Preferential Distribution of Anionic Sites on the Basement Membrane and the Abluminal Aspect of the Endothelium in Fenestrated Capillaries

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Abstract Cationized ferritin (CF) was injected interstitially to study the distribution of anionic sites on the basement membrane and abluminal aspect of the endothelium in the fenestrated capillaries of the mouse pancreas and intestinal mucosa.

Extensive, but uneven removal of the basement membrane was obtained by collagenase perfusion of the vasculature before CF labeling. In the absence of collagenase treatment, CF label was essentially restricted to the lamina rara externa of the basement membrane and occurred in clusters distributed in a relatively ordered planar lattice. After collagenase digestion, labeling of the lamina rara interna and of the abluminal aspect of the endothelium became possible. In the lamina rara interna, the CF label occurred in clusters with a distribution comparable to that found in the lamina rara externa. On the abluminal aspect of the endothelium, the plasmalemma proper was extensively, though variably, labeled. Coated pits were heavily labeled, whereas the membranes and stomatal diaphragms of plasmalemmal vesicles and transendothelial channels remained free of CF decoration. In contradistinction with the heavy labeling of their luminal aspects, the abluminal surface of the fenestral diaphragms were free of any CF decoration. The findings establish the existence of differentiated microdomains on the abluminal aspect of the endothelial plasmalemma and suggest that the capillary wall selects permeant macromolecules according to charge, in addition to size.

We have recently reported the existence of differentiated microdomains on the luminal surface of the endothelium in the fenestrated capillaries of the pancreas and intestinal mucosa of the mouse (45-47). These domains correspond to structural elements involved in transepithelial exchanges (e.g. plasmalemmal vesicles, transendothelial channels, stomatal diaphragms and diaphragmed fenestrae [31, 39]), and are characterized by a pronounced differential distribution of glycoproteins and proteoglycans. The new findings suggest that the macromolecules that permeate the endothelium are selected according to charge, in addition to size, and that cationic and anionic macromolecular solutes follow different paths across the endothelial layer of the capillary wall. In view of these results, it became of interest to find out whether similarly differentiated microdomains existed on the abluminal aspect of the endothelium, and whether anionic sites were also preferentially distributed on the basement membrane (basal lamina) of blood capillaries. In this paper we report the result of an investigation in which we have addressed these issues.

Materials and Methods

Animals and Materials

The animals used in this study were 25-30 g Swiss albino mice from the colony of the Institute of Cellular Biology and Pathology (Bucharest, Romania). Cationized ferritin (CF), pl 8.4, and native, horse spleen ferritin, pl 4.5, (x 6 crystallized, cadmium free) were purchased from Miles Laboratories (Elkhart, IN). Before administration, the ferritin solutions were dialyzed against 0.15 M NaCl for 48 h at 4°C. Anionized ferritin, pl 3.5, was prepared by N. Ghinea according to a slightly modified version of the method of Rennke et al. (37). Collagenase CLS type IV was from Worthington Biochemical Corp., Freehold, NJ, and papain type VI was purchased from Sigma Chemical Co., St. Louis, MO. Dulbecco’s phosphate-buffered saline (PBS) and minimum essential medium (MEM), 50-times concentrate, were obtained from GIBCO Laboratories (Grand Island Biological Co., Grand Island, NY). The solution used for perfusions was PBS, pH 7.2, supplemented with 5% MEM amino acids and 14 mM...
Experimental Protocol

**BASEMENT MEMBRANE AND INTERSTITIAL STRUCTURES:** For the detection of anionic sites on the basement membrane (peripheral aspect, primarily) we have applied the following procedure. (a) Under ether anesthesia and after laparotomy, 10 μl CF in saline solution (10 mg/ml) was injected into the loose connective tissue of the pancreas; the area turned light brown. (b) 5 min later, saline was infused in large excess (200-400 μl) in the same area until the tissue lost its brown color by dilution or removal of unbound CF. (c) This step was followed by fixation in situ with 2.5% glutaraldehyde in 0.1 M Na arsenate-HCl buffer, pH 7.2, or with a mixture of 1% formaldehyde and 2.5% glutaraldehyde in the same buffer. All solutions used in steps a to c were prewarmed at 37°C. (d) After 10-min fixation in situ specimens were collected and further processed for electron microscopy.

