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LYMPHATIC ENDOTHELIAL-INTERSTITIAL INTERFACE

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In comparing differences in blood vessels and lymphatics, the blood capillary endothelium has been shown to contain well differentiated domains of neven charge density and is surrounded by a basal lamina which acts as both a size and charge barrier in the formation of plasma ultrafiltrate (1-3). Unlike the blood capillary, the lymphatic capillary lacks a well differentiated basal lamina (basement membrane) and is in direct contact with the surrounding interstitial spaces (4,5). Since the lymphatic capillary serves a very specialized function of draining interstitial boundary fluid and plasma macromolecules, its lymphatic-interstitial boundary is specially adapted to provide for the passage of these substances from the interstitial spaces into the lymphatic capillary lumen. Therefore, its endothelial lining should facilitate rather than repel the transport of plasma macromolecules across the interstitial-lymphatic interface. Thus, the net electrical charge at its lymphatic interstitial boundary should be reduced in comparison to that of the blood-tissue interface. Glycosaminoglycan moieties of proteoglycans are major structural components of the extracellular matrix which also surround blood vessels as well as comprise the vascular wall (6). The different glycosaminoglycan moieties of glycoproteins are also highly negatively charged molecules and are present throughout the extracellular matrix and the surfaces of cells (7-9). Proteoglycans can be visualized in the electron microscope by using the ruthenium red method (10), which selectively binds to acidic polymers to give rise to an electron-dense label when combined with OsO_4. To further characterize the lymphatic endothelium and its subendothelium layer we have used the ruthenium red (RR) method as well as cationized ferritin (CF) to label anionic sites on the lymphatic capillary endothelium and its underlying subendothelial layer. In addition, the chemical nature of these components was also investigated.

MATERIALS AND METHODS

Animals

The experimental system takes advantage of the rich plexus of lymphatic capillaries and collecting lymphatic vessels located in the superficial and deep regions of the dermis in the ears of Swiss Albino mice as well as the central lacteals of the small intestine. In addition, the diaphragmatic lymphatics in mice were also investigated using CF as an electron dense label.

Ruthenium red staining

Following anesthesia, the blood vasculature was cleared by perfusing with a balanced salt solution and fixation was achieved by perfusing with a glutaral-

dehyde formalin mixture containing RR (11). After perfusion fixation, specimens of ears and small intestine were removed and cut in small slices and postfixed in OsO_4 with RR and processed for transmission electron microscopy (TEM). In some experiments the tissue was fixed by immersion in solutions containing RR and then processed for TEM.

Interstitial injection of cationic ferritin

Cationized ferritin (CF), PI 8.4 (Miles Laboratories, Elkhart, IN) was dialyzed before use for 48 hours at 4°C against 0.15M NaCl. After animals were lightly anesthetized with ether, 0.1mg of CF in 0.05ml PBS was injected interstitially into the margin of the ears using microinjection procedures carried out in our laboratory (12). Following interstitial injections, animals were sacrificed at 5 to 30 minutes and the tissue fixed in 2.5% glutaraldehyde in sodium cacodylate buffer. Tissue was postfixed in 2% OsO_4 in phosphate buffer and processed for TEM (13). To assess the effect of plasma on CF binding, thin slices of the mouse diaphragm were removed and cut into thin sections and washed in several changes of PBS to remove plasma proteins. The sections were then incubated in CF for 5 to 10 minutes and the tissue was then washed in PBS to remove excess label and subsequently fixed and processed for TEM.

Enzymate digestion

To demonstrate the nature of the anionic sites on the lymphatic endothelium and its subendothelial layer slices of dermal tissue were obtained from the ears of mice and incubated in hyaluronidase, 8,000 U/ml in 0.1M acetate at pH 5.4 for 30-45 minutes or in chondroitinase ABC, 2.4 U/ml in 0.1M Tris HCl at pH 7.4 at 37°C for 30-45 minutes. After incubation in enzyme the tissue was rinsed in buffer and then fixed in glutaradlehyde containing RR, or followed by incubation in CF (PI 8.4), postfixed in OsO_4 and processed for TEM.

