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# DISTRIBUTION OF CHARGED SITES ON LYMPHATIC ENDOTHELIUM

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## ABSTRACT

*The charge distribution on the luminal and abluminal aspects of fixed and living lymphatic endothelium was examined with particular emphasis on the endocytotic vesicular system and interendothelial junctions. Native ferritin (NF; pI = 4.5), when administered abluminally to perfused lymphatics, entered endocytotic vesicles and abluminal and luminal caveolae; NF was also found in intercellular channels. In contrast, NF when applied luminally was largely excluded from both luminal caveolae and intercellular channels. Cationic ferritin (CF; pI = 8.4) bound to the discontinuous basal lamina and to the abluminal plasma membrane, clustering preferentially around the stomata of abluminal caveolae. CF did not, however, bind to the plasma membrane of, or enter, either the vesicular system or intercellular channels, when administered abluminally. When added to the perfusion fluid CF bound to the luminal membrane and to the infundibula of intercellular channels. Ruthenium red (RR) and alcian blue (AB), both cationic stains, bound intensely to the luminal membrane and much less so to the abluminal surface, thus simulating the binding pattern of CF. Unlike CF, however, RR and AB bound to the membranes of abluminal and luminal caveolae with the same level of staining as to the plasma membrane to which they were attached. These results reflect a marked asymmetry in the membrane charge characteristics of endothelial cells.*

The passage of macromolecules across vascular endothelium is, in general, a function of both size (1,2) and net charge (3-6). Selectivity on the basis of these parameters appears to be, at least in part, a property of the endothelium, or of intimately related structures such as the basal lamina, which are interposed between blood or lymph and the interstitial milieu. The ability to discriminate between molecules of differing charges may be attributable to the charged nature of the endothelial plasma membrane itself: both electrophysiological (7) and tracer studies (8,9) have demonstrated a negatively charged surface. Moreover, studies of blood capillary endothelium by Simionescu et al (10) have revealed a complex patchwork of biochemically distinct microdomains, each with a characteristic charge and charge density, whose locations correspond to ultrastructural features implicated in macromolecular transport. Such correlations have fostered the idea of the capillary endothelium as a charge barrier, successful transit thus being dependent both on the size and charge of the transported molecule as well as on the size and charge of relevant endothelial structures.

Little comparable information is available concerning the existence and distribution of charged sites on lymphatic endothelium. Studies by Leak (11) on diaphragmatic lymphatics and by Charonis and Wisig (12) on lacteals have, however, yielded some intriguing information. The luminal surface of diaphragmatic lymphatics appears to possess numerous anionic sites whereas the abluminal aspect of lacteals possesses few, if any, such sites. Whether such charge asymmetry accurately reflects the situation in a single lymphatic vessel is unknown. Accordingly, it is our intent in the present study to define the charge distribution on both the abluminal and

luminal front of lymphatic endothelium, focusing attention on the topographical relations between charged sites and structures implicated in transendothelial macromolecular transport, specifically, intracellular channels and the endocytotic vesicular system.

## *MATERIALS AND METHODS*

**Lymphatics:** Isolated canine renal hilar lymphatics were used in these analyses. The suitability of such vessels for in vitro studies of macromolecular transport has been established by O'Morchoe et al (13). As revealed in the aforementioned study, these vessels are in many respects morphologically identical with lymphatic capillaries,i.e. they consist of little more than an attenuated, nonfenestrated endothelium and lack a continuous basal lamina. Moreover, these vessels can be isolated with little adherent connective tissue.

Mongrel dogs of either sex were anesthetized by intravenous administration of sodium pentobarbital (30 mg/kg), and the renal pedicle exposed through a loin incision. Unbranched segments of lymphatics emerging from the renal hilum were dissected clean of adhering tissue and cannulated at both ends in situ with polyethylene cannulae (PE 10, O.D. 0.61 mm). As measured in a previous study (13) the average length of these perfused segments ranged from 6 to 7 mm and had an average diameter between 0.3 and 0.5 mm. Lymphatics, with both cannulae in position, were excised and transferred to a perfusion chamber containing Dulbecco's Modified Eagles Medium (DMEM), in which temperature, pH and oxygen content were controlled. The inflow cannula was connected to a Sage syringe pump; the outflow cannula was positioned so that the perfusate did not contaminate the bathing fluid.

