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Ultracytochemical characterization of anionic sites in the wall of brain capillaries

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Summary

The chemical nature of anionic sites located on both fronts of the endothelial cells (ECs) and in the basement membrane (BM) of mouse brain capillaries was studied using tissue sections embedded in Lowicryl K4M and cationic colloidal gold. Before labelling with cationic probe, the sections were digested with the following enzymes: trypsin, papain, pronase E, proteinase K, collagenase, chondroitinase ABC, hyaluronidase, heparinase, heparitinase, neuraminidase and endoglycosidase H.

The results indicate that the negatively charged surface layer on the luminal front differs in chemical nature from that on the abluminal front of the EC. Anionic sites located on the luminal surface of the plasmalemma of the ECs are mainly contributed by sialic acid residues of acidic glycoproteins. On the contrary, the anionic domains on the abluminal front of the EC represent mixed proteoglycan and acid glycopeptides containing hydrophobic amino acids, sialic acid residues, and are rich in heparan sulphate-bearing glycosaminoglycans. The anionic sites of the BM are contributed in a substantial degree by chondroitin and heparan sulphate-rich glycosaminoglycans.

The effect of endoglycosidase H suggests that glycopeptides containing oligomannosyl residues linked to *N*-acetylglucosamine contribute in small degree in maintenance of the negative charge in the BM, but not on the surfaces of the EC.

These results show that brain endothelium bears surface anionic domains differing chemically from those described for some fenestrated and continuous endothelia. The distribution of anionic sites indicates that the discrimination against various negatively charged molecules takes place on both fronts of the ECs as well as in the BM of brain micro-blood vessels. The exact role of these domains in the function of the blood–brain barrier remains to be established.

Introduction

The negatively charged surface layer (anionic sites) on the plasma membrane (plasmalemma) of endothelial cells (ECs) is considered to be one of the important factors involved in the maintenance of blood–brain barrier (BBB) function (Houthoff *et al.*, 1984; Hardebo & Kåhrström, 1985). The opening of the BBB produced by positively charged substances is an important argument for the role of this surface layer in maintaining membrane integrity and in contributing to the endothelial barrier function (Nagy *et al.*, 1983; Vorbrodt *et al.*, 1981; Houthoff *et al.*, 1984; Hardebo & Kåhrström, 1985; Hart *et al.*, 1987; Strausbaugh, 1987).

It has been shown that, in bone marrow sinusoids and in visceral fenestrated capillaries, the anionic sites are located along the whole luminal endothelial surface (plasmalemma proper) except locations where plasmalemmal vesicles or transendothelial channels are present (De Bruyn *et al.*, 1978; Simionescu *et al.*, 1981; Palade *et al.*, 1982; Pino, 1984). Anionic sites – as visualized by cationized ferritin – were also observed on the abluminal surface of capillary endothelium, facing the basement membrane (Simionescu *et al.*, 1982; Charonis & Wissig, 1983). The presence of negatively charged domains in the basement membrane (BM), visualized by cationic ferritin as well as by other cationic probes (ruthenium red or polyethyleneimine) were also observed in various types of capillaries (Schurer *et al.*, 1978; Hopwood *et al.*, 1983; Thürauf *et al.*, 1983).

It was shown that the chemical nature of negatively charged domains located on the surface of the plasmalemma proper or in various transport-related structures vary in various types of fenestrated endothelia. This variety probably reflects the functional differences of the endothelium in different vascular beds (Simionescu *et al.*, 1981, 1985; Pino, 1986a).

The chemical nature of anionic sites in the components of the wall of brain capillaries, representing BBB-type of micro-blood vessels (MBVs), has not been investigated in detail. The present work is an attempt

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to use a new technical approach to this type of study, permitting the detection of anionic sites with cationic colloidal gold in the entire cross-section of the capillary wall, after embedding of tissue samples in hydrophilic resin Lowicryl K4M.

Materials and methods

Adult mice (IM or BALBc/J strains) of both sexes (six males and three females) were used in these experiments. Poly-L-lysine (PL) hydrochloride (MW 306 000) was obtained from Miles Laboratories (Naperville, Il). Chloroauric acid, trypsin type III, papain, pronase E, proteinase K, collagenase, chondroitinase ABC, hyaluronidase type IV S, heparinase, neuraminidase type X, endoglycosidase H and other chemicals were obtained from Sigma Chemical Company (St Louis, MO). Heparitinase from Flavobacterium heparinum was obtained from Seikagaku Kogyo Co Ltd, Tokyo, Japan. The hydrophilic embedding medium Lowicryl K4M was obtained from Polysciences Inc. (Warrington, PA).

Preparation of the tissue

Small pieces of cerebral cortex were taken after fixation of whole animals by perfusion with 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, supplemented with 3% polyvinylpyrrolidone (MW 40 000). Time of perfusion was approximately 15 min, followed by immersion fixation up to 2 h. After fixation, tissue samples were cut into small blocks (1–2 mm), immersed for 4 h in ice-cold 0.1 M NH_4Cl to block free aldehyde groups and embedded in Lowicryl K4M. After polymerization at low temperature under UV lamp, specimens were cut with a diamond knife on a Sorvall MT 5000 (DuPont) microtome and picked up on 75- or 100-mesh Formvar-carbon-coated nickel grids.