RESULTS

General organization of the lymphatic-interstitial interface

The lymphatic endothelium rests on an ill-defined subendothelial layer which separates it from dense bundles of collagen and elastin fibers, and cells of the surrounding connective tissue *(Fig. 1)*. This subendothelial layer is of a variable width (up to 0.5μm), and in routine preparations for electron microscopy it appears as a translucent band which contains anchoring filaments (8nm) *(Fig. 1)*. These insert on the plasmalemma and

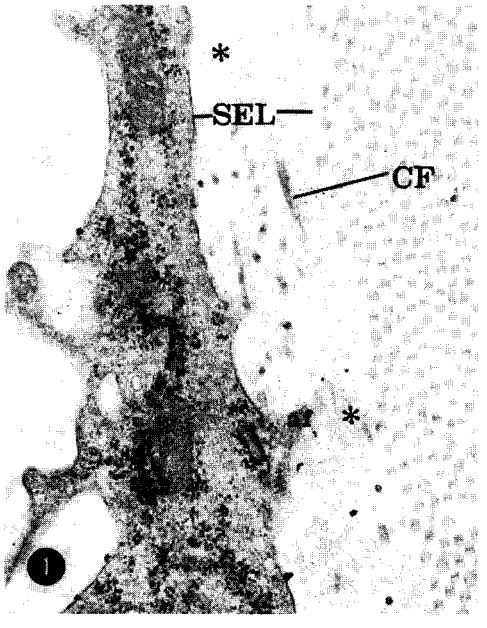
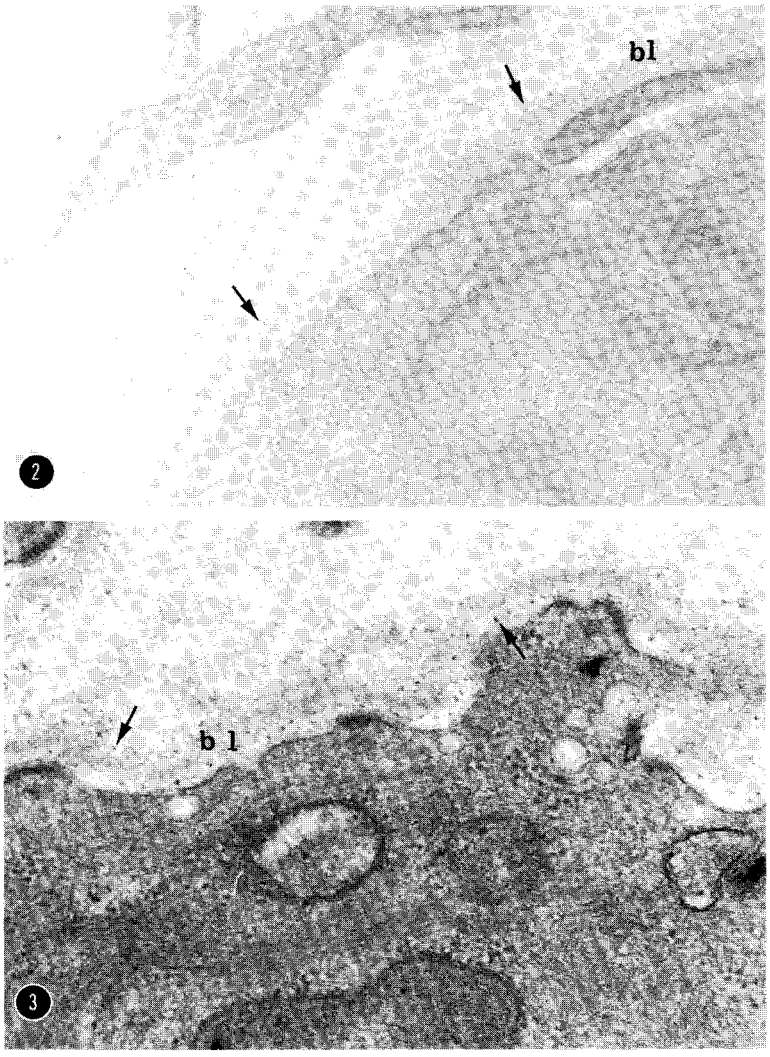