**Perfusion Techniques:** Two perfusion protocols were used in these studies. All lymphatics were placed initially in a perfusion chamber containing DMEM at 37°C, pH 7.0-7.3 and perfused with the same medium at a rate of 2 $\mu$l/min. In one set of experiments, the appropriate tracers were included in either the bathing fluid or the perfusate, allowed to interact with the living endothelium for ten minutes, rinsed (or perfused) with tracer-free DMEM for five minutes and fixed in cold (4°C) Karnovsky's fixative (14). Alternatively, lymphatics were fixed as soon as perfusate emerged from the outflow cannula. After fixation for one hour at 4°C, lymphatics were washed twice over ten minutes in 0.15M sodium cacodylate buffer, pH 7.3 and incubated in 0.1M ammonium chloride for 8-12 hours at 4°C to block fixation-induced aldehyde groups. They were then rinsed briefly in buffer and returned to the perfusion chamber, attached to the Sage pump as before and tracers administered either luminally or abluminally. The former protocol reveals binding and endothelial handling of the applied tracer in living vessels while the latter demonstrates the distribution of charged sites prior to the addition of the probe. The last protocol was necessitated by the finding that in some endothelia, binding of cationic molecules induces lateral migration of anionic sites (8).

**Tracers:** Five electron microscopically demonstrable probes of varying sizes and charges were used: (1) anionic, native ferritin (NF; pI = 4.5, Mr approximately 480,000, molecular diameter approximately 11nm) at a concentration of 10-30 mg/ml, (2) cationized ferritin (CF; pI = 8.4, Mr approximately 480,000, molecular diameter approximately 11nm) at a concentration of 0.5-1.0 mg/ml, (3) a cationic stain, ruthenium red (RR; Mr = 786, molecular diameter approximately 1.1 nm), (4) a second cationic stain, alcian blue (AB; Mr approximately 1,300, molecular diameter 2nm), and (5) tannic acid (TA; Mr approximately 1,700) a mixture of molecular species which non-specifically enhance the plasma membrane by binding to membrane-associated macromolecules. Procedures for the use of RR, AB and TA to delineate membrane-associated molecules were those of Luft (15), Behnke and Zelander (16) and Simionescu and Simionescu (17), respectively.

Native and cationized ferritin were added singly to either the abluminal or luminal surface of both living and fixed lymphatics. RR, AB and TA were administered simulta-

neously to both the abluminal and luminal surfaces of fixed lymphatics only since their visualization depends on the presence of the fixative.

For each probe and each perfusion protocol and minimum of three lymphatic segments, each from a different dog, was tested. Two additional lymphatics were used for each protocol in the case of abluminally administered NF. In these additional vessels NF was retained in the lumen by plugging both inflow and outflow cannulae. Thus a total of three lymphatics each were tested with RR, AB and TA, ten with NF and six with CF.

**Tissue Processing:** Following primary fixation, as described above, lymphatics exposed to either NF or CF were postfixed in 2% osmium tetroxide in 0.15M sodium cacodylate buffer, pH 7.3 for one hour at room temperature; postfixation procedures for vessels exposed to RR, AB and TA varied according to the demands of their respective techniques. All lymphatics were dehydrated through a graded acetone series and embedded in Epon 812. Thin sections were cut on a diamond knife and stained with bismuth subnitrate (18). This staining procedure specifically enhances the visualization of the ferritin molecule by staining the proteinaceous apoferritin portion of the molecule while leaving the tissue essentially unstained.

