Published March 1, 1984

Cultured Endothelial Cell Monolayers that Restrict the Transendothelial Passage of Macromolecules and Electrical Current

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ABSTRACT Bovine microvascular endothelial cells (BMECs) proliferated to confluence on the stromal surface of human amniotic membrane that had been denuded of its natural epithelium. The resulting cultures had the following characteristics: (*a*) The endothelial cells formed a thin, continuous monolayer and, like their in vivo counterparts, contained basal adhesion plaques and large numbers of cytoplasmic vesicles and 10-nm filaments. In addition, the endothelial cells elaborated a basement membrane-like structure. (*b*) The borders of the BMECs reacted with $AgNO_3$ to produce the "flagstone" pattern typical of endothelium stained with this reagent in vivo. (*c*) More than 90% of the zones of contact between endothelial cells examined 8 d after plating prevented passage of a macromolecular probe (wheat germ agglutinin conjugated to horseradish peroxidase) across the BMEC monolayer. (*d*) 8 d-old cultures displayed a transendothelial electrical resistance that averaged 69 ± 28 $\Omega \cdot cm^2$. Monolayers of BMECs maintained on amnion thus resemble in vivo endothelium in several respects and should provide a useful and relevant model for the in vitro study of various phenomena that occur at the microvascular wall.

The microvascular endothelium plays an important role in regulating the exchange of fluid, macromolecules, and cells between the blood and the extravascular tissue. Studies of the mechanisms that underlie these exchanges have been limited by the lack of a simple and relevant in vitro model of the microvessel wall. The basic requirements for such a model are a suitable strain of endothelial cells and a substrate on which a monolayer of the cells can be maintained in a well-differentiated state. For these monolayers to be useful in examining the transendothelial movement of materials and cells, it is important that they possess permeability characteristics that are similar to those of endothelium in vivo. That is, the endothelial cells must form intercellular junctions that exclude appropriate macromolecular probes and resist the passage of electrical current.

We have developed an in vitro model of a microvessel wall that consists of cloned bovine microvascular endothelial cells (BMECs)[1] cultured on connective tissue prepared from human amnion. BMECs adhere readily to this tissue and proliferate to form confluent monolayers, a result that also has been reported by Foltz et al. (1). We report here that the morphology of BMECs cultured on amniotic tissue, as assessed by light and electron microscopy, is similar to that of endothelial cells in vivo. More than 90% of the zones of contact between these BMECs were impermeable to a macromolecular tracer, wheat germ agglutinin conjugated to horseradish peroxidase. Moreover, monolayers of BMECs maintained on amniotic tissue developed a transendothelial electrical resistance. Our observations indicate that BMEC-amnion cultures closely resemble in vivo microvessel walls and thus should prove useful for examining a variety of questions regarding the passage of fluid, macromolecules, and cells across endothelial barriers.

[1] *Abbreviations used in this paper:* BMECs, bovine microvascular endothelial cells; HIDCS, heat-inactivated donor calf serum; αMEM, minimal essential medium, alpha modification; WGA-HRP, wheat germ agglutinin coupled to horseradish peroxidase.

MATERIALS AND METHODS

Isolation of Cells: Microvascular endothelial cells were isolated from bovine adrenal cortex according to Folkman et al. (2). A single presumptive endothelial cell was allowed to proliferate in tumor-conditioned medium prepared as described in reference 2 until a colony of several hundred cells was formed. A cloning ring (Bellco Glass, Inc., Vineland, NJ) was placed over the

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colony and cells were detached with 0.25% trypsin (1–300 from ICN Pharmaceuticals, Cleveland, OH) and 2 mM Na_2EDTA in PBS. The suspended cells were transferred to a 2-cm^2 tissue culture well (Costar, Cambridge, MA) and grown to confluence in tumor-conditioned medium. These cells were maintained in minimal essential medium, alpha modification (αMEM) (GIBCO Laboratories, Grand Island, NY), supplemented with 15% heat-inactivated donor calf serum (HIDCS) (Flow Laboratories, Inc., McLean, VA), penicillin (100 U/ml), and streptomycin (100 μg/ml), at 37°C in a humidifed 5% CO_2/95% air atmosphere and were routinely subcultured at a split ratio of 1:5. All Inc., Detroit, MI) in PBS before use (2).

The endothelial origin and purity of the BMEC strain were confirmed by immunofluorescent staining (3) using rabbit antiserum directed against bovine factor VIII antigen (a generous gift from Dr. Ed Kirby, Temple University School of Medicine); all cells examined clearly contained this antigen. It is likely that the endothelial cells we have isolated originated from the microvasculature, since the adrenal cortex is rich in capillaries and contains few larger vessels.

Preparation of Amniotic Tissue: Human amniotic tissue was prepared by a modification of the method of Liotta et al. (4). Placentas were obtained from normal vaginal and Cesarean deliveries at The New York Hospital, usually within 0.5 h after delivery. Under aseptic conditions, the amnion reflecta (that portion of the amnion that does not cover the placenta) was separated from the chorion by blunt dissection. The amnion was fastened to Teflon rings (16 mm I.D., 22 mm O.D., 9.5 mm high; manufactured by The Rockefeller University Instrument Shop, New York) with Viton (vinylidene fluoride-hexafluoropropylene) O-rings (C. E. Conover, Fairfield, NJ). The orientation of the rings with respect to the stromal and epithelial surfaces of the amnion is shown in Fig. 1. The Teflon rings and attached amniotic tissue were separated from the remainder of the amnion, washed extensively in PBS containing penicillin (500 U/ml) and streptomycin (200 μg/ml), and incubated with sterile 0.25 M NH_4OH for 2 h at room temperature. The epithelial layer was removed by gentle scraping with a rubber policeman, leaving behind the collagenous stroma (Fig. 1). The tissue was washed extensively with PBS to remove debris and was stored in PBS containing penicillin and streptomycin at 4°C until use.

