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Cellular mechanisms of the blood-brain barrier (BBB) opening to albumin-gold complex

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Summary. Cold lesion injury applied to mouse brain and infusion of hyperosmolar L(+) arabinose solution into rat carotid artery were used as extravascular and intravascular insults, respectively, leading to blood-brain barrier (BBB) disruption. To study the cellular mechanisms of the BBB opening, heterologous (bovine) and homologous (mouse and rat) albumin-gold complexes were used as a macromolecular tracer. Both insults rapidly induce the leakage of the blood-borne tracer, although the mechanisms of their action appear to be different. Cold lesion injury (cryoinjury) leads to the opening of interendothelial junctions and concomitantly to an endothelial-platelet reaction. This insult is followed by irreversible changes such as desquamation, degeneration and necrosis of the endothelial lining, formation of thromboses, and disruption of the basement membrane. Osmotic opening occurs through at least the four mechanisms (presumably temporal and reversible) that follow: 1) opening of a part of the junctional complexes; 2) the formation of transendothelial openings (interendothelial gaps or penetrating, crater-like excavations); 3) the uncontrolled passage of tracer particles through the cytoplasm of the injured endothelial cells; and 4) segmental denudation of the endothelial lining. The basement membrane appears to represent one of the main obstacles in the passage of blood-borne albumin-gold complexes to the extracellular space in the brain parenchyma.

Key words: Blood-brain barrier, Albumin-gold complex, Cryoinjury, Hyperosmolarity, Junctional complexes

Introduction

Since the pioneer work of Reese and Karnovsky (1967), horseradish peroxidase (HRP) has been widely used as a protein tracer in ultrastructural studies concerning macromolecular transport across the normal or impaired blood-brain barrier (BBB).

Although the transport of this tracer from the blood to brain parenchyma has been studied extensively, the problem of the cellular mechanisms involved in the opening of the barrier remains controversial (Rapoport, 1985; Balin et al., 1987; Broadwell, 1989).

In the present study we decided to use albumin as a macromolecular tracer because, contrary to HRP, this protein is one of the normal components of blood plasma which serves as a carrier for many important and metabolically active substances (Ghitescu et al., 1986; Milici et al., 1987). Besides that, the application of albumin-colloidal gold complexes is advantageous in that it provides excellent visibility for electron microscopy (EM) and allows easy visual tracking of the gold particles as they are crossing the vessel wall. This tracer is especially useful for studying the increased permeability of the impaired BBB because we have previously observed that in the rat (unpublished data) and in adult mouse brain microvasculature, no transport of bovine serum albumin (BSA) or mouse serum albumin (MSA) complexed with colloidal gold and injected intravenously (*in vivo* studies) or infused *in situ* (see below) occurs under normal conditions (Vorbrodt and Lossinsky, 1986; Vorbrodt et al., 1987).

Because albumin molecules can be complexed with colloidal gold particles of a wide range of sizes, the size effect of the tracer molecules on their transvascular passage can be easily studied. It is worth noting that after complexing with colloidal gold, the albumin does not change its biological properties (Handley and Chien, 1987; Milici et al., 1987; Villaschi, 1989).

The main objective of the present work is to gain insight into the ultrastructural events associated with the opening of the BBB to albumin as a representative of macromolecular solutes. We applied two widely used factors that affect BBB function. As an extravascular factor, a cold probe was applied to the surface of the mouse brain, producing cryoinjury. As an intravascular factor, a hyperosmolar solution of L(+)arabinose was infused into carotid artery of the rat.

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Materials and methods

Cold lesion injury

Fifteen adult (2- to 6-months old) BALBc/J mice of both sexes, weighing approximately 25 g, were used. Under Nembutal (sodium pentobarbital) anesthesia, the scalp was incised and the periosteum exposed. Focal cerebral freeze-lesions were made by applying a metal rod cooled to -50°C with dry ice and acetone for 1 min to the intact skull, similar to the method described by other investigators (Baker et al., 1971; Cancilla et al., 1972).

For macroscopic and light microscopic (LM) visualization of the leakage of blood vessels, some mice were injected intravenously with 2% Evans blue (EB) in Ringer's solution or with HRP (Sigma, type VI) dissolved in Ringer's solution or with HRP (Sigma, type VI) dissolved in Ringer's solution (10 mg/0.1 ml) just prior to induction of cold lesion. After circulation of HRP for 5 min or 1 h, the animals were perfused with our standard fixative (2% paraformaldehyde and 1% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, supplemented with 0.2M sucrose). The localization of HRP was cytochemically detected, using 3,3'-diamine benzidine (DAB), according to the method of Reese and Karnovsky (1967).

All other mice were perfused *in situ* with a solution of BSA or MSA complexed with colloidal gold (BSA-G and MSA-G, respectively) in Dulbecco PBS containing 14 mM glucose (DPBSG), similarly as in the technique described by Ghitescu et al. (1986). The final concentration of albumin was approximately 200 µg/ml, giving an absorbance of 1.16 at 525 nm wavelength. This solution was oxygenated and warmed up to 37°C before infusion. The vascular perfusion was performed as follows: a) under Nembutal anesthesia, an 18-gauge needle connected to a Harvard constant-pressure infusion pump was inserted into the left heart ventricle. The right atrium was cut open to allow drainage during the rest of the procedure. b) Blood was removed quickly by perfusing the vascular bed with warm DPBSG, followed by intermittent perfusion (up to 10 min) with a solution of albumin in DPBSG (see above). The temperature was maintained during the entire procedure at 37°C, using an overhead heating lamp. c) After the perfusion was completed the animals were sacrificed by decapitation at 30 or 45 min or 1, 24, and 48 h after cold injury. The brain was quickly removed and fixed by immersion in our standard fixative (see above) for at least 3 h. The samples from contralateral, uninjured brain hemisphere served as a control. During our pilot experiments, we observed that immersion fixation is superior to perfusion fixation because it does not wash the tracer from blood vessels. In this fixative, the desired brain samples were minced into small blocks (1 x 2 x 2 mm).

After fixation, tissue samples were washed in 0.1 M cacodylate buffer, pH 7.2; fixed in buffered 1% osmium tetroxide for 2 h; washed again and stained *en bloc* with 0.5% uranyl acetate (pH 5.0) for 1 h at room temperature; dehydrated in ethanol; and embedded in Spurr low-viscosity resin. Ultrathin sections were cut on a Sorvall (DuPont) MT-5000 ultramicrotome and observed in a Philips 420 electron microscope. Some sections were stained with lead citrate for 6 min at room temperature (22°C).