Preparation of cationic colloidal gold

Particles of colloidal gold with mean diameter of 14 nm were prepared according to Frens (1973). Cationic colloidal gold (CCG) was prepared according to the method of Skutelsky and Roth (1986) by complexing PL and colloidal gold, as described previously (Vorbrodt, 1987).

Digestion procedure

Ultrathin sections attached to nickel grids were placed in a moist chamber on a drop of the enzyme solution. The incubation conditions, i.e., the concentration of the enzyme, type and pH of the buffer, temperature and time of incubation are shown in Table 1. The choice of enzymes and incubation conditions were essentially based on the data presented by Pino (1986a, b) and Simionescu and colleagues (1981, 1982). The incubation was terminated by transferring the grids into 0.05 M Tris-HCl buffer-saline (TBS), pH 7.

Demonstration of anionic sites

Digested or nondigested (control) sections were transferred from TBS to 0.1 M glycine in TBS, pH 7 for 30 min, then to TBS, pH 2.1 supplemented with 0.02% polyethylene glycol MW 20 000 (TBS–PEG) for 5 min and finally were incubated at room temperature on a drop of a solution of CCG in TBS–PEG containing 0.1% bovine serum albumin. Final concentration of CCG was approximately 50 $\mu g\ ml^{-1}$ giving an absorbance of 0.70 at 525 nm wavelength. This solution was prepared immediately before incubation and was centrifuged for 10 min at $\times 400\,g$ for removal of the large aggregates of PL-gold complexes. Time of incubation at room temperature was 60 min. After labelling, the sections were washed three times (2 min each) in TBS and in distilled water and finally were counterstained with 4% aqueous uranyl acetate (3 min) and with lead acetate (Millonig, 1961) for 45 s. The sections were examined in a Philips 420 electron microscope.

Preembedding labelling of anionic sites

For comparative purposes, some fixed tissue samples were cut with a Sorvall TC-2 tissue sectioner (chopper) into 40 μm thick sections. In these sections the accessible anionic sites (essentially located on the luminal front of the ECs) were labelled with the cationic probe at room temperature for 45 min. The composition of the staining solution was the same as described above. After incubation and washing the sections were postfixed in buffered 1% OsO_4 for 1 h at 4° C, stained with 0.5% uranyl acetate (pH 5) for 1 h and finally embedded in a low viscosity Spurr (1969) resin.

Control incubation

In the control sections the anionic sites were blocked by incubation in a solution of nonlabelled PL (1 $mg\ ml^{-1}$ of TBS, pH 2.1) for 1 h at room temperature before incubation with CCG (as above).

Results

The results of our observations are summarized in Table 1.

Although the character of this study is morphological and descriptive, we decided to present the intensity of the labelling of both fronts of the EC with the arbitrary symbols described in Table 1. For this purpose, in digested and nondigested sections ten randomly chosen transversely sectioned cortical capillaries were photographed and the number of gold particles located on both luminal and abluminal surfaces of the ECs was counted. The obtained mean values (number of gold particles per 1 μm) were used for the expression of intensity of the labelling with minus or plus signs. These values should be taken as approximate, because in the Lowicryl K4M-embedded sections the outlines of the plasma membrane are frequently difficult to visualize and consequently locating the gold particles becomes difficult.

Control (undigested) sections

A typical localization of anionic sites labelled with cationic colloidal gold in normal mouse brain MBVs is shown in Fig. 1. After labelling of 'chopped' sections, only anionic sites located on the luminal surface of the capillary ECs are accessible for the cationic probe and are decorated with gold particles (Fig. 1A and C). As it