Culture of Cells on Amniotic Tissue: Amniotic tissue was washed once with αMEM containing 15% HIDCS and antibiotics before use. BMECs were plated on the stromal surface of the amnion (Fig. 1) at a density of 8×10^4 cells/ring (4×10^4 cells/cm^2 of tissue). Cultures were maintained in αMEM + 15% HIDCS, penicillin, and streptomycin. Spent medium was removed and replaced with fresh medium every other day. All BMECs examined in these experiments were used at the ninth passage following their initial isolation.

Silver Nitrate Staining of Cultures: BMEC cultures were stained with silver nitrate by a modification of the method of Poole et al. (5). Cultures were flooded sequentially with the following reagents: 5.0% glucose for 30 s, 0.25% $AgNO_3$ for 30 s, 5.0% glucose to rinse, 1.0% NH_4Br for 30 s, 5.0% glucose to rinse, 3.0% $CoBr_2$ for 30 s, 5.0% glucose to rinse, and 5.0% formalin to fix. All steps were performed at room temperature. Cells were counterstained with Wright's stain (Sigma Chemical Co., St. Louis, MO) for 10–15 min.

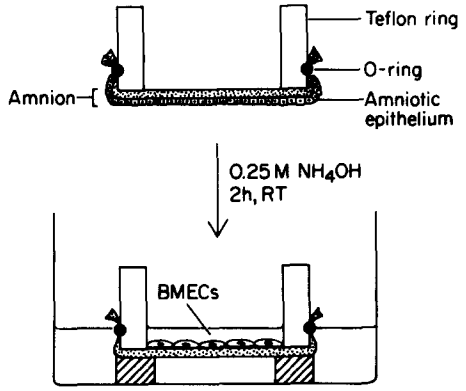

FIGURE 1 Preparation of BMEC-amnion cultures and apparatus for permeability and electrical resistance studies. Teflon rings were fastened to the stromal aspect of human amniotic membrane. Amniotic epithelium was removed as described in Materials and Methods, and BMECs were plated on the stromal side of the denuded amniotic tissue. For examining permeability of the cultures to proteins and for measuring transendothelial electrical resistance, the Teflon rings holding the cultures were placed on silicone rubber supports glued to the bottom of tissue culture wells, as shown in the lower portion of the figure. *RT*, room temperature.

Electron Microscopy: Cultures were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 1 h, postfixed in 1% OsO_4 in 0.1 M sodium cacodylate, pH 7.4, for 1 h, stained en bloc with 0.25% uranyl acetate in 0.1 M sodium acetate, pH 6.3, for 0.5 h, dehydrated, and embedded. Thin sections were stained with uranyl acetate and lead citrate before examination by electron microscopy.

Permeability Studies: Conjugates of wheat germ agglutinin and horseradish peroxidase (WGA-HRP) (Miles Laboratories, Inc., Elkhart, IN) were used to assess the permeability of monolayers of BMECs on amnion to macromolecules. Analysis of the WGA-HRP preparation by electrophoresis on a 5 to 16% gradient SDS-polyacrylamide gel under reducing conditions revealed a band of protein with a molecular weight of ~40,000, which probably represents unconjugated HRP. The conjugated material was distributed over a very broad range of molecular weights, and a considerable amount did not enter the gel (not shown).

Permeability studies were performed by placing WGA-HRP above the apical surface of BMEC-amnion cultures held in the apparatus shown in Fig. 1. To construct this apparatus, supports were fashioned by cutting rings (23 mm O.D., 14.5 mm I.D., 3 mm high) from silicone rubber sheeting (Ronsil Rubber Products, Belle Mead, NJ) and fastening them to the bottom of 35-mm diam tissue culture wells (Costar) with a nontoxic silicone glue (Dow-Corning Corp., Midland, MI). Each well was filled with 4.5 ml of αMEM + 15% HIDCS. BMEC-amnion cultures, held by Teflon rings, then were lowered onto the silicone rubber supports while 0.8 ml of WGA-HRP (200 μg/ml) in αMEM + 15% HIDCS was pipetted simultaneously over the surface of the cells. Each BMEC-amnion culture thus divided the tissue culture well into two compartments: an inner compartment containing medium with WGA-HRP and an outer compartment containing medium alone (Fig. 1). The liquid levels in the two compartments were approximately equal, thereby minimizing effects of hydrostatic pressure. Following incubation at 37°C in a 5% CO_2/95% air atmosphere for varying times, BMEC-amnion cultures were lifted from the wells at the same time that the WGA-HRP solution was removed by aspiration. The specimens were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 1 h and washed twice with saline for 10 min. The fixed tissue was incubated with 3,3′-diaminobenzidine tetrahydrochloride (1.5 mg/ml) (Polysciences, Inc., Warrington, PA) and 0.02% H_2O_2 (vol/vol) in 0.1 M Imidizole, 0.05 M Tris-HCl, pH 7.0, for 0.5 h, washed once with saline for 10 min, and postfixed in 1% OsO_4 in 0.1 M sodium cacodylate, pH 7.4, for 1 h. All of these incubations were carried out at room temperature. Samples then were dehydrated and embedded in Epon according to standard procedures. Unstained thin sections were examined in a Philips 300 or JEOL 100C transmission electron microscope. A control sample was processed identically, except that WGA-HRP was omitted from the medium placed above the culture. No electron-dense HRP reaction product was seen in this specimen.