Osmotic opening of the BBB

Ten adult (6- to 10-months old) Lewis rats of both sexes were used. A solution of 1.8 M L(+)arabinose (Sigma) in saline was freshly prepared each time, filtered through a Millipore filter with a pore diameter of 0.22 µm, and warmed up to 37°C. Under Nembutal anesthesia, the solution was infused through a polyethylene cannula into one internal carotid artery through the common carotid artery after the external carotid had been ligated (Brightman et al., 1973). The infusion was maintained for approximately 30 sec at a rate of 0.12 ml/sec (total volume was approx. 3.6 ml) with a Harvard infusion pump, as suggested by Rapoport et al. (1978). It was followed by slow infusion (90 sec) at a pressure approximately 30 mm Hg of 1 ml of solution of BSA-G or rat serum albumin-gold complex (RSA-G) in DPBSG (approx. 2 mg/ml), or by injection into the external jugular vein. The infusions were completed 5, 10, or 15 min after the start of infusion of hyperosmolar L(+)arabinose. During all infusions, the carotid bifurcation was observed with a low-power dissection microscope to ensure that the infusate passed through the internal carotid artery rather than in a retrograde direction down the common carotid.

Simultaneously, rats received an injection of 0.5 ml of 2% EB into a femoral vein. Immediately after infusion of L(+)arabinose, one rat received an injection of 1 ml of PBS containing 50 mg of HRP (Sigma, type VI) into the external jugular vein; circulation time of HRP was approximately 5 min. These additional tracers were used to monitor the effect of hyperosmolar L(+)arabinose on brain vasculature permeability.

To avoid washing out of the tracers, the brain was rapidly excised and fixed by immersion in our standard fixative (see above). The samples taken from contralateral brain hemisphere served as a control. The remainder of the procedure was identical to that described above in previous section.

Preparation of albumin-gold complexes

A monodisperse supension of colloidal gold with a mean particle diameter of 5 nm was prepared according to the procedure of Mühlpfordt (1982). As reducing agents, 1% trisodium citrate and 1% tannic acid were used. The preparation of 15-nm gold particles was based on the original procedure of Frens (1973), with 1% trisodium citrate as a reducing agent. The particle size in all colloidal solutions prepared was determined by EM.

The optimal amount of albumin necessary to stabilize a given amount of the colloidal gold was determined for each type (MSA, RSA, or BSA) and for each particle size of the colloidal gold, using 10% NaCl as a flocculating agent according to the method of Horisberger and Rosset (1977). In our hands, the concentration of albumin proposed by Ghitescu et al. (1986) was sufficient to stabilize colloidal golds with a particle size from 5 to 15 nm. The procedure was as follows: 50 ml of colloidal gold solution adjusted to pH 5.5 was rapidly mixed with 1 ml of the solution containing 6 mg of albumin and stirred, and after 1-2 min, 2 ml of 1% polyethylene glycol (M.W. 20,000) was added to stabilize the complex. The solution was centrifuged at 60,000 x g (for 15 nm gold particles) or at 100,000 x g (for 5 nm gold particles) for 45 min. The clear supernatant eventually containing unbound albumin was carefully discarded, and the red sediment was dissolved in 3 ml of PBS containing 0.1% polyethylene glycol. In the descriptions that follow, the sizes of the gold particles are referred to as follows: BSA-G5 for 5-nm gold particles and BSA-G15 for 15 nm gold particles (the same designations relate to MSA and RSA).

Results

Cold lesion injury

Cryoinjury affects a limited area of the cerebral cortex together with the pertinent vascular network. Macroscopically, the leakage of the affected blood vessels can be easily demonstrated by intravenously injecting a solution of Evans blue (EB). Thus, the use of this tracer facilitates the proper choice of cortical samples for subsequent ultrastructural examination.

For macroscopic and microscopic evaluation of the BBB opening, HRP intravenously injected just prior to cryoinjury also appears to be a useful tracer. After a short period of circulation (5 min), only the area directly affected becomes stained, indicating that these vessels located in this area are leaking. After longer circulation time (1 h), both the directly affected area and the adjacent neuropil become stained, indicating the spread of extravasated edematous fluid containing blood-borne tracer (Fig. 1).

At 30 min after the application of cryoinjury (this time includes 10 min intermittent perfusion of the vascular bed with albumin-gold complex), dramatic changes in the affected blood microvessels can be noted. These changes include a shrinkage of the endothelial cells (ECs), leading to the opening of intercellular junctions, and the formation of interendothelial clefts, which apparently constitute the main avenue for the escaping tracer (Fig. 2). Some ECs lose their contact with the subjacent basement membrane (BM) leading to the formation of narrow subendothelial clefts in which gold particles frequently appear (Figs. 2, 3). At this time, relatively few gold particles are crossing the BM; those particles that do so appear in a perivascular space.

In many sectioned capillaries, numerous platelets appear in the vessel lumen, leading to the formation of aggregates (microthrombi) containing red blood cells (Fig. 3). In some microvessels, the endothelial lining is disrupted or fragmented and detached from the BM. In these vessels, the formation of fibrin can be observed. It is worth noting that in a majority of affected microvessels, the continuity of the BM is preserved, although the outlines of some segments of this membrane become blurred and appear ill-defined (Figs. 3, 4).

At 45 and 60 min after cryoinjury, the ultrastructural signs of the damaging action of the applied insult on the microvessel wall are more pronounced. The endothelial lining shrinks, becomes attenuated, or is even discontinued. Many ECs are partially or totally detached from the BM and replaced by platelets. Some platelets are flattened and squeezed between the detached ECs and the BM (Fig. 5), whereas others keep their characteristic shape (Fig. 6). In a number of vessels the BM is swollen or even disrupted. In the vicinity of such disruptions or gaps, numerous albumin-gold particles appear outside the vessel wall, i.e., in a perivascular area (Fig. 6).

In the majority of affected vessels, the lumen is totally or partially obstructed by microthrombi composed of platelets, red blood cells, fibrin, and cell remnants. At 60 min, in the cortex near the margin of the freeze-lesion, many vessels show increased numbers of luminal and abluminal pits and presumptive plasmalemmal vesicles (PVs). Because only a single or a few gold particles are present in these structures and no gold particles appear in the perivascular space, these vessels are considered not to be leaking.

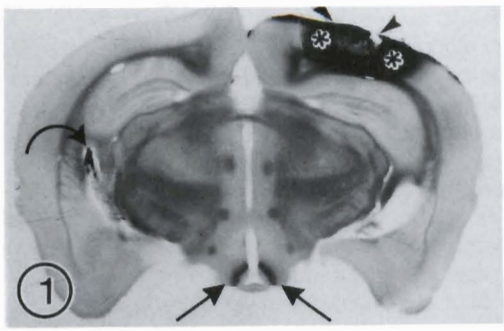
As the time progresses (in excess of 1 h), the injured cortical tissue directly exposed to the cold probe becomes necrotic; therefore, it cannot be used for ultrastructural examination.

