Differentiated Microdomains on the Luminal Surface of Capillary Endothelium: Distribution of Lectin Receptors

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ABSTRACT Lectins conjugated with either peroxidase or ferritin were used to detect specific monosaccharide residues on the luminal front of the fenestrated endothelium in the capillaries of murine pancreas and intestinal mucosa. The lectins tested recognize, if accessible, the following residues: $\alpha$-N-acetylgalactosaminyl (soybean lectin), $\beta$-D-galactosyl (peanut agglutinin [PA] and Ricinus communis agglutinin-120 [RCA]), $\beta$-N-acetylglucosaminyl and sialyl residues (wheat germ agglutinin [WGA]), $\alpha$-L-fucosyl (lotus tetragonolobus lectin), and $\alpha$-D-glucosyl and $\beta$-D-mannosyl (concanavalin A [ConA]). The labeled lectins were introduced by perfusion in situ after thoroughly flushing with phosphate-buffered saline the microvascular beds under investigation. Specimens were fixed by perfusion, and subsequently processed for peroxidase detection and electron microscopy. Control experiments included perfusion with: (a) unlabeled lectin before lectin conjugate; (b) labeled lectin together with the cognate hapten sugar, and (c) horseradish peroxidase or ferritin alone. Binding sites were found to be relatively homogeneously distributed on the plasmalemma proper, except for Lotus tetragonolobus lectin and Con A, which frequently bound in patches. Plasmalemmal vesicles, transendothelial channels, and their associated diaphragms were particularly rich in residues recognized by RCA and PA ($\beta$-D-galactosyl residues) and by WGA ($\beta$-N-acetylglucosaminyl residues). Receptors for all lectins tested appeared to be absent or considerably less concentrated on fenestral diaphragms. The results reported here extend and complement previous findings on the existence of microdomains generated by the preferential distribution of chemically different anionic sites (Simionescu et al., 1981, J. Cell Biol., 9:605-613 and 614-621).

We have recently demonstrated the existence of differentiated microdomains on the luminal aspect of the endothelium of fenestrated capillaries in the pancreas and intestinal mucosa of the mouse. The most striking differences were recorded between fenestral and stomatal diaphragms. Cationic ferritin (CF), perfused as a probe for anionic sites, did not bind to the stomatal diaphragms of the plasmalemmal vesicles and transendothelial channels, while binding heavily to fenestral diaphragms. Also remarkable was the high density of CF binding sites on the plasmalemma proper, coated pits and coated vesicles, and—by contrast—the absence of similar sites on the membrane of plasmalemmal vesicles and vesicle-derived transendothelial channels (31). All the anionic sites on the luminal aspect of the endothelium were found to be removable by perfusion with proteases of broad specificity, and additional tests with neuraminidase and glycosaminoglycan-degrading enzymes indicated that these sites belonged to either glycoproteins or proteoglycans. The anionic sites of the fenestral dia-

phragms, for instance, appeared to be contributed primarily by heparan sulfate-bearing proteoglycans (32).

We have extended these studies by using lectins with well-established specificities to find out whether the distribution of the glycoconjugates they recognize is also heterogeneous and, if so, to establish to what extent it can be correlated with the nonrandom distribution of anionic sites and with the structural differentiations present on the luminal aspect of the endothelium of fenestrated capillaries.

MATERIALS AND METHODS

Animals

Fifty-two male Swiss albino mice weighing from 20 to 30 g were used in these experiments.

Reagents

Peroxidase conjugates of peanut agglutinin (PA-P), Ricinus communis agglutinin (RCA-P), and concanavalin A (ConA-P).
tin 120 (RCA-P), and wheat germ agglutinin (WGA-P), ferritin conjugated
concanavalin A (Con A-F), unlabeled lectins and cationized ferritin, pl 8.4, were
purchased from Miles Laboratories, Inc., Elkhart, IN. Peroxidase conjugates of
Lotus tetragonolobus lectin (LT-P), and soybean agglutinin (SBA-P), the corre-
sponding unlabeled lectins, the monosaccharides used as hapten inhibitors of
lectin binding, horseradish peroxidase (HRP), native ferritin and protease type
XIV (Pronase E), were obtained from Sigma Chemical Co., St. Louis, MO.
Dulbecco's phosphate-buffered saline (PBS) and minimum essential medium
(MEM) amino acids (50 times concentrate) were from Gibco Laboratories, Grand
Island Biological Co., Grand Island, NY.

