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Lymphatic Removal of Fluids and Particles in the Mammalian Lung

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The structure and distribution of pulmonary lymphatics and their permeability to fluids and particulate materials have been investigated in the lungs of rats following fixation by combined intratracheal and vascular perfusion. In such preparations, the lymphatics remain in a distended state, and a close relationship to other structural components of the pulmonary interstitium is maintained. They were identified in regions with an abundant amount of connective tissue, forming an elaborate plexus within the pleura, the interlobular septum, peribronchial and perivascular areas. Recent data have shown that water-soluble molecules and particulate matter are removed from the interstitium along the lymphatic capillary (initial lymphatics) segment. It is distinguished by attenuated endothelial cells with extensively overlapping cell margins which are easily separated. We have studied this segment of the lymphatic vascular system following intratracheal injections of colloidal particles (ferritin and carbon) to determine the structural features responsible for the transport of large molecules and particulate materials across the lymphatic endothelial wall in the lung. The results showed that the tracer particles cross the lymphatic endothelial wall via the clefts of intercellular junctions. While the tracer particles were observed within vesicles, the question of transport across the lymphatic endothelium via plasmalemmal vesicles is still not settled since the number and size of vesicles containing tracer particles also increased with time. Intravascular injected dextran was also localized within the clefts of intercellular junctions and plasmalemmal vesicles. The results obtained with intratracheal and intravascular injected tracer substances are consistent with those observed in lymphatic capillaries for other tissues.

Physiologists have long realized the importance of the lymphatic vascular system in the removal of fluids, proteins, particulate components and cells that are not reabsorbed at the venular limb of the blood vascular system (*1*). Likewise, information from a number of studies has established that the lymphatic system serves primarily as a transport system designed to maintain homeostasis of the interstitial environment by draining excess fluids and proteins from the interstitium for its return to the blood stream (*1-6*). The importance of the lymphatic system lies in the fact that even normal blood capillaries are permeable to macromolecules in proportion to their molecular size and that these molecules, particularly proteins, disrupt the normal Starling pattern of exchange between capillaries and tissues. If left undisturbed, this would lead to a considerable accumulation of fluid within the interstitial spaces leading to edema and altering the hemodynamics of tissue fluids (*1*). By cannulating the large lymphatic trunks which drain various regions, much information has been obtained regarding the overall contribution of lymphatics, not only in the maintenance of fluid homeostasis for the various tissues throughout the body (*5, 7-9*), but also in regards to its role in the removal of hormones (*10*), of enzymes (*11-14*), of lipoproteins (*6*), as well as of cells (*15, 16*).

Although we now have some ideas regarding the overall contribution of the lymphatics to the maintenance of fluid homeostasis in various tissues of the body, the precise topographical organization, and ultrastructure of this drainage vascular system within the lung still remain unclear.

Notwithstanding the voluminous literature on the anatomy and pathology of pulmonary lymphatics, there have been few definitive studies at the ultrastructural level which consider: (a) their precise topographical arrangement and the ultrastructural basis of interstitial fluid and particulate removal, (b) the structural basis for lymph formation in the lung and the subsequent propulsion of lymph in a uni-

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directional stream toward collecting vessels, (c) their participation in pulmonary defense mechanisms and involvement in the genesis, and dissemination of various respiratory disease processes including the metastasis of lung cancer.

Just as there is a rich plexus of lymphatic vessels distributed throughout other areas of the body in which there is an abundant amount of connective tissue, the same is true for the lungs (*17*). In our efforts to gain a new perspective on the old problem of the structural organization of pulmonary lymphatics and their role in the removal of excess interstitial components, we have employed improved techniques of tissue preservation and combined transmission and scanning electron microscopy to study the topography and ultrastructure of pulmonary lymphatic vessels.

Materials and Methods

The lungs used in the study were from rats, mice, dogs, and guinea pigs. However, most of the illustrations in this presentation are from the lungs of rats. In an attempt to determine the fine structure of pulmonary lymphatics and their precise distribution within the pulmonary interstitial areas and their close proximity to the alveoli, use was made of combined intratracheal and vascular perfusion methods of fixation.

Surgical Procedure

For control animals, rats were anesthetized with an intravenous injection of sodium pentobarbital. The heart was exposed through a median sternotomy incision and heparin was injected intravenously. Saline containing heparin, and procaine were perfused through the lung by inserting a number 260 polyethylene tubing into the pulmonary artery through the right ventricle. The lungs immediately blanched as the saline displaced the blood in the pulmonary blood vessels. After 2-5 min, the saline was replaced with the fixative which consisted of a glutaraldehyde-formalin mixture (*18*) of 2.5% glutaraldehyde in phosphate buffer. At the same time that fixative was being perfused into the blood vessels a number 29 gauge needle was inserted into the trachea and the airway perfused with fixative. After perfusing the lung with fixatives for 15-30 min, the hardened lungs were resected and placed in fixatives for an additional 2 hr. The lungs were cut into small pieces and subsequently processed for transmission and scanning electron microscopy.

Experiments with Colloidal Ferritin and Carbon

In order to follow the movement of intratracheally instilled particles in the lungs of rats, suspensions of colloidal ferritin (~ 80 Å diameter) or colloidal carbon (~ 350 Å diameter) were administered via the trachea. After periods of 5, 15, and 30 min and 1, 3, 6, and 24 hr, the lungs were perfused as discussed above and small pieces processed for transmission electron microscopy.

Dextran Experiments

Dextrans of varying molecular weights (60,000 to 300,000) were injected via the saphenous veins of young adult rats. At 5, 15, 30 and 60 min, the lungs were perfused with saline followed by glutaraldehyde-formalin mixture in phosphate buffer at pH 7.4 and at 0°C (*19*).

Preparation of Lungs for Scanning Electron Microscopy

Following perfusion fixation, lungs were cut into small sections and post-fixed in 1% OsO_4 at 5°C for 1 hr, rinsed, and dehydrated in a graded series of alcohols to 100% and gradually infiltrated with Freon 113. The specimens were then processed by the critical point drying method (*20*, *21*) with Freon 13 with the use of a Bomar Critical Point drying machine. After drying, specimens were mounted on stainless steel studs with silver conducting paint and subsequently coated with a thin layer of carbon followed with a coating of gold palladium in a Hummer Coating machine. Specimens were observed in an ETEC-Auto-Scan scanning electron microscope.

Results and Discussion

Topography and Ultrastructure of Pulmonary Lymphatics

In order to provide a cohesive account of pulmonary lymphatics in relation to their role in the dynamics of lymph formation and the removal of particles and cells from the pulmonary interstitium, it is crucial to give a portrait of the lymphatic vascular system as it relates to the movement of fluids, plasma proteins, and cells across the blood-interstitial-lymphatic interface and the air-interstitial-lymphatic interface in the lung.

In considering the general organization and morphology of pulmonary lymphatics these vessels can be classified into two categories. There is a superficial plexus and a deep plexus of vessels which, unlike the accompanying blood vessels, do not comprise a circulatory system, but a system which subserves the lungs by providing a one way drainage