**ABLUMINAL ENDOTHELIAL SURFACE:** For surveying the abluminal aspect of the endothelium and the adjacent surface of the basement membrane, the following procedure was worked out. (a) The blood was washed out by retrograde perfusion (45) through the aorta with sPBS. (b) This step was followed by intermittent perfusion (as in reference 46) with collagenase (200 U/ml) over 30 min. Collagenase perfusion led to extensive, but uneven, detachment of the endothelium from the basement membrane, and thereby facilitated enzyme and tracer access to the abluminal aspect of the endothelium. (c) Excess collagenase was washed out by perfusion with sPBS. (d) CF was injected interstitially as in the previous procedure which was followed for all subsequent steps. All solutions used for either perfusion or interstitial injections were prewarmed at 37°C, and the animals were maintained at the same temperature (in a thermostat) for most of the duration of the experiment.

In some experiments, the microvascular beds were also perfused with CF (as in reference 45) introduced after collagenase removal and washed out before injecting the tracer into the interstitia, all other protocol steps being the same as above.

In still other experiments, native or anionic ferritin was substituted for cationized ferritin in protocols otherwise identical to those described above.

**ENZYMIC TESTS:** For enzymic treatments in situ, the procedure was the same as in the previous section up to and including removal of excess collagenase. This step was followed by the interstitial injection of 1% papain in saline solution; the incubation period was 15 min at 37°C. Excess enzyme was removed by infiltrating the tissue with sPBS or saline solution. The rest of the procedure (CF injection, fixation, etc.) followed the corresponding steps in the preceding section.

**Specimen Preparation for Electron Microscopy**

Tissue specimens, collected at a standard distance of ~1.5 mm from the site of tracer injection, were trimmed and their fixation was continued for 90 min in the same mixture used for the initial in situ fixation. After postfixation in 1% OsO4 in 0.1 M Na arsenate-HCl buffer, pH 7.2, for 90 min at 4°C, the specimens were transferred for 30 min to a solution of 0.5% galloylgluucose for mordanting (41) or 0.5% magnesium uranyl acetate for staining in block. The specimens were then processed through dehydration and embedding in Epon 812. The rest of the preparation procedure was as given in references 40 and 45.

**RESULTS**

**Basement Membrane**

It is generally assumed that the basement membrane of blood capillaries is a simple fibrillar layer (3, 13, 42), equivalent in principle to the lamina densa of the basement membrane of renal glomerular capillaries. The results reported in this paper indicate, however, that equivalents of the laminae rarae (externa and interna) can be recognized in the basement membrane of capillaries provided with a fenestrated endothelium. The same may also apply for other types of capillary vessels.

Without previous collagenase treatment, the injection of CF in the interstitia led to the labeling of the peripheral aspects of the basement membrane by clusters of ferritin molecules. These clusters varied noticeably in size—presumably on account of variations in tracer penetration into, and removal from, the tissue—but irrespective of the amount injected and the time allowed for binding, they remained discontinuous, located at center-to-center distances that ranged from 100 to 150 nm (Fig. 1). Grazing sections through basement membranes suggested that the clusters were distributed with a certain degree of order in a planar lattice in which they were spaced at distances of 80 to 100 nm and appeared interconnected by crisscrossing, fine fibrils (Fig. 2). Sections perpendicular to the capillary wall revealed primarily CF clusters located in the lamina rara externa and showed that the tracer penetrated to a varied but generally limited extent through the rest of the basement membrane. In most cases, only small and irregular deposits were detected in the lamina rara interna against the abluminal front of the plasmalemma (Fig. 1) or in the interstitia between the endothelium and pericytes (Fig. 3). In all cases, however, it was difficult to distinguish between clusters associated with the abluminal plasmalemma and clusters binding to the adja-

Abluminal Aspect of the Endothelium

Where the entire basement membrane was disorganized or completely removed as a result of collagenase treatment, CF was found to decorate the plasmalemma proper on the abluminal side of the endothelium. CF decoration varied from clustered (Fig. 10) to quasicontinuous (Fig. 11), but in general did not extend to the membranes of plasmalemmal vesicles (Figs. 10-12) and transendothelial channels (Fig. 12) and to their associated stomatal diaphragms (Figs. 11 and 12). In some cases, theintroits or infundibula leading to plasmalemmal vesicles were also not decorated (Figs. 8 and 11). As far as the plasmalemma proper and plasmalemmal vesicles were concerned, the decoration was, therefore, identical to that already described for the luminal aspect of the plasmalemma. Coated pits and derived coated vesicles appeared as heavily labeled by CF as their counterparts on the luminal aspect of the endothelium (Figs. 6 and 9).