Fig. 1. Electron micrograph of lymphatic capillary endothelium. A well defined basal lamina is lacking. However, the subendothelial layer contains numerous anchoring filaments (af) that attach to the abluminal (connective tissue) surface of the endothelium and extend into the surrounding interstitium. In addition, there is also a network of fine filaments (*) that form a boundary layer between the lymphatic capillary wall and the surrounding collagen bundles of the interstitium. (x46,500)

extend into the surrounding connective tissue between collagen and elastic fibers. In our earlier studies of these structures, it was suggested that the anchoring filaments provided a means of stabilizing the



*Figs. 2 and 3. RR staining sites in basal lamina (bl) of blood capillary (*Fig. 2, *x72,000) and basal lamina of epidermal epithelium (*Fig. 3, *x61,000). Mouse perfused with solution containing RR.*

irregularly-shaped and attenuated lymphatic endothelial wall and served to connect the endothelium to the surrounding interstitium (14). When RR was perfused via the blood vascular system its small size enables it to penetrate the blood capillary wall and gain access to the interstitium. The results from these studies showed that ruthenium red positive granules measuring 20-50nm in diameter appeared in regular pattern in the basal lamina of blood capillaries as well as the basal lamina of epidermal cells *(Figs. 2-3)*. However, its location in the lymphatic subendothelial layer was sparsely associated with the irregular basal lamina, with many areas lacking the RR positive granules *(Fig. 4)*. In specimens fixed by immersion with RR in the pre- and postfixation solutions, RR staining was observed within the lymphatic lumen, on the luminal surface of the plasmalemma, and within plasmalemmal vessels *(Fig. 5)*.

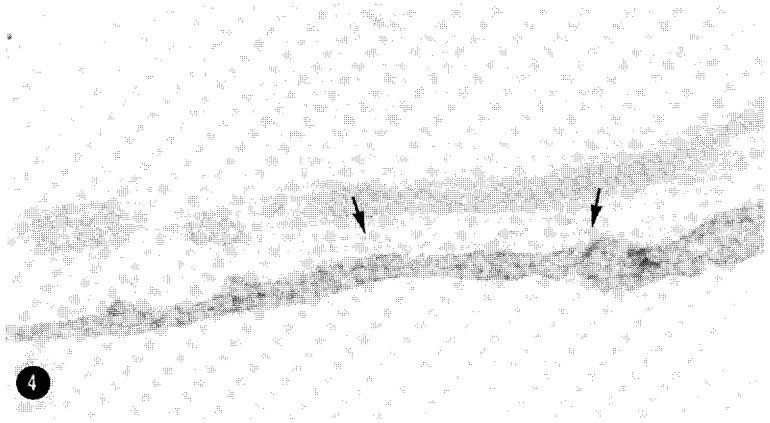


Fig. 4. RR staining sites (arrows) in subendothelial layer of lymphatic capillary. In some areas RR dense granules are aligned in a row of punctate densities, while in others they appear irregular or some areas of the subendothelial layer are unstained. Mouse perfused with solution containing RR. (x56,000)

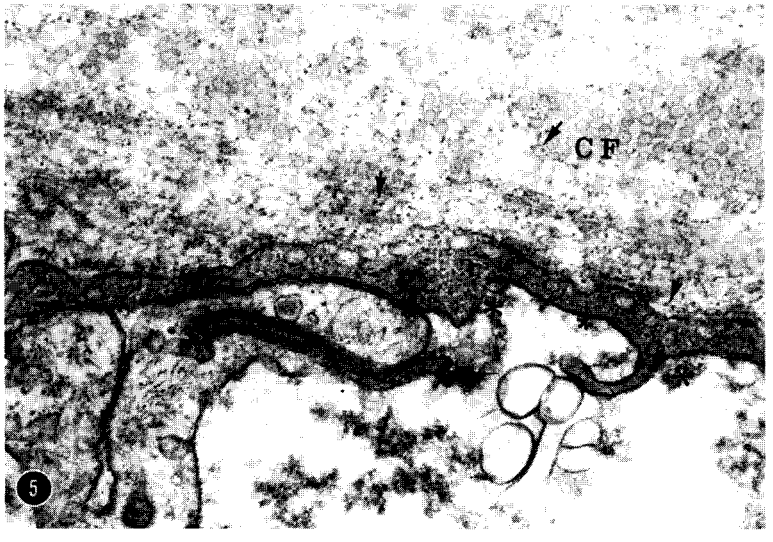


Fig. 5. This electron micrograph shows a portion of dermal tissue from mouse ear that was fixed by immersion in solution containing RR. There are numerous RR dense staining sites (arrows) in the subendothelial layer as well as throughout the interstitial area and on collagen fibers (CF). With the immersion procedure the RR also stains the luminal surface of the lymphatic capillary endothelial plasmalemma (*), but not its abluminal surface. (x25,500)