Electrical Resistance Measurements: Teflon rings holding BMEC-amnion cultures, with 2.0 cm^2 of exposed endothelium, were placed on silicone rubber supports as shown in Fig. 1. α MEM + 15% HIDCS was placed both above and below the cultures. 10 microamperes of current were passed across the cultures using Hg/HgCl electrodes connected to agar-3 M KCl bridges. Voltage changes were detected using agar-salt bridges connected to Hg/HgCl electrodes and a Keithley 600C electrometer; these bridges were placed 1.0 mm from either side of the culture. The resistance then was calculated from Ohm's Law. The symmetry of the electrodes and bridges used in these experiments was determined; the potential difference was zero.

For each group of samples, the resistance of a piece of amnion without BMECs also was measured. This background resistance was subtracted from the total resistance of each culture to determine the resistance of the BMEC monolayer itself. Background resistances ranged from 15 to 50 $\Omega \cdot cm^2$, with an average of 29 ± 14 $\Omega \cdot cm^2$. These differences in background resistance may reflect variations in the structure of the amniotic connective tissue. We have noted that pieces of connective tissue derived from a single amnion vary in thickness and in fibrillar content.

Those cultures that exhibited an electrical resistance above background were treated as follows. While the culture was still in the apparatus used for resistance measurement, the medium bathing the culture was removed and replaced with either 10 mM EDTA in PBS lacking divalent cations or 0.25% trypsin + 2 mM EDTA in the same buffer. The time required for the electrical resistance to fall to background level then was recorded.

RESULTS AND DISCUSSION

Culture of BMECs on Human Amnion

The human amniotic membrane at term is composed of three major layers (6): (*a*) a monolayer of epithelial cells which is in direct contact with the amniotic fluid; (*b*) a basal lamina that is tightly bound to the epithelium by an extensive system

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of hemidesmosomes; and (*c*) an underlying, avascular stroma that is loosely attached to the chorion. To prepare a suitable substrate for the culture of BMECs, the amnion was separated from the chorion, and the amniotic epithelium was removed by lysis in NH_4OH as described in Materials and Methods. BMECs then were plated on either side of the remaining amniotic tissue, that is, on the now exposed basal lamina or on the stromal surface that was originally adherent to the chorion. Electron microscopic observation of these cultures showed that either surface of the amnion supported the formation of a confluent monolayer of BMECs that, as discussed below, rested on a newly synthesized, discontinuous basement membrane. However, when cultivated on the basal laminar side, the BMEC monolayer and its underlying basement membrane appeared suspended 1 to 2 μm above the amniotic surface (Fig. 2). Furthermore, amniotic epithelial cell debris remained associated with the basal lamina. In an attempt to denude the basal lamina more completely, lysis of the amniotic epithelium was performed using 4% deoxycholate rather than NH_4OH. In addition, some samples of amnion were incubated with dithiothreitol (3 mg/ml in 50 mM Tris-HCl, pH 8.7) for 15 min before or after lysis of the epithelium, a procedure that has been shown to disrupt desmosomes (7). None of these treatments resulted in increased adherence of BMECs to the amniotic basal lamina. In contrast, BMECs grown on the stromal surface were in close contact with the amniotic tissue, and little debris was seen beneath the endothelial monolayer. Since the BMECs appeared more firmly attached to the stroma of the amnion than to the basal lamina, the cells were plated on the stromal side in all subsequent experiments.

Because the amnion is thin and translucent, it was possible to examine the proliferation of the BMECs on this tissue by phase microscopy. When seeded at a density of 4×10^4 cells/cm^2 of tissue, the BMECs became confluent within 3 to 4 d. At 8 d after plating, the confluent cells appeared more spread and attenuated than at 4 d. A cross-sectional view of a culture fixed at 8 d is shown in Fig. 3; the BMECs form a thin, flat, apparently continuous monolayer on the amniotic stroma.

Differences Between BMECs Grown on Amnion and on Plastic

There were two obvious differences between BMECs grown on gelatin-coated plastic dishes and those cultured on amniotic membranes. First, BMECs could be cultured on amnion for at least 22 d, whereas BMEC monolayers that were grown on gelatin-coated plastic began to detach from the substrate in sheets after only 7 to 10 d. Second, the borders of BMECs that were grown to confluence on amnion could be stained with $AgNO_3$ (Fig. 4*a*). The pattern of staining was very similar to that reported for aortic endothelium in vivo (5). No silver staining of cell borders was observed in cultures of BMECs that were maintained on gelatin-coated plastic under identical conditions (Fig. 4*b*).

Ultrastructural studies of $AgNO_3$ staining of endothelium in intact blood vessels (8) and of BMECs grown on amnion (not shown) have demonstrated that the silver granules produced by this technique are located in the intercellular spaces and subjunctional areas. However, the substances that react with $AgNO_3$ are not known. It has been proposed that chlorides that are bound to polycationic materials such as glycosaminoglycans are involved (8). Presumably, BMECs produce these materials when maintained on amnion but not when grown on plastic.

FIGURE 2 Electron micrograph of BMECs cultured on the basal laminar surface of amnion for 8 d. The endothelial cells are separated from the amniotic basal lamina by a distinct gap. The gap contains debris, which is presumably remnants of amniotic epithelium. Bar, 2 μm. × 6,500.