The situation was, however, entirely different at the level of
FIGURES 1-3 Decoration of the basement membrane and abluminal aspects of the endothelium by CF injected directly into interstitia before fixation *in situ* (see Materials and Methods). Fig. 1 shows clusters of CF decorating anionic sites indicated by short arrows in the lamina rara externa. Long arrows point to CF labeling of the lamina rara interna and abluminal surface of the endothelium. X 80,000. Fig. 2 shows an oblique section through the basement membrane and the endothelium. Clusters of CF in the basement membrane (bm) are indicated by short arrows. Note that many of these clusters appear to be interconnected by fibrilar strands. vc, large vacuole in the endothelium. X 130,000. Fig. 3 shows extensive decoration of anionic sites in the lamina rara externa by CF clusters (short arrow). Discontinuous decoration of the abluminal surface of the endothelium and of the lamina rara interna is indicated by the right arrow. The left arrow points to the decoration of the abluminal surface of the endothelium in a narrow interstitium between the latter and a pericyte. X 100,000.

All figures are micrographs of fenestrated capillaries and adjacent cells in a mouse pancreas. For all figures, the following general indications are used: I, lumen; e, endothelial cell; v, plasmalemmal vesicles; p, pericyte; bm, basement membrane (basal lamina); c, collagen fibril.
fenestral diaphragms whose abluminal side was generally free of CF labeling (Figs. 10-12). Often, but not always, the infundibula leading to such diaphragms were also free of CF decoration (Fig. 11). The luminal aspects of the same diaphragms is the site provided with the highest concentration of high affinity anionic sites encountered on either aspect of the endothelium (45). To rule out the possibility that the anionic sites of the fenestral diaphragms are sensitive to collagenase, and to check the asymmetric distribution suggested by our new findings, we examined tissue specimens collected from experiments in which CF was perfused through, as well as injected into the interstitia. In such cases, the abluminal surface of the fenestral diaphragms was found to be heavily decorated by CF, whereas their abluminal aspect appeared completely free of CF (Fig. 13).

The junctional elements of the endothelium and the abluminal segments of its intercellular spaces were generally free of CF labeling (Fig. 7).

Endothelial cell processes extending towards pericytes were covered by a continuous heavy layer of CF (not illustrated). The lamina rara externa (interstitial aspect) of pericytes, smooth muscle cells, Schwann cells (Fig. 14), and pancreatic exocrine cells (not illustrated) was found to be decorated by CF clusters, highly reminiscent of those seen on the basement membrane of blood capillaries. Comparable labeling was absent or rare on fibroblasts and macrophages (Fig. 14).

Papain Digestion

The introduction of papain in the interstitia (after collagenase perfusion) resulted in extensive, often complete, removal of basement membrane remnants. It also removed practically all anionic binding sites from the abluminal surface of the endothelium. In addition, it removed or disorganized CF binding sites on collagen fibrils, leaving instead large CF clusters apparently free in the interstitia (Figs. 15 and 16).

Native and Anionized Ferritin

No preferential labeling of the abluminal aspect of the endothelial plasmalemma, basement membrane, and connective tissue elements was obtained with either native or anionized ferritin.