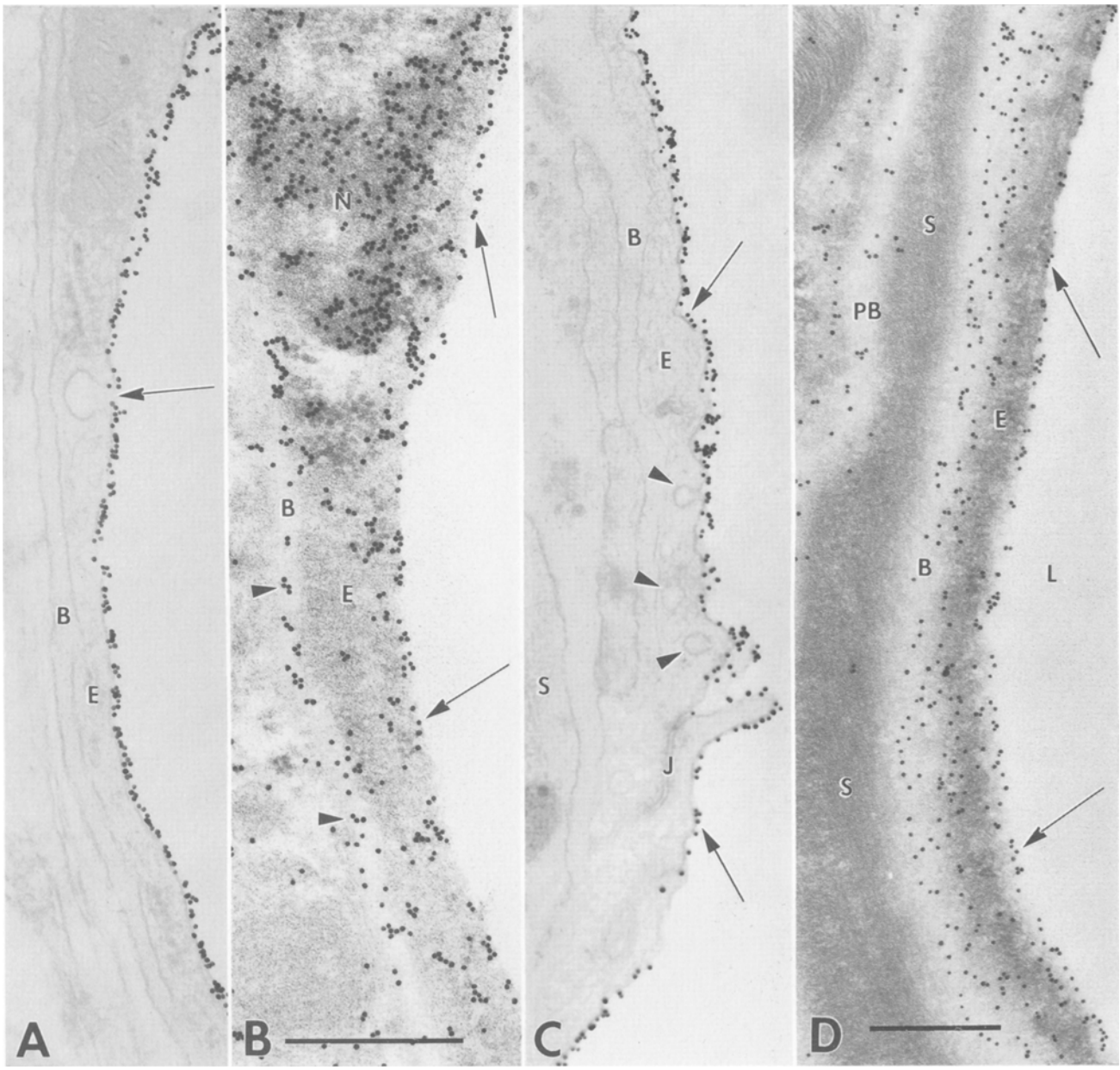
Table 1. The effect of enzymatic digestion on the intensity of labelling of anionic sites in the wall of mouse brain capillaries

Enzyme used	*Action*	*Incubation condition*	*Intensity of labelling of EC surfaces*		*Additional comments*
			Luminal	*Abluminal*	
None	—	—	++++	++	
Trypsin	Arg-Lys-	0.5 mg (5100 U) per ml DPBS 45 min, 37° C	++	+	
Papain	wide range protease	0.5 mg ml^{-1} acetate buffer pH 6.1, 60 min, 37° C	— (less than 1 particle per 5 μm)	+−	reduced labelling of the BM
Pronase E	bonds adjacent to hydrophobic amino acid residues	1 U ml^{-1} DPBS 45 min, 37° C	+++	+	
Proteinase K	hydrophobic amino acid residues	2.3 U ml^{-1} DPBS 45 min, 37° C	+	+	
Collagenase	Collagen	240 U ml^{-1} DPBS 60 min, 37° C	++++	++	loosened structure and reduced labelling of the BM
Chondroitinase ABC	Chondroitin sulphates	1 U ml^{-1} DPBS 90 min, 37°C	++++	++	reduced labelling of the BM
Hyaluronidase	Hyaluronic acid	2100 NFU ml^{-1} acetate buffer pH 5.4, 60 min, 24° C	++++	++	
Heparinase	Heparin	10 U ml^{-1} DPBS 60 min, 37° C	++++	++	
Heparitinase	Heparan sulphates	0.1 mg ml^{-1} DPBS 45 min, 42°C	++++	+	reduced labelling of the BM
Neuraminidase	Sialic acid	4 U ml^{-1} acetate buffer pH 5.4, 60 min, 37° C	+−	+	
Endoglycosidase H	Mannosyl (1–4)-β-$(GlcNac)_2$ linkage	0.1 U ml^{-1} acetate buffer pH 5.4, 60 min, 37° C	++++	++	

The intensity of the labelling was graded according to the number of gold particles per 1 μm of the EC surface: −, no labelling; +−, less than 1 gold particle; +, 1–5 particles; ++, 6–10 particles; +++, 11–15 particles; ++++, more than 16 particles per 1 μm.

can be easily noted, the main part of the luminal surface of brain capillary ECs represents the plasmalemma proper which is rather uniformly decorated with gold particles. In normal conditions, the number of transport-related structures in the capillary ECs, such as plasmalemmal pits and vesicles is negligible. These structures are visible only occasionally, and one can note that their limiting membranes are not labelled with gold particles (Fig. 1A: arrow, C: arrowheads).

In the Lowicryl-embedded sections the outlines of the plasmalemmal pits and vesicles are only rarely recognizable because of poor preservation and staining of membranous structures resulting from the omission of the osmium-containing fixative. On the contrary, in these sections the anionic sites present on the luminal and abluminal fronts of the ECs as well as in the BM and eventually on the plasma membrane of pericytes or perivascular astrocytic endfeet are accessible to the cationic probe (Fig. 1B and D). It is of interest that some negatively charged domains in the cell



The following symbols are used: B, basement membrane; E, endothelial cell; J, tight junction; L, vessel lumen; N, cell nucleus; S, smooth muscle cell. Scale bars represent 0.5 μm.