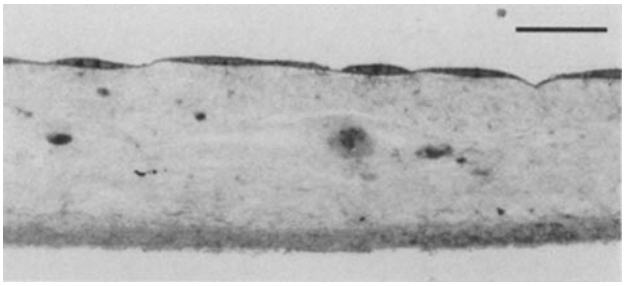

FIGURE 3 Light micrograph of a cross-section of an 8 d-old culture of BMECs grown on the stromal aspect of amnion. The darkly stained BMECs form a thin, flat, continuous monolayer on the stromal surface of the more lightly stained amniotic tissue. A relatively dense layer of the stroma underlies the basal lamina on the opposite face of the amnion; note the absence of intact amniotic epithelium on this face. Stained with 0.2% Azure A. Bar, 25 μm. × 475.

Ultrastructure of BMECs Cultured on Amnion

Examination by transmission electron microscopy of BMECs that were grown on amnion for 8 d revealed that they display many of the features that have been reported by others (2) and observed by us (not shown) to be characteristic of bovine adrenal cortical endothelium in situ. The apical and basal plasma membranes of the BMECs were smooth with few protrusions (Fig. 5). Electron-dense areas that resembled adhesion plaques were sometimes seen in the cytoplasm immediately adjacent to the basal plasma membrane (not shown). The cytoplasm of these cells also contained large numbers of 10-nm filaments (Fig. 5, *a* and *b*) and vesicles that averaged ~900 Å diam (Fig. 5*b*). Many of these vesicles opened onto the apical, basal, or lateral membranes. Often, the neck of such a vesicle was bridged by a thin diaphragm (Fig. 5*b*).

A thin, amorphous, apparently discontinuous basement membrane-like structure was seen beneath the BMEC monolayer (Fig. 5, *a* and *b*). This material was probably synthesized by the BMECs themselves, since amniotic membrane incubated in medium for 8 d in the absence of BMECs lacked this structure (not shown).

Where two cells met, their lateral membranes were almost always in close contact (Fig. 5, *a* and *c*). The configurations of the zones of intercellular contact ranged from simple abutments and overlaps of the lateral membranes to complex

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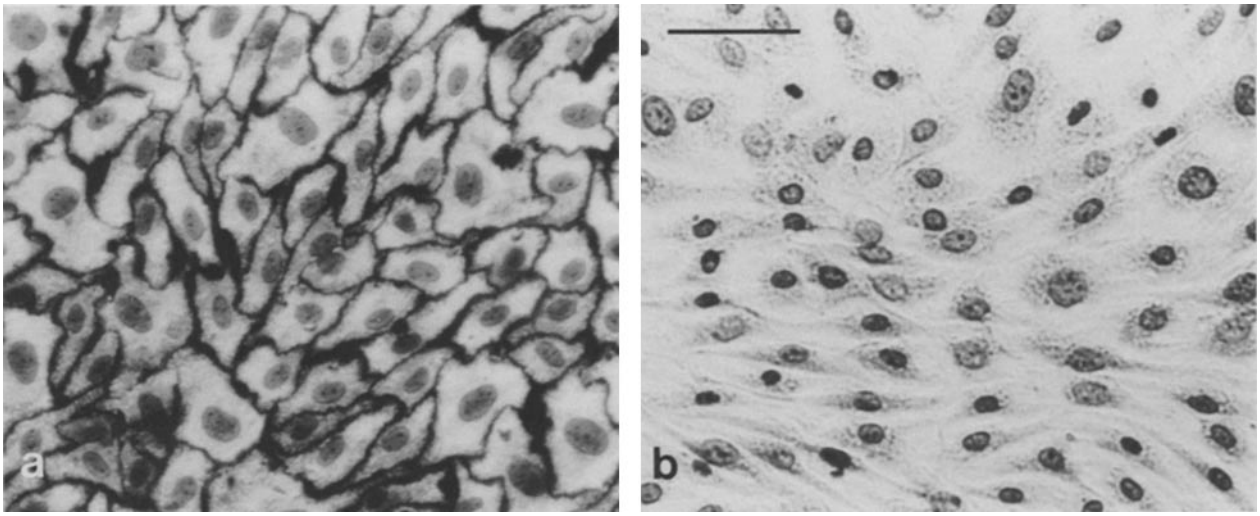

FIGURE 4 Light micrographs of BMECs grown on the stromal surface of amniotic tissue (a) or on gelatin-coated plastic (*b*) for 8 d and stained with $AgNO_3$. (*a*) Dense deposits of silver granules outline the borders of BMECs grown on amnion. (*b*) No staining is observed in cultures grown on plastic; counter-stained with Wright's Stain. Bar, 50 μm. × 350.

interdigitations of cellular processes. Structures that appeared to be junctional specializations often were seen within these zones (Fig. 5*c*). In the areas of specialization, the lateral membranes of adjacent cells approached each other closely but did not appear to fuse. Underlying these areas of close membrane apposition were concentrations of electron-dense material within the cytoplasm (Fig. 5*c*).

The ultrastructure of BMECs that had been grown on amnion was examined 4, 8, 16, and 22 d after plating. In general, the morphology of the cells changed little with time in culture. However, two exceptions were noted. First, BMECs in 4 d-old cultures were not as thin and well spread as those in older cultures. Second, gaps between cells were more frequent at 22 d than at earlier times. No multilayering of the cells was seen in any of the cultures.