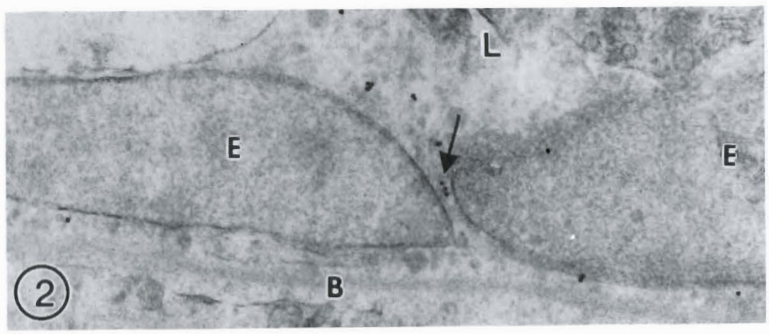
After 24 and 48 h, some tracer particles can be found in the neuropil adjacent to the necrotized tissue, indicating the spread of extravasated edematous fluid containing blood-borne tracer. The samples obtained do not contain any leaking vessels and consequently cannot be used for our study on the mechanism of the BBB opening.

In the course of these experiments, we noted that neither the original source of the albumin (BSA = heterologous, and MSA = homologous protein tracer) nor the size of the gold particles (G5 or G15) had any influence on the localization, fate, and pathway of the tracer in the affected microvessels.

Osmotic opening of the BBB

Osmotically induced opening of the BBB occurs rapidly and can be demonstrated macroscopically by intravenously injected EB as soon as a few minutes after infusion of hyperosmolar L(+)arabinose. The staining of

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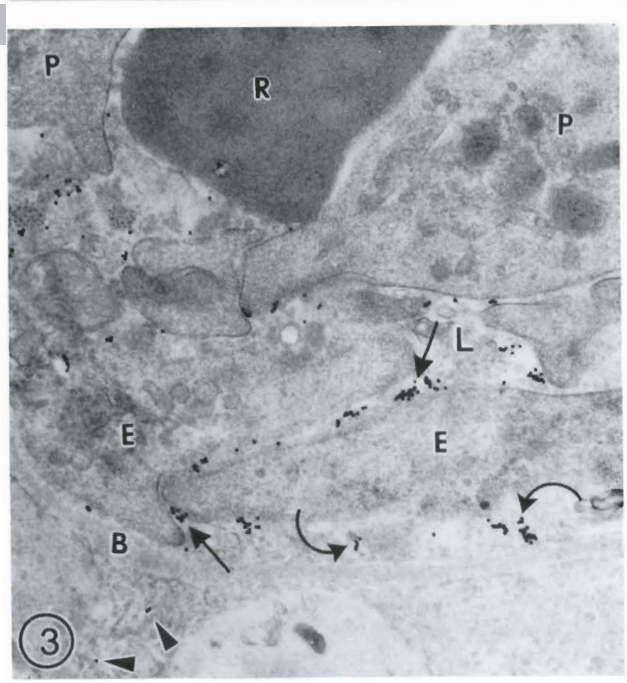
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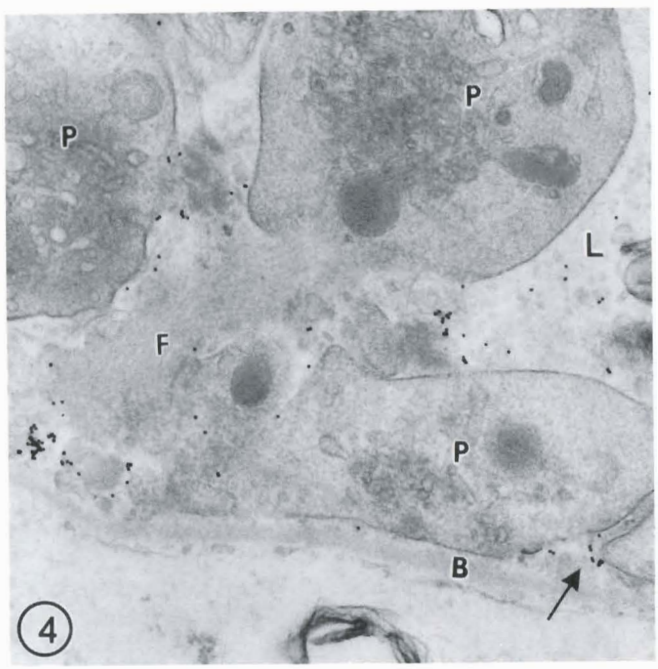
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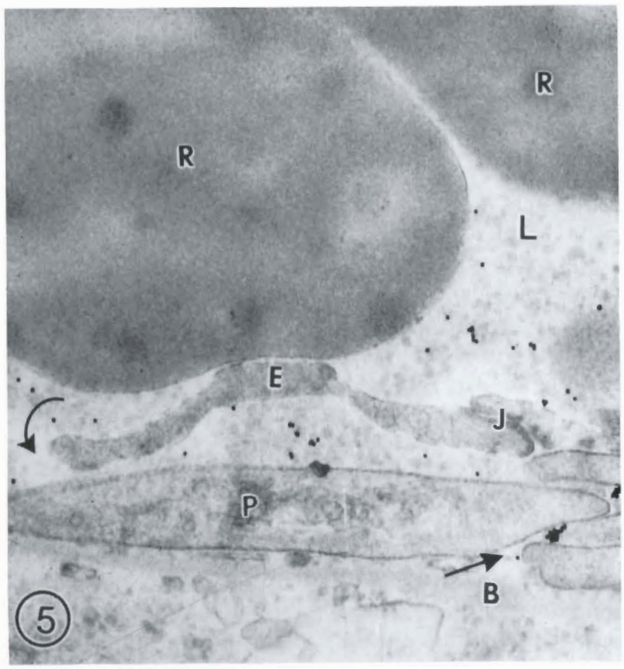
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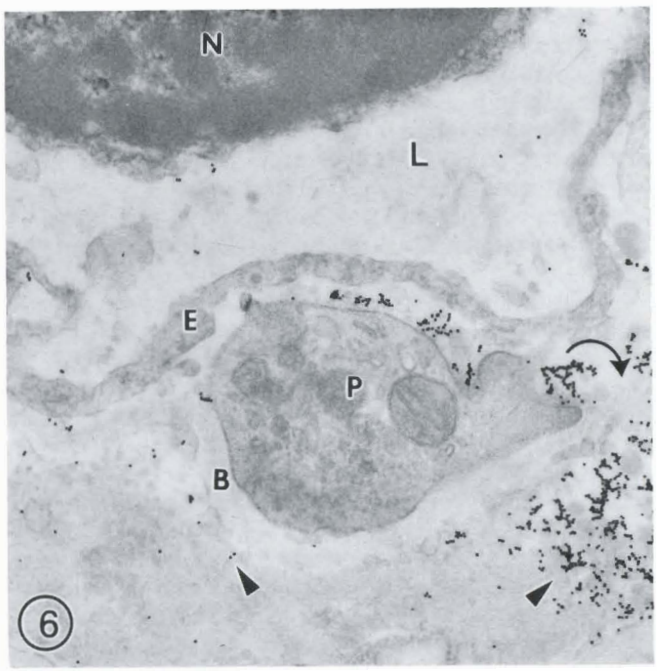
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the ipsilateral side of the brain is convincing evidence that the experimental procedures were correct and successful. It should be emphasized, however, that the staining of the cerebral cortex is not even, and on the uniformly stained background, several spot-like overcolorings can be noted (Fig. 7).

