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Lectin binding patterns in two cultured endothelial cell types derived from bovine corpus luteum

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Abstract Epithelial cells of different phenotypes derived from bovine corpus luteum have been studied intensively in our laboratory. In this study, specific lectin binding was examined for cells of type 1 and 3, which were defined as endothelial cells. In order to confirm differences in their glycocalyx at the light microscopic level, five biotinylated lectins were applied to postconfluent cultures which had been fixed with buffered paraformaldehyde or glutaraldehyde. Cells were not permeabilized with any detergent. Lectin binding was localized with a streptavidin-peroxidase complex which was visualized with two different techniques. The DAB technique detected peroxidase histochemically, while the immunogold technique used an anti-peroxidase gold complex together with silver amplification. Neither cell type 1 nor cell type 3 bound a particular lectin selectively, yet each cell type expressed a particular lectin binding pattern. With the DAB technique, diverse lectin binding patterns were seen, probably indicating either "outside" binding, i.e., a diffuse pattern, a lateral-cell-side pattern and a microvillus-like pattern, or "inside" binding, i.e., a diffuse pattern, and a granule-like pattern. With the immunogold technique, only "outside" binding was observed. In addition, the patterns of single cilia or of single circles were detected, the latter roughly representing 3-μm-sized binding sites for concanavalin A. When localizing them at the ultrastructural level, single circles corresponded with micron-sized discontinuities of the plasma membrane. Shedding vesicles were detected whose outer membrane was labelled with concanavalin A. Our results confirm the diversity of the two cell types under study. The "inside" lectin binding may be caused by way of transient plasma membrane openings and related to shedding of right-side out vesicles ("ectocytosis").

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Introduction

Lectins are proteins which bind selectively to simple or complex carbohydrates (Sharon and Lis 1989, 1993). Since the glycocalyx represents the exoplasmic carbohydrate portion of cell membrane proteins, it can be characterized by specific lectin labelling. Ideally, a specific lectin binds to the carbohydrate portion of a particular membrane protein so that it can be analysed further by biochemical separation techniques. The glycocalyx changes its carbohydrate components when cell function changes, for example during organogenesis or in metastasing cells (Sharon and Lis 1993). Lectins are involved in the interaction of endothelial cells with white blood cells by way of transmembranous adhesion molecules known as selectins which express a lectin-like domain on their exoplasmic face (Bevilacqua 1993; Bevilacqua and Nelson 1993). Thus, specific cell-cell adhesion occurs between the lectin-like domain and its carbohydrate ligand, both present either on endothelial cells or on blood cells. For example, lymphocytes express L-selectins, which adhere selectively to glycoproteins of endothelial cells, thereby triggering the first step of lymphocyte adhesion (Springer 1990, 1994; Ager 1994). It is likely that the glycocalyx of endothelial cells varies, in different organs, between the arterial and venous segments of the microvascular tree and with their functional activation during inflammation. Such heterogeneity in the glycocalyx may or may not be related to heterogeneity in endothelial cell morphology.

We have defined five different phenotypes of epithelial cells derived from bovine corpus luteum (Spanel-Borowski and van der Bosch 1990; Spanel-Borowski 1991). Morphological and functional studies have shown that the diverse morphology is not the product of cell transformation in culture but of constitutive differences (Fenyves et al. 1993, 1994). Since these cells behave like endothelial cells (expression of factor VIIIr antigen, endocytosis of acetylated low-density lipoprotein, and tubule formation), the five cell types were first assumed to be microvascular endothelial cells (Spanel-Borowski and

Table 1 Lectins used for receptor localization

Lectin	Abbreviation	Concentration (μg/ml)	Inhibiting sugar	Final concentration
N-Acetylgalactosamine/galactose group				
Ricinus communis I agglutinin	RCA I	5	Methyl-β-galactoside	0.4 M
Griffonia simplicifolia I agglutinin	GS I	10	Methyl-α-D-galactopyranoside	0.3 M
			N-Acetyl-D-galactosamine	0.3 M
Glucose/mannose group				
Lens culinaris haemagglutinin	LcH	10	–	
Succinyl *Canavalin ensiformis* agglutinin (concanavalin A)	Con A	50	α-Mannopyranoside	0.2 M
Sialinic acid group				
Limax flavus agglutinin	LFA	20	*N*-Acetylneuraminic acid	0.1 M

van der Bosch 1990). However, cells of type 2 are now known to represent a coculture of endothelial cells and desmin-positive cells (Fenyves et al. 1994), and cells of type 5 are diagnosed as immature granulosa cells (Spanel-Borowski et al. 1994a). Cells of types 1, 3 and 4 are still judged to be microvascular endothelial cells. Cells of type 1 have abundant cytokeratin filaments, while cells of types 3 and 4 do not (Fenyves et al. 1994). A similar distinction is also given for cultured endothelial cells isolated from bovine aorta (Spanel-Borowski et al. 1994b). The site of origin in the microvascular tree of the bovine corpus luteum is still unclear for endothelial cell types 1, 3 and 4. This problem has been addressed in the present study for endothelial cell types 1 and 3 only by looking for specific lectins which selectively bind to the cells in a manner analogous to an antigen-antibody reaction. It was assumed that such lectin specific could be used to localize the corresponding cells in histological sections.