Permeability of BMEC Monolayers to WGA-HRP

Initially, permeability of BMECs cultured on amnion to macromolecules was examined using horseradish peroxidase (HRP). HRP has been used to assess vessel wall permeability in vivo. Studies in the mouse have indicated that nearly all endothelial intercellular junctions in capillaries of brain (9), intestinal mucosa (10), lung (11), and diaphragm (12) exclude this probe. In contrast, 15–20% of the junctions in postcapillary venules of mouse diaphragm are permeable to HRP (13). To study the permeability of BMEC monolayers to this tracer, HRP in culture medium was added to the apical side of BMECs that had been grown on amnion for 8 d. After 5 min at 37°C, the cultures were fixed without prior washing and incubated in 3,3′-diaminobenzidine and H_2O_2 to detect peroxidase. Examination of the cultures by transmission electron microscopy revealed little or no HRP reaction product in the amniotic stroma beneath the endothelial monolayer. However, deposition of reaction product along the apical plasma membranes of the BMECs was also very light, raising the possibility that the HRP was washed away during fixation of the specimens. Therefore, HRP linked to the lectin wheat germ agglutinin (WGA-HRP) was used in subsequent permeability studies; Simionescu et al. (14) have shown that this conjugate is bound avidly by endothelial plasma membranes in vivo.

To determine the optimal time of incubation of WGA-HRP with BMEC-amnion cultures for these studies, WGA-HRP was added to the apical surface of the BMECs as described in Materials and Methods. The cultures were incubated at 37°C for 2, 5, 10, or 20 min and then were processed for electron microscopy. After 2 min of incubation, WGA-HRP reaction product was seen bound continuously along the apical plasma membranes of the BMECs and also within vesicles opening to the apical surface (Fig. 6*a*). After 10 or 20 min of incubation, vesicles filled with reaction product were seen at the lateral and, more rarely, at the basal plasma membranes. The extracellular spaces directly beneath such vesicles often contained reaction product as well, as if the vesicles had transported the WGA-HRP across the cytoplasm and discharged their contents to the exterior of the cells. To avoid confusing true junctional permeability with this appar-

FIGURE 5 Electron micrographs of BMECs grown on the stromal surface of amniotic tissue for 8 d. (a) Two BMECs resting on basement membrane-like material (arrowheads). The ends of the zone of contact between the cells are indicated by arrows; densities that may represent junctional specializations are seen within this zone (see also Fig. 5c). Large numbers of 10-nm filaments course parallel to the plane of the section in the cell to the right. (*b*) Vesicles that open onto the basal or apical plasma membranes are often separated from the extracellular space by thin diaphragms (arrows). The amorphous basement membrane-like structure that underlies this cell is indicated by arrowheads. A group of 10-nm filaments is seen in cross section just below the mitochondria in the right-hand portion of the cell. (*c*) A portion of an intercellular junction formed by interdigitation of cell processes. In some areas, the membranes of the two cells involved approach each other very closely (arrows); in these areas of close apposition the adjacent cytoplasm contains electron-dense material. Bars, 0.25 μm. × 35,000 (*a*); × 59,500 (*b*); × 92,500 (*c*).

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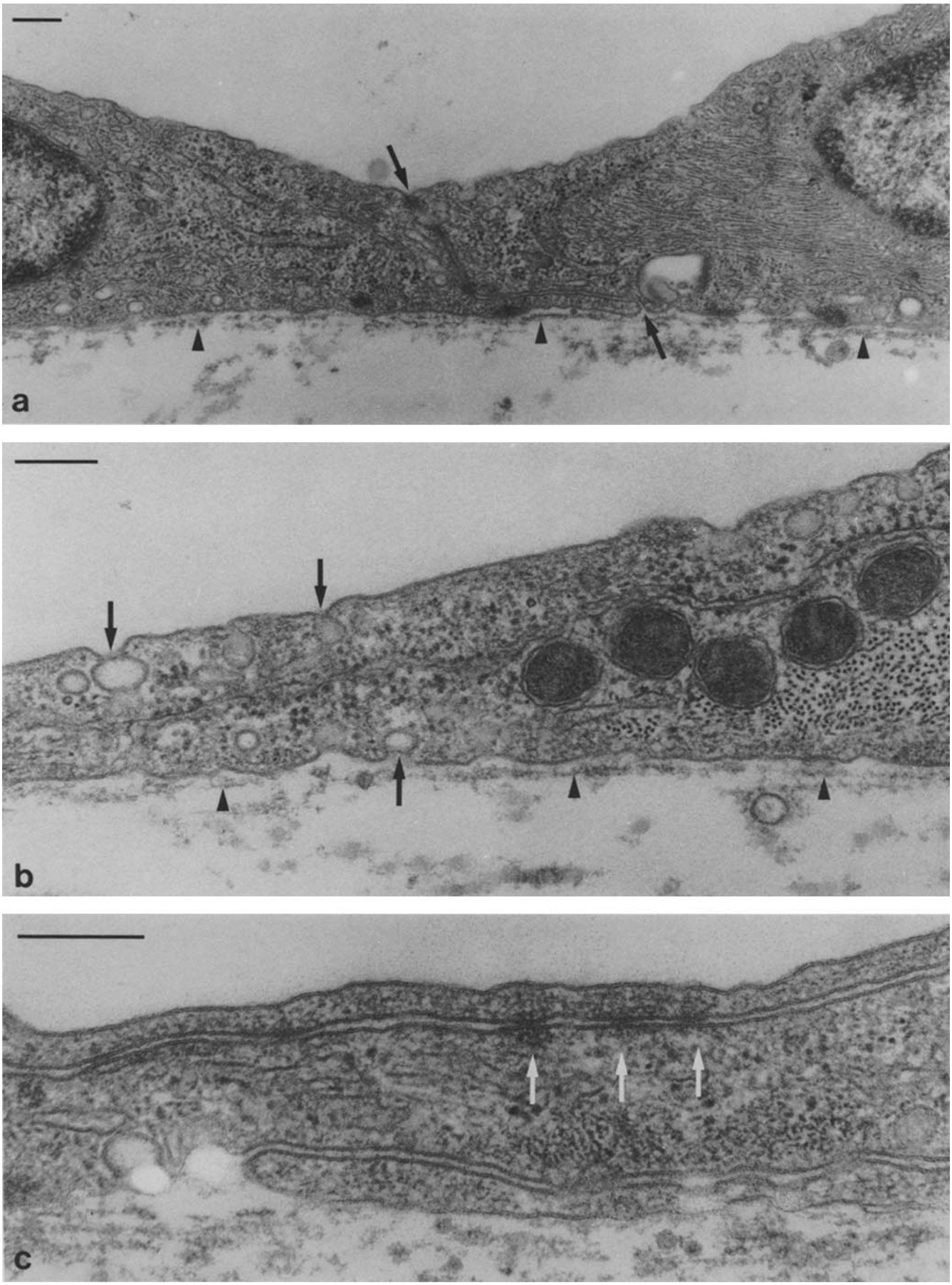