The spotty staining of the ipsilateral hemisphere becomes evidently more pronounced after the application of HRP as a macromolecular tracer (Fig. 8). Such a localization of the reaction product for HRP can be considered crucial evidence that the leakage of blood-borne tracer does not occur through the entire vascular network but only through particular segments of the network, which are presumably especially sensitive to the applied insult. The observed dissemination of leakage also indicates that the subsequent ultrastructural examination must be precisely targeted at particular affected segments of the microvessels.

Because the vast majority of the vascular network in cerebral cortex is represented by capillaries, the profiles of these microvessels are most numerous in examined specimens. The most conspicuous evidence of the action of hyperosmolarity, observed as early as 5 min after infusion of L(+)arabinose, relates to tight junctional modification. It should be emphasized, however, that structural modifications can be noted only in a small number of junctional complexes, predominantly in capillaries.

In the majority of examined microvessels, the junctional complexes are composed of two peripheral tight junctions (luminal and abluminal) and one or a few additional junctions separated by longer or shorter segments of interendothelial cleft, also called pools or lacunae of the extracellular space (Brightman et al., 1973).

One of the modifications consists in the enhanced binding of BSA-G or MSA-G particles to the EC surface at the luminal opening of the interendothelial junction. In these junctions, the intercellular cleft becomes distended, and some tight junctions that link the ECs are open (Fig. 9).

In some dilated junctions, groups of gold particles appear within the junctional complex, suggesting their passage through the interendothelial cleft (Fig. 10). As a result of the tortuosity of the junctional complexes, only short segments of the distended clefts with gold particles are visible in thin-sectioned ECs (Figs. 11-13). In some ECs, a few PVs are present, but they usually do not contain gold particles, indicating that vesicular transport of the tracer either does not occur at all or is negligible (Fig. 11).

Frequently, small groups of gold particles appear at the abluminal mouth of the junctional complex; presumably, it represents a final stage of their transendothelial journey from the vessel lumen (Fig. 12). It is noteworthy that even in close proximity to the site of leakage, one can find apparently unaffected junctional complexes sealed with a few tight junctions (Fig. 13).

However, in many segments of the affected microvessels, leakage of the tracer occurs through passageways other than junctional ones. The ultrastructural details of some passageways are so elusive that they cannot be precisely defined, and their existence can be noted only by their presence in the vicinity of the extravasated gold particles (Fig. 14).

Occasionally, funnel-like recesses, deep lacunae, or channel-like, tubular profiles are located in close proximity to swollen mitochondria in the EC's

Figs 1- 20. The following symbols are used in the micrographs: B, basement membrane; E, endothelial cell; F, fibrin; J, junctional complex; L, vessel lumen; M, mitochondrion; N, cell nucleus; P, platelet; R, red blood cell; S, smooth muscle cell; V, plasmalemmal vesicle.

Fig. 1. Coronal section of a mouse brain 1 h after cold lesion injury followed by intravenous injection of HRP. The two arrowheads point to the surface of the cerebral cortex exposed to cold lesion. The two asterisk indicate the spread of extravasated edematous fluid containing blood-borne peroxidase. Leakage of HRP from the vessels supplying the circumventricular organs such as the choroid plexus (curved arrow) and median eminence (arrows) is also noticeable. x 6,5

Fig. 2. A segment of the capillary wall located in the area of cold lesion injury (30 min) is shown. Gold particles are present in the vessel lumen (L), and some of them are presumably passing through the incercellular cleft (arrow) between swollen endothelial cells (E). A few gold particles also appear in a narrow cleft bertween the ECs sloughing off and the basement membrane (B). x 36,000

Fig. 3. Cross-sectioned microvessel from the same specimen as shown in Fig. 2. In the lumen of the vessel (L), a plug (microthrombus) composed of platelets (P) and red blood cell (R) is formed. The MSA-G particles are presumably passing through an opened intercellular junction (arrows) between the affected ECs. Several gold particles (curved arrows) appear in a cleft between the ECs and the basement membrane (B). A few gold particles, which presumably have already crossed the BM, appear in the perivascular area (arrowheads). x 22,200

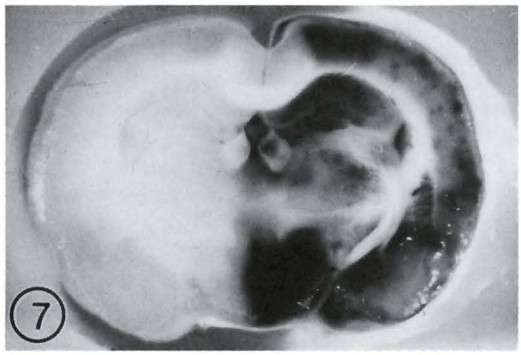
Fig. 4. In this microvessel (45 min after cryoinjury), the endothelial lining is substituted by the platelets (P), which participate in the production of fibrin (F). Some gold particles (arrow) appear in the vicinity of the basement membrane (B). x 27,200

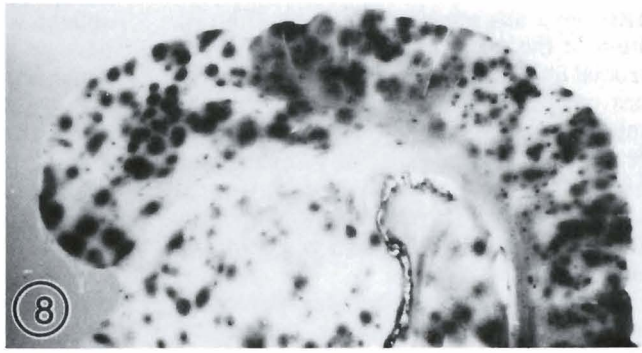
Fig. 5. A segment of a microvessel 1 h after cryoinjury is shown. Endothelial cells (E) appear shrunken and, presumably in the process of sloughing, are separated from the basement membrane (B) by a flattened platelet (P). Several gold particles are squeezed between adjacent platelets (arrow). The endothelial lining is discontinuous (curved arrow), although the tight junction (J) remains. x 27,000