When CF of PI 8.4 was interstitially injected in the dermis of mice, the dense particles were observed within the subendothelial layer in the form of dense patches separated by gaps of varying widths *(Fig. 6)*. For the most part CF particles were separated from the endothelial plasmalemma. Since the presence of plasma proteins and/or albumin has been shown to interfere or block the binding of negative sites to cationic probes (15), we incubated sections of ear tissue and diaphragms in PBS to remove plasma proteins prior to incubation in solutions with cationic tracers. In these preparations there was a binding of CF to the luminal surface of the lymphatic endothelium in the form of dense

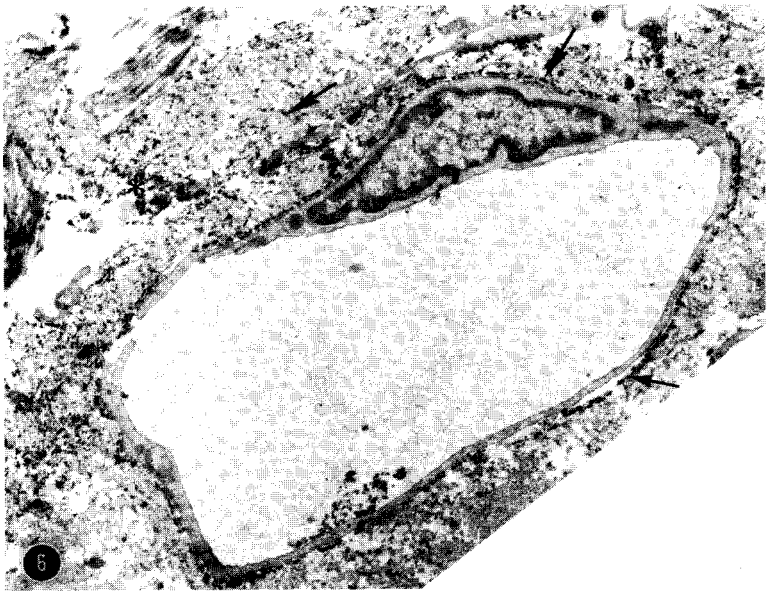


Fig. 6. This electron micrograph illustrates the labeling of CF at 5 minutes after interstitial injection. The tracer labels the interstitial spaces, collagen fibers in a regular pattern and is localized in the subendothelial layer (arrows) in dense patches but separated from the endothelial plasmalemma by a narrow space. The lumen is filled with plasma proteins and the luminal surface of the endothelium shows very little labeling of the CF tracer. (x18,000)

patches, but very few anionic sites were observed on the connective tissue front of the lymphatic endothelium *(Fig. 7)*. In addition, CF particles also decorated extensively the clefts of intercellular junction and labeled collagen fibers in a periodic fashion *(Fig. 7)*.

Enzyme digestion

The identity of proteoglycans and anionic sites was confirmed by the cytochemical method of employing selective extraction with specific enzymes followed by rinsing in buffer and then fixation of the tissue in glutaraldehyde containing ruthenium red, or followed by incubation in cationized ferritin (PI 8.4), postfixed in OsO_4 and processed for transmission electron microscopy. When we examined the effect of specific enzymes on the subendothelial layer and the lymphatic endothelium it was observed that the electron dense label was no longer demonstrable after incubation with either the hyaluronidase or the chondroitinase ABC *(Figs. 8-10)*.