Material and methods

Cell isolation and culture

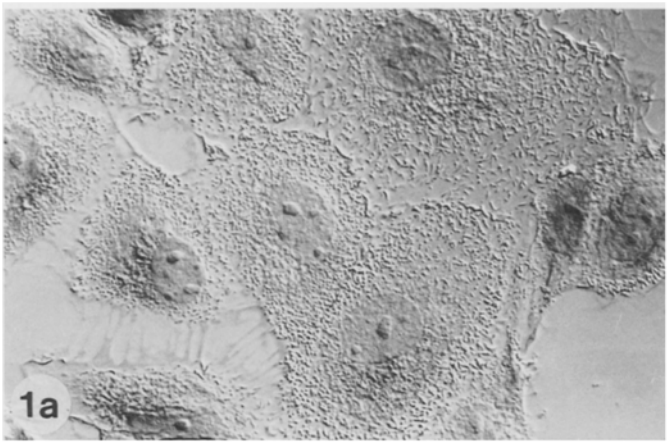
Epithelial cells were isolated from the bovine corpus luteum and cultured as described (Spanel-Borowski and van der Bosch 1990). In brief, cells obtained by mechanical disintegration were separated using a 50% Percoll (Pharmacia, Freiburg, Germany) density gradient. The fraction above the erythrocyte band contained the cells under study. They were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Basel, Switzerland) and nutrient mixture HamF12 (Gibco) mixed together 1+1 with 15 mM HEPES, 22 mM $NaHCO_3$ and 5% fetal calf serum (Myoclone, Gibco) added. Cultures were maintained in a humid atmosphere with 5% CO_2 at 37° C. Endothelial cell types 1 and 3 were mechanically purified from contaminating cells according to Spanel-Borowski and Fenyves (1994). The morphology of each cell type had been characterized in detail (Spanel-Borowski and van der Bosch 1990; Spanel-Borowski 1991; Fenyves et al. 1993; 1994). In order to study the binding of the lectins at the light or at the electron microscopic level, respectively, cells of the second or third passage were seeded onto 12-mm glass cover slips or onto Thermanox cover slips (Flow, Meckenheim, Germany), placed in the wells of a 24-well culture plate and then coated with a 1% solution of collagen type 1 (Vitrogen 100, Collagen Corp., Palo Alto, Calif., USA) before use. Postconfluent cultured cells were tested with five biotinylated lectins (E-Y Laboratories, Burgdorf, Switzerland). Lectin localization was detected by biotin-streptavidin binding. For the histochemical technique, a streptavidin-biotin horseradish peroxidase complex was used. The complex contains one streptavidin molecule which binds three biotin molecules each labelled with one peroxidase molecule; thus the primary binding site between streptavidin and the biotinylated lectin is amplified threefold. Horseradish peroxidase (HRP) was detected histochemically. For the immunogold technique, streptavidin-HRP binding was visualized with an antibody against HRP, at both light and electron microscopic level. For both techniques, each lectin was tested at least four times using cells from different isolation procedures.

Histochemical technique

For the histochemical technique, the following steps were carried out, each being separated by three 5-min washes in 0.01 M phosphate-buffered saline (PBS), pH 7.2: (1) Fixation of cells with 2% buffered paraformaldehyde at 21° C for 30 min. (2) Inactivation of endogenous peroxidase activity with 3% H_2O_2 – 10% methanol, both in PBS. The solution was freshly prepared, applied for 3 min, and the reaction stopped with a PBS buffer wash. (3) Incubation with the specific biotinylated lectin diluted with PBS according to Table 1 at 21° C for 60 min. Only the lectin GSI required 0.5 mM $CaCl_2$ for binding (Porter et al. 1990). (4) Incubation with the streptavidin-biotin-HRP complex (code no. RPN 1051; Amersham, Zurich, Switzerland), diluted 1:50, applied at 21° C for 30 min. (5) Detection of HRP with 0.05% of 3,3′-diaminobenzidine-tetrahydrochloride dihydrate solution (DAB; Aldrich, Basel, Switzerland) in PBS with 0.03% H_2O_2 in the dark for 15 min. (6) Counterstaining with hematoxylin and embedding in Kaiser's gelatine. Step 2 was optional, since lectin binding was found to be the same with or without enzyme inactivation.

Fig. 1 Lectin binding patterns for cell type 1 (**a–c**) and cell type 3 (**d–f**) using *non-permeabilized* cells and the DAB technique. Figures **a** and **d** are considered as "outside" labelling, while the others are interpreted as "inside" labelling. ×1000. Cells of type 1 show **a** the microvillus-like pattern by binding GS I, **b** the central diffuse reaction by binding Con A, or **c** the perinuclear granular reaction with RCA I. Cells of type 3 developed **d** the moderate lateral-cell-side response with RCA I (*arrow*), **e** the diffuse pattern with Con A, or **f** the diffuse granule-like reaction with LFA (*arrow*)