The monosaccharide specificities of the lectin conjugates and the conditions
under which the latter were used are given in Table I.

Experimental Procedure

The basic experimental protocol involved the following steps: (a) Retrograde perfusion in situ through the abdominal aorta with PBS, pH 7.2, supplemented
with 5% MEM amino acids and 14 mM glucose, and gassed with 95% O₂ and 5%
CO₂. In the rest of the text, this solution will be referred to as sPBS (supplemented
PBS). The perfusate was prewarmed to 37°C, and the perfusion was carried out
for 5 min at a rate of 6-8 ml/min. As already established (31, 32), this step
insured the effective removal of the blood from the microcirculatory beds
investigated in our experiments, while maintaining unaltered the morphology
(and presumably the function) of the endothelium. (b) Perfusion of lectin-
conjugates in sPBS (at concentrations and under conditions given in Table I)
carried out intermittently for periods of 1 min separated by intervals of 5 min for
a total duration of maximum 20 min. (c) Perfusion with sPBS to remove unbound
lectin-conjugates. (d) Fixation by perfusion for 10 min with 2% glutaraldehyde
in 0.1 M Na cacodylate-HCl or 0.1 M Na arsenate-HCl buffer, pH 7.4. (e) Collection
of specimens from the jejunum and the pancreas.

Bound peroxidase-lectin conjugates were detected by the Graham-Kamovsky
procedure (9) as modified in Simionescu et al. (27). Tissue processing for electron
microscopy was carried out as described in preceding reports (27, 28).

The specificity of lectin binding was tested by competitive inhibition with the
appropriate monosaccharide after step c, or unlabeled lectin before the same step.
These reagents were perfused at concentrations given in Table I and under
conditions described in the previous section. Nonspecific binding of lectin tags
was tested by perfusion with either unconjugated horseradish peroxidase or
unconjugated ferritin.

In a separate series of experiments, the microvascular beds were fixed by
perfusion for 10 min with 2% glutaraldehyde buffered as above, followed by
quenching with 0.1 M glycine for 5 min. After the quenching solution was flushed
out, lectin conjugate was administered as in the standard protocol.

In experiments designed to test the general nature of the compounds recog-
nized by lectins, the administration of either WGA-P or PA-P was preceded by
intermittent perfusion with a solution of pronase (5 ml, 5 U/ml in a balanced salt
solution pH 7.4) for 20 min at 37°C.

In a separate set of RCA-P or WGA-P experiments, CF was introduced either
before or after step c, to find out whether bound lectins interfere or not with
subsequent CF binding. The conditions for CF perfusion were those given in
reference 31.

RESULTS

General Findings

All the lectins tested were found to bind to the luminal aspect of the capillary endothelium and in all cases the distri-
bution of the binding sites was reproducibly and consistently heterogeneous (Figs. 1 through 9). In all cases, there was no
evidence of lectin access and binding to the tissue aspect of the
plasmalemma and its associated plasmalemmal vesicles, to the
basement membrane, and to structures present in the immedi-
ate vicinity of the endothelium. We assume that the lectin
conjugates transported across the endothelium during the step
b of our experimental protocol were diluted during steps c and
d and concomitantly washed out from structures and spaces
located on the tissue side of the endothelium.

We did not detect any change in the characteristic structural
differentials on the luminal plasmalemma ascribable to the
ligands. Moreover, we did not find any evidence of ligand-
induced clustering or patching, on the same plasmalemma (by
comparing lectin distribution before and after glutaraldehyde
fixation).