DISCUSSION

While there are similarities between blood and lymphatics, major differences also exist in both their function and structural make up. Along the microcirculatory segment of the blood vascular system, gases, plasma macromolecules and cells permeate the blood endothelial lining to enter the interstitial spaces throughout the body. To regulate a selective passage across its wall, the blood vascular endothelium is provided with both size and charge barriers which restrict flow from its luminal surface according to need by specific tissues and regions of the body (16). It has been demonstrated by a number of workers that a charge barrier exists on the blood front as well as its underlying basal lamina (basement membrane) (17). To carry out its specialized function of draining fluids, macromolecules and cells from the interstitium the

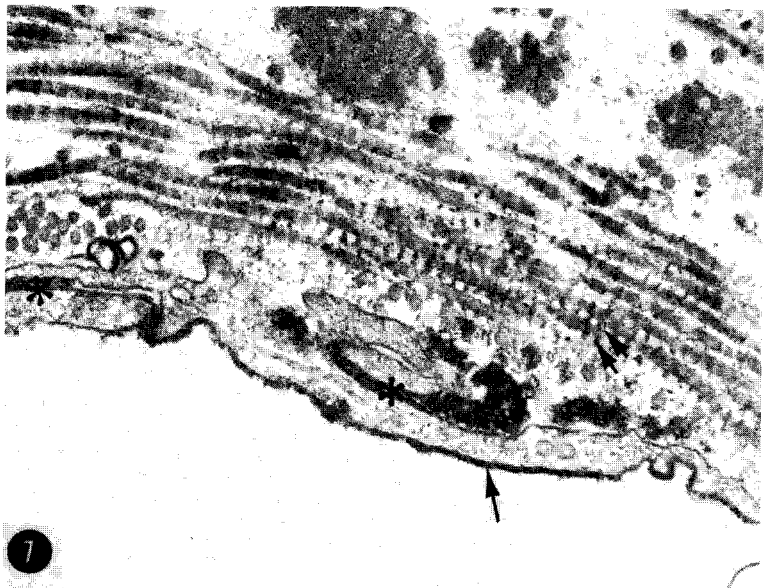
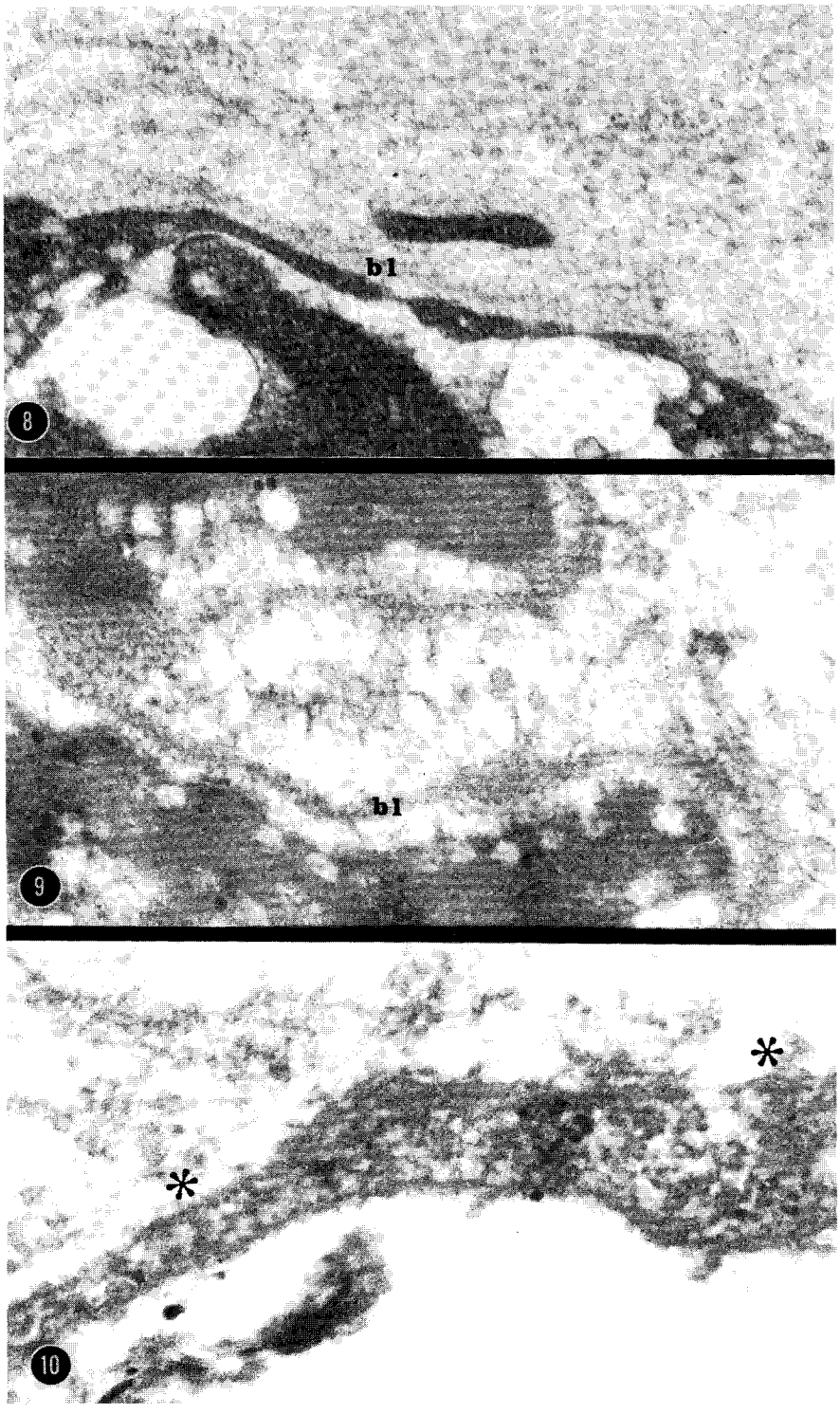