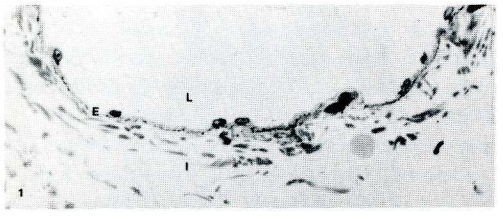


*Fig. 1: Portion of the wall of a renal hilar lymphatic, following isolation and perfusion in vitro. Note the extreme thinness of the intima and almost complete lack of adventitia. L = lymphatic lumen; E = endothelium; I = interstitium. X 400*

## *RESULTS*

The appearance of a portion of the wall of a renal hilar lymphatic after perfusion is shown in Fig. 1. The extremely thin nature of the vessel and almost total lack of adherent connective tissue is readily apparent. This latter point was considered of paramount importance since the presence of substantial extra-intimal elements might pose a barrier to the free diffusion of the various probes used in the present study.

**Native Ferritin (NF)** In living lymphatics abluminally applied NF was found in the adherent interstitium, within endocytotic vesicles and their abluminal and luminal caveolae and free in the lymphatic lumen (Fig. 2). NF was also contained in inter-

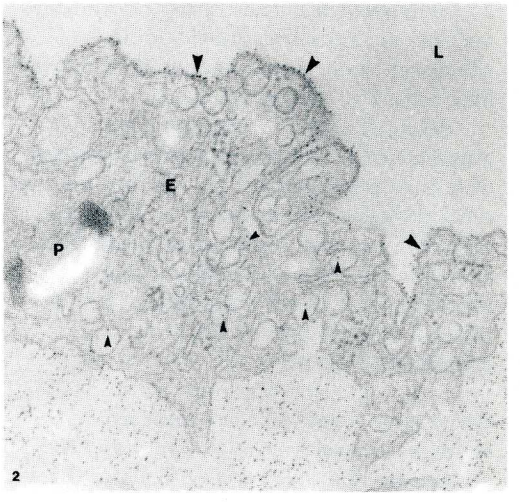


*Fig. 2: Portion of the endothelium of a living lymphatic to which NF was added abluminally during perfusion. NF is seen abluminally, and at low concentration in vesicles and intercellular channels (small arrows), and adherent to the luminal membrane (large arrows). L = lymphatic lumen; E = endothelium; P = paracrystalline inclusion. X 75,000*

cellular channels and vesicles opening off these structures (Fig. 2, arrows). Although NF did not appear bound to any of the aforementioned structures, luminally it was observed in intimate contact with the intercaveolar membrane (Fig. 2, large arrows), despite extensive flushing with tracer-free DMEM.

Included in the perfusate, i.e. applied luminally, NF was largely excluded from both luminal caveolae and intercellular channels, even when these latter lacked specialized junctional complexes (Fig. 3a and b, arrows). As was the case with abluminally applied NF which had apparently traversed the endothelium, NF directly applied to this surface appeared bound to it (Fig. 3a and b). The exclusion of this tracer, when applied luminally, from luminal caveolae and intercellular channels was dramatically confirmed in lymphatics in which the tracer was retained in the lumen during processing (Fig. 3c and d).

When applied abluminally to fixed lymphatics, NF entered only a few abluminal caveolae and intercellular channels; only rarely was it observed free in the lumen. Applied luminally, its distribution was identical to that in living endothelium.

**Cationized Ferritin:** When applied abluminally to living endothelium, CF bound quasi-periodically to both the endothelial and interstitial aspects of the attenuated basal lamina (Fig. 4a). Direct binding, at low density, to the abluminal plasma membrane was observed as well, most of the tracer clustering preferentially around the stomata of abluminal caveolae (Fig. 4b). CF