Fig. 2a, b Lectin binding patterns in *permeabilized* cells of type 1 as obtained with the immunogold technique. The patterns appear to indicate the "inside" labelling. ×800. **a** The perinuclear granular pattern which is detected by RCA I binding is comparable to Fig. 1c. **b** The central diffuse pattern which is seen by binding of Con A is reminiscent of Fig. 1b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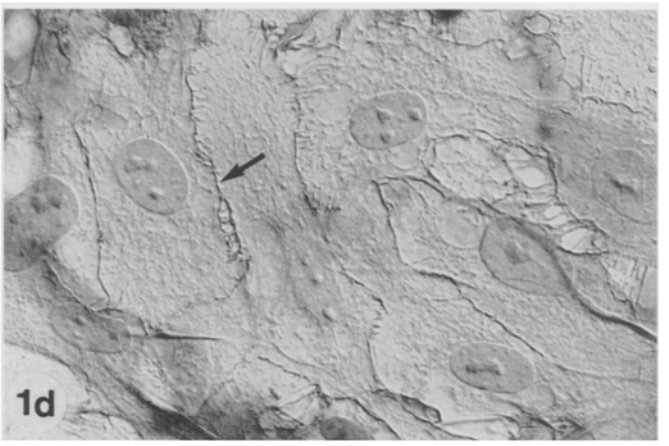
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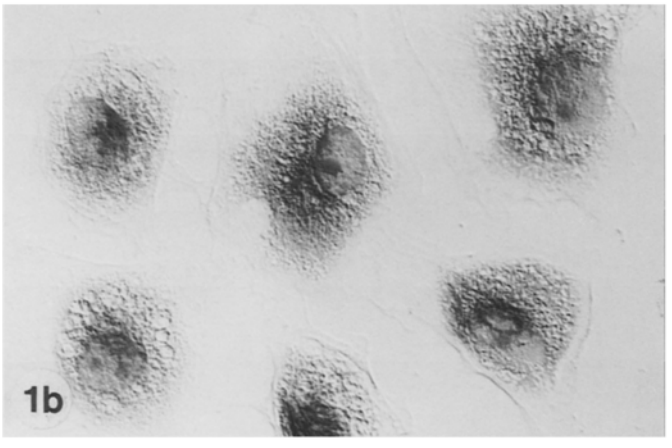
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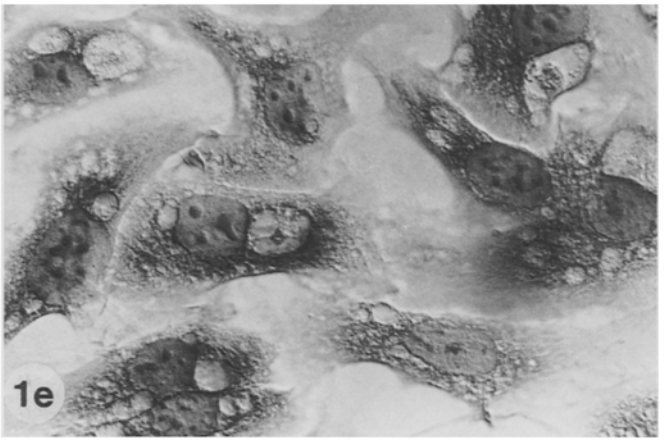
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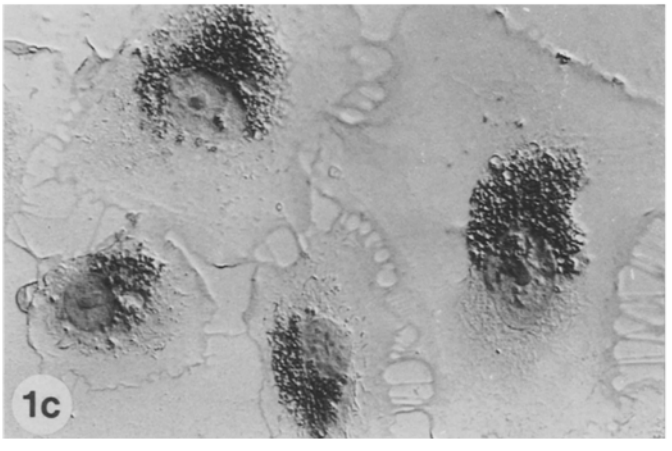
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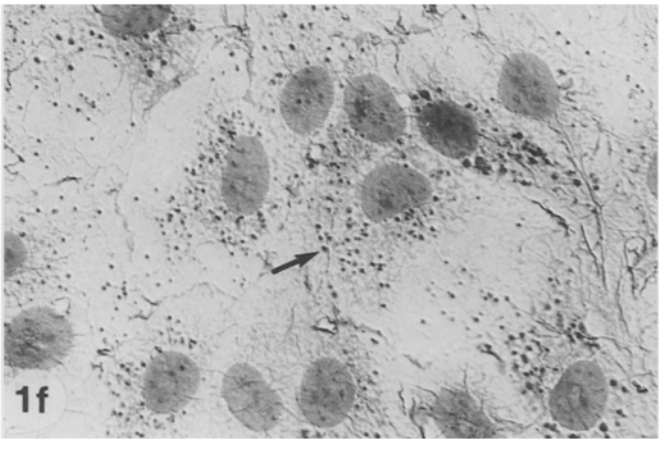
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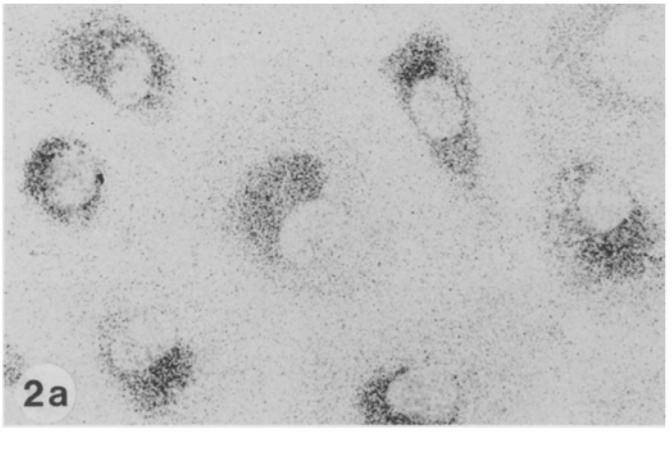
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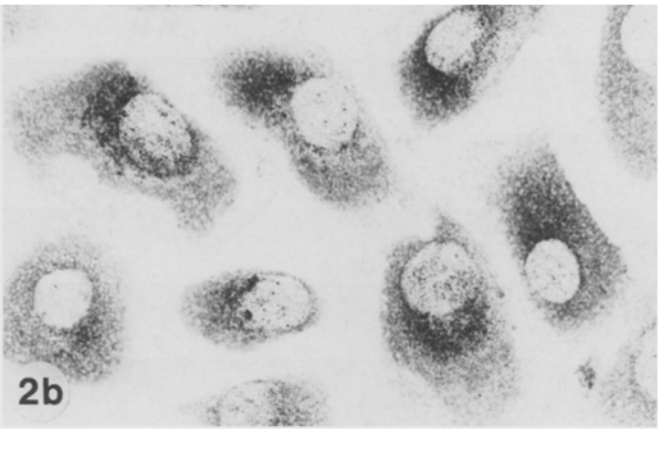
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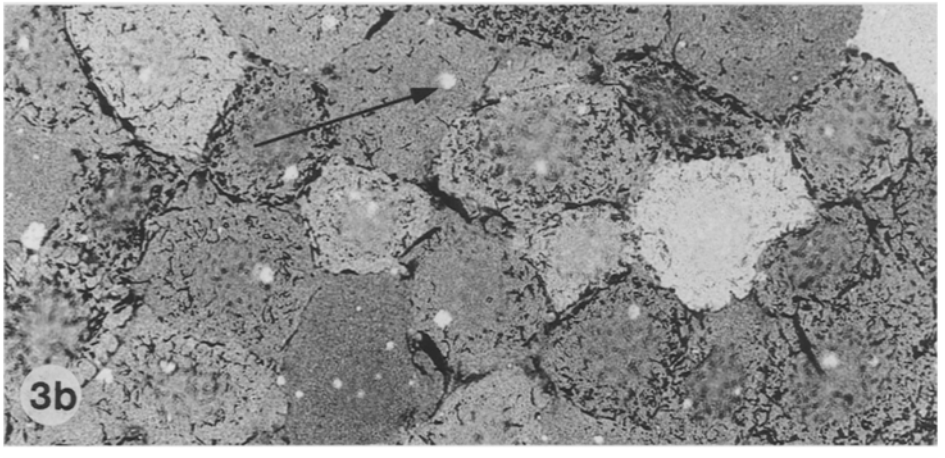
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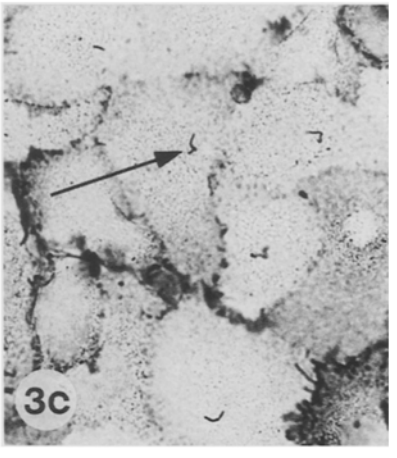
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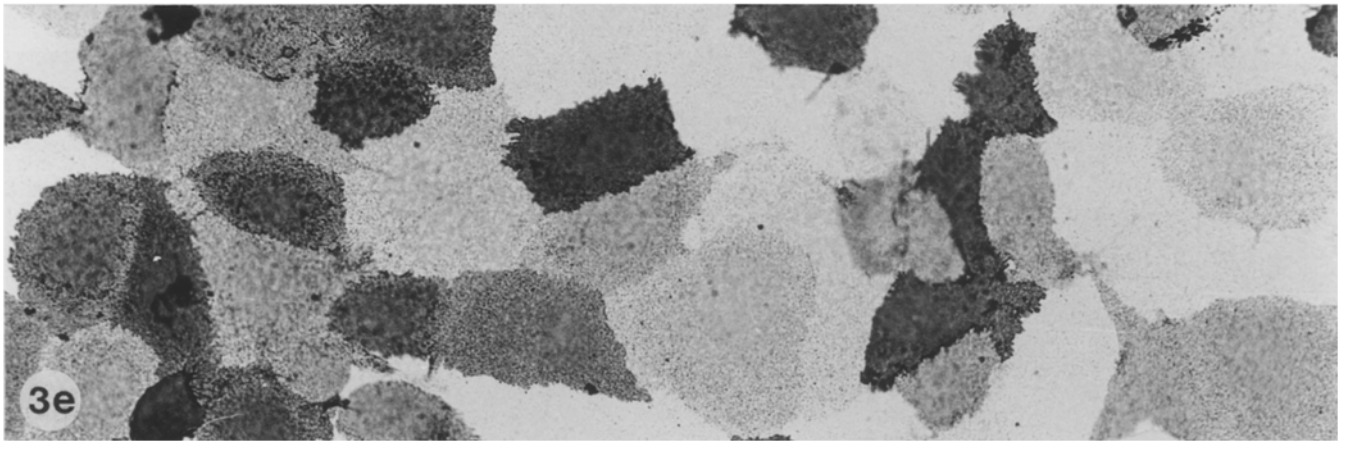
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Immunogold technique