Figures 5-9  Progressive states of disorganization of the basement membrane as a result of collagenase perfusion in situ. Fig. 5 shows that CF clusters in the lamina rara interna (i) can still be recognized along most of this segment of the perimeter of the vessel. Only a few clusters of the lamina rara externa have survived the enzyme treatment (e). The lamina densa is extensively disorganized. CF decorates quasicontinuously the abluminal aspect of the endothelium (a) to which it has gained access as a result of basement membrane digestion. Note that the abluminal aspect of the fenestral diaphragms (f) is not decorated by CF. x 100,000. Fig. 6 shows two parallel rows of CF clusters in the lamina rara externa (e) and interna (i) that can be recognized along most of this endothelial segment. CF has gained access to the abluminal aspects of the endothelium where it heavily decorates a coated pit (cp) and discontinuously part of the plasmalemma (a). As in Fig. 5, the abluminal aspect of a fenestral diaphragm (f) is not decorated by CF. x 73,000. Fig. 7 shows few remnants of the CF clusters in the lamina rara externa (e) and more numerous clusters in the lamina rara interna (i). The abluminal aspect of the endothelium is extensively decorated by CF (a), with the exception of a transendothelial channel (c) and the abluminal aspect of a fenestral diaphragm (f). Note the absence of labeling in the intercellular space leading to a junction (j). x 85,000. Fig. 8 shows that, as in Fig. 7, a transendothelial channel (c) and the abluminal aspect of a fenestral diaphragm are not decorated by CF. In addition, the introit (asterisk) to a plasmalemmal vesicle and the stomatal diaphragm of the latter are not labeled by CF. x 93,000. Fig. 9 shows large CF clusters in the lamina rara interna and extensive CF decoration of the abluminal surface of the endothelium. Note the heavy labeling of a coated pit (cp) and the absence of label on the abluminal aspect of a fenestral diaphragm (f). x 117,000.
FIGURES 10 and 11  Examples of complete (or nearly complete) removal of the basement membrane by collagenase perfusion, a condition which gives CF full access to the abluminal aspect of the endothelium. Fig. 10 illustrates discontinuous labeling (d) of the abluminal surface of the endothelium. Note that the abluminal aspect of all fenestral diaphragms and most plasmalemmal vesicles and their stomatal diaphragms are unlabeled. CF labeling of plasmalemmal vesicles opened on the abluminal aspect in the extreme right part of the figure is a rare finding; it may be ascribed to incomplete removal of lamina rara interna remnants. c: collagen fibrils still decorated by CF. × 65,000. Fig. 11 is an illustration at higher magnification of the extensive decoration of the abluminal surface of the endothelium by clusters (a) or monolayers (a₁) of CF, and of the absence of similar decoration on the abluminal surface of fenestral diaphragms (f₁), stomatal diaphragm of a plasmalemmal vesicle (v₁), and the plasmalemmal area leading to another fenestral diaphragm (f₁), missed by the plane of the section. × 180,000.

DISCUSSION

Basement Membrane

Our findings indicate that the basement membrane of the fenestrated capillaries of the murine pancreas and intestinal mucosa is provided with clusters of anionic sites distributed with a certain degree or order in its two laminae rarae. The situation is highly reminiscent of that described by Caulfield and Farquhar (8) and Kanwar and Farquhar (19) in renal glomerular capillaries, except that in the fenestrated capillaries we have examined, the planar lattices formed by the clusters of anionic sites appear to be less regular.

In the laminae rarae of the basement membrane of the glomerular capillaries of the rat, the anionic sites were shown to be contributed primarily by heparan sulfates (20, 21) presumably as parts of sulfated proteoglycan molecules (22). But small amounts of chondroitin sulfates and hyaluronic acids (26) have also been detected in isolated glomerular basement membrane preparations. More recently, entactin, a high molecular weight (Mᵣ 158,000) sulfated glycoprotein (6), originally isolated from the intercellular matrix of a line of murine carcinoma cells (12), was localized by immunofluorescence in the basement membrane of many capillary beds in rats and mice (1). Other potential contributors of anionic sites are usual, nonsulfated sialoglycoproteins. At present, the only information concerning the chemistry of anionic sites in the basement membranes of the murine fenestrated capillaries we have examined comes from the results obtained by pronase digestion in situ. All anionic sites are sensitive to this protease of broad specificity; hence, all of them appear to be accounted for by proteoglycans and/or glycoproteins.

The relatively regular distribution of anionic sites in the laminae rarae of the basement membranes of fenestrated capillaries may reflect an underlying order in the lamina densa itself, which cannot be detected at present by other means. Yet, an ordered distribution could also be achieved or stabilized by interactions in the plane of each lamina rara as suggested, in fact, by the work of Kanwar and Farquhar in the case of glomerular basement membranes (19). The last assumption is in keeping with the finding that a certain amount of ordered
FIGURES 12 and 13  Fig. 12 is an illustration of the decoration of the abluminal aspect of the endothelium (a), the lack of decoration of the abluminal aspect of a fenestral diaphragm (f) and of the membranes and stomatal diaphragms of a transendothelial channel (c), and a plasmalemmal vesicle (v) opened on the abluminal front of the endothelium. x 100,000. Fig. 13 shows CF labeled specimen by perfusion as well as by interstitial injection (after collagenase digestion of the basement membrane). The luminal aspect of the endothelium is heavily and extensively labeled (al). A fenestral diaphragm (f) is asymmetrically labeled: heavy CF deposits on its luminal aspect, no CF on its abluminal surface. The labeling of the abluminal aspect of the endothelium is discontinuous (a) and lighter (in this case) than usual. x 125,000.

