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LIGHT AND ELECTRON MICROSCOPY OF THE STRUCTURAL ORGANIZATION OF THE TISSUE-LYMPHATIC FLUID DRAINAGE SYSTEM IN THE MESENTERY: AN EXPERIMENTAL STUDY

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ABSTRACT

Supplementing vital microscopy and histophysiologylogy, we examined using combined light and electron microscopy the tissue fluid-lymphatic drainage system of the mesentery isolated from guinea pigs, rabbits, and tree shrews. In silver impregnated tissue, different types of lymphatics and blood vessels were able to be distinguished along with argyrophilic and argyrophobic structures in the connective tissue. Some initial lymphatic pathways were interrupted by non-endothelialized tissue zones thus forming separate but discrete vascular "islands". After carbon labeling of the lymphatic collectors, carbon particles were seen to escape from the initial lymphatic lumen at various sites. Electron microscopy revealed wide apertures in the lymphatic endothelial cells of these microvessels. These morphological findings support the concept of an "open" prelymphatic-lymphatic system in the mesentery. The special histometrical features exhibited by a flat membranous organ like the mesentery are discussed in terms of physiologic function of mesenteric tissue fluid transport.

The principal organization and structural basis of the origin of the lymphatic system have generally been explained in two ways. One concept views the gaps and spaces between the formed elements of the tissue ("Saftkanälchen") (1) as directly connected to the lymph vessels (2). The other regards access to the lymphatic system as restricted by a discrete endothelial membrane within the tissue (3-5). Recent findings by transmission electron microscopy (TEM) seem to favor the latter concept. This ultrastructure technique when applied to various organs reveals the extremely minute true vascular elements of the lymphatic system (i.e., the initial lymphatics) as lined by a continuous endothelium and thereby separate from prelymphatic pathways (for review, see 6,7). On the other hand, a system of small prelymphatic channels has been described in the brain and eye where true lymphatics are absent (8). Special morphological conditions and a high degree of permeability for certain substances exist at the interface between the peritoneal cavity and initial lymphatics in the subdiaphragmatic peritoneum (9). At that site, the lymphatic drainage is recognized as connected directly with the peritoneal cavity via broad stomata (10, 11).

Furthermore, a complicated system of tissue channels that range in width from a few micrometers to 50μm has been observed in the *in situ* mesentery of the rabbit and cat (12). These channels are readily visible under incomplete dark field illumination (13) and are scattered as a network over the mesentery. Some of these tissue channels often accompany

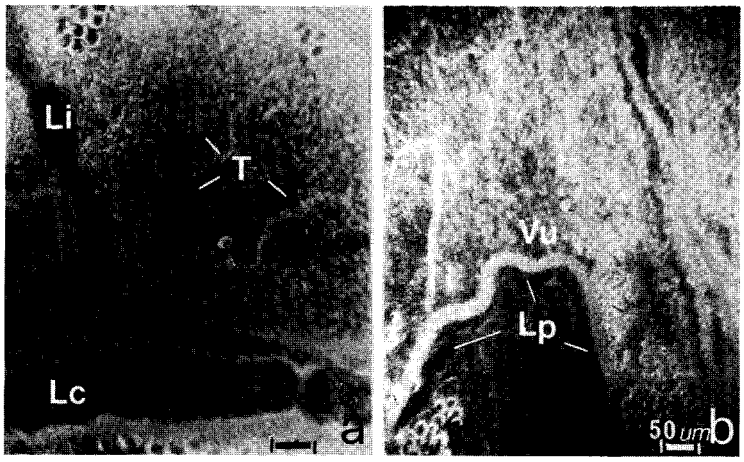

*Fig. 1. Light micrographs (**a,b**) from the rabbit mesentery. **a**) With incomplete dark field illumination, the ducts of the lymphatic drainage system are displayed as black channels while the blood vessels appear as bright granular structures. Note the network of small "tissue channels" (T) and the initial lymphatic (Li) joining a collecting lymphatic (Lc). **b**) A paravascular lymph channel (L_p) is contiguous with a collecting venule (Vu) (see Hauck, G 12,29).*