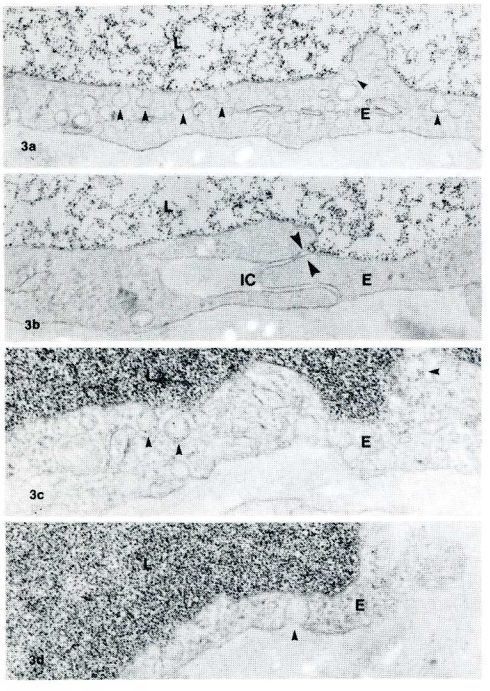


*Fig. 3: Endothelium from living lymphatics to which NF was added luminally by perfusion and retained therein during fixation and processing. L = lymphatic lumen; E = endothelium; IC = intercellular channel. a, b: Vessel perfused at a NF concentration of 10 mg/ml. NF is seen free in the lumen, and adherent to the luminal membrane. Note its exclusion from luminal caveolae (Fig. 3a) and from an intercellular channel which apparently lacks a luminal junctional complex (Fig. 3b, arrows). X 73,000. c, d: Vessel perfused at a concentration of 30 mg/ml. NF is largely excluded from luminal caveolae (Fig. 3c, arrows). A single vesicle apparently forming a thoroughfare channel from which NF is excluded is shown on Fig. 3d. This was the only example of such a structure seen in the present study. X 75,000*

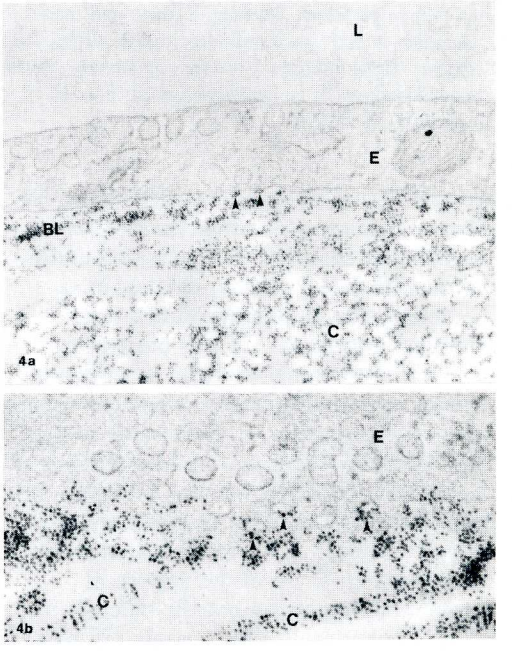


*Fig. 4: Portions of two living lymphatics to which CF was applied abluminally during perfusion. L = lymphatic lumen; E = endothelium; BL = basal lamina; C = collagen. a: CF is seen decorating both aspects of basal lamina, as well as adhering to the collagen. No CF is seen in abluminal caveolae and the abluminal membrane is devoid of tracer, except as indicated by arrows. X 75,000. b: Direct binding of CF to the plasma membrane in a section tangential to the abluminal surface. CF is clustered preferentially around, but not over, the stomata (arrows). X 80,000*

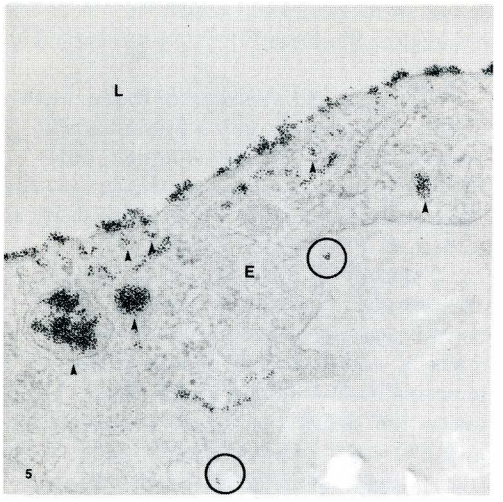


*Fig. 5: Distribution of luminally applied CF after a ten minute perfusion. Patching of tracer and internalization in both small and large vesicles (arrows) is evident. Circle indicates CF which apparently crossed the endothelium. L = lymphatic lumen; E = endothelium. X 75,000*