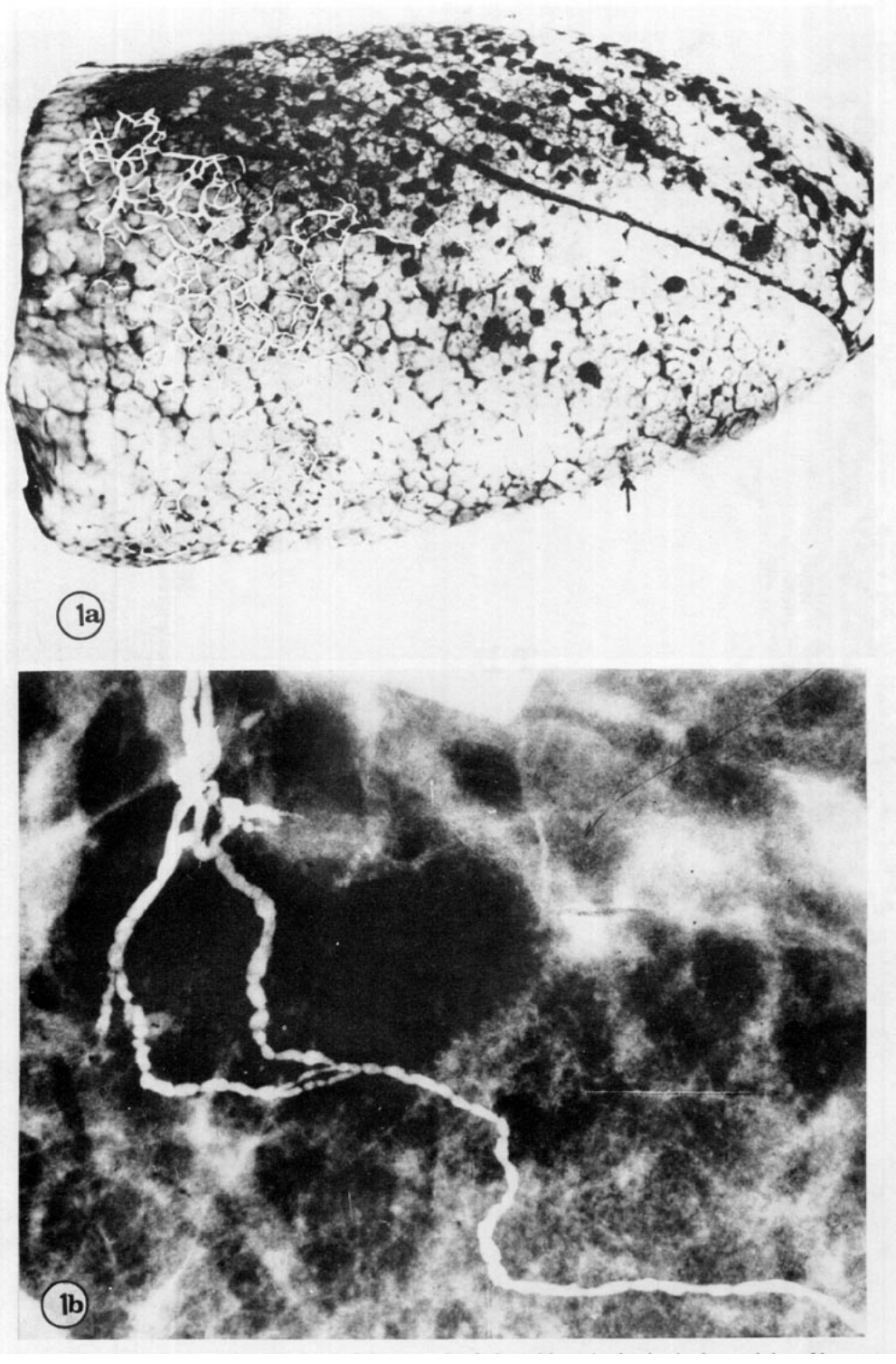

FIGURE 1. (*a*) Radiograph illustrating a rich network of pleural lymphatics in the lower lobe of human lungs; (*b*) the periodic constrictions along the length of the vessels indicate the location of valves. Data of Trapnell (*22*).

FIGURE 2. Light micrograph which shows the position of lymphatics (L) in relation to a terminal bronchiole (TB) and the adjacent alveoli (*). × 900. Data of Leak (25).

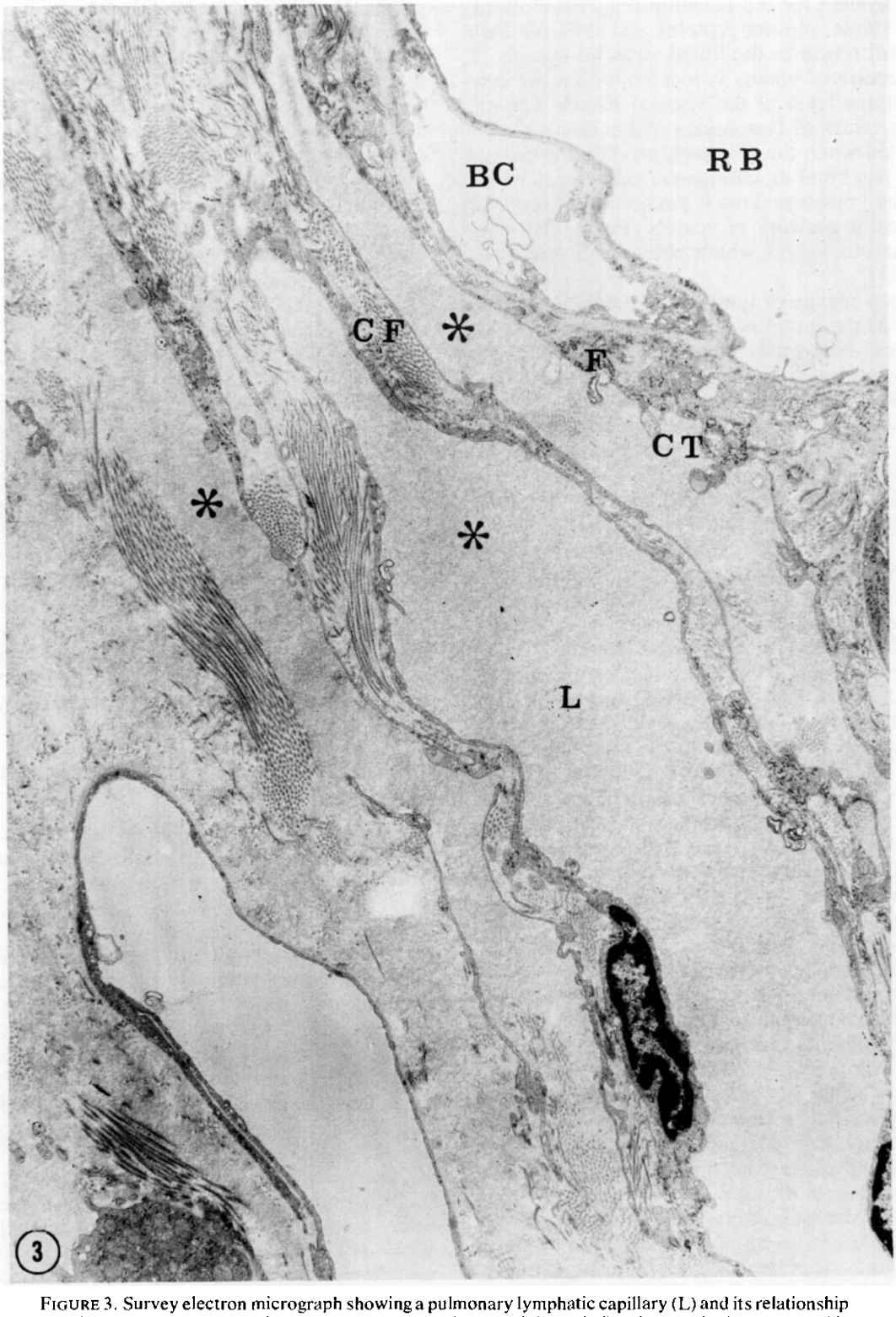

FIGURE 3. Survey electron micrograph showing a pulmonary lymphatic capillary (L) and its relationship with a respiratory bronchiole (RB). A grey flocculant precipitate (*) fills the lymphatic lumen, and is also located in the connective tissue. The lymphatic capillary is separated from the respiratory bronchiole by a blood capillary (BC) and a band of connective tissue (CT) which contains a thin process from fibroblasts (F) and collagen fibrils (CF). × 7000.

vascular system for the constant removal of extravascular fluids, plasma proteins and cells for their subsequent return to the blood vascular system.

The superficial plexus is located within the connective tissue layer of the visceral pleura. The extensive network of lymphatics within this region is appreciated when these vessels are filled with vital dyes (trypan blue) or radiopaque substances which outline the vessels making it possible to observe an anastomosing network of vessels (Fig. 1) that contain numerous valves which point in all directions (*17, 22, 23*).

The deep plexus of lymphatic vessels is also referred to as the intrapulmonary or parenchymatous lymphatics. It consists of an interconnecting network of vessels which surround bronchi, pulmonary arteries and veins. It is also generally agreed that a rich plexus abounds within the interlobular septa. These vessels are thought to provide interconnections between the deep and superficial lymphatics as indicated by a flow of injected dyes from the pleura to the deep parenchymatous lymphatic plexus (*22, 24*). Although it is generally agreed that lymphatics abound within the connective tissue sleeves surrounding pulmonary blood vessels and bronchi the existence of alveolar lymphatics or their presence at the air-blood-barrier is still unclear.

By using improved micro-injection techniques combined with vascular perfusion, a rich plexus of lymphatics can be identified within the connective tissue sleeve which extends to the terminal and respiratory bronchioles. These lymphatics are also juxtaposed to alveoli and are separated from the air space only by the alveolar epithelium and a thin layer of adjoining connective tissue. At this level of the bronchial tree, the lymphatics are of a smaller caliber than those surrounding the large bronchi. The lymphatics in these regions are extremely thin-walled and are situated at the sites of fluid formation which if left to accumulate would lead to pulmonary edema (*24*). The smaller and thin-walled lymphatics (10 μm in diameter) represent the initial segment of the pulmonary lymphatic vascular system, i.e. lymphatic capillary. Although the continuous layer of endothelial cells is extremely attenuated, many areas between adjacent cells lack adhesion devices. Therefore, adjacent cells are easily separated and can readily accommodate the movement of interstitial fluids and particles into the lymphatic lumen. Therefore, from a functional standpoint pulmonary lymphatics begin as blind end saccules or tubes within a thin band of connective tissue at the level of terminal and respiratory bronchioles.