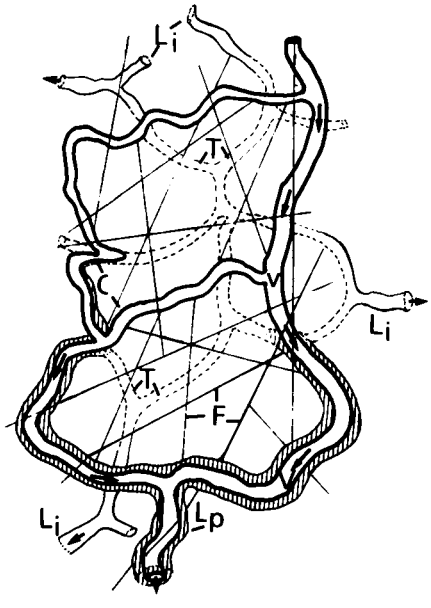

Fig. 2. Schematic diagram of the general organization of the tissue fluid lymphatic drainage system in the mesentery based on vital microscopy. Prelymphatic tissue channels (T), paravascular lymphatic channel (L_p), initial lymphatic (L_i), F=elastic fibers (also see Hauck, G 13,27).

collecting venules and small veins *(Fig. 1)*. In contrast to larger lymphatics, no endothelial lining is detected along the fine tissue channels. After injection of a fluorescent dye, the tissue channels and lymphatics of the mesentery are both highlighted and no barrier is detected between the two systems. These observations have suggested a comprehensive system of preformed tissue channels and lymphatic vessels as depicted schematically in *Fig. 2.*

Some questions, however, remain unanswered by vital microscopy. What kind of structural organization do the tissue channels of the mesentery have? Are they lined by endothelium? And is there an endothelial barrier between simple tissue channels and true lymphatics? Finally, is the lymphatic-tissue drainage system in the mesentery a special situation only representative of that organ complex?

In a preliminary study, both small and larger tissue channels adjacent to readily recognizable lymphatic vessels were seen in fixed conventionally stained mesenteric tissue by light microscopy under incomplete dark field illumination (14,15). The findings suggested that there were tissue channels both with and with-

out an endothelial lining. By TEM, it was demonstrated that some of the tissue channels displayed a discontinuous endothelium. Nonetheless, a clear determination of the relation of these tissue channels to the lymphatic drainage system of the mesentery was not possible. In the present study, the earlier approach has now been extended with more detailed examination and utilizing additional techniques. Specifically, silver impregnation of the mesentery and carbon labeling of lymphatic structures were applied. The specimens were ultimately examined by scanning and electron microscopy (SEM and TEM).

MATERIALS AND METHODS

Fourteen adult guinea pigs, four rabbits, and two tree shrews (*Tupia belangeri*) were used. Under deep ether anesthesia, the peritoneal cavity was opened and the small and large intestine were mobilized and freed from the mesenteric root. The removed organ was carefully stretched out, pinned on a polystyrol plate by hedgehog spines and bathed in warm Ringer's solution. Accordingly, investigations were done in a "supravital state". Each segment, whether mesojejunum, mesoileum, or mesocolon, were examined by light microscopy. Using incomplete dark field illumination, it was possible to identify lymphatic pathways by detecting their special morphological properties, namely, dark and empty lumina, absent or only sparsely developed wall structures, and widely varying diameters. Only those areas (1-3 of each preparation), containing lymphatic structures were further investigated.

Labeling with carbon

Carbon was injected to demonstrate the lymphatic pathways of the fresh mesentery. The marker (finely dispersed Burri ink) was administered by a special micropipette with a small tip (5-15μm) handled by means of a micromanipulator. The micropipette was inserted into the lumen of a lymphatic so that the ink easily filled the lymph vessels both retrograde to the initial part and antegrade toward the collectors. The injection pressure was controlled by a mercury manometer and calibrated up to 100mmHg. This pressure was necessary to overcome the resistance of the extremely tapered tip of the micropipette. Only a small amount of carbon solution was injected to avoid overfilling and vascular damage. After light microscopic examination and photographic recording of the injected tissue, the specimen was fixed in 2.5% glutaraldehyde for TEM and SEM. Some specimens were dehydrated in isopropyl alcohol, stained after Masson-Goldner, and mounted on slides for light microscopy.