Fig. 1. Demonstration of anionic sites in nondigested sections of adult mouse brain with CCG. (A) This section was labelled with gold before embedding (preembedding technique). Only the anionic sites located on the luminal surface of the EC (plasmalemma proper) are accessible to the cationic probe. The limiting membrane of the plasmalemmal vesicle opened to the vessel lumen remains unlabelled (arrow). × 55 500. (B) Thin section of mouse brain embedded in Lowicryl K4M and labelled with CCG is shown (postembedding technique). The luminal surface of the plasmalemma proper is more intensely labelled (arrows) than the abluminal surface of the capillary EC. The basement membrane (B) is labelled with numerous gold particles (arrowheads). In the cell nucleus (N) the chromatin is strongly labelled. Gold particles scattered throughout the EC cytoplasm indicate the presence of some unrecognizable anionic domains which became accessible to the cationic probe. × 55 500. (C) A section of an arteriole labelled with CCG (preembedding technique). Only the luminal surface of the EC is irregularly labelled (arrows). In the EC cytoplasm relatively numerous vesicles are present (arrowheads). A tight junction between adjacent ECs is visible (J). × 40 500. (D) A section of an arteriole labelled with CCG (postembedding technique) is shown. The decoration of the luminal surface of the EC (arrows) is intense, although less regular than in majority of capillaries. The labelling of subendothelial (B) and peripheral (PB) laminae of the basement membrane is relatively strong, whereas the cytoplasm and plasmalemma of smooth muscle cell (S) remain almost unlabelled. × 40 500.