For the *immunogold technique* (Roth et al. 1992a, b), HRP was localized with anti-HRP antibodies labelled with 4-nm gold particles. The latter were detected by the silver enhancement procedure as follows: (1) Fixation either with 2.5% glutaraldehyde in 0.01 M PBS at 21° C for 30 min, to obtain non-permeabilized cells, or, for permeabilized cells, with a glutaraldehyde solution containing 0.2% Triton X-100 (Merck). (2) Breaking of aldehyde cross links with 0.05 M sodium borohydride in PBS (10 min), following blocking of non-specific binding with 2% milk powder diluted in 0.05 M TRIS-buffered saline (TBS), pH 7.6 for 20 min; in the case of permeabilized cells, two TBS washes with 0.1% Triton X-100 followed by a simple TBS wash were carried out. (3) Incubation with the specific lectin overnight at 4° C (Table 1). (4) Incubation with streptavidin-HRP (no. 016-030-084; Jackson Immunoresearch, Geneva, Switzerland) diluted 1:400 with 1% milk powder in TBS (30 min). (5) Incubation with anti-HRP-gold complex (no. 123-185-021; Jackson Immunoresearch) diluted 1:50 (45 min). (6) Postfixation of the anti-HRP-gold complex with 1% glutaraldehyde in PBS (30 min). (7) Silver enhancement, carried out in siliconized glass moulds as indicated on the instruction sheet from Jackson Immunoresearch. Between each of the above steps a thorough buffer wash with TBS was carried out.

Controls for both techniques involved either incubation without biotinylated lectin or incubation with biotinylated lectins mixed 1+1 with their related inhibiting sugar (final sugar concentrations are indicated in Table 1).

Lectin binding at the electron microscopic level

For this study, non-permeabilized fixed cells were treated with the immunogold technique. After preembedding staining using 6- and 12-nm gold particles, and after postfixation with 1% glutaraldehyde followed by 1% OsO_4 in PBS (1 h, 4° C), monolayers were embedded in Epon 812, according to Spanel-Borowski (1991). For polymerization, the Thermanox cover slips were transferred into appropriate silicone rubber moulds. Ultrathin sections were cut perpendicular to the monolayer, mounted on Formvar-coated copper slit grids, and contrasted with uranyl acetate and lead citrate before the cells were viewed with a Philips CM 10 electron microscope.