The unidirectional flow process begins at the tissue-lymph interface with the uptake of interstitial fluids and plasma proteins by the smallest and more permeable vessels, the lymphatic capillaries. From these channels, the lymph is propelled into an extensive system of collecting vessels whose continuity is frequently interrupted with lymph nodes that serve as a filtering or screening system. These vessels contain valves which prevent the regurgitation of lymph. The collecting vessels usually follow the overall distribution of the arteries and veins within the lung. Lymph drained from the collecting vessels enters the main lymphatic vessels, which drain toward the hilar regions of the lungs.

Upon superficial examination of respiratory bronchioles and the adjoining alveoli in paraffin sections, the general organization of the connective tissue is unimpressive. However, on closer inspection of 1 μm-thick Epon sections, a plexus of lymphatic vessels can be detected at the light microscopic level (Fig. 2). The content of precipitated lymph along with occasional lymphocytes or macrophages make it easier to differentiate lymphatics from blood vessels. Electron micrographs of perfused lung tissue confirm the presence of precipitate within the lymphatic lumen (Fig. 3). In addition, the irregularity of its wall and close topographical relationship to the adjoining alveolar space (saccule) is also appreciated. In such areas the lymphatic capillary is separated from the air space by the alveolar wall and a thin band of connective tissue. In some areas blood capillaries may be interposed between the lymphatic vessel and alveolar saccule.

Ultrastructure of Pulmonary Lymphatic Capillaries

The endothelial cells of the pulmonary lymphatics, like those in other tissues, are extremely attenuated over large areas. At this level of organization, these vessels resemble lymphatic capillaries as no smooth muscle cells were observed in their walls. In addition, they lack a continuous basal lamina and there were numerous anchoring filaments that extended into the surrounding connective tissue (Fig. 4). The adjacent cell margins are extensively overlapping, and the intercellular cleft is of variable widths and occasionally accommodates the passage of lymphocytes from the interstitium (Figs. 5 and 6). It is likely that plasma proteins, as well as other cells, also gain access to the lymphatics in this manner.

Other salient features of the endothelial cells include numerous plasmalemmal invaginations, and many microfilaments occur throughout the cytoplasm (Fig. 7). Recent studies of Lauweryns et al, (*26*) demonstrated that the 5-6 nm filaments in pulmonary lymphatic endothelial cells formed the characteristic arrowhead complexes when reacted with heavy meromyosin, which suggest that these filaments represent actin.

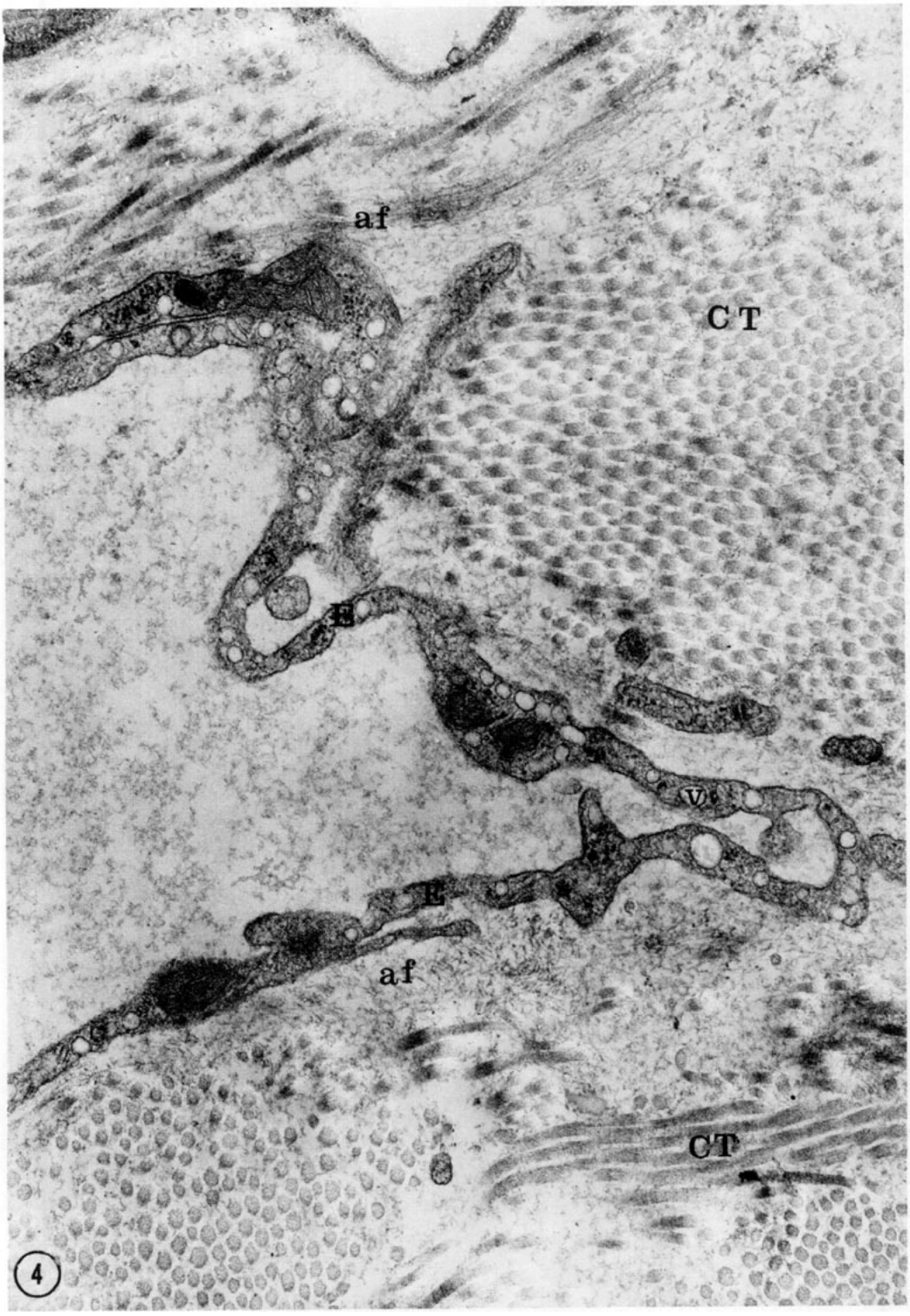

FIGURE 4. Portion of a pulmonary lymphatic capillary illustrating its close relation to the adjoining connective tissue (CT). Numerous anchoring filaments (af) extend from the abluminal surface and project into the surrounding connective tissue. The endothelium (E) contains plasmalemmal invaginations and vesicles (v).