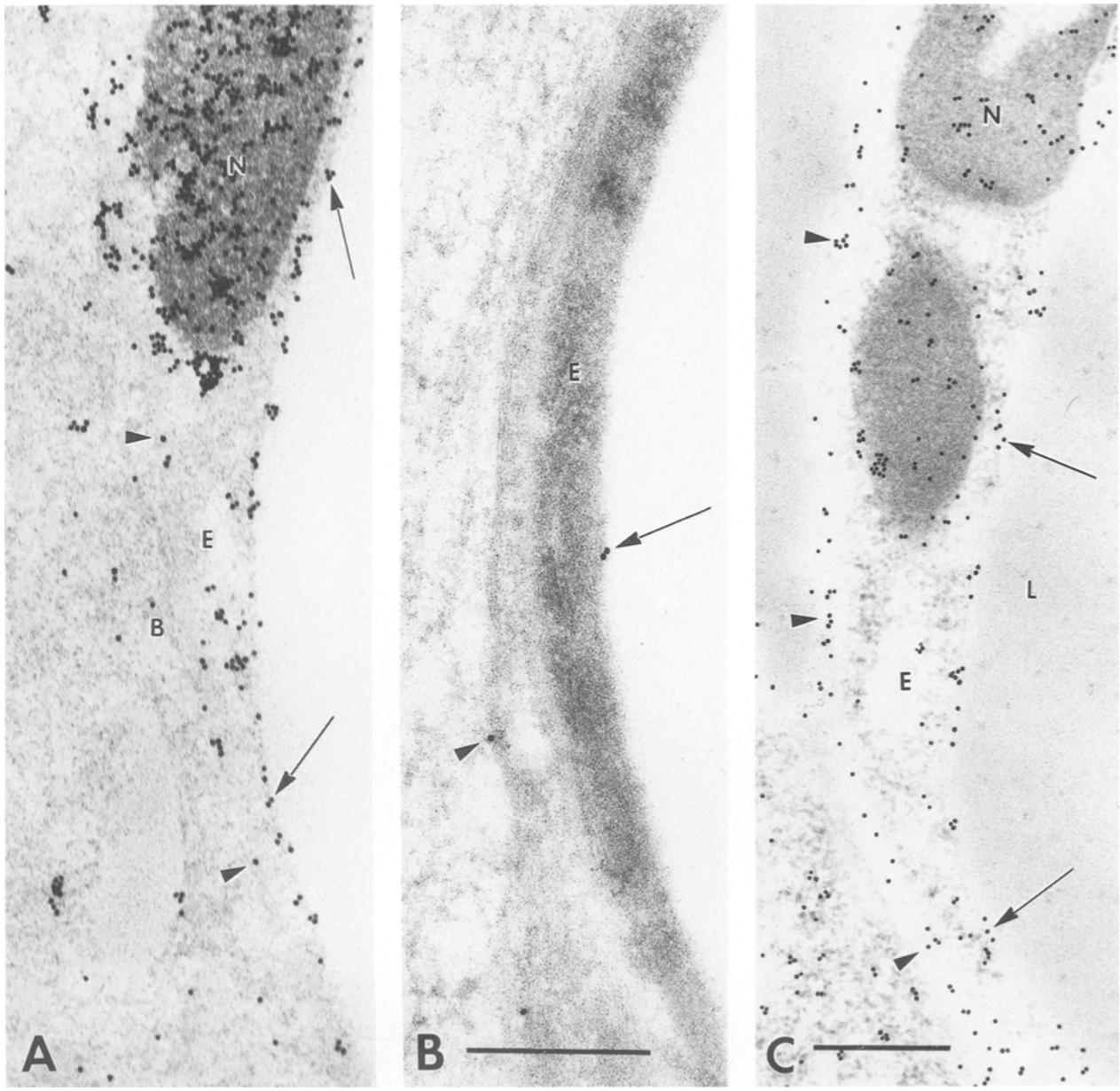


Fig. 2. The effect of some proteolytic enzymes on the distribution and intensity of the labelling of anionic sites in Lowicryl-embedded mouse brain is shown. (A) Trypsin digestion results in a reduction of the labelling with gold particles of both luminal (arrows) and abluminal (arrowheads) fronts of the EC, as well as the BM (B) of the capillary. The labelling of the nuclear material remains intense (N). × 55 500. (B) Papain digestion results in almost total removal of anionic sites except for a few solitary gold particles scattered on the luminal surface of the EC (arrow) and in the area of the BM (arrowhead). × 55 500. (C) Digestion with proteinase K causes the loosening of the structure of the EC and BM. The labelling of the luminal (arrows) and abluminal surfaces of the EC is evidently reduced and irregular. The BM remains labelled with numerous gold particles (arrowheads). The labelling of the nuclear chromatin (N) is slightly reduced. × 40 500.

nucleus (presumably in the chromatin) and inside the EC cytoplasm are also labelled. As a rule, the intensity of the labelling of the luminal plasmalemma is evidently higher than that on the abluminal front of the EC in capillaries (Fig. 1B) and in arterioles (Fig. 1D). The exact recognition of gold particles attached to the abluminal front of the EC from other gold particles located in the adjacent BM is frequently very difficult.

The labelling of the luminal surface of the plasmalemma proper of the EC in arterioles is less regular than in capillaries. It results presumably from the presence of more numerous pits and plasmalemmal vesicles deprived of the anionic sites in these vessels (Fig. 1C and D). In a majority of venules labelling of the abluminal front of the EC and BM is similar to that observed in capillaries, whereas decoration of the