Results

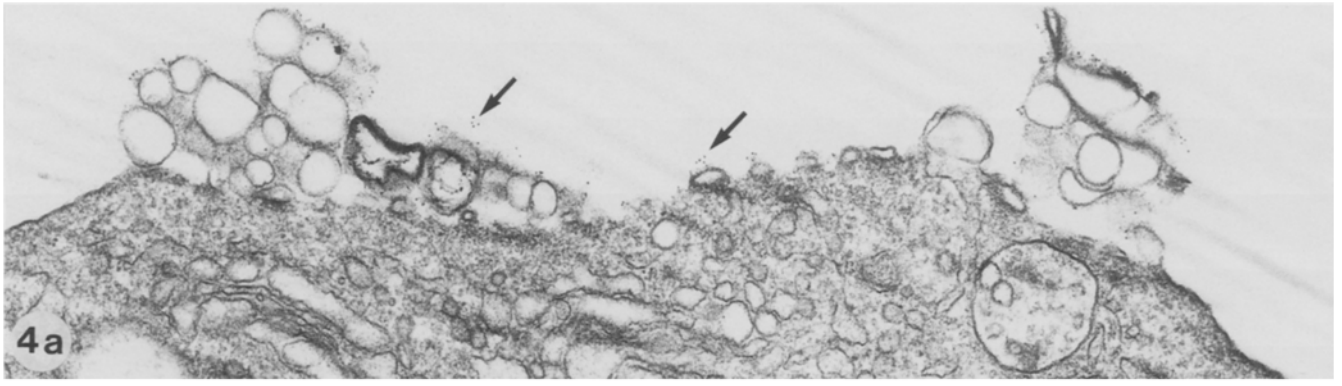
The tested lectins bound specifically to endothelial cells types 1 and 3; neither controls conducted without lectins nor controls carried out with the lectin simultaneously with its inhibiting sugar gave a positive signal. No particular lectin selectively labelled a specific cell type. Compared to the immunogold technique, the DAB technique appeared to be inferior, causing an irregular staining of the monolayer with cell areas of weak response alternating with those of moderate response. The estimated numbers of cells with moderate or weak responses were not reproducible for the same lectin when applied to monolayers derived by different isolation procedures. The irregular binding responses may have been due to lectins causing an "inside" binding (see below). In contrast, with the immunogold technique, the lectin bindings were usually distinct, homogeneous, of high contrast and of excellent resolution.

The DAB technique seemed to reveal specific lectin bindings on the outside of the membrane of non-permeabilized cells, such as the microvillus-like pattern for cells of type 1 (Fig. 1a), and the lateral-cell-side pattern with short filipodia seen for cells of type 3 (Fig. 1d). Additionally, the DAB technique appeared to reveal lectin binding patterns inside the cell, since nuclei or large intracytoplasmic vacuoles were in focus together with the adjacent positively responding cytoplasm (Fig. 1b, c, e, f). The "inside" lectin binding by way of a diffuse pattern was uniformly developed in monolayers of cell types 1 (Fig. 1b), while areas with low or absent lectin binding were obvious in cells of type 3. The latter finding was termed monolayer heterogeneity. The diffuse patterns of cell types 1 and 3 might still represent "outside" or "inside" binding to lectin receptors. A granule-like pattern developed in the perinuclear region of cells of type 1 (Fig. 1c). Granule-like bodies were diffusely distributed in cells of type 3 (Fig. 1f).

For the immunogold technique, the observed patterns seemed to indicate "outside" lectin labelling for *non-permeabilized* cells. Three patterns were related to those described for the DAB technique: the microvillus-like pattern for cells of type 1 (Fig. 3b) and the diffuse pattern for cells of types 1 and 3 (Fig. 3b, e). The diffuse "outside" pattern was evenly expressed all over the apical side of the cell. The diffuse pattern appeared strikingly after binding of *Limax flavus* agglutinin (LFA) in cultures of cell type 3 and was heterogeneous, with strongly responsive cells adjacent to non-responsive ones (Fig. 3e). Two additional patterns, either of single circles or of single cilia, were displayed. One to three complete or incomplete circles were regularly detected on the apical sides of cells types 1 (Fig. 3a). The lectin concanavalin A (Con A) selectively bound to the "circles". The average circle diameter was 2.6 μm for cells of type 1 and smaller for cell types 3 (Fig. 3a, d). The frequency of single circles changed with the cell type: each type 1 cell showed such structures, whilst few type 3 cells expressed single circles. The patterns of single cilia were selectively detected with *Griffonia simplicifolia* I agglutinin (GS I) and *Lens culinaris haemagglutinin* (LcH) in cells of type 1 (Fig. 3c).

The assumption that "outside" and "inside" labelling occurred was supported by the findings of the immunogold technique carried out with *permeabilized* cells. Permeabilized cells of type 1 demonstrated the perinuclear granule-like pattern, and the diffuse pattern was distinct when the plane of the nucleus was in focus (Fig. 2a, b). The "outside" lectin binding patterns of microvil-

