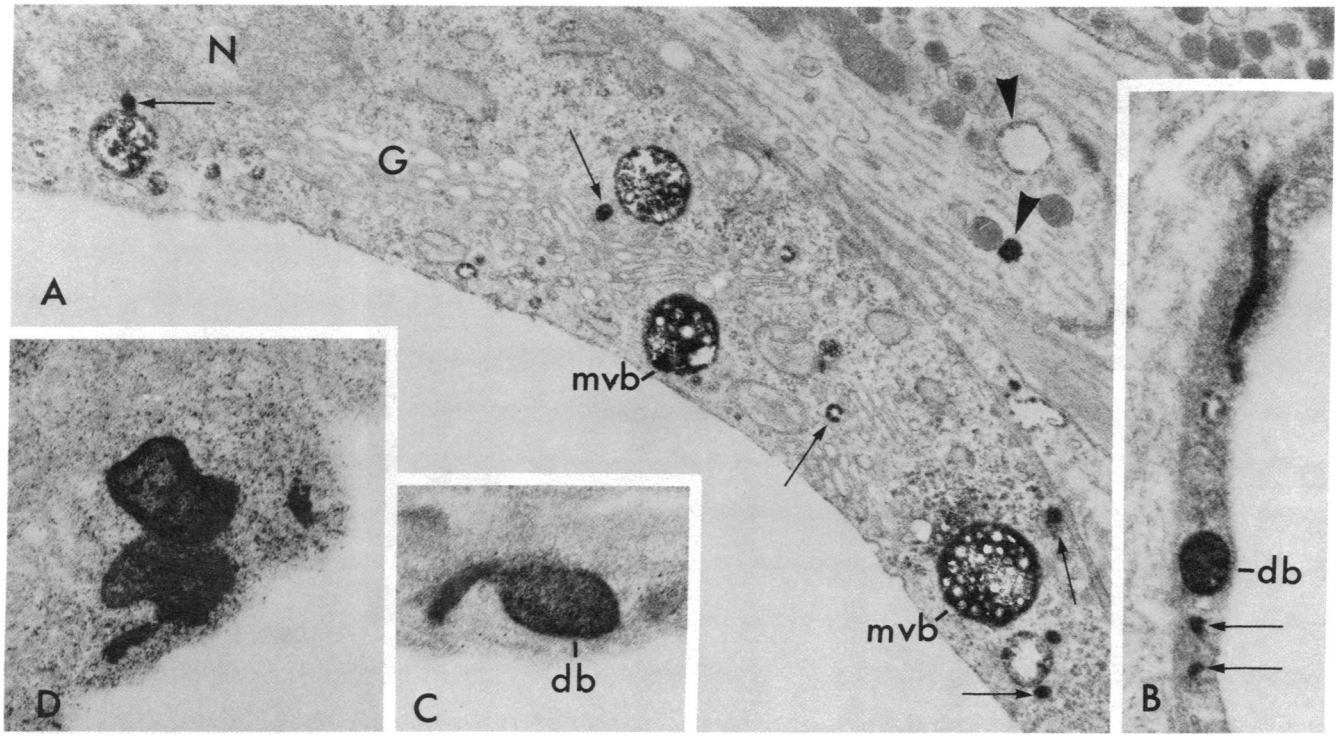

FIG. 1. Vesicles (*A* and *B*; arrows), dense bodies (*B* and *C*; db), multivesicular bodies (*A*; mvb), and blunt-ended tubules lying free in the cytoplasm or attached to dense bodies (*B* and *C*) within cerebral capillarly endothelial cells contain peroxidase after intravenous administration of the protein. Dense bodies and some tubules exhibit acid hydrolase activity (*D*). Arrowheads in *A* indicate peroxidase-labeled organelles undergoing retrograde axoplasmic transport in axons of the hypothalamoneurohypophysial system passing through the supraoptic nucleus (see refs. 12 and 13). G, Golgi apparatus; N, nucleus. (*A*, 10 hr after peroxidase injection, ×30,000; *B*, 2 hr after peroxidase injection, ×45,000; *C*, 2 hr after peroxidase injection, ×60,000; *D*, trimetaphosphatase activity; ×50,000.)