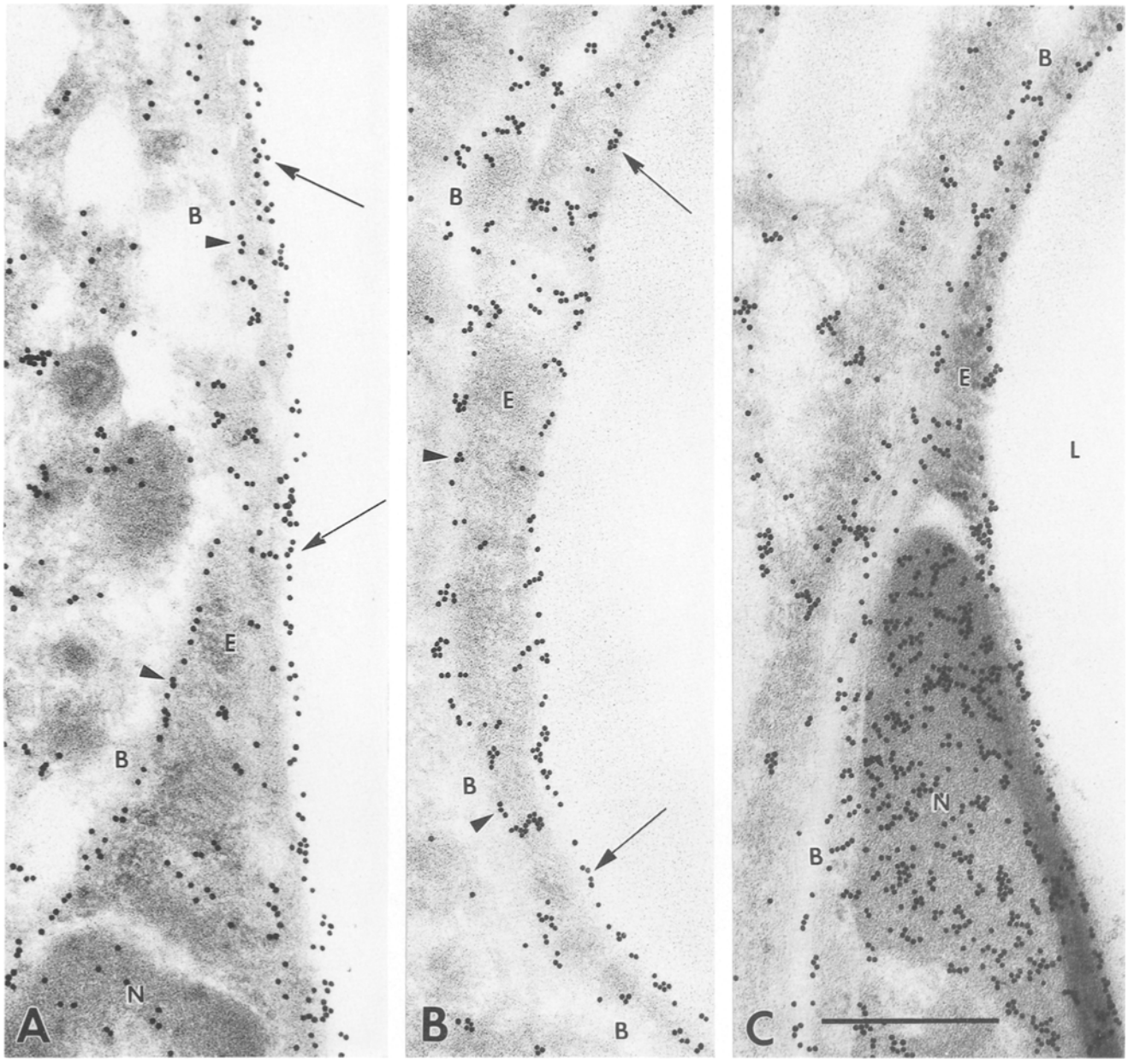


Fig. 3. (A) Collagenase digestion does not noticeably affect the decoration of the luminal (arrows) and abluminal (arrowheads) fronts of the EC, whereas the structure of the BM (B) is loosened and shows only weak and irregular labelling. × 55 500. (B) Chondroitinase ABC does not noticeably affect the labelling of the luminal (arrows) and abluminal (arrowheads) fronts of the EC. The labelling of some segments of the BM (B) is diminished. × 55 500. (C) Digestion with heparinase does not diminish the labelling of anionic sites on both fronts of the EC. The labelling of nuclear chromatin (N) and of the BM (B) is not noticeably affected by the enzyme. × 55 500.

luminal surface of the EC is less regular and less intense. These differences existing among various types of brain MBVs were described previously (Vorbrodt, 1987).

The labelling of anionic sites is completely abolished in sections exposed to unlabelled PL prior to incubation with CCG.

Digested sections

Because of poor visibility of plasmalemmal pits and vesicles in the Lowicryl-embedded tissue sections and their rare appearance in normal brain MBVs, our description of the EC surfaces is essentially related to the plasmalemma proper.

Trypsin reduces binding of gold particles to both surfaces of the EC rather moderately (approximately by half). The labelling of the BM is also diminished, although the elimination or reduction of the labelling is not uniform, i.e. in some segments of the BM it is more pronounced than in others. The labelling of