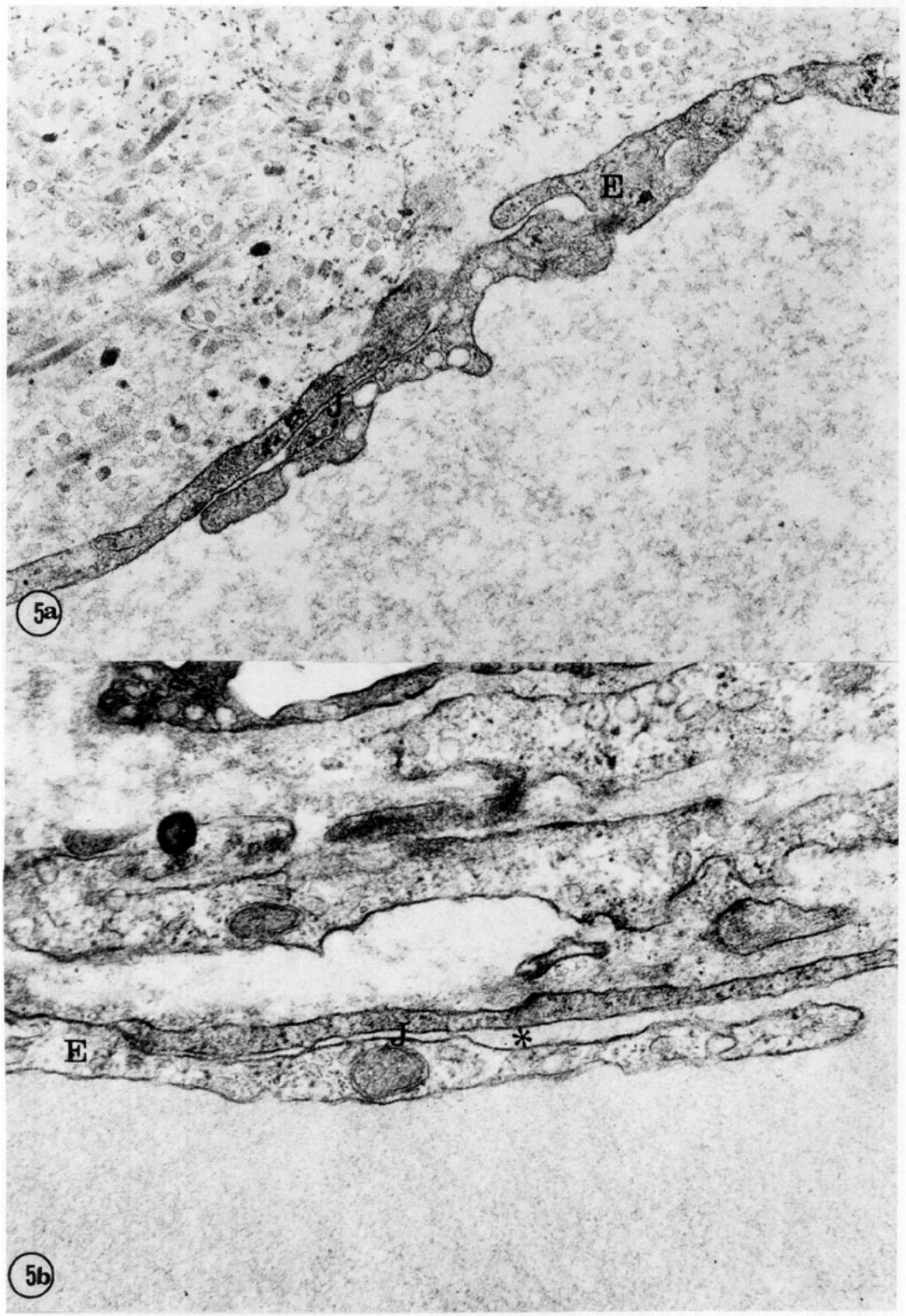

FIGURE 5. Extensive overlapping of adjacent endothelial cells (E) at intercellular junctions (J) is illustrated in these electron micrographs. The width of the intercellular cleft (*) is also variable. Both × 56,000.

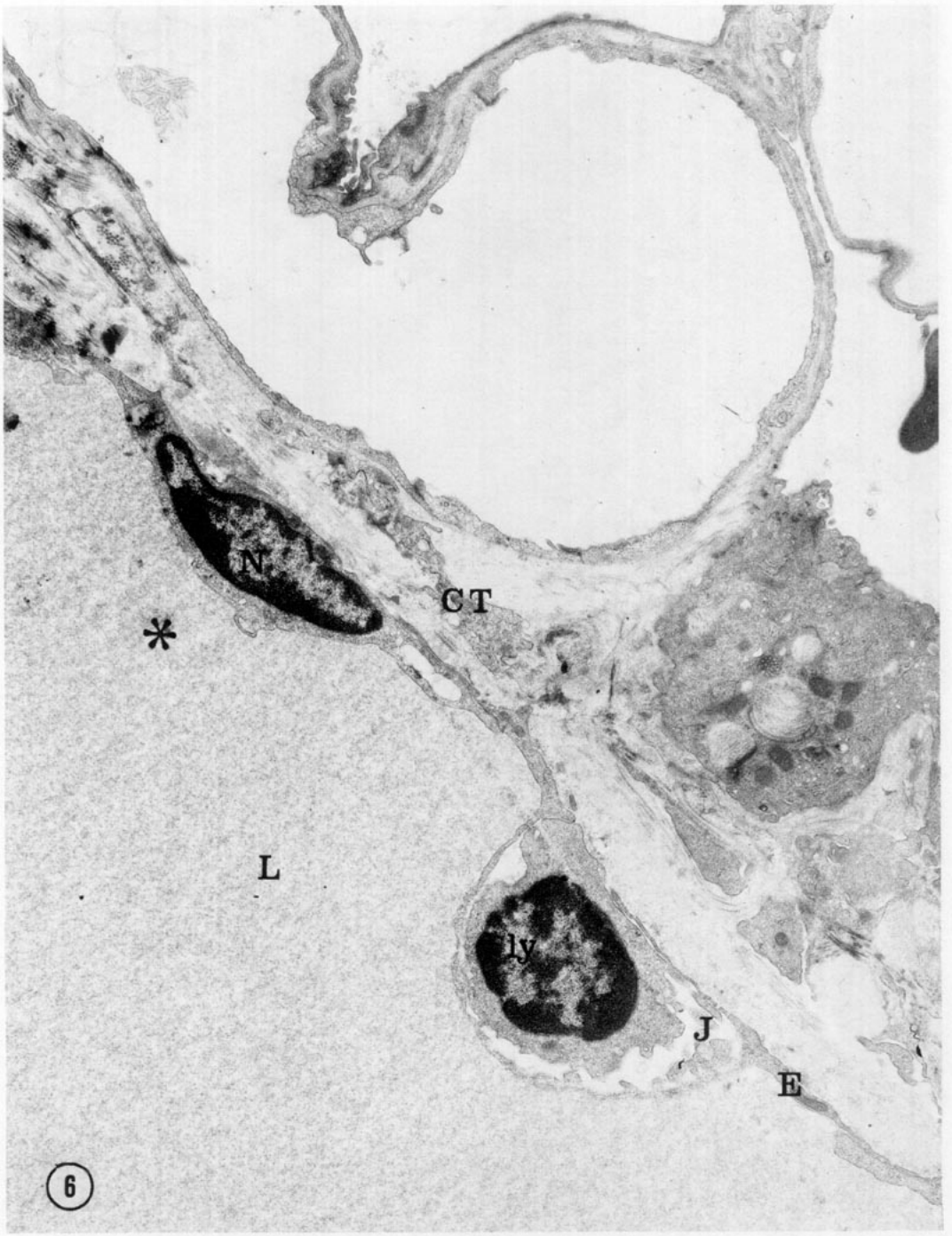

FIGURE 6. Portion of a juxta-alveolar lymphatic capillary (L) whose lumen is filled with an electron dense precipitate (*). A lymphocyte (ly) is located within the cleft of an intercellular junction (J). The lymphatic endothelium (E) is extremely attenuated except in areas occupied by the nucleus (N). The lymphatic capillary is separated from the alveolar sac by a thin band of connective tissue (CT). × 7600.