Impregnation with $AgNO_3$

After short rinsing with distilled water, the experimental preparation was placed for 24 hrs in a dish with 1% $AgNO_3$ at 2°C. Thereafter, it was rinsed in distilled water and exposed to bright sunlight until the mesentery turned distinctly brown. The procedures of rinsing with distilled water, placing the specimen into 1% $AgNO_3$ solution (for 60 min at 60°C), and exposure to light were then repeated to improve the tissue contrast. Thereafter, after rinsing with distilled water, the specimen was dehydrated with isopropyl alcohol, cleared with xylene, mounted on a large slide, and embedded with resin (Depex) under cover glass. The examination of the whole mount preparation was carried out with a light microscope using bright and dark field illumination (for further details of the silver impregnation technique see ref. 16).

TEM and SEM

For TEM small pieces were excised from the freshly dissected mesentery and fixed with 2.5% glutaraldehyde in phosphate buffer for two days at 4°C. In some instances, the mesentery was prefixed *in situ* by injecting 1% glutaraldehyde into the peritoneal cavity before dissecting the mesentery. Portions were also obtained from mesentery with lymphatics having

been labeled beforehand by carbon. Thus, it was possible to select special areas for examination as the most distal part of an initial lymphatic, or at the zones where carbon particles had escaped from the lymphatic vascular lumen. Another place of interest examined was where a lymphatic ran closely with a blood vessel such as a collecting venule or an arteriole. The fixed specimens were washed overnight in phosphate buffer (pH 7.2) at 2°C, post-fixed in 2.5% buffered osmium tetroxide for 1 hr, dehydrated in graded-acetone up to 70% acetone, and stained with 0.5% and 1% phosphotungstic acid for 1 hr. After dehydration in acetone, the specimens were embedded in Vestopal-W, cut with glass knives on a Reichert ultramicrotome, and viewed by a Zeiss EM 9 at an accelerating voltage of 60kv.

For SEM the fixed tissue of the mesentery was divided into small pieces by using a razor blade as horizontally as possible thereby obtaining a large field of the connective tissue zone for improved view into the lumen of the tissue channel and adjacent vessels. The fragments were dehydrated in a graded series of ethyl alcohol, transferred to freon 11 and 13, dried in a critical point apparatus, mounted on stubs with conducting carbon and sputtered with gold. The specimens were observed in the Leitz AMR 1200 scanning electron microscope at an accelerating voltage of 25kv.

RESULTS

Although the histologic appearances are similar to each species, the findings in the guinea pig mesentery are primarily reported because in this rodent the mesentery is rich with lymphatics (for specific morphologic features of the mesentery, see also ref. 16).

Light microscopy of whole mount preparations

In whole mount preparations of the unstained mesentery or of those stained with common dyes, the fluid drainage system is readily seen under incomplete

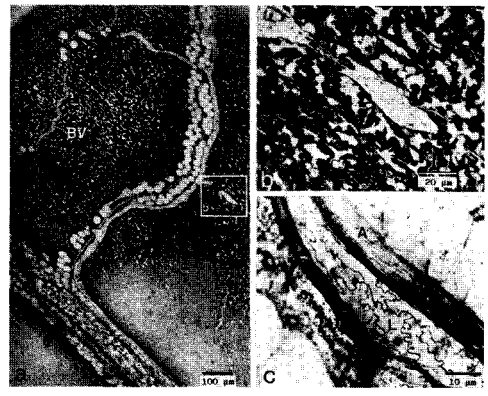

Fig. 3. ***a****: Silver-treated mesentery of guinea pig (small intestine) showing vascular structures of both the blood (BV) and lymphatic system (LV). Here, but more distinctly in* ***b*** *and* ***c****, lymphatics are identified by their typical serrated pattern of the endothelial layer.* ***b****: Enlarged area from* Fig. 3**a** *showing an initial lymphatic as a segment ("lymphatic island") separate from the main lymphatic vessels. Spotlike concentrations of silver precipitations along the otherwise finely demarcated endothelial boundaries indicate zones of open junctions. The connective tissue outside the lymphatic is composed of dark (argyrophilic) and bright (argyrophobic) structures.* ***c****: Photomicrograph of a silver-stained specimen showing an initial lymphatic (L) contiguous with an arteriole (A).*