1 *A* and *B*). The vesicles and perhaps smaller vacuoles most likely represent internalized luminal surface membrane involved in delivering their membrane and pinocytosed peroxidase to the multivesicular and dense bodies. No more than a total of five multivesicular and dense bodies were seen in a single thin section of a capillary endothelial cell. Both types of organelles were located frequently in the vicinity of the cell nucleus and Golgi apparatus (Fig. 1*A*). The peroxidase-labeled tubular profiles were usually oriented parallel to the walls of the endothelial cell; they were either free in the cytoplasm or attached to labeled dense bodies (Fig. 1 *B* and *C*). The tubules were not observed to form patent, communicating channels

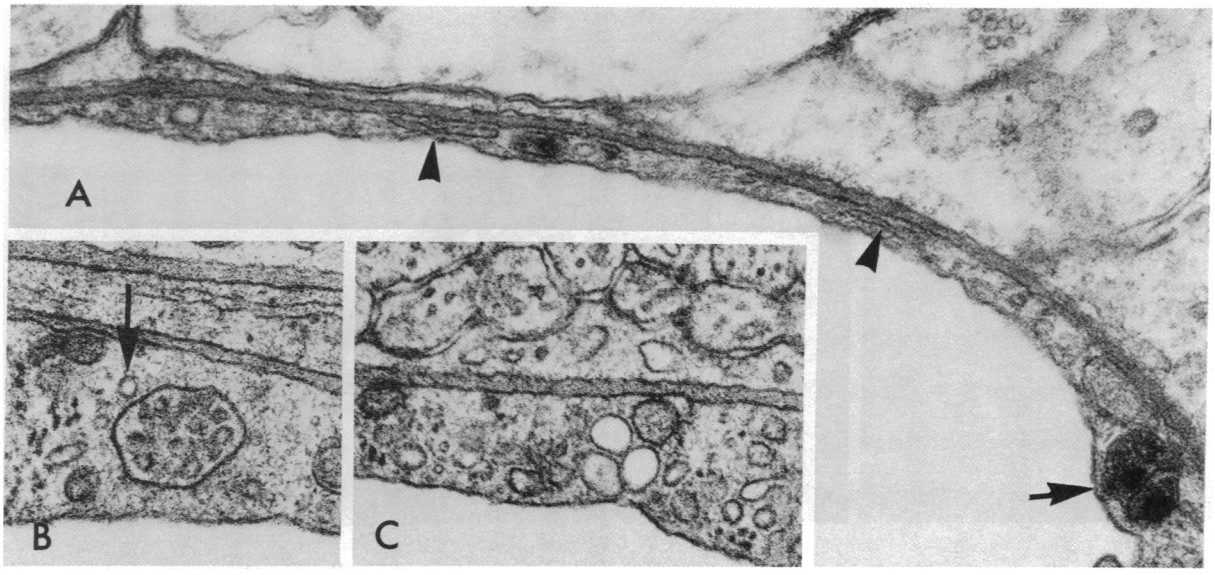

FIG. 2. Organelles morphologically similar to those containing peroxidase in Fig. 1 are common in capillary endothelial cells from mice not intravenously injected with peroxidase. (*A*) Dense body (arrow); tubules (arrowheads). (×25,000.) (*B*) Vesicle (arrow) adjacent to a multivesicular body. (×47,000.) (*C*) Numerous vesicles and vacuoles, some of which may represent tubules cut in cross section. (×60,000.)