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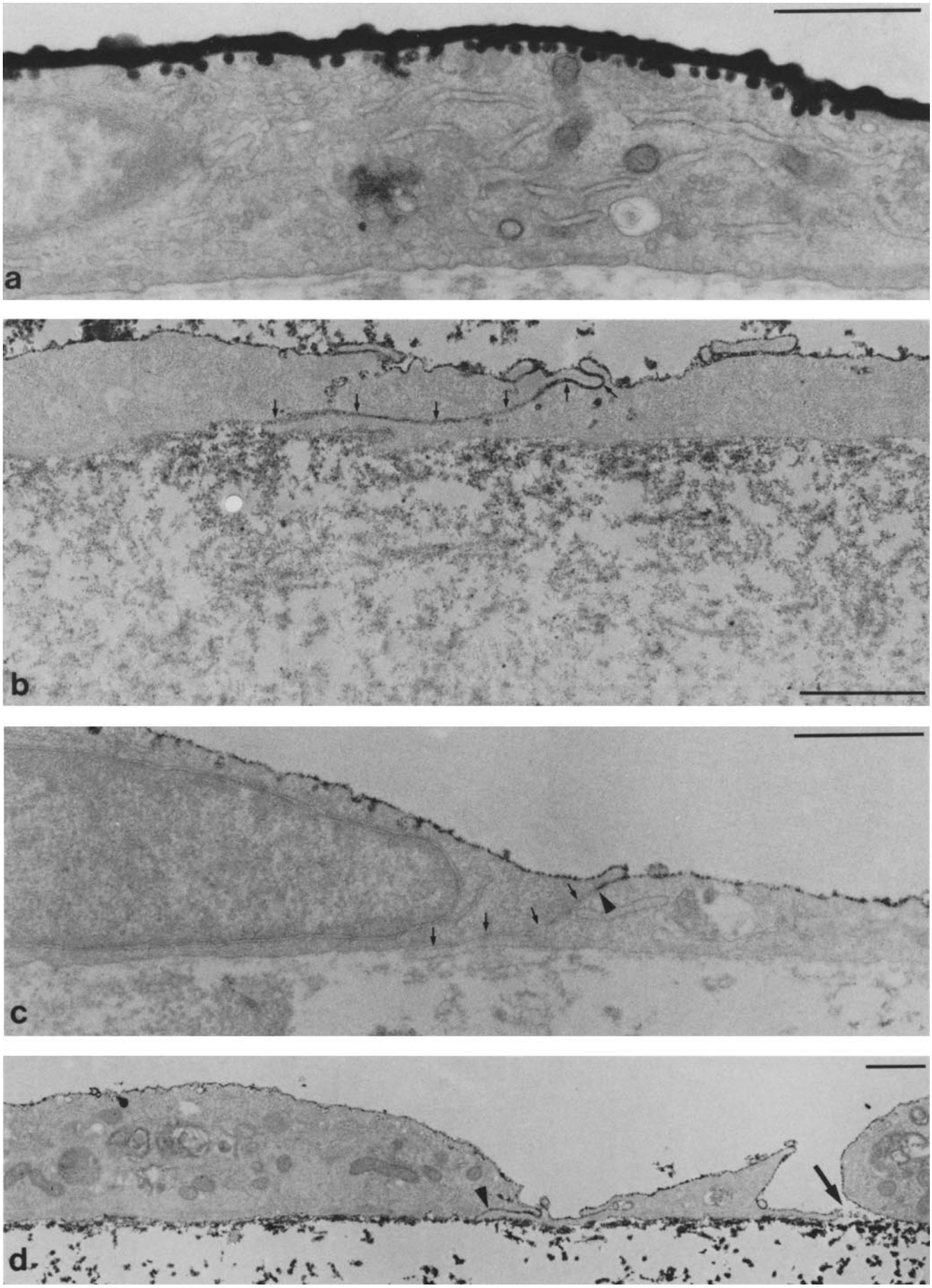

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ent transcellular transport of WGA-HRP, we decided to evaluate penetration of tracer through zones of intercellular contact after 5 min of incubation, a time when very few vesicles filled with reaction product appeared at the lateral membranes.