Fig. 7. Electron micrograph showing CF labeling in diaphragmatic lymphatic capillary. The specimen was rinsed in PBS to remove plasma proteins prior to incubation in CF. The tracer decorates the luminal plasmalemma (arrows), the clefts of intercellular junctions (*) and collagen fibers (double arrows) in a periodic pattern. However, very little label occurs on the basal surface of the lymphatic endothelial plasmalemma. (x40,000)

lymphatic capillary endothelium should facilitate rather than restrict the passage from the interstitium into the lymphatic lumen. From the studies carried out in our laboratory as well as the work of others it has been shown that the lymphatic capillary endothelium is provided with loosely adherent intercellular junctions, and lacks a definitive underlying basal lamina. Therefore, its structural makeup allows direct access of the interstitial plasma ultrafiltrate as well as cells to the lymphatic lumen (4,14,18).

The studies reported here demonstrate that the lymphatic capillary endothelium possesses an uneven charge density on its luminal surface; however, there was a very sparse distribution pattern of anionic sites observed in its underlying subendothelial layer. The results from these cytochemical studies demonstrated that 20 to 50nm diameter RR-positive granules were associated with irregular segments of basal lamina. However, the subendothelial layer contained very few RR-positive granules. This finding is different than that found in the basal lamina underlying blood capillaries which contain an array of discrete anionic components along both surfaces of the lamina densa (11,19-20).

When CF was injected interstitially the label was found within the subendothelial layer in the form of dense patches separated by gaps of varying widths and little to no labeling occurred on the endothelial plasmalemma. This lack of binding to the endothelium can be attributed to the presence of plasma proteins and albumin throughout the interstitium and within the lumen. However, a regular binding of CF particles to the luminal surface of the endothelium was demonstrated in diaphragmatic lymphatic endothelium when plasma proteins were removed prior to incubation in solutions with the CF label. These results showed that there were very few anionic sites on the abluminal (connective tissue) surface of dermal lymphatics and confirm our earlier studies with diaphragmatic lymphatics (13). Using isolated canine renal



Figs. 8-10.

*Figs. 8-10. Electron micrographs showing basal lamina (bl) of blood capillary endothelium (*Fig. 8, *x56,000), the epidermal epithelium (*Fig. 9, *x48,500 and the subendothelial layer of the lymphatic capillary (*Fig. 10, *x85,000). The tissue was incubated in hyaluronidase, rinsed in PBS and then processed in a solution containing RR. No RR dense staining sites were observed in the basal lamina of blood capillaries, the epidermal cells, or the subendothelial layer (*) of the lymphatic endothelium.*

lymphatics (lymphatic collecting vessels), Jones et. al. (21) demonstrated that CF did not bind to the plasma membrane when administered abluminally, but when added in the perfusion fluid CF did bind to the luminal membrane and infundibula of intercellular channels. In our previous studies (13), we observed extensive CF labeling throughout the cleft of intercellular junctions in lymphatic capillaries. This may reflect a functional difference betweeen the lymphatic collecting vessels and lymphatic capillaries, as the latter vessels begin as blind end tubes and serve a major function in providing for the continuous removal of interstitial fluids, ions and small solutes. Other regions of high concentrations of anionic sites have also been associated with increased permeability to water and small solutes (3).