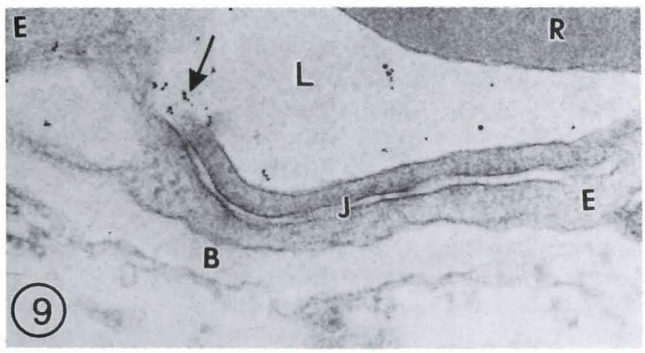
Fig. 6. Another microvessel from the same specimen as shown in Fig. 5. The platelet (P) is interposed between a shrunken, detached EC (E) and the basement membrane (B). Curved arrow points to an apparently destroyed segment of the BM, where leakage of the albumin occurs. Numerous extravasated albumin-gold particles (MSA-G15) are located in the perivascular neuropil (arrowheads). x 24,600

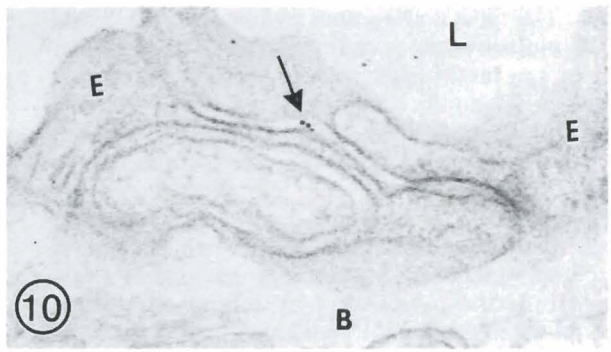
cytoplasm. These putative passageways, frequently containing many gold particles, appear at the transition site where the cytoplasm embracing the mitochondria becomes thinner (Fig. 15). The concomitant presence of numerous gold particles on the abluminal side of the vessel wall in the neighboring perivascular space is an indication that the examined segment of the microvessel becomes permeable to the injected macromolecular tracer.

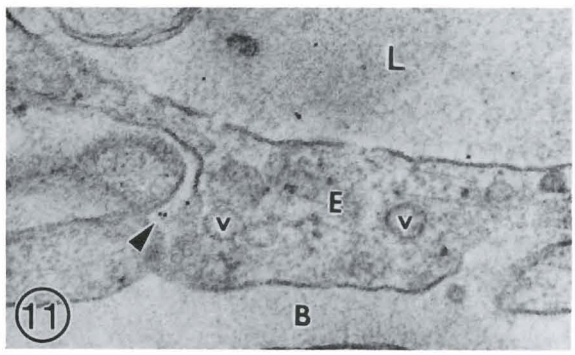
Interestingly, arterioles appear to be less sensitive to the hyperosmolarity in that the ultrastructural pattern of all of the components of their walls remains unchanged (Fig. 16). The interendothelial junctions are not affected, and the majority of luminal and abluminal pits and PVs

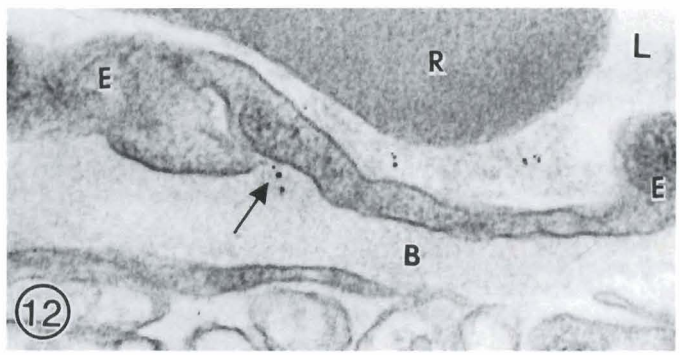


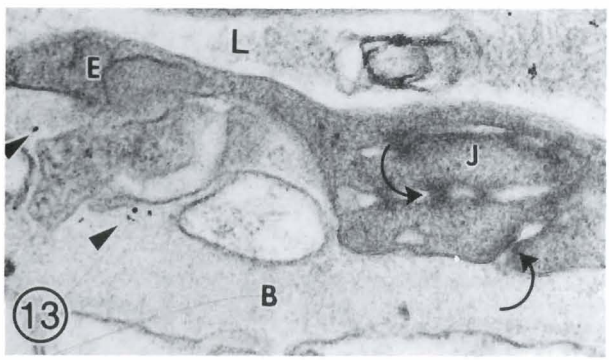


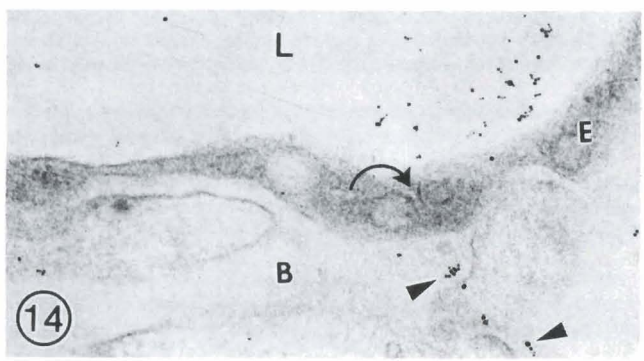












do not contain gold particles. Only a few gold particles located in elongated or oval-shaped compartments resembling endosomes can be noted inside the EC body.

Another type of leakage, frequently observed at 10 or 15 min after infusion of hyperosmolar L(+)arabinose, occurs through degenerating or necrotizing ECs. Ultrastructurally, it is manifested by blurred outlines and deterioration of cytoplasmic membranous structures, swelling of mitochondria, and vacuolization of the endoplasmic reticulum. The cytoplasm of these ECs is inundated by great numbers of albumin-gold particles, some of which also cross the BM (Figs. 17, 18).

In some vessels, the adjacent ECs become completely separated, forming a wide gap with the BM exposed (Fig. 19). One can assume that such a gap represents a direct passageway for rapid extravasation of blood-borne solutes. It is difficult, however, to determine whether such a gap is formed at the site of the interendothelial junction or whether it represents a cavitation of the EC body induced by the applied insult.