Fig. 3 Lectin binding patterns in cell type 1 (**a–c**) and cell type 3 (**d, e**) using *non-permeabilized* cells and the immunogold technique. This response is judged as "outside" lectin labelling. **a–e** ×800, **f** ×2000. **a** Cells of type 1 develop patterns of single circles with Con A (*arrow*). (**b**) Cells of type 1 exhibit the microvillus-like pattern by binding of GS I. The frequency of "microvilli" and thus the intensity of lectin binding differs from cell to cell. Additionally, single circles without any response are apparent (*arrow*). **c** The pattern of single cilia is disclosed by GS I binding (*arrow*). **d** In cells of type 3, the single circles caused by Con A binding (*arrow*) are less frequent than shown in Fig. 3a. **e** The diffuse pattern and the monolayer heterogeneity by LFA binding is displayed by responsive and non-responsive cells of type 3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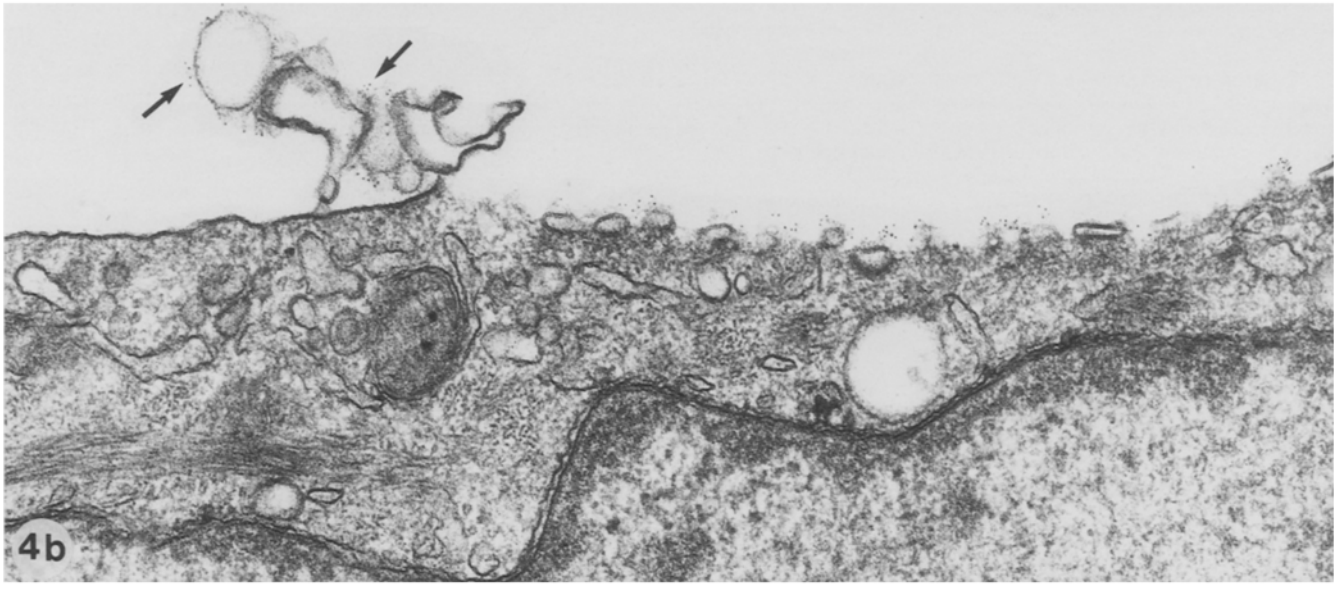
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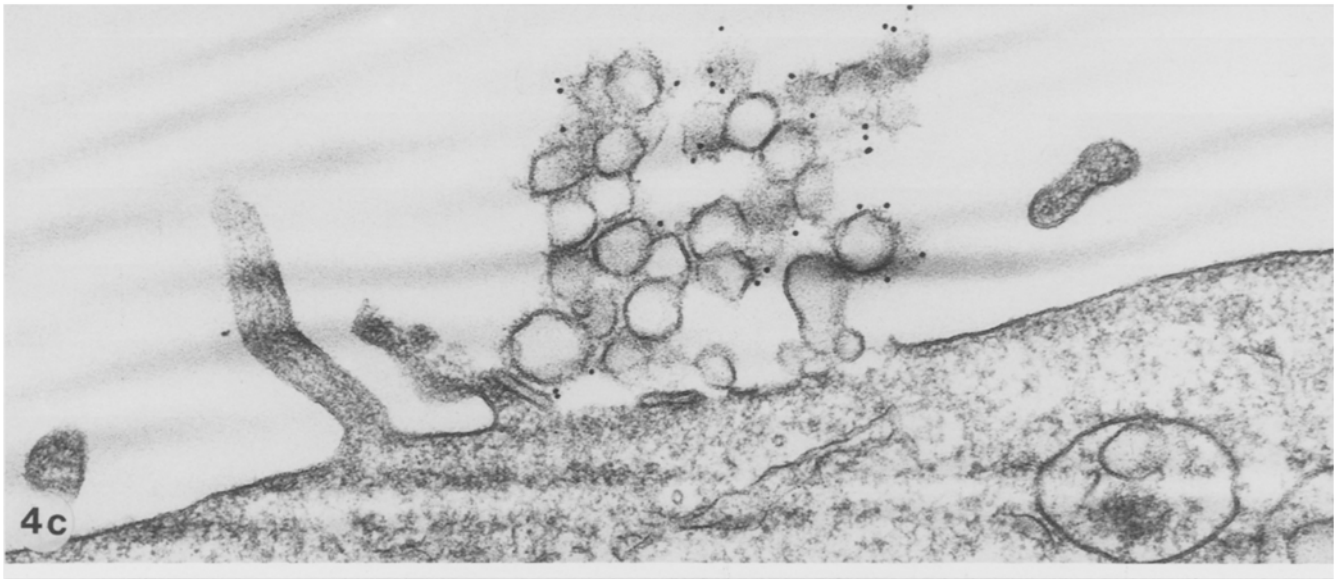
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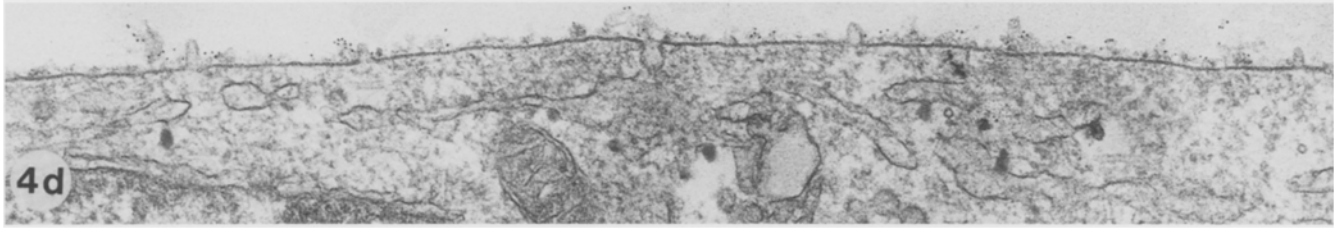
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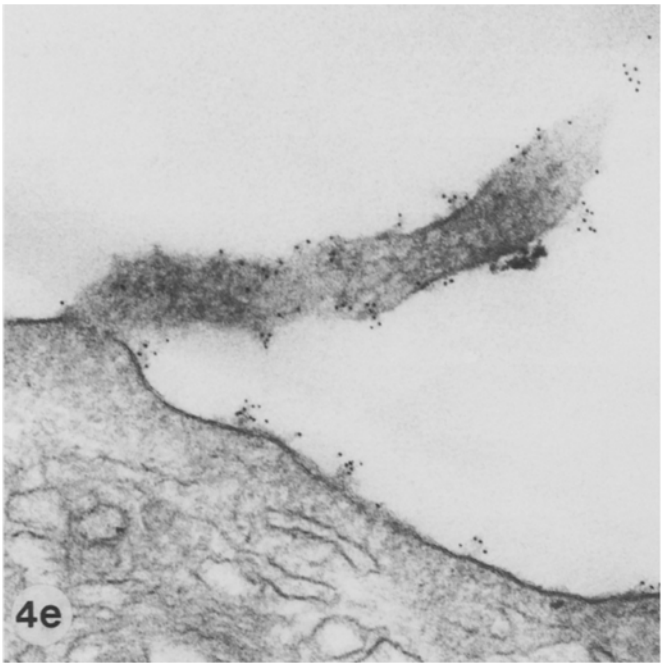