dark field illumination. However, a clear distinction cannot be made between simple tissue spaces and true vascular structures. The mesentery treated with $AgNO_3$ displays intracellular boundaries of the endothelium as visible clear black lines *(Fig. 3**a**)*. Thus, a clear distinction is possible not only between tissue channels without an endothelial lining and lymphatic vessels, but also between different types of vessels of both the lymphatic and blood vascular system taking into account the characteristic appearance of the endothelial cells. Narrow elongated endothelial cells, for example, are characteristic of an arteriole, broad polygonal endothelial cells for blood capillaries, and rhomboid formed endothelial cells for venules and small veins. For initial lymphatics, a typical pattern consists of broad cellular territories with wavy boundaries, whereas in collecting lymphatics, small and extend-

ed cellular outlines with tapered endings are evident (17,18). Valve structures arranged at regular distances are a further feature of true lymphatics.

On the other hand, in the endothelial layer of initial lymphatics even the zones of open junctions functioning as tiny inlet valves can be recognized as spot-like structures along the endothelial borders *(Fig. 3b)*. The fiber system of the connective tissue is also clearly noted in silver-treated mesentery. The ground substance reveals two components, which markedly differ in regard to their affinity for silver. One pattern shows dark argyrophilic architecture and the other is argyrophobic. These two components probably correspond to both phases of the connective tissue ground substances often described as the "sol" and "gel" phase. At several sites, the argyrophobic tissue portion fuses directly with the lumen of the initial lymphatic and probably represents a direct link between the two systems.

In tracing the lymphatic pathways of the silver-stained tissue back to its origin, we noted that some initial lymphatics in the most distal part gradually lose their endothelial lining and finally continue into a space or channel without any distinct endothelial contour. Apart from such a situation, initial lymphatics also arise with marked bulb-like outpocketings. Small and broad outpouchings are also seen as branching structures at different locations in the course of an initial lymphatic. The endothelial line pattern of other initial lymphatics becomes interrupted for a short distance and is there replaced by structural elements of connective tissue. In the course of lymphatic zones with a well-established endothelial silver-lined pattern and those without such a marking can be seen to alternate several times. Such preparations favor the existence of "lymphatic islands" isolated from the main lymph vascular pathway *(Fig.3b)*. In its proximal part, the initial lymphatics enter the adipose tissue and run together with the supporting mesenteric blood vessels. In this area, the lymphatics are frequently found in close proximity to a venule or small vein, and sometimes to an arteriole *(Fig. 3c)*.

After injection of carbon into a mesenteric lymphatic, the tracer filled the lumen in both a backward and forward direction *(Fig. 4a)*. However, carbon

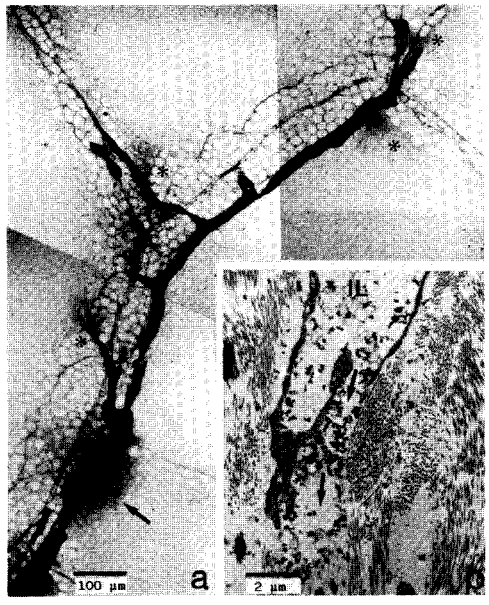

Fig. 4. a: Photographic montage of guinea pig mesentery (small intestine). The lymphatic system is labeled by carbon particles. The site, where the carbon tracer was injected is indicated by the arrow. At some places (asterisks), carbon has escaped from the lymphatic vascular lumen creating a finely dispersed pattern of carbon particles in the tissue. b: Transmission electron micrograph of a similar preparation showing an initial lymphatic (IL) with a wide, direct communication between the lumen and the tissue (arrows). A large amount of carbon has escaped from the initial lymphatic through this interface and has entered the tissue zone.