Binding of conjugated lectins was prevented by previous
perfusion with unconjugated lectins, and eliminated by com-
petition with hapten monosaccharides, thereby establishing the
specificity of the binding reactions. Nonspecific binding of
lectins through their tags was found to be negligible in experi-
ments in which nonconjugated HRP or ferritin replaced the
corresponding lectin conjugate in the second step of the exper-
imental protocol. Under the conditions of these experiments,
HRP or ferritin were detected only in interiorized vesicles or
vacuoles (Fig. 10) and were absent from any structure exposed at,
or open to, the luminal aspect of the endothelium.

Plasmalemma Proper

The plasmalemma proper was heavily and quasicontinu-
ously marked by deposits of the reaction product of all the
HRP-conjugated lectins we tested (Figs. 1-9, 11). In the ma-
jority of cases, the deposits appeared to be particulate with an
occasional suggestion of regularly spaced distribution (Figs. 4
and 5). The intensity of the labeling varied from lectin to lectin:
it appeared pronounced, but uneven (size and local density of
reaction product deposits), in the case of WGA-P (Figs. 1-6), a lectin specific for N-acetylglu-
saminyl and sialyl residues, and of the galactosyl-specific
lectins: RCA-P (Fig. 7) and PA-P (Figs. 8 and 9), and more even, in the case of WGA-P (Figs. 1-6), a lectin specific for N-acetylglu-
saminyl and sialyl residues, and of the galactosyl-specific
lectins: RCA-P (Fig. 7) and PA-P (Figs. 8 and 9), and more even, in
the luminal aspect of the endothelium.

Plasmalemmal Vesicles and
Transendothelial Channels

Lectin binding sites were demonstrated with HRP-conju-
gated on the membrane of all vesicles and channels open on
the luminal front, irrespective of the presence or absence of
stomatal diaphragms. The labeling was particularly heavy and
often more intense than that of the plasmalemma proper in the
case of WGA-P (Figs. 1-6), a lectin specific for N-acetylglu-
saminyl and sialyl residues, and of the galactosyl-specific
lectins: RCA-P (Fig. 7) and PA-P (Figs. 8 and 9). By contrast,
the labeling of the plasmalemmal vesicles was nearly equal to

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**Table 1**

| Lectin Conjugates* and Conditions Used in Mapping Reactive Groups on the Luminal Surface of the Capillary Endothelium (Mouse Pancreas and Jejunal Mucosa) |
|----------|-----------------|-----------------|-----------------|-----------------|
| Lectin   | Monosaccharide  | Specificity     | Inhibitors used | Control experiments |
|----------|-----------------|-----------------|-----------------|-----------------|
| Peanut agglutinin (PA-P) | β-D-Gal | 0.0-0.4 M β-methyl-D-galactoside |
| Ricinus communis agglutination-120 (RCA-P) | β-D-Gal | 0.2 M β-methyl-D-galactoside |
| Wheat germ agglutinin (WGA-P) | β-GlCNAC, NANA | 0.3 M N-acetylglucosamine |
| Soybean agglutinin (SBA-P) | α-GalAC, β-D-Gal | 0.2 M N-acetylgalactosamine |
| Lotus tetragonolobus lectin (LT-P) | α-L-Fuc | 0.2 M l-fucose |
| Concanavalin A (Con A-F) | α-D-Glc, α-D-Man | 0.2 M o-methyl-α-D-mannopyranoside |