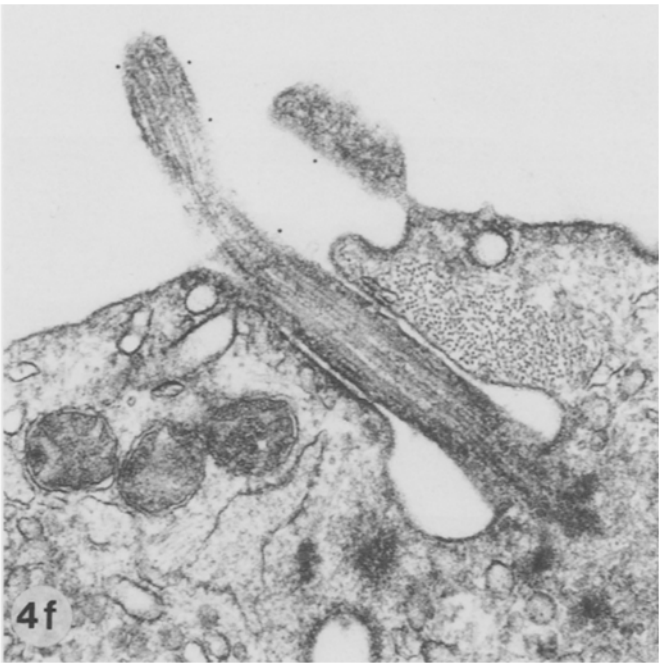

Fig. 4a–d At the ultrastructural level, the different lectin binding patterns are shown for cells of types 1 and 3. Non-permeabilized cells are treated with the immunogold technique, which indicates "outside" labelling. **a** In cell type 1, the pattern of single circles is shown by Con A binding. The plasma membrane appears to be discontinuous. Electron-lucent vesicles of different size have accumulated irregularly. The outside vesicle membrane is labelled with 6-nm gold particles (*arrows*). ×46500 **b** Comparable to **a** . The gold particles are seen distinctly around vesicles (*arrows*) of the defective plasma membrane region. No gold particles are apparent on the intact cell membrane. ×54600 **c** Comparable to **a** and **b**, yet gold particles are of 12 nm size. ×74100 **d** In cell type 3, the diffuse pattern is displayed by LFA binding; rather uniformly distributed 6-nm gold particles are exhibited on the glycocalyx. ×54600. **e** In cell type 1, the microvillus-like pattern is made apparent by GS I binding. ×81900. **f** In cell type 1, a single cilium, developing from a deeply invaginated plasma membrane, is weakly labelled by GS I. ×51300

lus-like appearance, of single cilia or of single circles were not found.

Taken together, cell-specific *lectin binding patterns* occurred in *non-permeabilized* cells which were either dependent or independent of the technique applied. A microvillus-like pattern (Figs. 1a, 3b), a diffuse pattern (Figs. 1b, e, 3b, e) and a lateral-cell-side pattern (Fig. 1d) appeared with the DAB technique and the immunogold technique. A granule-like pattern of either perinuclear or diffuse appearance occurred only with the DAB technique (Fig. 1c, f). Patterns such as single circles (Fig. 3a, d) or single cilia (Fig. 3c) were exhibited with the immunogold technique only.

The light microscopic impression of "outside" labelling in non-permeabilized cells of type 1 and 3 was confirmed for the pattern of single circles using ultrastructural localization (Fig. 4a–c). Positively responding microdomains were detected with Con A binding. They were similar in diameter to the light microscopic single circles. Interestingly, the microdomains represented a defective plasma membrane. Small electron-lucent vesicles were often aligned over the defective area, continuous with the still intact plasma membrane, and large vesicles tended to accumulate at the periphery of the defective microdomains. The outer vesicular membrane was labelled with gold particles. No Con A labelling was noticed apart from vesicles. The intact plasma membrane was labelled by one of the other five lectins, showing either the diffuse pattern (Fig. 4d), the microvillus-like pattern or the single cilium (Fig. 4e, f).

Figure 5 summarizes the different lectins yielding the particular lectin binding patterns in cells of types 1 and 3, depending on whether the DAB or the immunogold technique was used.

Discussion

None of the five tested lectins bound selectively to either of the two endothelial cell types under study. Thus, we had to give up our aim to detect particular lectins for the subsequent in situ localisation of the endothelial cell types. Their origin in the bovine corpus luteum needs to be analysed further with more appropriate tools such as specific monoclonal antibodies. The present outcome is nevertheless of interest, since the data show cellular variations in particular lectin binding sites depending on the technique applied. The immunogold technique is the method of choice for "outside" lectin binding due to its high resolution, sensitivity and reproducibility. Roth et al. (1992a, b) were the first to demonstrate the excellence of the immunogold technique when studying lectin binding on paraffin sections of human biopsy samples.

We have introduced the term "inside" and "outside" lectin binding to describe the visual impression that some lectins are able to bind to moieties within the cell