In addition to the structural lesions described above that are induced by hyperosmolarity, in some vessels, especially in the area of bifurcations at capillary-venular junctions or in venules, a segmental denudation of the endothelial lining occurs (Fig. 20). It appears after a longer time interval, i.e., at 10 or even 15 min after infusion of hyperosmolar L(+)arabinose. In the vicinity of such endothelial denudation, the albumin-gold particles are infiltrating and crossing the exposed BM. In the vessel lumen, in addition to groups of platelets, various remnants of the cell (mitochondria, nuclei, dense bodies, vacuoles) are also present indicating that hyperosmolarity can induce fast degeneration and disintegration of some sensitive cells.

No noticeable differences in the fate or localization of injected tracers related to the origin of the albumin (BSA-G or RSA-G) were noted. The size of the gold probe, however, appears to have some effect on the passage across the BM: smaller particles (G5) complexed with albumin penetrate this membrane more easily than do larger particles (G15).

Discussion

The main findings of this study are the following:

(A) Both applied insults, i.e., cryoinjury and hyperosmolarity, open the barrier, resulting in the passage of gold-labeled albumin from blood to brain parenchyma. Concomitantly, this barrier was also opened to additional tracers such as EB and HRP, which were used for macroscopic and light-microscopic evaluation of increased BBB permeability. As is generally believed, EB forms a complex with albumin (Brightman et al., 1973; Klatzo et al., 1980; Wolman et al., 1981), and consequently, the blue staining of the brain parenchyma can be considered additional evidence of the opening of the BBB to blood-borne albumin.

Because the leakage is rapid and intense after the application of both insults, one can assume that the openings are formed very rapidly and are of rather high diameter; this assumption offers a feasible explanation for why rapid passage of a large volume of solutes occurs. It should also be emphasized that only occasionally are a few plasmalemmal pits or vesicles containing solitary gold particles observed inside the EC's cytoplasm. Consequently, one can conclude that the mechanism of transendothelial vesicular transport does not appear to cause the relatively fast leakage of the affected segments of the vascular network we observed.

Thus, one can expect that other types of passageways for macromolecular solutes were formed in the affected

Fig. 7. Coronal section of a rat brain fixed 5 min after infusion of 1.8 M L(+)arabinose into the right carotid artery with concomitant i.v. injection of 2% EB. Limitation of the staining exclusively to the ipsilateral hemisphere indicates opening of the BBB. x 4

Fig. 8. Coronal section of the ipsilateral hemisphere of rat brain 5 min after infusion of hyperosmolar L(+)arabinose into the carotid artery followed by i.v. injection of HRP. Numerous focal extravasations of blood-borne peroxidase are scattered throughout the entire hemisphere, although they are more dense in the cerebral cortex. x 7,5

Fig. 9. A segment of a capillary wall from the rat cerebral cortex 5 min after infusion of hyperosmolar L(+)arabinose and RSA-G5. An agglomeration of numerous gold particles (arrow) in the vicinity of the luminal opening of the intercellular junction (J) is shown. x 55,500

Fig. 10. Another microvessel from the same specimen as shown in **Fig. 9.** Three gold particles (arrow) are shown within the cleft of the junctional complex. x 69,000

Fig. 11. A segment of a microvessel shown 10 min after infusion of hyperosmolar L(+)arabinose. A few gold particles (BSA-G5) are located within the intercellular cleft (arrowhead), whereas plasmalemmal vesicles (v) do not contain the gold particles. x 69,000

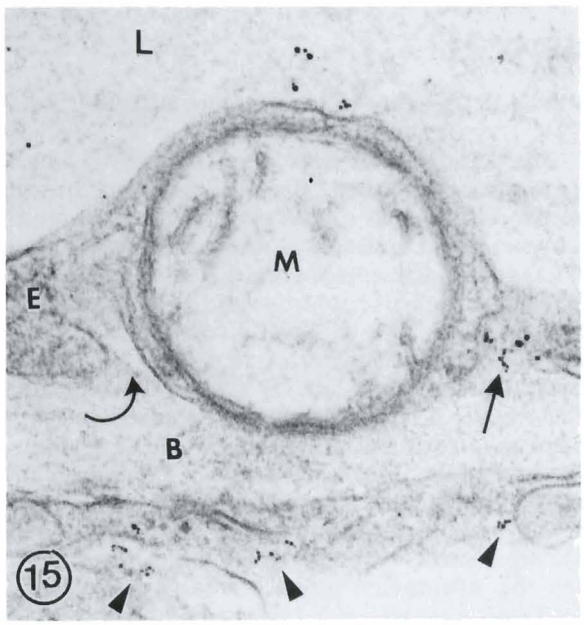
Fig. 12. The same specimen as shown in **Fig. 11.** In this microvessel, a few gold particles are located in the vicinity of the abluminal mouth of the interendothelial junction (arrow), suggesting their passage from the vessel lumen. x 69,000

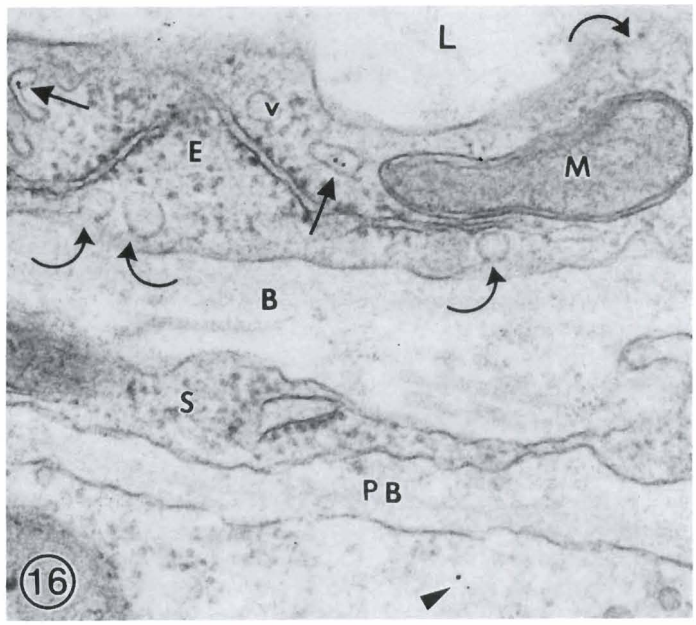
Fig. 13. A segment of the wall of a cortical microvessel is shown 10 min after osmotic opening and infusion of RSA-G5. Although there are a few gold particles (arrowheads) on the abluminal side of the EC, the junctional complex (J) is sealed in several locations (curved arrows). x 56,000