* All lectins were conjugated to horseradish peroxidase except for concana-
  valin A which was conjugated with ferritin. 
† For each lectin, two concentrations (200 μg/ml and 500 μg/ml) were tested
  by intermittent perfusion carried out for 20 min at 37°C (see text for further
details).
Capillary endothelium (mouse pancreas) after in situ perfusion with wheat germ agglutinin-horseradish peroxidase conjugates (WGA-P) followed by removal of unbound lectin-conjugate by perfusion with SPBS. For all figures, l marks the lumen, e, endothelium, v, plasmalemmal vesicles, bm, basement membrane, ps, pericapillary space, c, collagen fibrils. Fig. 1 shows that WGA-P reaction product labels the luminal front of plasmalemma proper (p), and marks with relatively heavy deposits plasmalemmal vesicles and their diaphragms, (v). In contrast, the labeling of fenestral diaphragms (f) is either faint or absent. No reaction product is detected in the subendothelial spaces, basement membrane (bm) or pericapillary space (ps); X 70,000. Fig. 2 shows capillary endothelium with stomatal diaphragms (arrows) more heavily labeled than the adjoining plasmalemma proper (p); j, junction; X 75,000. Fig. 3 shows an example of sharp difference between the intense decoration of the vesicle membrane (v) and particularly its stomatal diaphragm (arrow), and lack of labeling of the fenestral diaphragm (f); X 68,000. Fig. 4 shows that the coated pit or coated vesicle (cv) is less heavily labeled by WGA-P reaction product than the adjacent plasmalemma proper (p); X 68,000. Fig. 5 shows that plasmalemmal vesicles open on the blood front, and especially their stomatal diaphragms are intensely decorated by WGA-P reaction product (arrows). Note the discontinuous, apparently particulate character of the labeling of the plasmalemma proper (p). The arrows point out plasmalemmal vesicles open on the tissue front. They are unlabeled for reasons given on page 4; X 100,000. Fig. 6 shows heavy labeling of the plasmalemma proper, plasmalemmal vesicles and (especially) their stomatal diaphragms (arrows), and absence of WGA-P reaction product along the abluminal segment of an intercellular space (is) (see also Fig. 2); X 100,000.
that of the plasmalemma proper in the case of SBA-P (Fig. 11) and LT-P (micrographs not shown).

**Stomatal Diaphragms**

The diaphragms associated with vesicles and transendothelial channels were heavily labeled by all lectin conjugates. Reaction product deposits on these diaphragms were often more abundant than on the plasmalemma proper and vesicle membrane in specimens treated with conjugated lectins specific for N-acetylglucosaminy l residues, e.g., WGA-P (Figs. 3, 5 and 6) or galactosyl residues, e.g., RCA-P (Fig. 7) and PA-P (Figs. 8 and 9). Less pronounced deposits were obtained with other conjugated lectins.

**Coated Pits and Coated Vesicles**

All lectin conjugates marked the membrane of coated pits and coated vesicles (some of the latter presumably still open on the luminal front), but the labeling appeared to be noticeably less intense than that of the plasmalemma proper and of the plasmalemmal vesicles (compare Fig. 4 to Figs. 2, 3, 5, and 6).

**Fenestral Diaphragms**

The only structures on the luminal aspect of the endothelium that appeared either lightly labeled or unlabeled by the lectin conjugates tested were the fenestral diaphragms. Light labeling was generated by RCA-P (Fig. 7) and PA-P (Fig. 8), but no significant binding was detected in the case of WGA-P or SBA-P (Figs. 1 and 3, and Fig. 11, respectively).

After perfusion with pronase, WGA-P and PA-P did not bind to any of the structures labeled in controls (no protease), e.g. plasmalemma proper, plasmalemmal vesicles, transendothelial channels, and stomatal diaphragms.

WGA-P and PA-P binding did not interfere with the subsequent decoration of the plasmalemma proper and of fenestral diaphragms by cationized ferritin (Figs. 12, 13).

In previously fixed specimens, the general binding pattern of lectin conjugates was comparable to that observed when fixation followed lectin administration.

**DISCUSSION**

**Lectin Specificity**

At present, the specificity of a lectin is defined primarily by...
FIGURE 10. Capillary endothelium from a control experiment in which horseradish peroxidase alone was perfused under the same conditions as in experiments with lectin conjugates. With the exception of a large endocytic vacuole (vc), none of the endothelial structures is decorated by HRP-reaction product. × 63,000.

FIGURE 11. Labeling of the luminal aspect of the capillary endothelium given by the reaction product of a soybean agglutinin-horseradish peroxidase conjugate: the decoration of plasmalemma proper (p) is of equal intensity to that of plasmalemmal vesicles (v), and no preferential labeling of stomatal diaphragms is detected. The labeling of fenestral diaphragms (f) is either light or absent. The insets compare the labeling of the luminal aspects of the endothelium with cationized ferritin in a, and soybean agglutinin-horseradish peroxidase conjugate in b: cationized ferritin marks heavily the fenestral diaphragms and lightly the plasmalemma, whereas SA-P gives an opposite pattern of labeling; × 68,000. Insets, × 113,000.