did not, however, enter into or bind to the plasma membrane of either the caveolae or intercellular channels. Following luminal perfusion with CF, a patchy, discontinuous binding to the luminal membrane was observed. Moreover, CF was present in many of the luminal caveolae as well as in vesicles and canalicular-like structures lacking obvious connections to the luminal surface (Fig. 5). Dense binding to the infundibula of intercellular channels was also observed. In a limited number of cases, images suggesting complete transit of the endothelium were observed (Fig. 5, circle) the pattern of luminal binding was markedly different in fixed lymphatics. In these vessels, CF bound at high density with a continuous distribution and was excluded from luminal caveolae and cytoplasmic vesicles (Fig. 6a and 6b). These results strongly suggest that CF binding in living endothelium induces rapid lateral migration of the anionic sites into patches and subsequent internalization. The binding of CF to the infundibula of intercellular channels was identical to that observed in living endothelium, as was the pattern of abluminal binding.

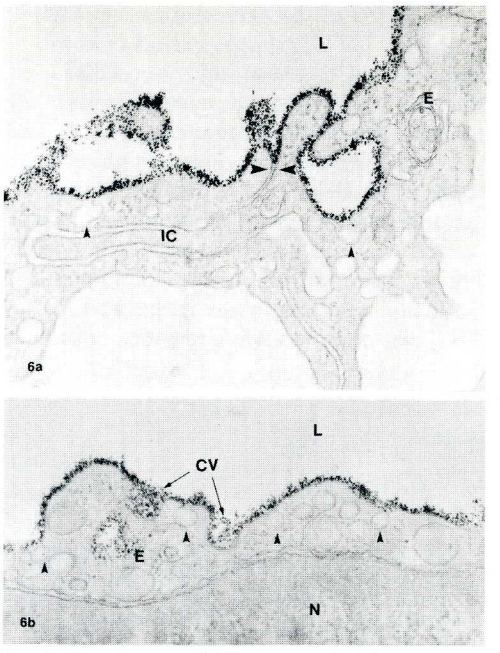


*Fig. 6: Distribution of DF when applied luminally to a pre-fixed lymphatic. L = lymphatic lumen; E = endothelium; N = nucleus; IC = intercellular channel. X 75,000. a: Continuous distribution of tracer is demonstrated, as is its exclusion from intercellular channels and luminal caveolae (arrows). b: Binding of CF to luminally disposed coated pits (CV), but not the luminal caveolae of uncoated vesicles (arrows).*

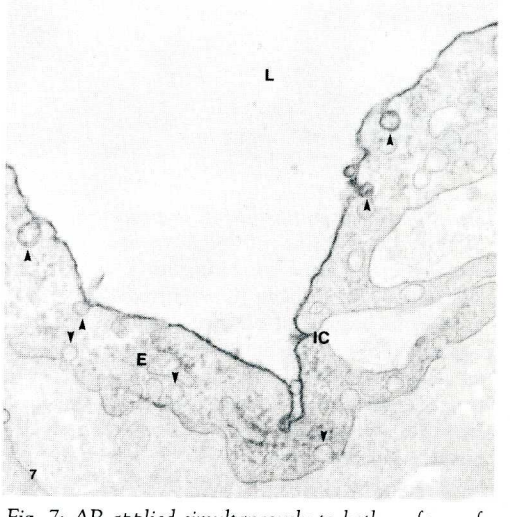


*Fig. 7: AB applied simultaneously to both surfaces of a fixed lymphatic. The difference in intensity of binding between the two surfaces is clearly evident. L = lymphatic lumen; E = endothelium; IC = intercellular channel. X 75,000*

**Ruthenium red (RR) and alcian blue (AB):** The abluminal and luminal pattern of binding of RR and AB were similar and approximated the results obtained with CF on fixed lymphatics (Fig. 7, 8). With both of these cationic stains, the luminal membrane stained intensely; abluminal membrane staining was markedly less intense, especially in the case of AB. Binding of these stains has been shown to be semi-quantitative; staining intensity may therefore be taken as a indication of charge density. Unlike CF, however, RR and AB bound to the membranes of abluminal and luminal caveolae with the same intensity of staining as the plasma membrane with which they were apparently continuous. Indeed, the vast majority of so-called cytoplasmic vesicles stained with either abluminal or luminal intensity, indicating surface attachment.