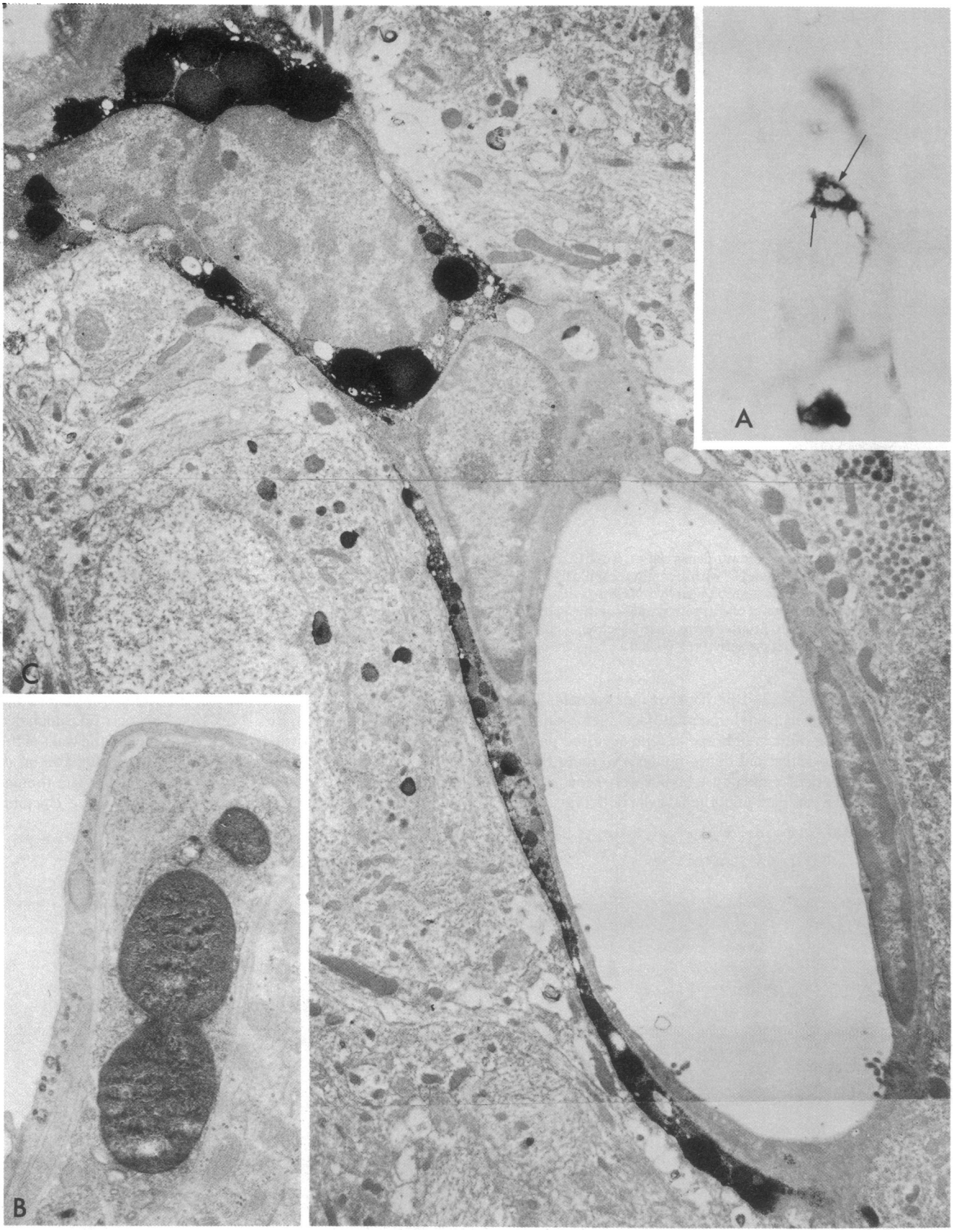

FIG. 3. Two hours after intravenous administration of peroxidase, pericytes associated with capillaries and arterioles contain peroxidase reaction product in granules (*A*, arrows, ×135) at the light microscopic level and in dense bodies ultrastructurally (*B*, ×20,000). Pericyte cell bodies and processes exhibit an impressive concentration of acid phosphatase-positive lysosomes (*C*, β-glycerophosphate, ×9500). Note the lysosome size in the pericyte cell body compared to that in a neuronal cell body adjacent to the pericyte.

between the luminal and abluminal surfaces of the endothelial cell. In this regard, peroxidase reaction product was never seen in the abluminal perivascular space in any of the injected animals.

Cerebral capillaries in nonincubated sections examined for normal morphology contained electron-dense lysosome-like dense bodies and electron-lucent multivesicular bodies, vacuoles, tubules, and vesicles (Fig. 2). Many of these structures may correspond to those derived presumably by direct internalization of cell surface membrane in the peroxidase-exposed material.

Pericytes located on the abluminal surfaces of cerebral capillaries and arterioles throughout the brain and particularly in cortical areas exhibited numerous peroxidase-positive granules (Fig. 3A). Ultrastructurally the pericytes contained peroxidase-positive vesicles, vacuoles, and dense bodies, the latter measuring 0.1–2.0 μm in diameter (Fig. 3B). The labeled organelles were clustered mainly in the vicinity of the cell nucleus; some of the smaller dense bodies were located in processes emanating from the cell proper. In mice receiving multiple intravenous injections of peroxidase and fixed 12–24 hr after the first injection, the pericyte cytoplasm at both light microscopic and ultrastructural levels was filled diffusely with reaction product.

Acid hydrolase activity was identified within lysosomes in cerebral capillarly endothelial cells and in pericytes (Figs. 1 *D* and 3 *C*). The lysosomes in each cell type were identical in appearance, size, concentration, and cellular distribution to the peroxidase-positive multivesicular and dense bodies seen in similar cells from the intravenously injected mice. Tubular profiles displaying acid hydrolase activity were rare in capillaries. Occasionally, an acid hydrolase-reactive lysosome and tubule were confluent (Fig. 1*D*).

In our hands, the best substrate for demonstrating acid hydrolase activity in cerebral microvasculature was trimetaphosphate. Although cytidine 5′-monophosphate and β-glycerophosphate were particularly useful in revealing acid phosphatase activity in neuronal GERL‡ and lysosomes (Fig. 3*C*), both substrates were considerably less effective for demonstrating this enzyme activity in cerebral vessels from the same material. All three substrates were useful in revealing acid hydrolase activity in pericytes.