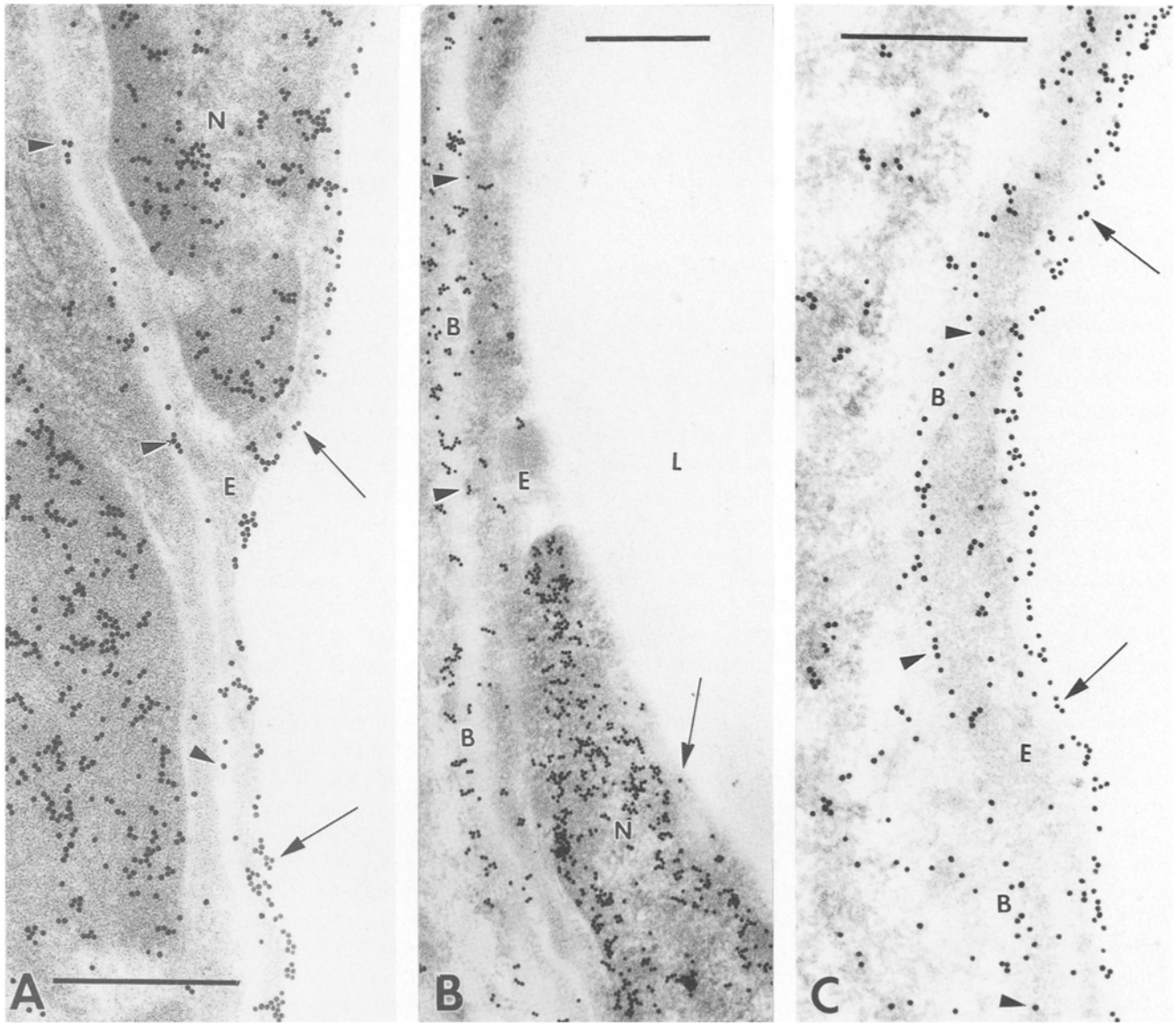


Fig. 4. (A) Digestion with heparitinase does not affect noticeably the labelling of the luminal surface of the EC of brain capillary (arrows), whereas labelling of the abluminal front of the EC and of the BM (arrowheads) is evidently reduced. Labelling of nuclear material (N) is not affected. × 55 500. (B) After digestion with neuraminidase the labelling of anionic sites on the luminal surface of the EC is almost completely abolished except few solitary gold particles (arrow). On the contrary, labelling of the abluminal front of the EC (arrowheads) and of the BM (B) is almost unchanged. This enzyme also does not affect the labelling of anionic domains in the cell nucleus (N). × 37 000. (C) Digestion with endoglycosidase H does not affect the labelling of either luminal (arrows) or abluminal (arrowheads) surfaces of the EC, although labelling of the BM (B) becomes focally diminished. × 55 500.

nuclear structures is not affected or is slightly enhanced (Fig. 2A).

Papain, which has broader specificity than trypsin completely removes anionic sites from both luminal and abluminal aspects of the ECs, as well as from the BM and adjacent structures (Fig. 2B). It also reduces binding of CCG to nuclear and intracytoplasmic negatively charged domains.

Pronase E and proteinase K also partially remove anionic sites, although the effect of the latter is more pronounced. Proteinase K causes a loosening of the structure of the EC cytoplasm and diminishes the staining capacity of all components of the vessel wall. The enzyme also changes the labelling of the luminal plasmalemma from a nearly continuous to a definitely patchy or discontinuous appearance (Fig. 2C). The labelling of abluminal front of the EC is also reduced, whereas the decoration of the BM is almost unaffected. Labelling of intranuclear structures is slightly reduced.

Collagenase does not affect the decoration with gold particles of either luminal or abluminal aspects of the EC, whereas it strongly diminishes the labelling of the BM, the contour of which becomes blurred (Fig. 3A). The labelling of the interface between the abluminal front of the EC and adjacent BM is slightly enhanced, suggesting the unmasking of anionic sites in this area.

Chondroitinase ABC does not reduce the labelling of anionic sites on either luminal or abluminal fronts of the EC, although the character of the labelling is occasionally changed from a continuous to a patchy appearance (Fig. 3B). The labelling of the BM is slightly reduced and less regular than in undigested sections. This irregularity is manifested by the appearance of BM segments with weak or no labelling.

Digestion with hyaluronidase does not noticeably change the decoration with cationic gold particles of the BM or of either front of the EC. After digestion with heparinase the labelling of both fronts of the EC as well as the BM is not noticeably affected. Labelling of nuclear chromatin is also unaffected (Fig. 3C).

Heparitinase, representing another glycosaminoglycan-degrading enzyme, does not reduce the labelling of the luminal front of the EC, although some discontinuity of the label and patchy distribution of anionic sites is seen (Fig 4A). On the contrary, the labelling of the abluminal front of the EC as well as of the BM is evidently reduced. The character of the labelling of these structures is also changed as manifested by a discontinuous and patchy appearance. This enzyme does not affect the binding of gold particles to nuclear structures.

After digestion with neuraminidase labelling of the luminal surface of the EC is almost completely abolished. Only a few single gold particles are seen, scattered on or near the luminal plasmalemma. Labelling of nuclear chromatin and intracytoplasmic structures remains unchanged (Fig. 4B). The binding of gold particles to the abluminal front of the EC is only slightly diminished. The labelling of the BM remains almost unaffected, although some changes manifested by the formation of small aggregates of gold particles or patchy appearance of the label can be noted.

Endoglycosidase H does not affect the labelling of either luminal or abluminal fronts of the EC (Fig. 4C). After treatment with this enzyme, the binding of gold particles to the BM acquires a less regular and slightly patchy character. In some MBVs the labelling of the BM is focally diminished or even abolished.

Discussion

The effects of proteolytic enzymes suggest that the negatively charged domains on the luminal plasmalemma of the EC are mainly of acidic glycoprotein nature. Digestion with trypsin is only partly effective in removal of these anionic sites. Such a partial effect of trypsin can be explained by rather narrow specificity of this protease, which cleaves specifically peptide bonds on the C terminal side of positively charged residues (arginine and lysine).

By contrast, papain has much broader specificity than trypsin and consequently is extremely effective in causing almost complete removal of CCG-binding sites. Proteinase K is a potent protease acting on carboxyl groups of aromatic or hydrophobic amino acid residues that cannot be degraded by pronase E. The latter enzyme acts on the bonds adjacent to hydrophobic amino acid residues. Thus, the stronger effect of proteinase K than of pronase E on the label of anionic sites can be explained by the different mechanisms of their actions mentioned above.