**Tannic acid (TA):** TA bound both abluminal and luminal membranes with equal intensity (Fig. 9a and 9b). As was the case with RR and AB, the membranes of both abluminal and luminal caveolae and those of many of the apparently cytoplasmic vesicles were likewise labelled.

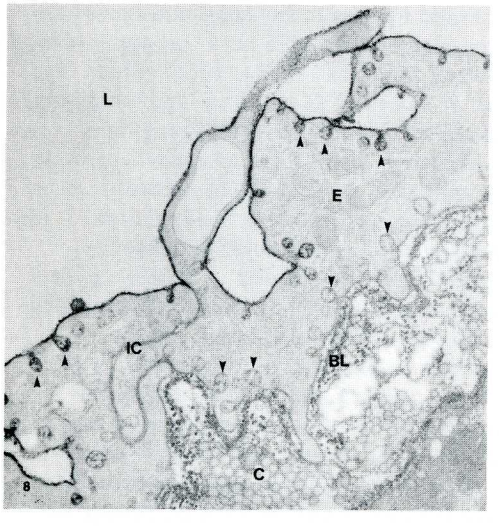


*Fig. 8: RR applied simultaneously to both surfaces of a pre-fixed lymphatic. The difference in intensity of surface and caveolar membrane staining is apparent (arrows). L = lymphatic lumen; E = endothelium; BL = basal lamina; IC = intercellular channel; C = collagen. X 110,000*

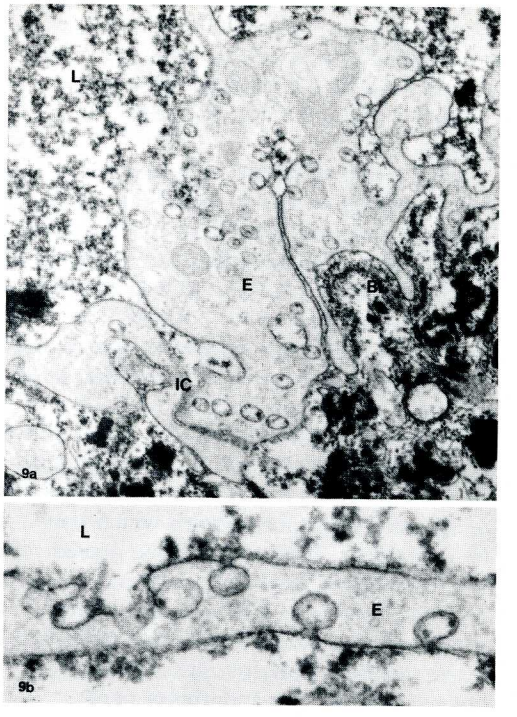


*Fig. 9a and b: Binding of TA to the surfaces of a pre-fixed lymphatic. No difference in the intensity of binding between the two surfaces is apparent. The discontinuous nature of the basal lamina is especially conspicuous in these preparations. L = lymphatic lumen; E = endothelium; BL = basal lamina; IC = intercellular channel. a X 50,000; b X 110,000*

## *DISCUSSION*

The data presented herein provide evidence for an asymmetry of both charge sign and density between the abluminal and luminal aspects of lymphatic endothelium, and reveal specific distributions of these sites relative to ultrastructural features implicated in macromolecular transport. As demonstrated by charged ferritin decoration, the luminal plasma membrane is richly invested with both cationic and anionic sites, although the latter vastly outnumber the former. No obvious topographical separation of cationic and anionic sites on this surface was apparent; both were uniformly distributed over the entire surface, excluding caveolar membranes. The abluminal plasma membrane evidenced relatively few charged

domains of any description. No cationic sites were discernible and anionic sites, as demonstrated by CF binding, were largely confined to the periphery of caveolar stomata.