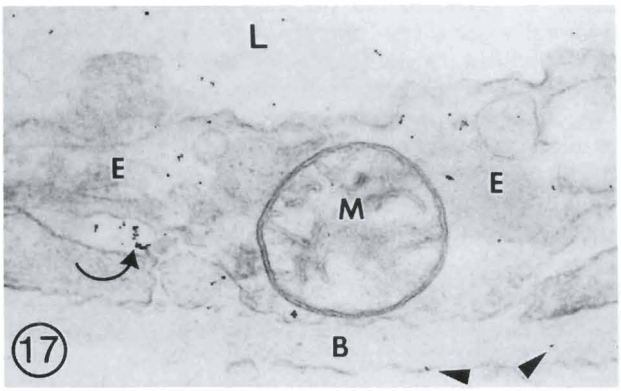
Fig. 14. Another microvessel from the same specimen as shown in **Fig. 13.** Several gold particles present in a perivascular space (arrowheads) suggest that the leakage of the tracer apparently occurs through a narrow passageway resembling a transendothelial channel (curved arrow). x 58,000

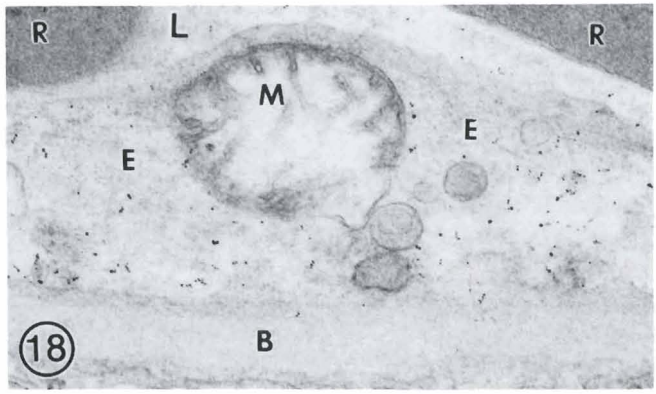
blood vessels that were exposed to the applied insults. Among them, the following four mechanisms should be considered:

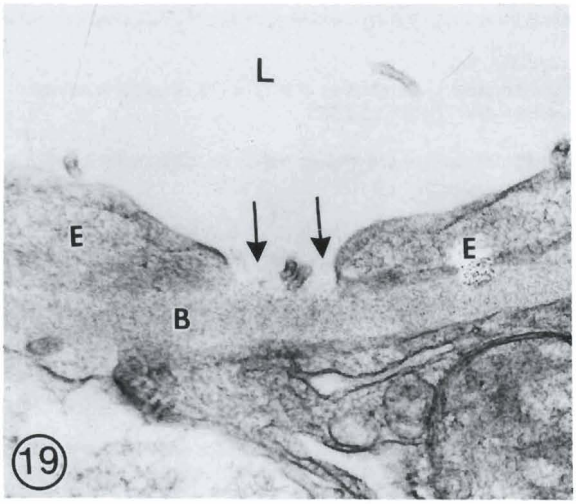
1) Opening or modification of interendothelial junctions. Many authors believe that the modification of interendothelial junctions represents a basic mechanism of the opening of the BBB after application of cryoinjury (Baker et al., 1971; Cancilla et al., 1972; Nagy and

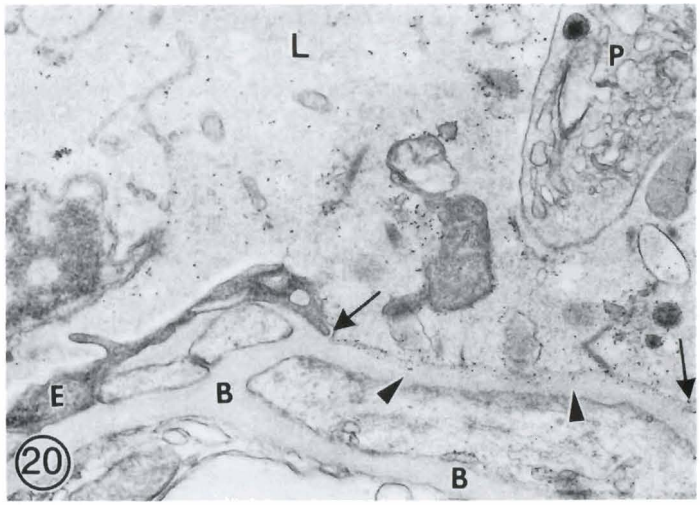












Brightman, 1986), hyperosmolarity (Brightman et al., 1973; Lehtosalo et al., 1982; Rapoport, 1985; Rapoport and Robinson, 1986; Brightman, 1989), or other types of insults (Nagy et al., 1983). This notion, however, can be seriously undermined by the following facts: a) only a limited number of the junctional complexes are modified by the infusion of hyperosmolar solutions; b) even interendothelial clefts partially filled with HRP were always separated by one or a few tight junctions (Brightman et al., 1973; Brightman, 1989); c) our own observations indicate that the gold particles, even located in the interendothelial clefts of presumably opened junctions, never form continuous bead-like rows linking luminal and abluminal estuaries of the junctional complexes.

Thus, our observations suggest that only some of the junctional complexes constitute passageways for macromolecular solutes after administration of hyperosmolar solutions, and their opening seems to be limited in number. Consequently, only a small portion of the leaking tracer can utilize this route for crossing the BBB.

On the contrary, cryoinjury induces total opening of the junctional complexes, which constitute relatively wide gateways for escaping albumin complexed with even larger (G15) gold particles. Such an opening, however, seems to be the first stage of a rapidly progressing degeneration of the endothelial lining in the affected vascular network. Thus, it cannot be considered an example of temporal (or reversal) opening of the tight junctional complexes, as is postulated in the case of the osmotic opening of the BBB (Rapoport et al., 1972, 1980).

2) Focal disruption of the endothelial continuity. Focal disruption of the continuity of the endothelial lining can occur essentially by two mechanisms: a) the separation of adjacent ECs and the formation of wide gaps with concomitant exposure of the BM, as was demonstrated by Nagy et al. (1983) these gaps are presumably formed at the site of the interendothelial junctions or b) the formation of deep, crater-like excavations, which penetrate the EC's body and form funnel-like perforations that can be considered an excellent passageway for leaking blood-borne solutes. These openings have a rather large caliber and could be an efficient gateway for rapid escape of various solutes from the blood plasma. Similar crater-like openings were observed with scanning EM in the ECs of brain microvessels of mice with chronic relapsing experimental allergic encephalomyelitis (Lossinsky et al., 1989, 1991). The appearance of various openings, including transendothelial channels, has been a topic of controversy (Balin et al., 1987; Broadwell, 1989) with other models of brain injury studied by our group (Lossinsky et al., 1983; Vorbrodt et al., 1983) and reviewed recently by Wisniewski and Lossinsky (1991).