seemed to stop short of the most distal part of an initial lymphatic. At some sites, the carbon permeated the lymphatic wall spreading into the surrounding tissue. Within the tissue, the carbon did not form a homogenous mass but scattered along or between collagen fibers in a beam-like pattern emanating from the site of injection. This phenomenon was restricted to a few spot-like zones which in course of a lymphatic appeared at regularly distances. Although carbon

marking detects only a limited but larger portion of the lymphatic system, the findings correspond to those specimens examined after silver staining.

Electron microscopy

SEM cross-sections of the mesentery basically consist of two superficial sheets of peritoneum and an in between layer of loose connective tissue *(Fig. 5)*. Bundles

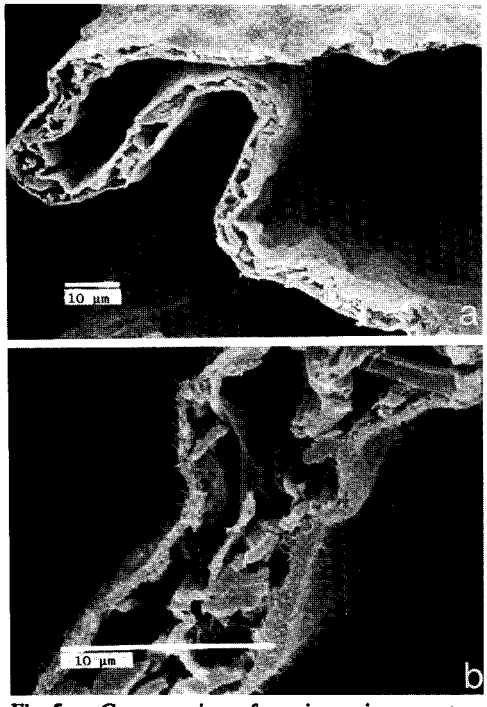

Fig. 5. a: Cross-section of a guinea pig mesentery. The tissue has been fixed in situ *by injecting 1% glutaraldehyde solution into the abdominal cavity. The two mesothelial layers covering a zone of connective tissue are well seen in this scanning electron micrograph. b: At higher magnification, many channel-like and irregularly formed spaces without an endothelial lining are found in the connective tissue, which contains various fibers, fibroblasts, and migrating cells.*

of collagen and elastic fibers traverse the zone of connective tissue in divergent directions separated by broad spaces. Although channel-like structures appear at some sites, they could not be traced over a long distance by SEM. There are numerous cells, such as fibroblasts and

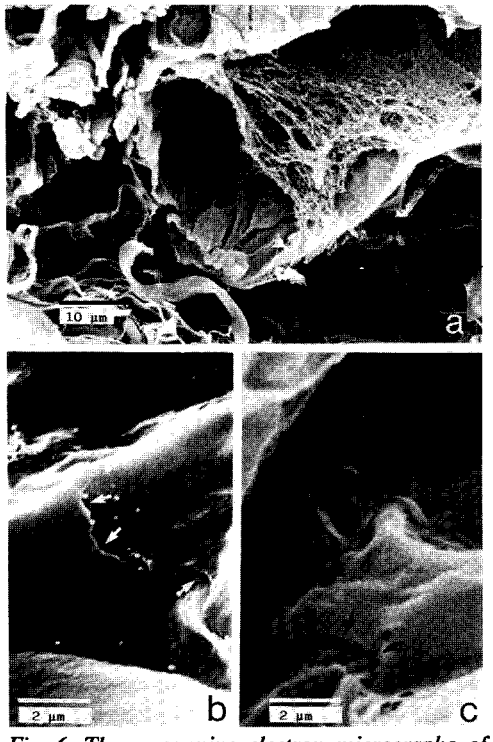

Fig. 6. These scanning electron micrographs of mesenteric connective tissue display an initial lymphatic. a: One part of the thin lymphatic vascular wall has been removed thereby allowing a view directly into the vessel lumen. Most anteriorly is seen a rough endothelial profile from protruding cells and cellular processes partly covered by flocculent residues of lymph. b: Wavy borderlines appear as characteristic elements of an initial lymphatic in this high-power scanning electron micrograph. Pocket-like structures are seen at the sites of open junctions (arrows). c: At specific sites wide openings permit a direct view of the connective tissue fiber system from the lymphatic vascular lumen (arrows).