FIGURE 14  Postcapillary venule. CF clusters (arrows) decorate the interstitial surface of the basement membrane of a pericyte and of a Schwann cell. Notice that the plasmalemma of an adjacent fibroblast (fb) is not labeled by CF. n, nerve endings, still surrounded by Schwann cell cytoplasm (sc); e, endothelium. Short arrows point out the basement membrane between the pericyte and the endothelium. x 27,000.

distribution is still detectable in cases in which the lamina densa seems to be removed or extensively disorganized (Figs. 5–7) as a result of collagenase treatment. The pronounced sensitivity of the lamina densa to collagenase is in keeping with the presence of collagen IV in all basement membranes so far examined (23). The collagenase used in our experiments had, however, residual, general proteolytic activity that could have contributed to the extensive degradation of that lamina.

Less extensive observations indicate that similar clusters of anionic sites can be demonstrated on the interstitial surface of the basement membrane of pericytes, smooth muscle cells, Schwann cells and pancreatic exocrine cells.
FIGURE 15 Example of complete removal of basement membrane and anionic sites from the abluminal aspect of the endothelium by pronase digestion. CF clusters apparently free in the connective tissue matrix are marked by arrows. x 60,000.

FIGURE 16 Pronase-treated specimen. The periodic decoration of collagen fibrils is lost; CF clusters (arrows) appear randomly scattered in the connective tissue matrix. x 72,000.

Charonis and Wissig have recently recorded the presence of anionic sites labeled with ruthenium red on the abluminal aspect of the basement membrane of muscle capillaries (9, 10) and Charonis et al. (11) have apparently localized heparan-sulfate-containing proteoglycans in the basement membrane of capillaries by immunohistochemistry.

Collagen Fibers

Small CF clusters were found periodically distributed (repeat unit ~60 nm) along collagen fibrils interconnecting them in phase in collagen bundles. The polyanions revealed by the decoration appear to bind to specific sites on individual collagen fibrils, since CF labeling is restricted to 20-nm-wide bands that recur in the same location along each fibril. These polyanions may play a role in the organization of collagen bundles, but their chemical nature and the molecular interactions involved in this apparent cross-linkage remain obscure at present. Sulfated proteoglycans are known to bind to collagen fibrils (27). Periodic decoration of collagen fibrils with other cationic probes or dyes has been mentioned in the literature.

Endothelium

On the tissue front of the endothelial plasmalemma, the distribution of anionic sites is similar to that already documented for the luminal aspect (45). The plasmalemma proper is heavily labeled by CF either continuously or in clusters. In contrast, the membrane of plasmalemmal vesicles and transendothelial channels opened on the tissue front is not labeled, and the same generally applies to the stomatal diaphragms of these structures. The differential distribution described is most clearly seen in cases in which the basement membrane, including the lamina rara interna with its own anionic clusters, is completely removed from the endothelium.

The absence or low concentration of anionic sites on plasmalemomal vesicles and transendothelial channels reinforces conclusions already advanced in previous studies (45-47). The limiting membrane of the vesicles is chemically distinct from the plasmalemna proper and appears to retain its distinctive character in spite of the repeated fusion-fission events implied in vesicular transport (2, 43, 44). Given the continuity of the fluid bilayers of the corresponding membranes, the existence of differentiated microdomains and the presence of sharp edges and sharp bends at vesicular stomata (30) appear as unexpected, hard to explain, structural features. Randomization of molecular components by lateral diffusion in the plane of two fused membranes, and rapid elimination of sharp edges upon vesicle fusion should occur unless prevented by appropriate means. At present these means remain obscure. Structural findings suggest, however, that in endothelial cells the plasmalemna proper—rather than the plasmalemomal vesicles—is stabilized by interactions with extensive, fibrillar infrastructures located on its cytoplasmic aspect (45).