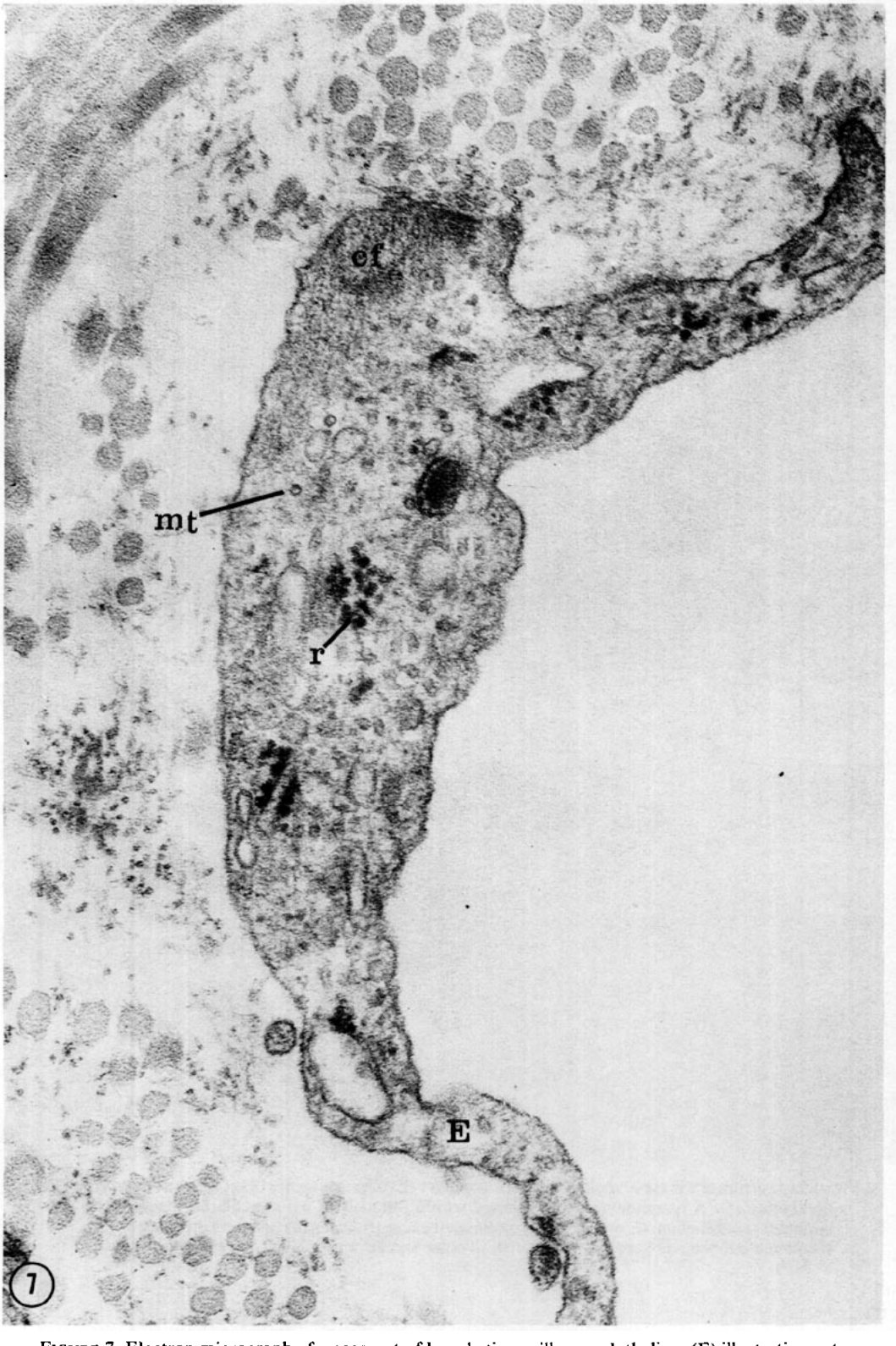

FIGURE 7. Electron micrograph of a segment of lymphatic capillary endothelium (E) illustrating cytoplasmic filaments (cf), microtubules (mt) and ribosomes (r). × 77,700.

Pulmonary Collecting Lymphatic Vessels

Proximal to the terminal bronchiole the thickness and complexity of the surrounding sleeve of connective tissue are gradually increased. Likewise, the plexus of peribronchiolar and perivascular lymphatics also become more elaborate (Figs. 8 and 9). The lymphatic vessels have a larger diameter and a thicker wall consisting of a continuous layer of endothelial cells which are held in close apposition by maculae adherents. The wall of the larger vessel is distinguished by the periodic occurrence of valves. The valves consist of leaflets of endothelial cells which project into the lumen as folds (Fig. 10). The two layers of cells are separated by a thin band of connective tissue consisting of collagen and elastic fibrils and an occasional fibroblast (Fig. 10). The regular spacing of valves and constriction at the base of each gives a beaded appearance which is characteristic of the distended lymphatic collecting vessel. When observed in the scanning electron microscope the bileaflet nature is readily apparent in three dimensional relief (Figs. 9 and 11). It is evident that the endothelial cells are reduplicated as a double layer of cells which encircle the lumen of the vessel. The folds are separated along the midline to form paired leaflets which appear as two thin cusps. The surface of each leaflet is lined with flattened endothelial cells. The leaflets project into the lumen at an angle such that their free edges fit together as a miter joint without fusing with each other (Fig. 11). The valves project into the lumen in the direction of fluid flow, which provides for a free movement of lymph toward the larger vessels and lymphatic trunks.

The wall of the collecting vessel is also distinguished by the presence of smooth muscle cells. Located in the tunica media, the smooth muscle cells may vary from single cells arranged in an incomplete spiral, to several complete layers of smooth muscle cells (Fig. 12). The smooth muscle cells are connected to each by communicating (gap) junctions and make contact with the endothelium by myoendothelial intercellular junctions (Fig. 13).

Trace Experiments

Pulmonary lymphatics also share the distinction of engulfing large molecules and particulate matter from the surrounding interstitium. Advantage is taken of this phagocytic property, not only to label pulmonary lymphatics, but also to ascertain the mechanisms involved in the movement of fluids and large particulate substances across the air-tissue-lymph interface as well as the blood-tissue-lymph-interface.

In an attempt to monitor the uptake of colloidal particles from the alveolar spaces, ferritin and colloidal carbon suspensions were instilled into the trachea of young adult rats. In samples of lungs observed at 15 and 30 min after tracer was instilled in the trachea, large amounts of ferritin and carbon could be seen in the lumen of the bronchial tree (Figs. 14 and 15). Tracer particles were also observed in vesicles within alveolar macrophages and squamous cells of the alveolar epithelium (Fig. 16). At time periods of up to 3 hr, the tracers were observed within vesicles in macrophages located in the interstitium and in vesicles within the lymphatic endothelium (Figs. 17 and 18). For periods of up to 6 months following intratracheal injections of collodial carbon the tracer was observed as large accumulations within autophagic vacuoles in the lymphatic endothelium as well as in macrophages located in the lymphatic lumen and in pulmonary lymph nodes. Although ferritin particles were observed free within the interstitium, this was not the case with the carbon tracer, which was observed within vesicles in macrophages and lymphatic endothelium.

Intravascular Injection of Tracer Substances

Although interstitial injections of tracer substances permit the uptake of tracer to be first removed by the lymphatics before their appearance is observed in the blood vascular system, the problem still exists for the development of some trauma due to unphysiological pressure in the vicinity of the injection site in the case of interstitial injection, or the production of a mild inflammatory response as indicated by the immigration of macrophages and neutrophils to the site of irritation. This possibility is overcome by using intravenous injections of tracer particles or of substances which can be rendered electron dense for visualization in the electron microscope by chemical means once the tissue has been fixed and processed. This difficulty can be avoided by using the procedures of Simionescu and Palade (*19*) in which dextran is injected intravenously. Dextrans of varying molecular weights (60,000 to 300,000) were injected via the tail or saphenous veins in young adult rats. It is well documented that intravascular injected dextran is able to pass across the blood capillary wall and is removed from the interstitium by lymphatic vessels for return to the systemic circulation (*4*). In order to observe dextran mainly in the interstitium and the lymphatic vessels, the lungs were perfused with saline to free the blood vessels of injected dextran solutions, leaving the tracer in the interstitial areas and within lymphatics. After fixation with a glutaraldehyde-formaldehyde mixture in phosphate buffer at a pH 7.4 at 0°C (*19*), dextran particles are retained in a homogenous distribution in the plasma