wandering cells, throughout the tissue. Occasionally, vascular elements like blood capillaries and small lymphatics appear on the cut plane. At higher magnification, both types of vessels are readily distinguished based on their special morphologic features: i.e., blood capillaries form endothelial tubes of about 10μm or less in diameter and are lined with a compact basement membrane. In contrast, small lymphatics have widths of 15μm and greater and exhibit a very thin wall, which

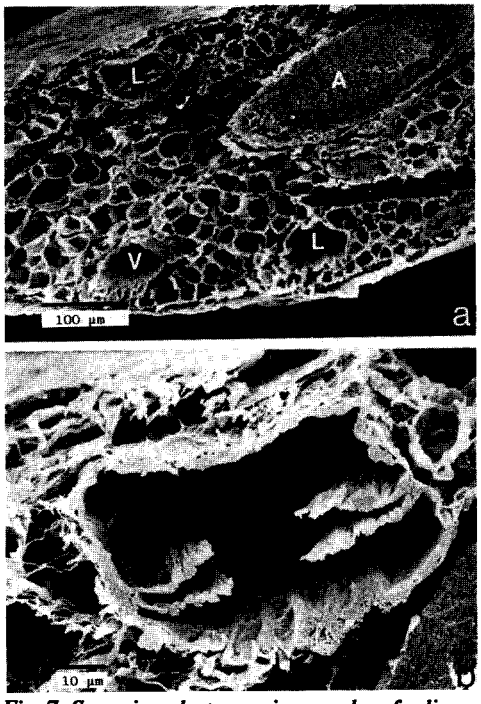

Fig. 7. Scanning electron micrographs of adipose tissue from the mesentery of guinea pig. ***a****: The structures of the supplying blood vessels (A,V) and the draining lymphatics (L) appear in this cut plane.* ***b****: Closeup of the collecting lymphatic shown in the upper part of* Fig. 7**a**. *Note the compact lymphatic vascular wall and the valve flaps protruding into the lymphatic lumen.*

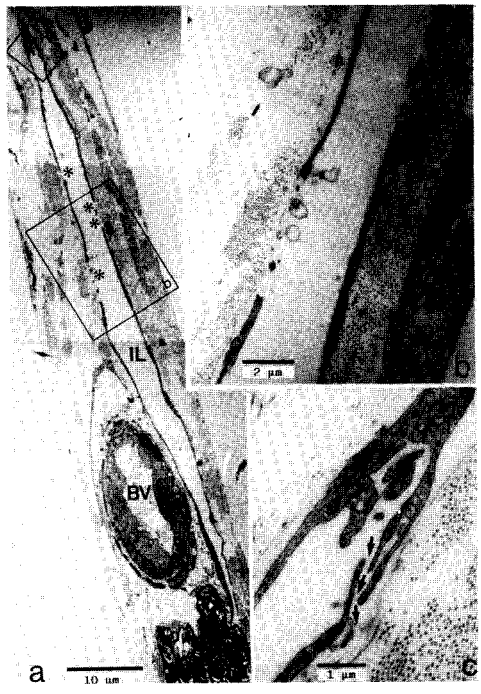

Fig. 8. ***A****: Transmission electron micrograph of an initial lymphatic of the mesentery in guinea pig. The outlines of an initial lymphatic (IL) appear in this photographic montage adjacent to a blood vessel (BV). Note the attenuated endothelial lining of the lymphatic and its sporadic discontinuity (asterisks).* ***b****: Sectional area (inset) of* Fig. 8**a** *showing open connections between the lymphatic vascular lumen and the adjacent tissue at higher magnification.* ***c****: In this enlarged area (inset) of* Fig. 8**a**, *cytoplasmic structures protruding into the lymphatic lumen can be seen. There is also an open junction (arrows) with an overlapping cytoplasmic flap.*