After incubation with hyaluronidase or chondroitinase ABC, the irregular patches of RR positive granules were no longer demonstrable in the subendothelial layer. Since hyaluronate has been shown to cause a high degree of hydration in a number of biological and experimental systems, its concentration in the lymphatic subendothelial layer suggests that it may also be involved in the overall process of fluid and cellular transport from the interstitium to and across the lymphatic capillary wall. Thus, the charge asymmetry of the lymphatic endothelium could give directionality to the lymphatic endothelium that would favor transport from the connective tissue to its luminal surface.

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REFERENCES

1. Chang, RL, WM Dean, CR Robertson, MM Brenner: Permeability of the glomerular capillary wall. III. Restricted transport of polyanions. Kidney Int. 8 (1975), 212.
2. Rennke, HG, Y Patel, MA Venkatachalam: Glomerular filtration of proteins: Clearance of anionic, neutral and cationic horseradish peroxidase in rats. Kidney Int. 13 (1978), 278.
3. Simionescu, N, M Simionescu, GE Palade: Differentiated microdomains on the luminal surface of the capillary endothelium. I. Preferential distribution of anionic sites. J. Cell Biol. 90 (1981), 605.
4. Fraley, EE, L Weiss: An electron microscopic study of the lymphatic vessels in the pinel skin of the rat. Am. J. Anat. 109 (1961), 85.
5. Leak, LV, JF Burke: Ultrastructural studies on the lymphatic anchoring filaments. J. Cell Biol. 36 (1968), 129.
6. Wright, TN, R Ross: Proteoglycans in primate arteries. I. Ultrastructural localization and distribution in the intima. J. Cell Biol. 67 (1975), 660.
7. Comper, WD, TC Laurent: Physiological function of connective tissue. Physiol. Rev. 58 (1978), 255.
8. Toole, BP: Glycosaminoglycans in morphogenesis. In *Cell Biology of the Extracellular Matrix*, Hay, E (Ed.), Plenum Press, New York (1983), 259.
9. Hascall, VC, GK Hascall: Proteoglycans. In *Cell Biology of the Extracellular Matrix*, Hay E (Ed.), Plenum Press, New York (1983), 39.
10. Luft, JH: Ruthenium red and violet. I. Chemistry, purification, methods for use for electron microscopy and mechanism of action. Anat. Res. 171 (1971), 347.
11. Charonis, AS, Wissig, SL: Anionic sites in basement membranes. Differences in their electrostatic properties in continuous and fenestrated capillaries. Microvasc. Res. 15 (1983), 265.
12. Leak, LV: Studies on the permeability of lymphatic capillaries. J. Cell Biol. 50 (1971), 300.
13. Leak, LV: Distribution of cell surface charges on mesothelium and lymphatic endothelium. Microvasc. Res. 31 (1986), 18.

14. Leak, LV, JF Burke: Fine structure of the lymphatic capillary and the adjoining connective tissue area. Am. J. Anat. 118 (1966), 783.
15. Thurauf, N, R Dermietzel, P Kalweit: Surface charges associated with fenestrated brain capillaries. I. *In vitro* labeling of anionic sites. J. Ultrastruct. Res. 84 (1983), 103.
16. Renkin, EM: Multiple pathways of capillary permeability. Circ. Res. 41 (1977), 735.
17. Farquhar, MG: Structure and function in glomerular capillaries. Role of the basement membrane in glomerular filtration. In *Biology and Chemistry of Basement Membrane*, Kefalides, NA (Ed.), Academic Press (1978), 43.
18. Casley-Smith, JR: How the lymphatic system works. Lymphology 1 (1968), 77.
19. Vaccara, CA, JS Brody: Structural features of alveolar wall basement membrane in the adult rat lung. J. Cell Biol. 91 (1981), 427.
20. Kanwar, YS, MG Farquhar: Anionic site in the glomerular basemment membrane. J. Cell Biol. 81 (1979), 137.
21. Jones, WR, CCC O'Morchoe, HM Jarosz, P O'Morchoe: Distribution of charged sites on lymphatic endothelium. Lymphology 19 (1986), 5.

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