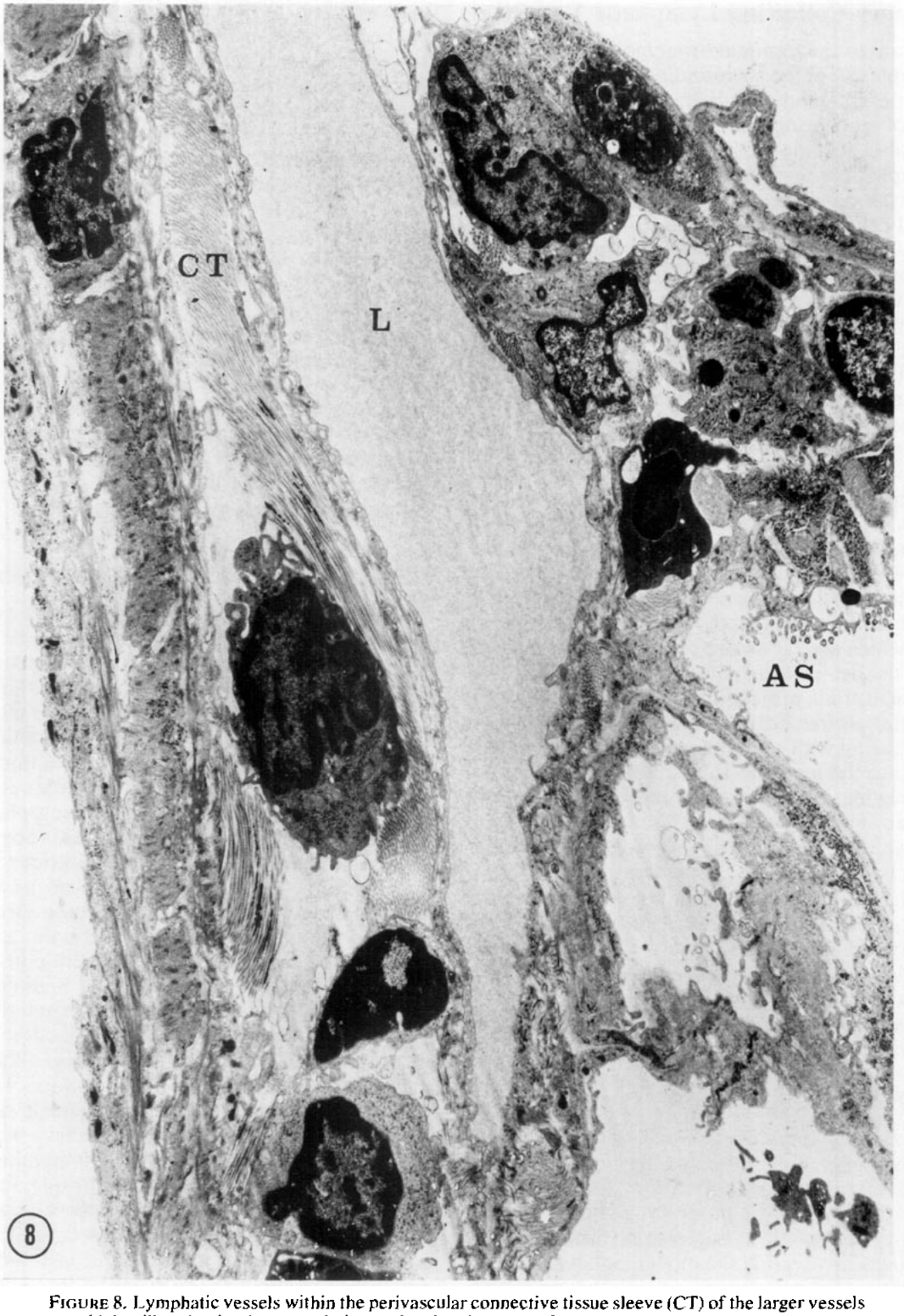

FIGURE 8. Lymphatic vessels within the perivascular connective tissue sleeve (CT) of the larger vessels which still maintain close proximity to the alveolar sac (AS). × 7350.

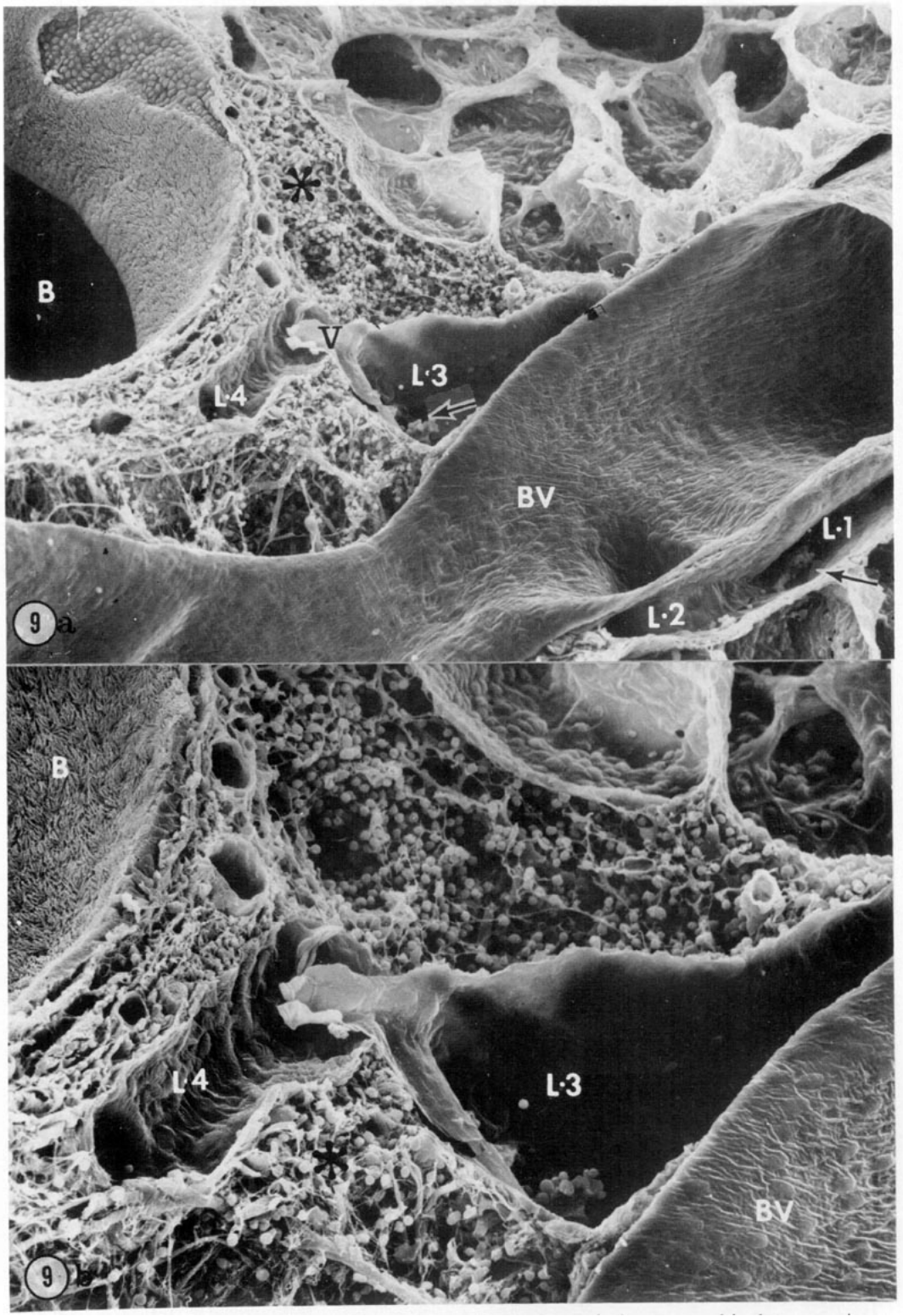

FIGURE 9. The branching of peribronchial and periarterial vessels is demonstrated in these scanning electron micrographs. Parts of lymphatic vessels are seen at L1 and L2 which contain a clump of cells (arrow). L3 also contains a clump of cells (arrow) and forms an anastomosis with L4 by way of a valve (V). Lymphoid tissue (*), similar to that observed in scanning images of lymph nodes surround the lymphatic vessels. Bronchial (B) and blood vessel (BV) are as marked. (*a*) × 250; (*b*) × 500. Data of Leak (*25*).

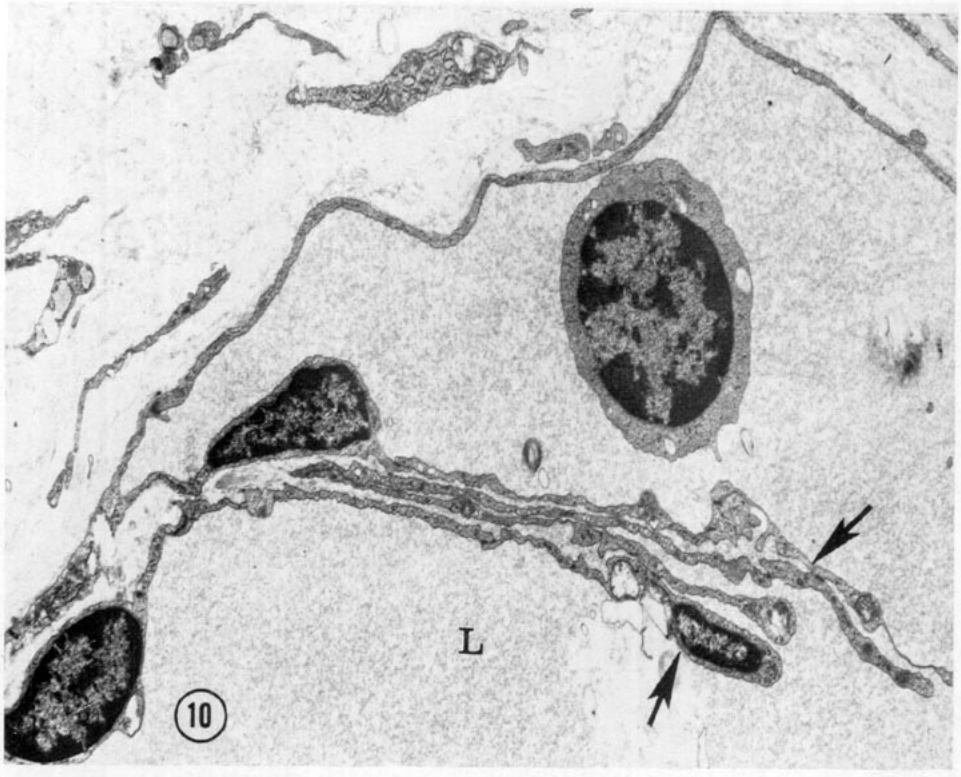

FIGURE 10. Electron micrograph of collecting vessels illustrating the appearance of valves (arrows) which arise as a reduplication of the endothelium which folds into the lymphatic lumen (L). (*a*) × 3000; (*b*) × 8000. Data of Leak (*25*).