3) Passage of macromolecules into and through the EC cytoplasm. The disseminated passage across the cell cytoplasm can occur in injured or degenerating segments of the ECs. Such passage was observed by the authors, who applied HRP as a macromolecular tracer in various models of BBB disturbances (Baker et al., 1971; Houthoff and Go, 1980; Nagy et al., 1983; Houthoff et al., 1984). We report here that only in experiments with hyperosmolar L(+)arabinose did we observe widespread inundations of EC's cytoplasm by albumin-gold complexes, especially when G5 particles were used. This observation suggests that the movement of albumin-gold complexes through the EC body is size dependent, at least to some degree. On the contrary, we did not observe such a mechanism to occur in cold lesion injury in which shrunken and desquamated ECs were not

Fig. 15. A small portion of the wall of a leaking microvessel 5 min after osmotic opening is shown. The arrow points to gold particles in a passageway presumably representing a transendothelial channel located at close proximity to a swollen mitochondrion (M). The opening of another empty, funnel-like indentation (curved arrow) is visible on the other side of the mitochodnrion. Numerous extravasated gold particles (BSA-G15) are scattered in the perivascular space (arrowheads). x 70,000

Fig. 16. A portion of the wall of an arteriole 10 min after infusion of hyperosmolar L(+)arabinose is shown. Although there is a single gold particle in the perivascular area (arrowhead), this vessel is rather unaffected, because the fine structural details of the cell cytoplasm are well preserved. A few gold particles are located in cytoplasmic organelles presumably representing endosomes (arrows), whereas numerous luminal and abluminal pits (curved arrows) and plasmalemmal vesicles (v) do not contain the tracer particles. x 56,000

Fig. 17. A portion of the capillary wall is shown 10 min after infusion of L(+)arabinose with swollen EC cytoplasm (E). The EC body is inundated with numerous irregularly scattered particles of BSA-G15, suggesting uncontrolled passage of the tracer across the affected endothelial lining. Several gold particles appear in a distended interendothelial cleft (curved arrow); some gold particles are shown on the distant side of the BM (arrowheads), suggesting that they have crossed this membrane. x 40,000

Fig. 18. A portion of a capillary wall 15 min after infusion of hyperosmolar L(+)arabinose with an injured EC is shown. A mitochondrion (M) is swollen, and the electron-lucent cytoplasm is inundated with numerous RSA-G5 particles, x 50,000

Fig. 19. A microvessel present in the same specimen as shown in Fig. 18 demonstrates a complete separation of adjacent endothelial cells (arrows). x 46,000

Fig. 20. Another microvessel (presumably a venule) from the rat cerebral cortex 15 min after infusion of L(+)arabinose is shown. The two arrows point to the denuded segment of the vessel wall where the endothelium is completely sloughed away. The adjacent segment of the wall is covered by the EC (E), which shows signs of damage including shrinkage and attenuation. Arrowheads point to gold particles infiltrating and presumably crossing the BM. In the vessel lumen (L), a platelet (P) and some remnants of detached ECs are present. x 19,000

invaded by the tracer.

These differences presumably result from the mode of action of hyperosmolarity, which is less damaging to the cell structure and function that cyroinjury and consequently allows more time for the full manifestation of increased permeability of the various cytomembranes. This assumption is supported by the fact that such type of cell injury was observed more frequently 15 min after infusion of L(+)arabinose than after shorter time intervals. Because only a few gold particles appear concomitantly in the BM and none appear in the perivascular area, one can conclude that the passage of albumin-gold complexes occurs faster through the cytoplasm of the affected ECs than through this membrane. The BM probably represents one of the main structural obstacles for escaping albumin. It is not known whether the size of the albumin-gold particles is a limiting factor of their passage through the BM, because this structure was observed to be easily flooded by other macromolecular tracers, especially by HRP (Baker et al., 1971; Cancilla et al., 1972; Brightman et al., 1973; Lossinsky et al., 1983; Vorbrodt et al., 1985; Balin et al., 1987; Broadwell, 1989).

4) Endothelial denudation. In some blood vessels, especially in venules or at capillary-venular junctions, we observed a portion of the endothelial lining sloughing away, leaving relatively long segments of the BM exposed to the bloodstream. In these segments of the vasculature, the infiltration of the BM by albumin-gold particles can be considered structural evidence of BBB opening. Thus, endothelial denudation appears to be one of the mechanisms that should be taken into consideration when osmotic opening of the BBB is discussed. It is obvious that the exposure of the BM promotes rapid platelet reaction, leading to the formation of platelet plugs and thromboses (Rosenblum, 1986).

The process of endothelial denudation in cryoinjured vessels evidently differs from that observed in hyperosmolarity. Platelet aggregation occurs before endothelial sloughing, and one can assume that platelet-endothelial interaction starts before the effect of cryoinjury becomes morphologically discernible at the ultrastructural level. Such a sequence of events bears a resemblance to the observations of Rosenblum (1986) and Rosenblum and Povlishok (1987) on platelet-endothelial interaction induced by excitation of intravascular fluorescein.

(B) The applied insults differ fundamentally in their mode of action on brain microvasculature: a) Cold lesion injury as an extravascular factors affects limited areas of the cerebral cortex together with the pertinent vascular network. All vessels in this area are affected, and their cellular components undergo rapid changes leading to degeneration and necrosis. Consequently, this type of injury is a useful and efficient method for inducing vasogenic brain edema to study its spreading and mechanisms of resolution (Klatzo et al., 1980; Wolman et al., 1981; Vorbrodt et al., 1985). However, this method is too brutal to be used for studying the cellular mechanisms of BBB opening because it induces irreversible injury, ultimately leading to endothelial cell death. b) On the contrary, the intravascular factor such as infusion of hyperosmolar L(+)arabinose induces widespread but disseminated changes in almost the entire vascular network in the ipsilateral brain hemisphere. This dissemination indicates that only particular segments of the vascular network are leaking, that this network is not uniformly sensitive to the applied insult, and that there exist *loci minoris resistentiae*, i.e., sites of lessened resistance. Thus, one can assume that the affected and non-affected vascular segments are located side by side, creating excellent conditions for the process of cellular reparation. Indeed, according to Rapoport et al. (1980) and Rapoport (1985), the osmotic opening is reversible, and the restoration of BBB function starts as soon as 30 min after application of the insult. Unfortunately, the structural events associated with the restoration of BBB integrity are not well known, and this problem requires detailed ultrastructural study.

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