The results obtained with the two cationic stains support and extend the results obtained with cationized ferritin. An important difference between the two techniques is the decoration of caveolar, as well as many vesicular membranes with both RR and AB. Aside from indicating the continuous nature of the plasma membrane, the membranes of abluminal or luminal caveolae and many of the so-called cytoplasmic vesicles, these findings indicate that the caveolar and vesicular membranes share the same sign and charge density as the membrane with which they are contiguous. While it might be argued that the reduced intensity of abluminal staining is attributable to poor penetration of these probes rather than a true difference in the number of anionic sites, the lack of any substantial extra-endothelial tissue, together with the discontinuous basal lamina and the equal intensity of staining obtained with the comparably sized TA makes this hypothesis unlikely. Exclusion of CF from the vesicular system and from intercellular channels may therefore involve additional solute-endothelial interactions, as well as charge. For example, endothelial associated glycosaminoglycans may substantially reduce the effective diameters of the caveolar stomata, thus excluding the ferritin on the basis of size. Indeed, the fiber matrix model of Curry and Michel (19) attributes primacy to the molecular sieving properties of the glycocalyx in blood capillary filtration.

Binding of the polycationic ligand, CF, has been demonstrated to result in the lateral migration and internalization of the anionic site in some endothelia, but not in others. For example, Pelikan et al (8), using both primary and transformed endothelial cell cultures from human umbilical veins, and Skutelsky and Danon (9) using guinea pig aorta and vena cava explants, observed rapid lateral migration of anionic sites; no such redistribution was observed in either mouse pancreatic (20) or pulmonary (21,22) capillaries. Whether these results reflect species differences or those associated with physiological functions dependent on vessel type is unclear. In instances where membrane mobility has in fact been demonstrated, lateral migration frequently occurs allowed by endocytosis and transendothelial transport (8,9). It has been postulated that the cross-linking of mobile anionic sites by CF induces this lateral migration as well as stimulating a complex cellular response ending in endocytosis (23,24). While CF induced endocytosis may thus in some respects mimic the interaction and fate of physiologically important ligands and their receptors, the phenomenon per se is of uncertain significance, especially since the majority of the plasma macromolecules postulated to transit the endothelium are anionic. Moreover, receptor mediated endocytosis of specific ligands has been demonstrated to involve coated vesicles (25,26); CF induced endocytosis clearly does not, as can be seen in Fig. 5.

Important similarities and differences of potential functional import are observed in the distribution of charged sites between lymphatic endothelium and that previously reported for blood capillary endothelium (see Simionescu et al, 1982 for a comprehensive review). Both endothelia possess a high density of anionic sites on their luminal aspects, which, in the case of blood vessels, are postulated to contribute to endothelia nonthrombogenicity (7) as well as function as a charge barrier which both affects and possibly effects endothelial permeability (10,20,27,28). In addition to numerous anionic sites, lymphatic endothelium exhibits a limited number of cationic sites on its luminal surface; similar coexistence of both anionic and cationic sites occurs on the luminal surface of rabbit aortic vasa vasorum (29), but not in pancreatic or pulmonary capillaries (10,21,22). Unlike blood capillary endothelium, which has a high density of abluminal anionic sites as well, lymphatic endothelium exhibits few anionic sites on its abluminal membrane, leading to a pronounced asymmetry of charge density.