DISCUSSION

Using peroxidase and acid hydrolase cytochemistry, we have demonstrated that under normal conditions cerebral capillary endothelial cells pinocytose blood-borne protein. This exogenous protein is directed, in part, to dense bodies and multivesicular bodies believed by us and other workers (12, 13, 19–21) to be lysosomal precursors (phagosomes) or *bona fide* secondary lysosomes. The failure of previous investigators (1–4) to obtain similar results is most likely a consequence of utilizing low concentrations of peroxidase, short postinjection survival periods, or both. Cerebral capillary lysosomes and their hydrolytic enzymes would function as a deterrent to the transcapillary transport of specific blood-borne substances. Such enzymatic regulation of transport would be similar to that of cerebral capillary monoamine oxidase and dopa-decarboxylase activities in regulating the entry to brain of monoamine precursors from blood (22).

Pinocytosis of peroxidase into cerebral capillary endothelial cells and vesicular transport of the protein through these cells are reported to be stimulated by a variety of insults to the brain (see refs. 23 and 24 for review). The 30 mg peroxidase dose we have used does not appear toxic to cell membranes or to the animals. Westergaard and Brightman (4) have administered up to 50 mg of peroxidase intravenously in mice with no observable damage to cerebral endothelial cells and neurons. A comparison of cerebral capillary endothelial cells from our injected and noninjected mice indicates that no new classes of organelles were induced by cellular exposure to peroxidase (Figs. 1 and 2). Peroxidase uptake by cells in general is nonspecific and associated with the internalization of cell surface membrane; therefore, protein or perhaps any large molecular weight substance within the extracellular fluid bathing a cell could conceivably be taken into that cell.

The peroxidase- and acid hydrolase-reactive, blunt-ended tubules attached to lysosomal dense bodies or lying unattached in the cytoplasm of the cerebral capillary cell are identical to those described by others in the myeloid sinusoidal endothelium (25), neurons (12, 13, 19), and anterior pituitary cells (20). These studies suggest that the peroxidase-positive tubules may differ in their derivation and function from those containing acid hydrolase activity. The peroxidase-labeled tubules could acquire their membrane from internalized cell surface membrane in the form of pinocytotic vesicles and vacuoles. Such tubules would then deliver their contents and membrane to lysosomal dense bodies and multivesicular bodies for degradation. Tubules containing acid hydrolase activity may be primary lysosomes of GERL origin and may ferry acid hydrolases to presumptive secondary lysosomes, or they may take their membrane directly from secondary lysosomes and thus represent an additional form of secondary lysosome.

That the peroxidase-positive tubules in cerebral capillaries are related to endocytosed cell surface membrane is further suggested by additional studies in our laboratory in which dimethyl sulfoxide has been used to open the blood–brain barrier to peroxidase and chemotherapeutic drugs (26). The cerebral endothelial cells from mice coinjected with dimethyl sulfoxide and peroxidase exhibit a proliferation of peroxidase-labeled tubules, vesicles, and vacuoles when compared to control mice not receiving dimethyl sulfoxide. A similar proliferation of these peroxidase-labeled structures has been reported by other investigators after damage to the blood–brain barrier (27, 28); these authors have proposed that the tubules function as transendothelial channels whereby direct communication is established between the vessel lumen and the perivascular space. This interpretation is highly speculative, because the tubules have yet to be seen interconnecting the luminal and abluminal walls of the endothelial cell.

Whether or not vesicular transport of peroxidase through cerebral capillary cells occurs in our material remains unresolved. Although some peroxidase-laden vesicles were located close to the abluminal capillary wall, there was no indication of vesicles fusing with the plasmalemma to form peroxidase-coated abluminal pits; peroxidase was never seen in the perivascular clefts, an observation that could be attributed to the bulk flow of extracellular fluid (11). Nevertheless, pericytes associated with cerebral microvasculature contained peroxidase-reactive lysosomes. An important function of the pericyte may involve phagocytosis of blood–borne large molecular weight substances that have passed through or between cerebral endothelial cells (11, 29–31). Pericytes may very well serve as the first line of defense once the blood–brain barrier has been breached. The pericyte cytoplasm in our material appeared diffusely filled with peroxidase only in mice receiving multiple intravenous injections. Such cytoplasmic labeling would suggest that the pericyte was somehow damaged, possibly at the time of fixation. An al-

‡ GERL is an acronym for *G*olgi-associated smooth *e*ndoplasmic *r*eticulum from which primary *l*ysosomes may arise (14, 18).

ternative speculation is that the pericyte imbibed so much peroxidase that lysosomal sequestration of the protein exceeded the rate of degradation, causing the lysosomes to rupture and spill their contents into the cytoplasm (11).

On the basis of evidence presented here, the definition of the blood–brain barrier must be expanded. In addition to circumferential belts of tight junctions between adjacent endothelial cells and a relative inability of these cells to transcellularly transport significant amounts of protein within vesicles, consideration should be given to the lysosomal system of organelles within capillary endothelial cells and pericytes.

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