Permeability of BMEC intercellular spaces to WGA-HRP was quantitated using duplicate or triplicate cultures maintained on amnion for 4, 8, 16, or 22 d. For each culture, a total of approximately 100 intercellular spaces from three to five separate areas of the BMEC monolayer was examined by

TABLE I

Permeability of Intercellular Spaces in BMEC-Amnion Cultures to WGA-HRP

Age of culture	Sample no.	Area no.	No. of intercellular spaces			Total no. impermeable junctions/total no. counted
			Impermeable	Permeable	Not sure	
d						
4	1	1	19 (68)*	9	0	
		2	10 (34)	14	5	48/117 (41)‡
		3	7 (30)	12	4	
		4	12 (32)	21	4	
	2	1	35 (88)	4	1	
		2	17 (85)	1	2	
		3	18 (69)	3	5	87/106 (82)
		4	17 (85)	2	1	
8	1	1	23 (96)	1	0	
		2	35 (76)	9	2	
		3	26 (96)	1	0	131/146 (90)
		4	47 (96)	2	0	
	2	1	22 (92)	2	0	
		2	19 (95)	0	1	84/96 (88)
		3	18 (75)	3	3	
		4	25 (89)	2	1	
	3	1	16 (94)	0	1	
		2	21 (100)	0	0	
		3	11 (100)	0	0	108/111 (97)
		4	28 (93)	2	0	
		5	32 (100)	0	0	
	1	1	27 (87)	4	0	
		2	29 (85)	4	1	81/100 (81)
		3	25 (71)	8	2	
	2	1	21 (57)	10	6	
		2	19 (66)	8	2	
		3	14 (48)	13	2	78/122 (64)
		4	24 (89)	3	0	
22	1	1	20 (67)	7	3	
		2	12 (60)	8	0	
		3	13 (45)	12	4	59/102 (58)
		4	14 (61)	7	2	
	2	1	28 (80)	6	1	
		2	11 (39)	17	0	
		3	6 (30)	12	2	54/116 (47)
		4	9 (27)	23	1	

BMECs were plated on amnion (4×10^4 cells/cm^2 of tissue) and cultured for the indicated times. After addition of WGA-HRP to the apical surface of the BMECs for 5 min at 37°C (described in Materials and Methods), cultures were fixed and processed for electron microscopy. Intercellular spaces were scored as permeable to WGA-HRP if they allowed the tracer to penetrate to the stroma beneath the cells or impermeable if they restricted its passage. Each thin section scored was taken from a separate area of the culture; three to five thin sections were evaluated for each culture.

* No. of impermeable junctions expressed as a percentage of total junctions scored for each area is given in parentheses.

‡ No. of impermeable junctions expressed as a percentage of the total junctions scored for each culture is given in parentheses.

FIGURE 6 Electron micrographs of BMECs cultured on amnion for 4 (*b*) or 8 (*a*, *c*, and *d*) d, at which times medium containing WGA-HRP was added to the apical side of the cells. (*a*) Incubated with WGA-HRP for 2 min. Reaction product is bound continuously to the apical plasma membrane and fills vesicles along the apical surface. (*b–d*) Incubated with WGA-HRP for 5 min. (*b*) An intercellular space that is permeable to WGA-HRP. Reaction product fills the entire length of the intercellular cleft (marked by arrows) and is deposited in the underlying stroma. (*c*) An intercellular space that is impermeable to WGA-HRP. Reaction product penetrates to the point indicated by the arrowhead; the remainder of the intercellular cleft (arrows) is free of reaction product. (*d*) Culture that was treated with 10 mM EDTA for 10 min prior to addition of WGA-HRP. Two cells remain in close contact but the intercellular cleft is permeable to WGA-HRP (arrowhead). On the right, the cells have retracted to form a gap (arrow). Heavy deposits of reaction product are seen throughout the amniotic stroma. On the right-most cell, reaction product can be seen bound to the basal as well as the apical plasma membrane. All sections are unstained. Bars, 1.0 μm. × 27,000 (*a*); × 24,500 (*b*); × 23,000 (*c*); × 11,000 (*d*).

transmission electron microscopy and scored for permeability to WGA-HRP (Table I). Intercellular spaces were judged permeable to WGA-HRP if they allowed the tracer to penetrate across the BMEC monolayer to the collagenous stroma beneath (Fig. 6*b*). Spaces that were scored as impermeable either excluded the tracer completely or, more commonly, permitted penetration of the WGA-HRP for a short distance (Fig. 6*c*). In the latter instances, penetration of the tracer often ended at a constricted area of the intercellular cleft. Presumably, these constrictions correspond to the junctional specializations described above (Fig. 5*c*).

At 4 d, when the BMECs were just reaching confluence, there was considerable variation in permeability between duplicate cultures. In one, 82% of the intercellular spaces were impermeable to WGA-HRP, but in the other, only 41% were impermeable. The greatest impermeability to WGA-HRP was seen at 8 d; an average of 92% of the intercellular spaces excluded WGA-HRP in the three cultures evaluated at this time. At 16 d, an average of 72% of the intercellular spaces were impermeable to WGA-HRP in the two cultures examined. At 22 d, gaps frequently were observed between cells, and, as expected, many of the intercellular spaces were permeable to WGA-HRP. As seen in Table I, permeability to WGA-HRP sometimes varied from area to area within a single culture. The least variability was noted in 8 d-old cultures.

The length of a given zone of intercellular contact was not correlated with its capacity to exclude WGA-HRP. Short areas of contact that excluded tracer often were observed; conversely, some areas of contact that involved extensive overlapping of cell processes were permeable to WGA-HRP along their entire length (Fig. 6*b*).

Incubation of the BMEC-amnion cultures with 10 mM EDTA in αMEM + 15% HIDCS for 10 min at 37°C caused the cells to retract from one another and round up. WGA-HRP added to these EDTA-treated cultures was seen bound around the entire periphery of the cells and was deposited in the collagenous stroma beneath (Fig. 6*d*). It is possible that those BMEC intercellular spaces that appeared impermeable to WGA-HRP actually lacked receptors for the lectin. However, the ability of an EDTA-treated cell to bind WGA-HRP at all points along its plasma membrane makes this interpretation unlikely.