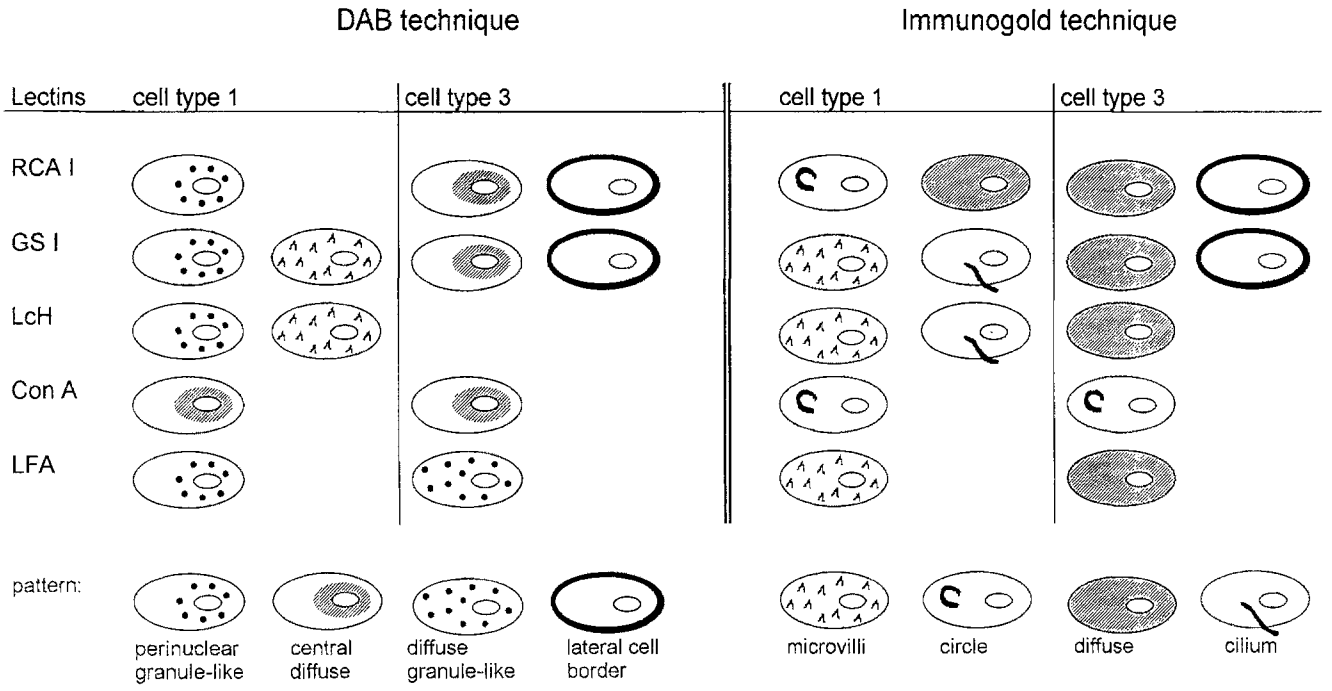

Fig. 5 Summary of the lectin binding patterns for two particular endothelial cell types as obtained by two different localization techniques using no cell membrane permeabilization

or to the glycocalyx, i.e., to the exoplasmic side of the cell membrane. When, for the DAB technique, "inside" binding is found to be diffuse, with granules in either a perinuclear or a diffuse arrangement, it appears to indicate binding to carboyhdrate components of the cytoplasm or of particular organelles. For example, the diffuse granule-like pattern (type 3 cells with LFA; Fig. 1f) or the perinuclear granule-like pattern (type 1 cells with *Ricinus communis* I agglutinin (RCA I); Fig. 1c) might indicate binding to specific granules or to the Golgi apparatus. This is supported by the observation that, in cultured endothelial cells, specific granules and the Golgi apparatus are labelled with RCA I or wheat germ agglutinin (Hormia et al. 1984). The lectin Con A is reported to vary in its labelling. It binds to the perinuclear area of fibrocytes in a diffuse way (Virtanen et al. 1980), to the endoplasmic reticulum of rearly rat spermatocytes (Martínez-Menárguez et al. 1993), and to the vesicles of endothelial cells (Hormia et al. 1984).

These reports by others on intracellular lectin receptor localization have employed cell permeabilization, which we largely omitted. Our speculation that the DAB technique possibly provides "inside" lectin binding in cells of types 1 and 3 requires a mechanistic explanation. In the literature, cultured fibrocytes, endothelial cells and other cell types are reported to undergo transient plasma membrane blebbing with subsequent shedding of right-side-out membrane vesicles (Lee et al. 1993; Tabibzadeh et al. 1994). The phenomenon, termed ectocytosis, appears to be related to micrometre-sized plasma membrane defects. These transient membrane defects are considered to be one way in which intracellular factors without signal sequences for secretion, e.g. fibroblast growth factor and interleukin-1 (McNeil and Khakee 1992; Yu and McNeil 1992; McNeil 1993), are transported outside. A transient defect in our endothelial cells could provide access for biotinylated lectins and for the detection reagents applied with the DAB technique. This would explain why "inside" lectin labelling was observed with the DAB technique only. The anti-HRP-gold complex used for the immunogold technique must remain outside because of the size of the gold particles (4 nm), explaining why only "outside" lectin binding was seen. The micrometre-sized cell membrane defects with shedding of vesicles seen here give strong support for such a mechanism. It is not clear whether the membrane defects are induced during fixation. If so, very special microdomains have to be claimed which are susceptible to minimal surface alteration. The accumulated electron-lucent vesicles with "outside" Con A labelling appear to mark such very specific microdomains of the cell membrane and the closely adjacent cytoplasm. The micrometre-sized plasma membrane defects appear to be related to the pattern of Con A-positive single circles. It follows that the frequency and size of transient plasma membrane openings would be indicated by the pattern of single circles. These occurred regularly in cell type 1. The pattern of single circles was seldom seen in cell type 3, where "circles" were the smallest. This may explain why "inside" monolayer staining was regularly developed in cells of type 1, but irregularly in type 3 cells.

Monolayer heterogeneity in lectin binding signifies that, within a particular culture, cell areas with different densities of lectin receptors are seen which appear to be either outside or inside the cell. According to other reports (Mills and Haworth 1986; Augustin-Voss et al. 1992) such monolayer heterogeneity may express the bi-

ological variability between cells, depending on their age or functional stage. In addition, the "outside" monolayer heterogeneity may indicate a specific subpopulation. This suggestion is of particular interest for cells of type 3, which are probably derived from the microvascular tree of the corpus luteum. They can be clearly differentiated into cells with or without binding of LFA. The lectin LFA has a high affinity to sialinic acid groups. These are important carbohydrate moieties of specific transmembranous molecules responsible for selectin-mediated adhesion of granulocytes to postcapillary venules by way of lectin receptor interactions (Bevilacqua 1993; Bevilacqua and Nelson 1993; Rosen 1993). The culture environment may have activated LFA-positive subtypes. The lectin binding patterns differently developed in cells of types 1 and 3 confirm what is already known for these cell types. Microvilli or single cilia have been observed in cells of type 1 at the ultrastructural level (Spanel-Borowski and van der Bosch 1990; Spanel-Borowski 1991). Thus, the observed lectin binding patterns accord with previous suggestions that the two endothelial cell types do not result from morphological plasticity in a changing culture environment, but represent truly independent cells (Fenyves et al. 1993, 1994).

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