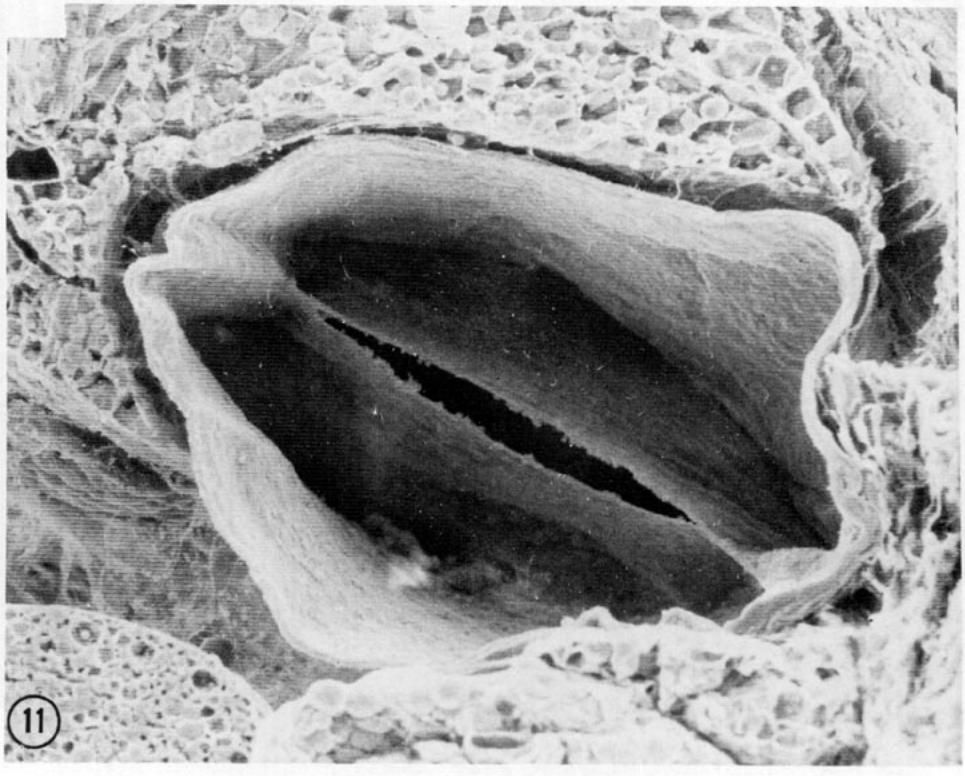

FIGURE 11. This scanning electron micrograph illustrates the appearance of a valve as seen in a cross section of a lymphatic duct. A pair of leaflets extend from the wall in a circumferential fashion and project into the lumen of the vessel at such an angle that their free edges fit together like a miter joint. × 240.

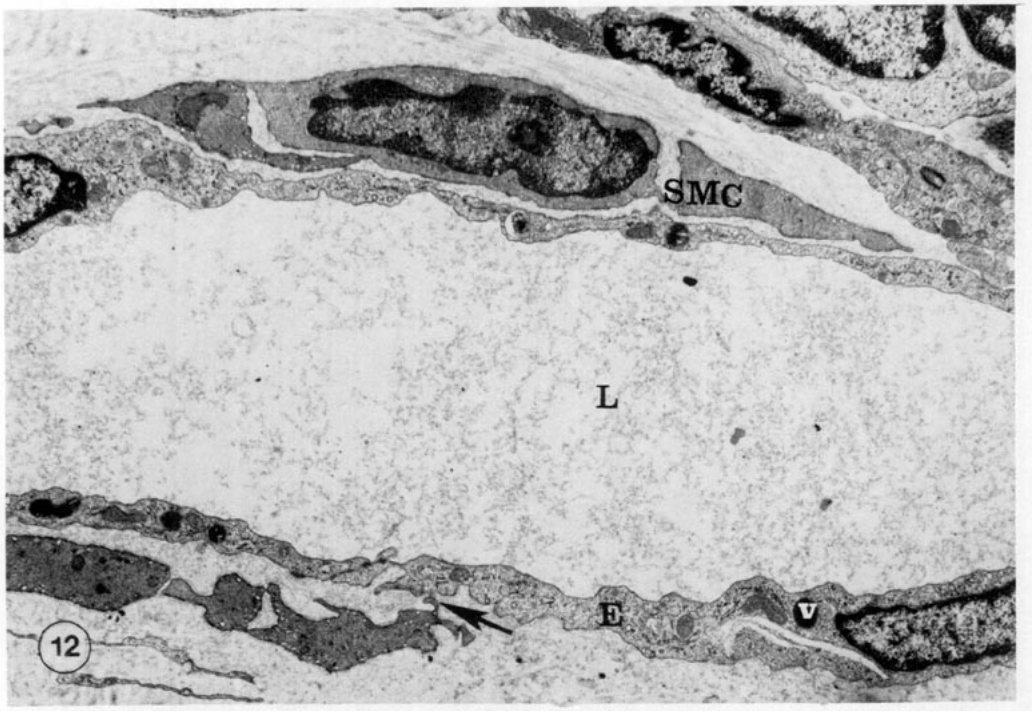

FIGURE 12. Portion of collecting lymphatic with closely associated smooth muscle cells (SMC), some of which make contact with the endothelial cells (arrows). The lumen (L) of collecting vessel also contains a dense precipitate. The endothelial cells (E) contain the usual complement of organelles including large vesicles (v) with an electron dense substance. × 9,570.

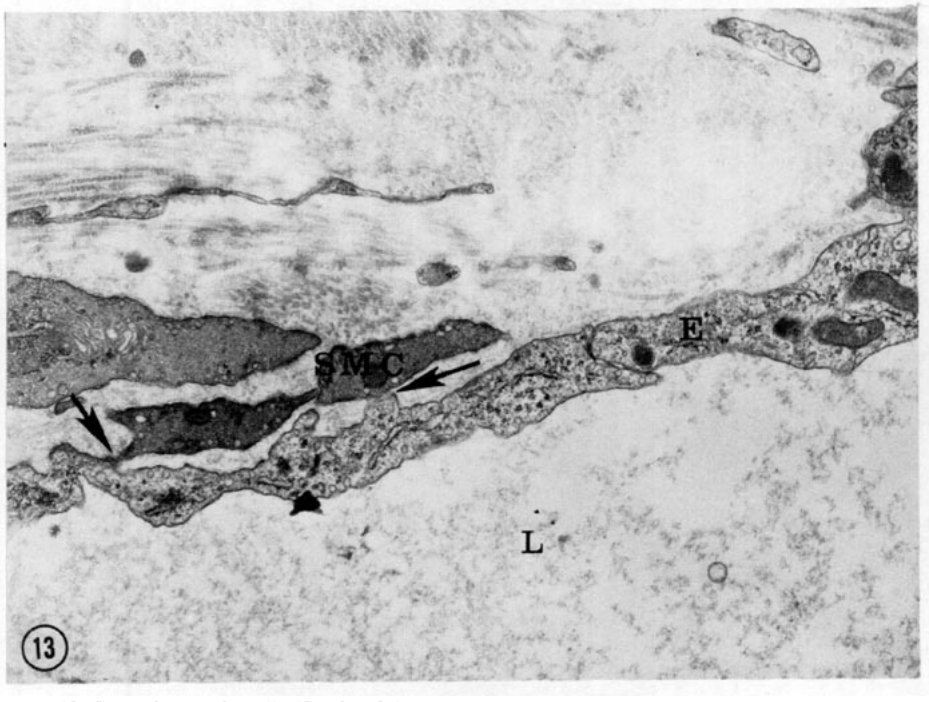

FIGURE 13. Smooth muscle cells (SMC) of the tunica media and the lymphatic endothelial (E) cells of collecting vessels are closely apposed to each other to form myo-endothelial junctions (arrows). × 15,048.