Among other enzymes, only neuraminidase shows unequivocal, dramatic effect on the luminal plasmalemma manifested by almost total removal of anionic sites. The effect of glycosaminoglycan-degrading enzymes such as chondroitinase ABC or heparitinase is insignificant and consists of a modest aggregation of anionic sites.

Thus, one can conclude that the anionic sites located on the luminal front of the plasmalemma proper of brain ECs are mainly contributed by terminal sialic acid residues of acidic glycoproteins. The remaining, small fraction of anionic domains is probably contributed by heparan sulphates.

The anionic domains located on the abluminal front of the ECs are not only less abundant but also differ in their chemical nature from those present on the luminal plasma membrane. These negatively charged domains are removed by papain, partially removed by proteinase K and neuraminidase, and strongly affected by heparitinase. Such findings indicate that they represent a proteoglycan/acid glycopeptide mixture, containing hydrophobic amino acids (sensitive to proteinase K), sialic acid residues and are rich in heparan sulphate-bearing glycosaminoglycans.

The luminal plasmalemma of the EC of brain micro-blood vessels can be compared to the luminal surface of the plasmalemma proper of continuous or fenestrated endothelia in other types of microvasculature.

The muscle capillaries of heart and diaphragm represent another type of continuous endothelium of non-BBB type. This endothelium differs from that of BBB type because it shows lower sensitivity of anionic sites to neuraminidase digestion, although the effect of heparinase and heparitinase is also negligible (Simionescu *et al.*, 1985). High sensitivity of the luminal plasma membrane of brain endothelia to neuraminidase digestion, manifested by almost total removal of the anionic sites, differentiates it from the plasmalemma proper of such fenestrated endothelia as in bone marrow sinusoids (De Bruyn *et al.*, 1978; De

Bruyn & Michelson, 1981), in mouse pancreas and intestinal mucosa (Simionescu *et al.*, 1981) and in the choriocapillaris (Pino, 1986a, b).

On the contrary, the anionic domains present on the luminal plasmalemma proper in both brain endothelium and in the fenestrated endothelium of mouse visceral organs (Simionescu *et al.*, 1981) are similar in respect to the relatively marked effect of proteolytic enzymes and negligible effect of heparinase, chondroitinase ABC and hyaluronidase. The luminal plasmalemma proper of the ECs of choriocapillaris differs from that of brain microvessels by high sensitivity to heparinase and heparitinase digestion (Pino, 1986b).

The negatively charged domains in the BM of brain micro-blood vessels are rather conspicuous and clearly demonstrated by the intense and relatively uniform labelling with gold particles. In the arterioles, the anionic sites of subendothelial lamina of the BM are more abundant than in the peripheral lamina surrounding (embracing) the smooth muscle cells (Fig. 1D). The negative domains in the BM are removed by papain, and to a lesser degree by trypsin, pronase E and proteinase K. The difference suggests that they are contributed by mixed population of protease-susceptible glycoproteins and proteoglycans, and not by glycolipids. The strong effect of collagenase indicates that the collagen – presumably collagen IV (Maxwell *et al.*, 1984) – is one of the main components of the basal lamina.

The contribution of terminal sialic acid residues to the maintenance of the negative charge of the BM seems to be negligible as indicated by the inconspicuous effect of neuraminidase digestion. The effect of hyaluronidase and heparinase is also rather dubious. On the contrary, both chondroitinase ABC and heparitinase evidently remove at least a considerable part of anionic domains in the BM. Their removal indicates that they are contributed to a substantial degree by chondroitin and heparan sulphate-rich glycosaminoglycans, similar to other types of vessels (Farquhar, 1981; Charonis *et al.*, 1983).

The effect of endoglycosidase H, although inconspicuous and limited to some segments of the BM, indicates that the glycopeptides containing oligomannosyl residues linked to *N*-acetylglucosamine (Tai *et al.*, 1975) contribute only to a small degree to the maintenance of the negative charge in this structure.

These observations indicate also that the BM is not only a mechanically supporting structure (scaffold) of the capillary wall but presumably plays also an important role as a negatively charged screen or filter taking part in controlling the movement of various negatively charged solutes between blood and brain parenchyma. Such a suggestion has already been expressed by several authors in relation to other vascular beds (Farquhar, 1981; Simionescu *et al.*, 1982; Charonis & Wissig, 1983; Dermietzel *et al.*, 1983; Hopwood *et al.*, 1983).

It is of interest that the BM of brain microvessels, in contradistinction to non-BBB microvasculature, does not show the quasiperiodic staining of anionic sites with ruthenium red. According to Schmidley (1987) and Schmidley and Wissig (1986) it could be due to a relative paucity of anionic sites or may be the result of the compact architecture of this peculiar BM which impairs the penetration of ruthenium red. The authors express the interesting notion that the BM of the BBB, like the endothelium, is structurally – and perhaps functionally – unique. In our study, the periodic arrangement of the anionic sites in the BM was not observed, although the problem of penetration was evaded by the use of ultrathin sections where anionic sites became accessible to the cationic probe.

The above described special features of anionic domains of brain endothelia, differing from those in other vascular beds, are probably a reflection of their special barrier function. The exact role of these chemical domains, located on both fronts of the brain endothelium as well as in the BM, in the function of the BBB remains to be established.

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