the monosaccharide that inhibits its binding to a cognate receptor (6, 12, 13, 18–20, 25, 26). Each lectin appears to recognize a specific monosaccharide residue located either at the nonreducing end of an oligosaccharide chain or in an accessible, subterminal position (6, 12, 13). Most lectins have more than one binding site; some lectins have affinity for more than one residue (e.g. WGA) (17, 24); and, in a few cases, binding depends in part on the immediate neighbors of the specific residue in the oligosaccharide chain (13). Other factors, such as ionic and hydrophobic interactions, membrane properties, and environmental conditions, play secondary roles (13). Lectin receptors are known to be either glycoproteins, or glycolipids (6, 12, 13, 19, 22, 25, 26). Therefore, by itself, lectin binding cannot provide information beyond this level on the chemistry of cognate receptors. Moreover, in in situ experiments like the ones we have conducted, lectin binding depends on the accessibility of receptors which can be hindered by other chemical structures (6, 22, 26). The information provided by
our results is therefore limited essentially to the existence of lectin receptors with accessible specific residues in terminal or subterminal positions on the luminal surface of the vascular endothelium investigated. Results obtained after perfusion with pronase suggest that glycoproteins rather than glycolipids are the molecular species recognized by WGA and PA. On these receptors, the lectins tested are expected to recognize monosaccharide residues present in either mannosyl-rich or complex oligosaccharide chains N-glycosidically linked to asparaginyl residues of glycoproteins. In addition, some of them, especially the N-acetylgalactosaminyl-specific soybean agglutinin should bind to mucine-type chains, O-glycosidically linked to seryl or threonyl residues of glycoproteins. In the case of the latter the information available at present is too limited to allow any firm correlation (see, for instance, reference 11).

**Differentiated Microdomains**

Our results indicate that the distribution of lectin receptors is characteristically heterogeneous; it defines microdomains which appear to be related to specific structural differentiations on the luminal surface of the endothelium in the fenestrated capillaries examined.

**Plasmalemma, Plasmalemmal Vesicles, and Stomatal Diaphragms**

The plasmalemma proper has receptors for all lectins tested and the same applies for the membranes of plasmalemmal vesicles (when accessible), and transendothelial channels and, especially, for stomatal diaphragms associated with both structures. These diaphragms are particularly heavily labeled by HRP-conjugated WGA and only slightly less heavily labeled by RC and PA. The results are in sharp contrast to those reported for the distribution of anionic sites: the latter were detected on the plasmalemma proper, but were absent from the membranes and diaphragms of both plasmalemmal vesicles and transendothelial channels (31, 32). This difference in binding activities suggests that the local receptors are either mannosyl-rich-like, or mucine-like oligosaccharides, and that complex oligosaccharide chains or chains rich in sialyl residues are missing from (or present in undetectable concentrations on) the membranes and diaphragms of both plasmalemmal vesicles and transendothelial channels. Assuming that there are no problems of accessibility, this interpretation is in keeping with the finding that WGA, a lectin that recognizes N-acetylglucosaminyl residues, as well as sialyl residues, labels stomatal diaphragms, structures that are not provided with high concentrations of anionic sites (Fig. 14).

**Coated Pits**

The luminal aspect of coated pits and (when accessible) coated vesicles was also labeled by all lectins, without any detectable preference, but the labeling was generally less pronounced than that of plasmalemmal vesicles.

**Fenestral Diaphragms**

The fenestral diaphragms represent special microdomains:
they were not labeled (or only slightly labeled) by any of the lectins tested. The local absence of lectin receptors is in sharp contrast to the high local concentration of anionic sites (insets in Fig. 10) contributed by heparan sulfate or heparin (32). These findings suggest that the lectins used do not recognize, or do not have access to, the monosaccharide residues of the corresponding proteoglycans.