Uncoated endocytotic vesicles comprise

a prominent feature of both blood and lymphatic endothelium, and as demonstrated by CF decoration, appear devoid of anionic sites in both sorts of endothelium. That this is not actually the case in lymphatic endothelium is indicated by the binding of two small cationic probes, ruthenium red and alcian blue, to caveolar and vesicular membranes. Although Simionescu et al (20) failed to demonstrate binding of alcian blue to the caveolae of pancreatic capillaries, studies using ruthenium red by Shirahama and Cohen (30) on myocardial capillaries and Chien et al (31) on arterial endothelium resulted in clear vesicular labelling. Perhaps the most significant finding to emerge from the pattern of binding of these small probes is that the membranes of caveolae and vesicles bear approximately the same charge density as the membrane with which they are apparently continuous. Thus anionic macromolecules approaching the abluminal surface of the lymphatic would encounter little endothelial charge barrier to their entrance into abluminal caveolae. Moreover, the clustering of anionic sites around the stomata of these structures might, through repulsion, serve to concentrate anionic molecules over the charge-free stomata, thereby facilitating their entrance into the caveolae and subsequent transport (21). Anionic molecules already in the lumen, however, would be repulsed by the high density of negative charges associated with the luminal membrane, and their egress prevented. The high density of negative charges clustered around the luminal infundibula of intercellular channels may likewise functionally close these structures, even in the absence of occluding junctional complexes. Thus the asymmetry of charge between the two faces of the lymphatic vessel could impart directionality to vesicular transport, favoring abluminal transport and precluding leakage. Such net unidirectionality in the transendothelial transport of protein is, of course, fundamental to lymph formation via a vesicular mechanism. The results obtained with anionic ferritin and living lymphatic endothelium in the present study are consistent with such an hypothesis.

Recent, more detailed studies in our laboratory using horseradish peroxidase (HRP), a protein of similar size and charge to plasma albumin, applied either abluminally or luminally to living isolated lymphatics show a clear adluminal directionality with respect to the transport of this protein (32). Applied luminally, it was confined to the vessel lumen and to luminally communicating caveolae. In addition to providing convincing evidence for unidirectional transport, the presence of luminally applied HRP within luminally communicating vesicles support the concept of a size-dependent component in the transport process as well.

The organization of the endocytotic vesicular system in relation to its postulated functions has recently come under scrutiny. As originally conceived, free macromolecule-laden vesicles were thought to shuttle between endothelial surfaces (33). Recent ultrastructural analyses of both blood (31, 34, 35, 36); and lymphatic (37) endothelium have, however, demonstrated that many, if not all, apparently discrete vesicles are part of complex vesicular racemes extending from either the abluminal or luminal membrane; physiological studies by Clough and Michel (38) on the earliest distribution of tracer in living mesenteric capillaries support this plan of organization. Restriction of particular membrane-associated molecular species to either the luminal or abluminal membrane, as was demonstrated in the present study, becomes mechanistically simpler in the absence of membrane mixing through vesicular shuttling. In the simplest scheme, macromolecular translocation would occur when abluminally and luminally attached vesicles fuse to form transient, short-lived thoroughfare channels. As discussed above, such channels would provide a one-way passage for anionic molecules i.e. passage into the lumen but not out of it. Long-lived transendothelial channels such as those described by Simionescu et al (17) in blood capillaries are rare in lymphatic endothelium.

A final and necessary consideration is the extent to which the present findings apply to true lymphatic capillaries. Detailed

ultrastructural studies of the canine renal lymphatic system have revealed few structural differences between intrarenal and hilar vessels (39,40); like the former, hilar lymphatics are comprised of little more than endothelium and an attenuated basal lamina. Most importantly, recent studies by O'Morchoe et al (13) have demonstrated that isolated hilar lymphatics, like "lymph-forming" lymphatic capillaries, transport interstitially (i.e. abluminally) applied protein into the vessel lumen in vitro. Moreover, nearly 50% of the protein thus transported involves a cytoplasmic, temperature sensitive mechanism such as vesicular transport. These structural and functional similarities, in conjunction with the suggestive charged tracer studies of Leak (11) and Charonis and Wissig (12), raise the possibility that charge asymmetry is a characteristic of lymphatics in general. Models seeking to explain the mechanism of translymphatic transport thus cannot ignore the possibility of endothelial charge barriers.

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