consists mainly of an endothelial layer covered by a sparse filamentous network. On the luminal surface the lymphatic endothelium contains many prominent cells with extending processes *(Fig. 6a)*. Between opposing endothelial cells wavy boundaries are visualized along with alternating tight and open junctions *(Fig. 6b)*. The open junctions are overlapped by cytoplasmic "flaps" thereby forming small pockets. At some places, apertures measure a few micrometers or more and occur along the endothelial boundaries so that the fiber connective tissue becomes visible from the luminal aspect *(Fig. 6c)*.

Collecting lymphatics are readily detected in the adipose tissue zone together with supporting vasculature of the mesentery *(Fig. 7a)*. The wall of collecting lymphatics is distinctly thicker than that of the initial lymphatics. There are also well established intraluminal valves in the precollectors and collectors *(Fig. 7b)*. The endothelial cells here display elongated rhomboid shapes, which resemble those of venules rather than that of initial lymphatics.

The ultrastructure of the initial lymphatic system is distinctly shown in TEM. In most parts the endothelial lining is extremely attenuated *(Fig. 8)*. The perinuclear zones protrude into the lymphatic lumen. There are also many cytoplasmic flaps and a variety of processes extending from the luminal surface. Small vesicles,

tubules, granules, and fine filaments are found in the cytoplasm. Intracellular pores are, however, not demonstrable. Tight junctions appear as structures of the intracellular border, but more commonly there are open junctions with overlapping cytoplasms *(Fig. 8c)*. Especially remarkable is the finding of broad apertures with openings up to 10μm in the endothelial lining *(Fig. 8a,b)*. With such large apertures, the initial lymphatic lumen freely communicates with the tissue space. A well defined basal lamina is not seen in this area although at some places a network of fine filaments is visible covering the outer endothelial surface of the initial lymphatic. In mesenteries where lymphatic pathways are labeled with carbon, dark particles escape only where the initial lymphatic is devoid of an endothelial lining *(Fig. 4b)*. Commonly, the carbon particles spread diffusely within the connective tissue except where the tissue space abuts directly against the initial lymphatic wall. Here, the carbon particles are concentrated and arranged in a narrow line suggesting a connecting channel between the connective tissue and the vessel's lumen.

TEM clarifies that where a lymphatic courses in close proximity to a venule or an arteriole, the facing vascular walls almost abut one another. At this site, the zone of connective tissue is reduced to barely a few micrometers. Occasionally, the basal lamina of the blood vessel is even in intimate contact with the lymphatic outer vascular wall.

DISCUSSION

The present study combining light and electron microscopy (TEM and SEM) was carried out to clarify the morphology of the tissue-lymphatic drainage system of the mesentery which still contains perplexing features for physiologists and morphologists alike. In the *in situ* mesentery, there is an intricate network of fluid draining channels which connect to initial lymphatics without restrictive barrier (12). This observation, however, is difficult to reconcile with the common notion that the lymphatic vascular system is blindly closed against the interstitium by a continuous endothelial barrier (6,7,19). If that were true, fluorescent dye should enter the mesentery from the blood circulation, spread diffusely throughout the connective tissues and reach the initial lymphatics from different directions. Vital microscopy, however, shows that stained interstitial fluid moves obviously along preformed prelymphatic-lymphatic ducts which represent the key part of the "low resistance tissue pathways" (20). There is no doubt that this system also exists in isolated supravital and fixed tissue. In these specimens, however, only true vascular structures (i.e., initial lymphatics, precollectors, and lymphatic collectors) are clearly delineated by light and electron microscopy and, after labeling with carbon, can be examined at specific sites of interest. Although initial mesenteric lymphatics display morphologic features which correspond to initial lymphatics of other body regions (14,20-23), the presence of comparatively large apertures between tissue spaces and the tissue lymphatic lumen seems particularly conspicuous in the mesentery. This finding documented by TEM and SEM explains both the escape of carbon particles into the tissue in the injected supravital specimens and the vital microscopic observations of fluid passage freely from the minute tissue channels into an established vascular system. In this context, the origin of the mesenteric lymphatic system can be regarded as "open" towards the interstitium although the precise morphologic nature of the prelymphatic-lymphatic interface could not be elucidated in other places, as "prelymphatics" the nonendothelialized spaces and channels of the interstitium which ultimately join true lymphatics have been defined (24-26). Neither silver impregnated tissue under light microscopy nor the specimens of the mesentery studied by electron microscopy structurally differentiate these tissue spaces, so that they can be referred to with certainty as prelymphatic channels. Because, however, most connective tissue ground substance has been removed in