TABLE II

*Electrical Resistance of BMEC Monolayers Cultured on Amniotic Tissue**

Age of culture	Culture no.	Resistance	Mean resistance of BMEC monolayers‡
d		Ω·cm²	Ω·cm²
6	1	0	
	2	7	
	3	8	0
	4	8	
	5	12	
	Amnion alone	15	
7	1	36	
	2	45	
	3	55	12 ± 15
	4	70	
	5	85	
	Amnion alone	50	
8	1	68	
	2	70	
	3	112	69 ± 28
	4	117	
	5	127	
	Amnion alone	30	
9	1	40	
	2	50	
	3	54	46 ± 34
	4	60	
	5	126	
	Amnion alone	20	
10	1	26	
	2	30	
	3	40	7 ± 6
	4	40	
	Amnion alone	28	

* BMECs were plated on amnion (4 × 10⁴ cells/cm² of tissue) and cultured for the indicated times. Electrical resistances of the cultures and of samples of amniotic tissue incubated for the same time periods without BMECs were measured as described in Materials and Methods.

‡ For each time point, the background resistance of a sample of amniotic tissue alone was determined and subtracted from the resistance of each culture to yield the resistance due to the BMEC monolayer itself. In calculating the mean resistances, cultures that displayed resistances equal to or less than the background resistance were considered to have zero resistance. The mean of the five samples of amnion alone was 29 ± 14 Ω·cm². ± SD.

Electrical Resistance of BMEC Monolayers Grown on Amnion

To examine the permeability of BMEC monolayers on amnion to ions, transendothelial electrical resistances of 6–10 d-old BMEC-amnion cultures were measured as described in Materials and Methods (Table II). None of the five cultures examined at 6 d after plating displayed any resistance above the background resistance of amnion alone. At 7 d, three of five cultures examined exhibited electrical resistances above background. By 8 d, all five cultures measured had electrical resistances above background; furthermore, the average resistance was highest (69 ± 28 Ω·cm²) at this time. Addition of EDTA or trypsin to all cultures that displayed resistance above background resulted in a lowering of the resistance to background levels within 2 to 20 min. Both of these reagents disrupt BMEC intercellular contacts (Fig. 6*d*; data not shown for trypsin). Therefore, the resistances observed depended on the presence of intact BMEC monolayers.

Crone and his colleagues (15, 16) have performed in vivo measurements of electrical resistances of capillaries in frog brain and mesentery. The average resistance of 8 d-old BMEC-amnion cultures is approximately 19-fold less than that of frog brain capillaries (1,295 Ω·cm²; reference 15) but 37-fold greater than that of frog mesenteric capillaries (1.85 Ω·cm²; reference 16).

Madri et al. (17) have employed human amnion as a culture substrate for microvascular endothelial cells isolated from rat epididymal fat pad. The behavior of these cells on amnion differed in several respects from that of the strain of BMECs that we examined. As discussed above, the BMECs that we employed did not adhere tightly to the denuded amniotic basal lamina. In contrast, the rat cells used by Madri et al. were separated from the basal lamina by only 10 to 20 nm in some areas. In other areas, the separation was greater (100 nm). Unlike BMECs, the rat endothelial cells grew on am-

niotic basal lamina in multilayers three to four cells deep and formed complex tubular structures. When plated on the stromal aspect of the amnion, the rat cells migrated into the stroma. BMECs, on the other hand, grew only on the surface of the stroma and did not invade the amniotic tissue. The reasons for these differences in behavior between the bovine and rat microvessel endothelial cells are unclear.

Several other investigators have designed in vitro systems to examine the passage of neutrophils (18, 19), tumor cells (1, 20, 21), or fluid and macromolecules (22) across endothelium. In the majority of these studies, the permeability characteristics of the endothelial monolayers employed were not reported. McCall et al. (22), however, examined the permeability properties of cultures of porcine aortic endothelial cells grown on porous polytetrafluoroethylene membranes. Although confluent monolayers of these cells reduced the rate of fluid flow across the membranes, they did not restrict markedly the passage of albumin. In contrast, Taylor et al. (19) found that monolayers of bovine aortic endothelial cells cultured on gelatin-coated polycarbonate filters did form a barrier to the transendothelial flow of albumin. We attempted to culture BMECs on similarly prepared filters but found that the cells did not adhere well and became multilayered in areas (M. B. Furie and S. C. Silverstein, unpublished observations). Our results and those of Madri et al. (17) and Taylor et al. (19) suggest that various types of endothelial cells may behave differently from one another when grown on the same substrate. For BMECs, amniotic tissue appears to be a particularly suitable substrate, since it allows the cells to express many of the properties that distinguish endothelial cells in vivo.

We would like to thank Drs. Judah Folkman and Bruce Zetter for their generous advice regarding the culture of microvascular endothelial cells, Ms. Linda Milks for carrying out resistance measurements, Mr. Alex Fulop and Ms. Judy Adams for excellent help in preparing specimens for electron microscopy, Dr. Samuel Wright for critical reading of the manuscript, and Miss Betty Broyles for skillful typing.

This work was supported by National Cancer Institute fellowship CA 06732 and grant DRG-409-F from the Damon Runyon-Walter Winchell Cancer Fund to M. B. Furie and United States Public Health Service grant AI 08697 to S. C. Silverstein.

A preliminary report of portions of this work was presented at the Twenty-second Annual Meeting of The American Society for Cell Biology, December 1982 (23).

Received for publication 26 August 1983, and in revised form 18 November 1983.

Note Added in Proof: In a recent report, Bowman et al. (*Annu. Neurol.*, 1983, 14:396–402) have demonstrated that confluent monolayers of bovine brain microvascular endothelial cells cultured on collagen-coated nylon mesh retard the transendothelial flow of [^{14}C]sucrose.

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