The distribution of anionic sites on coated pits and coated vesicles on the abluminal front of the endothelium was also found to be similar to that already described on the luminal front. Coated pits (38) represent a type of differentiated microdomain basically different from plasmalemomal vesicles. In their case, the mobile element (the vesicular carrier), rather than the receiving membrane, appears to be stabilized by interaction with the characteristic geodetic cage (14) (or basket [18]) formed by clathrin and associated proteins (32-34). For
a more extensive discussion of problems connected with the control of membrane specificity in vesicular transport, the reader should consult reference 29.

The only clear and striking difference between the two fronts of the endothelium concerns the fenestral diaphragms which appear to be free of anionic sites on their abluminal aspect, although their luminal surface has the highest concentration of high affinity anionic sites so far recorded on the capillary endothelium. These sites are provided primarily by heparan sulfates or heparin (46). As already discussed (45, 46), anionic macromolecules are expected to be preferentially transported across the endothelium via plasmaembrillar vesicles and transendothelial channels and to be discriminated against by diaphragmed fenestrae. The new findings reinforce this assumption for plasmaembrillar vesicles, but the asymmetric character of the fenestral diaphragms suggests that the permeability of these structures may be different in the two opposite directions. These diaphragms may favor the movement of uncharged or positively charged macromolecules from the plasma to the interstitial fluid, but the same may not apply for the opposite direction.

The microdomains described in this and the preceding papers (45–47) appear as local differentiations in membranes that share a common continuous bilayer. The endothelium has, however, additional microdomains in which a lipid bilayer is absent. In such domains, exemplified by fenestral and stomatal diaphragms, we can assume that the stability of the domain is ensured by the interaction of the ectodomains of integral membrane proteins either among themselves or with peripheral proteins of an extracellular variety. This type of organization could explain the chemical and structural stability of the diaphragms as well as the sharp bends generated by their insertion in a fluid membrane. Sharp bending may be generated, however, by other means in other situations, e.g., the rims of the diaphragm-free fenestrae of renal glomerular capillaries and of the discontinuities or lacunae found in the endothelium of liver sinusoids.

The chemical nature of the anionic sites found on the abluminal aspect of the endothelial plasmalemma remains unknown. Evidence already published suggests that the anionic sites on the luminal aspect of the endothelium are contributed by a mixed population of glycoproteins and sulfated proteoglycans (46). The same may apply for the abluminal aspect. Substantial contributions from gangliosides can be ruled out since all anionic sites are removed by pronase.

Cultured endothelial cells synthesize and secrete sulfated proteoglycans (4, 5, 15), and liver cells produce two versions of heparan sulfate-containing proteoglycans: one integrated in cell membranes, and the other apparently secreted (24, 25). Endothelial production of proteoglycans diversified along this line could explain the presence of anionic sites on the abluminal plasmalemma as well as on the basement membrane and subjacent elements of the connective tissue matrix.

Taken in their ensemble, the findings reported in this paper, indicate that the capillary wall comprises a series of charge barriers located at the level of the endothelial plasmalemma and of the two laminae rarae of the basement membrane. The existence of such charged barriers in the wall of blood capillaries in general could explain a number of "unexpected" findings already recorded in the literature. In dog-paw capillaries, for instance, the permeability for transferrin (effective molecular radius \( a_E = 4.3 \text{ nm} \)) is higher than for albumin (7); the latter is smaller (\( a_A = 3.5 \text{ nm} \)), but has a lower \( z_E \). The presence of an anionic screen on fenestral diaphragms could account for the fact that the permeability of fenestrated capillaries for plasma proteins (most of them anionic) is not significantly higher than that of capillaries with a continuous, nonfenestrated endothelium (16, 17, 35, 36). Finally, the presence of a quasi continuous screen of negative charges in the laminae rarae of the basement membrane could explain the finding that in skin and muscle capillaries albumin diffuses much more slowly from the interstitial fluid into the plasma than from the plasma into the interstitial fluid (28). Discrimination against anionic macromolecules is generally expected on account of our recent findings (45, 46, and this paper), but the molecular size beyond which charge restrictions begin to operate remains to be established for each barrier and for each direction across each barrier.

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