With these new results, the differences between stomatal and fenestral diaphragms become greater than previously shown (31). In a recent review (23) we have postulated—on the basis of their morphological similarity—that the two structures are developmentally related, a fenestral diaphragm being the single remaining stomatal diaphragm of a transendothelial channel collapsed to minimal pathlength. This postulate must be reassessed in the light of the differences already described (31) or now recorded. The two structures may represent the outcome of two entirely different morphogenetic processes, or they may derive from a common structural precursor, with subsequent modifications of surface chemistry in the case of the fenestral diaphragms.

**Microdomain Stabilization**

The existence of differentiated microdomains (e.g. plasmalemma proper vs. plasmalemmal vesicle vs. coated pits) on a continuous lipid bilayer (expected to be fluid at the temperature of the body) raises a number of questions as to the various means by which endothelial cells (or any other cells) prevent the randomization of molecular components from one microdomain to another. The probable role of stabilizing interactions within the membrane, or between microdomain components and stabilizing infrastructures has already been discussed (31). The new findings stress the importance of this question, since they demonstrate that differences among microdomains involve a wider variety of components than previously known.

At first sight, the fenestral diaphragms may appear to be a special case basically distinct from the microdomains of the plasmalemma, since their organization does not comprise a lipid bilayer. The morphology of the fenestral diaphragms indicates, however, that they are firmly anchored in the plasmalemma, and that they generate enough tension to bend the entire cell membrane, bilayer included, at a sharp angle along their anchoring perimeters. This type of anchoring may involve transmembrane proteins whose ectodomains (which are part of the structure of the diaphragm) are secured in place by interactions among themselves and/or the components (proteins?) of the central knob of the diaphragm. Like the other microdomains of the plasmalemma, the fenestral diaphragms, might be generated by transmembrane proteins except that, in their case, the domain might be stabilized by interaction with extracellular macromolecules (e.g. the proteins of the knob) rather than by interaction with an intracellular infrastructure. It remains to be seen, however, to what extent this assumption could be extended to other kinds of fenestrated endothelia, to other kinds of fenestral diaphragms, and to other cases of sharp angle bending of membranes.

**Functional Implications**

The heavy concentration of lectin receptors, most probably glycoproteins, on the membranes of plasmalemmal vesicles, transendothelial channels and associated stomatal diaphragms may impart a certain degree of selectivity to the movement of proteins from the blood plasma to the interstitial fluid. It was recently shown by Williams et al (37) that the endothelia of microvessels, isolated from rat epididymal fat pads, take up glycosylated albumin in preference to albumin. The uptake, measured by microfluorometry (35), was assumed to indicate preferential retention of albumin (as opposed to glycoproteins) in the blood plasma of the intact animal, and to explain microvascular “leakage” of albumin (presumably glycosylated) in diabetics. Freshly isolated endothelial cells are known to take up macromolecules by “constitutive micropinocytosis” as an expression of vascular transport (29).

At present it is of interest to extend the type of surface mapping we have recently carried out, to the tissue front of the endothelium. The object is to find out whether differentiated microdomains connected with specific structural elements exist or not on this front and (if present) to what extent they are similar to those observed on the luminal aspect of the endothelium. A similar or identical differentiation pattern would suggest that the plasmalemmal vesicles should be considered as specific membrane carriers (or containers) that shuttle, without losing their membrane specificity, between the two aspects of the plasmalemma as suggested by experiments reported in reference 33.

1 Albumin and glycosylated albumin were conjugated to different fluorophores.
Observations Already Recorded in the Literature

 Lectins were introduced in the early 1970's as means of making directly (1, 2) or indirectly (8, 15, 17) receptor sites on cell surfaces. More recently lectin-ferritin and lectin-peroxidase conjugates have been used extensively for mapping oligosaccharide moieties on the surface of a variety of cells (7, 14-16, 20, 26) or on membrane fragments from cell homogenates (10). This experimental approach was extended to the endothelium of the microvasculature of the lung (3), kidney (4, 5), heart (34), and to the endothelium of the aorta (36). Using a variety of labeled lectins (concanavalin A, WGA, RCA) these experiments have revealed a general, usually uniform labeling of the endothelial plasmalemma and their associated vesicles. Differentiated microdomains were not identified presumably because the experimental conditions used, the resolution attained, and the types of endothelia examined were different from those involved in our study.

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