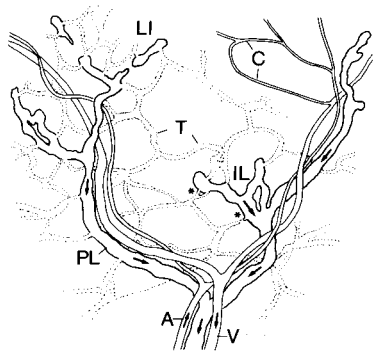

Fig. 9. Diagram of the fluid drainage system of the mesentery according to histological and electron-optical studies. T=tissue channel, LI=initial lymphatic, IL=isolated initial lymphatic segment, PL=paravascular lymphatic, C=blood capillary, A=arteriole, V=collecting venule. Sites where tissue channels communicate with the lumen of the initial lymphatics are indicated by an asterisk.

fixed-dried specimens, the microstructure of the prelymphatic channels in mesentery is not necessarily comparable to tissues *in vivo*. Thus, the argyrophobic areas flanked by argyrophilic zones in silver-treated mesentery do not fully correspond to a system of low and high resistance tissue-lymphatic pathways. Other special histochemical studies are needed. TEM of "tissue channels" that appear in dark field as nonendothelialized "twigs" of a carbon-labeled lymphatic generally reveal that most of these channels are already small endothelial lined tubes (for further detail, see 16).

Based on the silver-stained mesentery, the appearance of initial lymphatics as "vascular islands" should be stressed. Whether these structures relate to ongoing proliferation of vascular units which at an advanced stage fuse to form a final continuous lymphatic, or, alternatively, are portions of the prelymphatic channels already lined by endothelium and thus have the characteristics of a true lymphatic is not resolvable at this stage.

The phenomenon whereby a lymphatic runs in close proximity to a venule or arteriole needs to be examined in light of the hypothesis that plasma proteins can pass from a post-capillary venule directly into a lymphatic without diffusing through the interstitium (27,28). This physiologic concept is supported by light and electron microscopic findings of the present experimental study. The overall architectural arrangements of prelymphatic channels, lymphatics, and blood vessels of the mesentery based on current histology is shown in *Fig. 9*. In general, there is a remarkable conformity between the diagram shown in *Fig. 2* deduced formerly from vital microscopy and that of *Fig. 9*. The latter scheme, however, shows other direct interconnections between the interstitium and along the entire course of initial lymphatics before joining the collecting lymphatics.

In a more general sense, the mesentery is an organ of extreme thinness and may exhibit its own peculiar conditions for fluid interchange in the connective tissue and may not necessarily be comparable to fluid exchange in a solid organ. In other words, the special spatial dimensions of an organ need to be taken into account as important factors regulating topography, distribution, and cellular differentiation of the tissue-lymphatic fluid drainage system. The thin mesentery without muscular elements with sparse blood vessels may have limited fluid-exchange capacity compared with other large organ systems. On the other hand, propulsive features missing in mesentery may be offset by a highly permeable and even partly incomplete lymphatic endothelium thereby facilitating the movement of liquid along the prelymphatic-lymphatic system whenever microvascular fluid overload develops. Similarly, spontaneous contractile behavior of bulb-like initial lymphatic structures described in the bat wing (28,29) can be interpreted under the functional aspect. For flat bat wing tissue, this is an alternative way to facilitate the movement of tissue fluid and lymph in a well-defined membrane organ system.

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