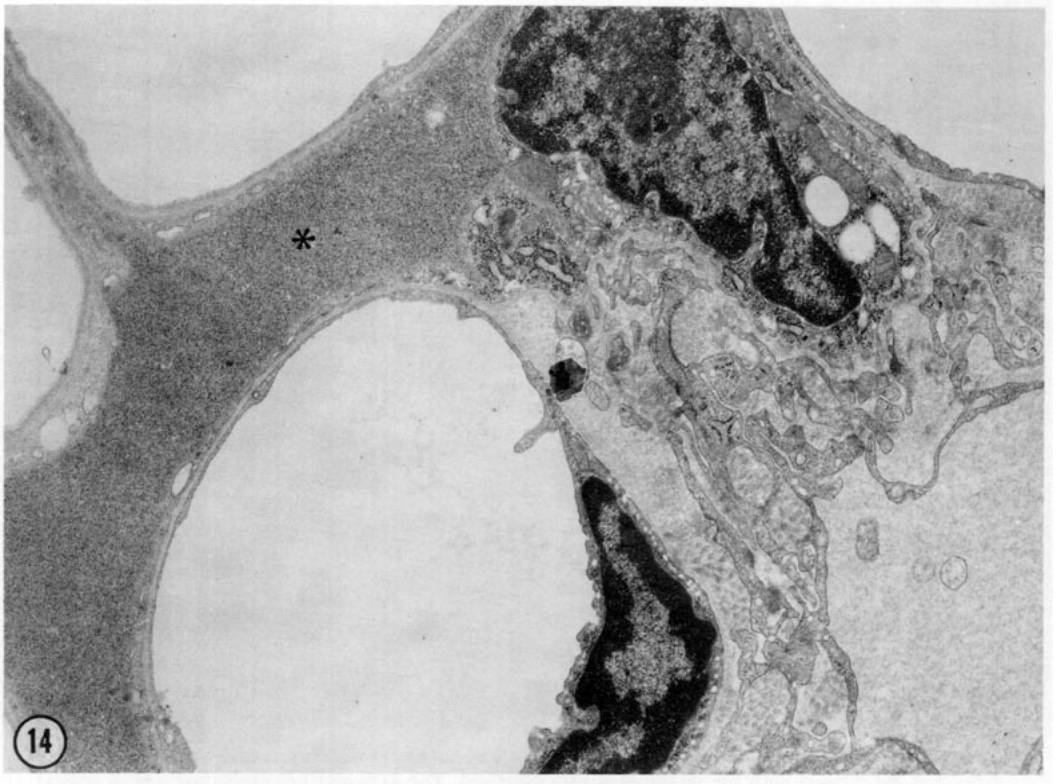

FIGURE 14. Electron micrograph depicting the presence of ferritin particles (*) within alveoli. × 20,000. Data of Leak (25).

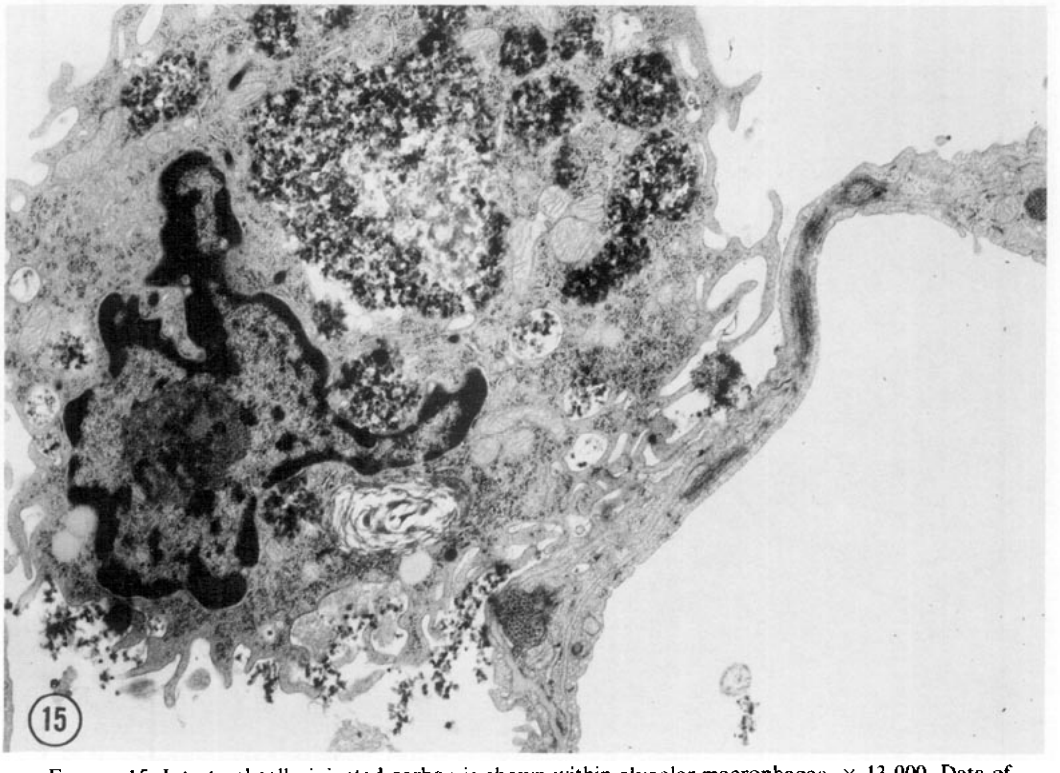

FIGURE 15. Intratracheally injected carbon is shown within alveolar macrophages. × 13,000. Data of Leak. (25).

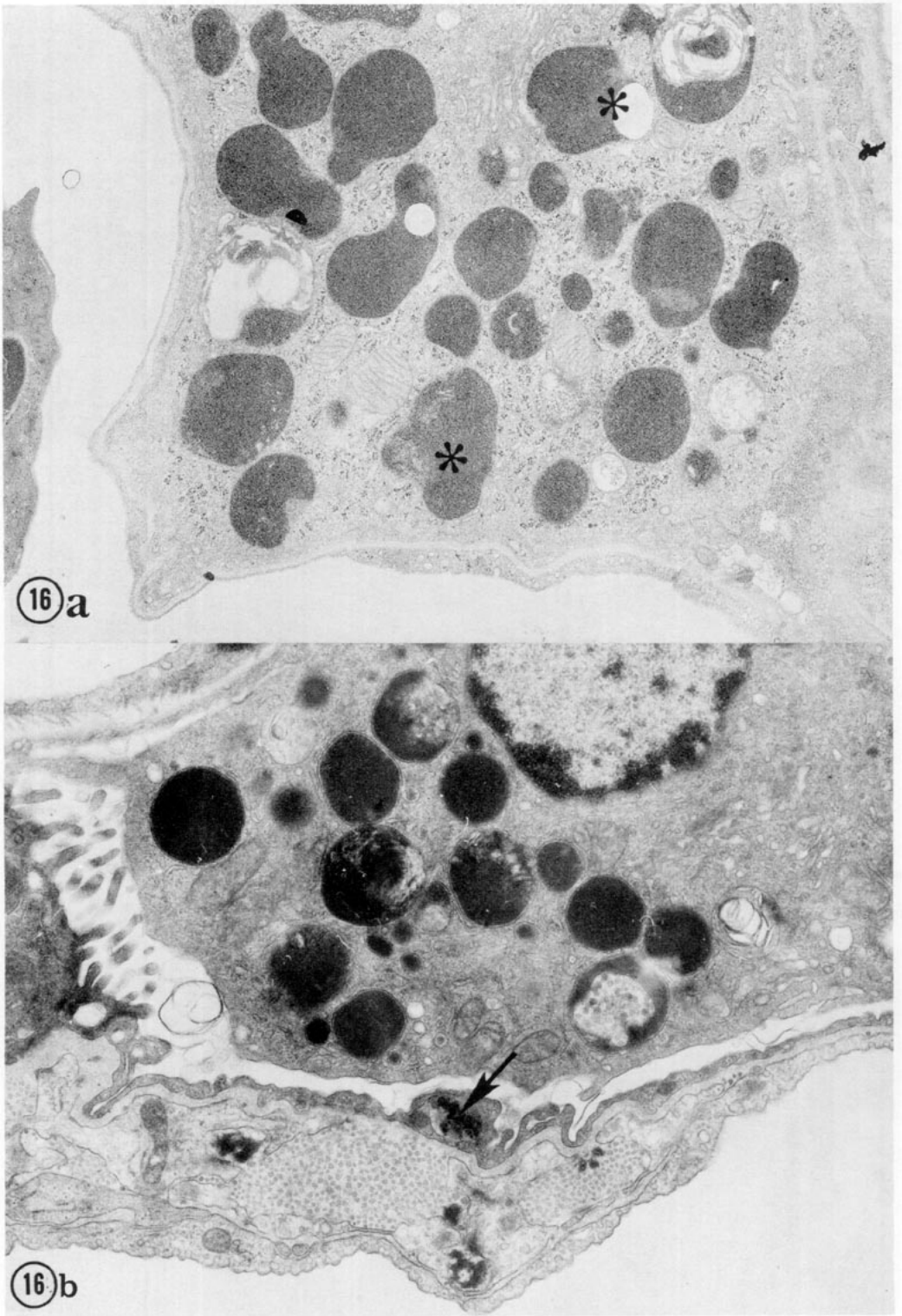

FIGURE 16. (*a*) Portion of alveolar macrophage which contain numerous vesicles that are filled with ferritin particles (*), × 12,400; (*b*) carbon tracer particles (arrow) are observed in a vesicle within the epithelium lining of the alveolar wall, × 15,378.

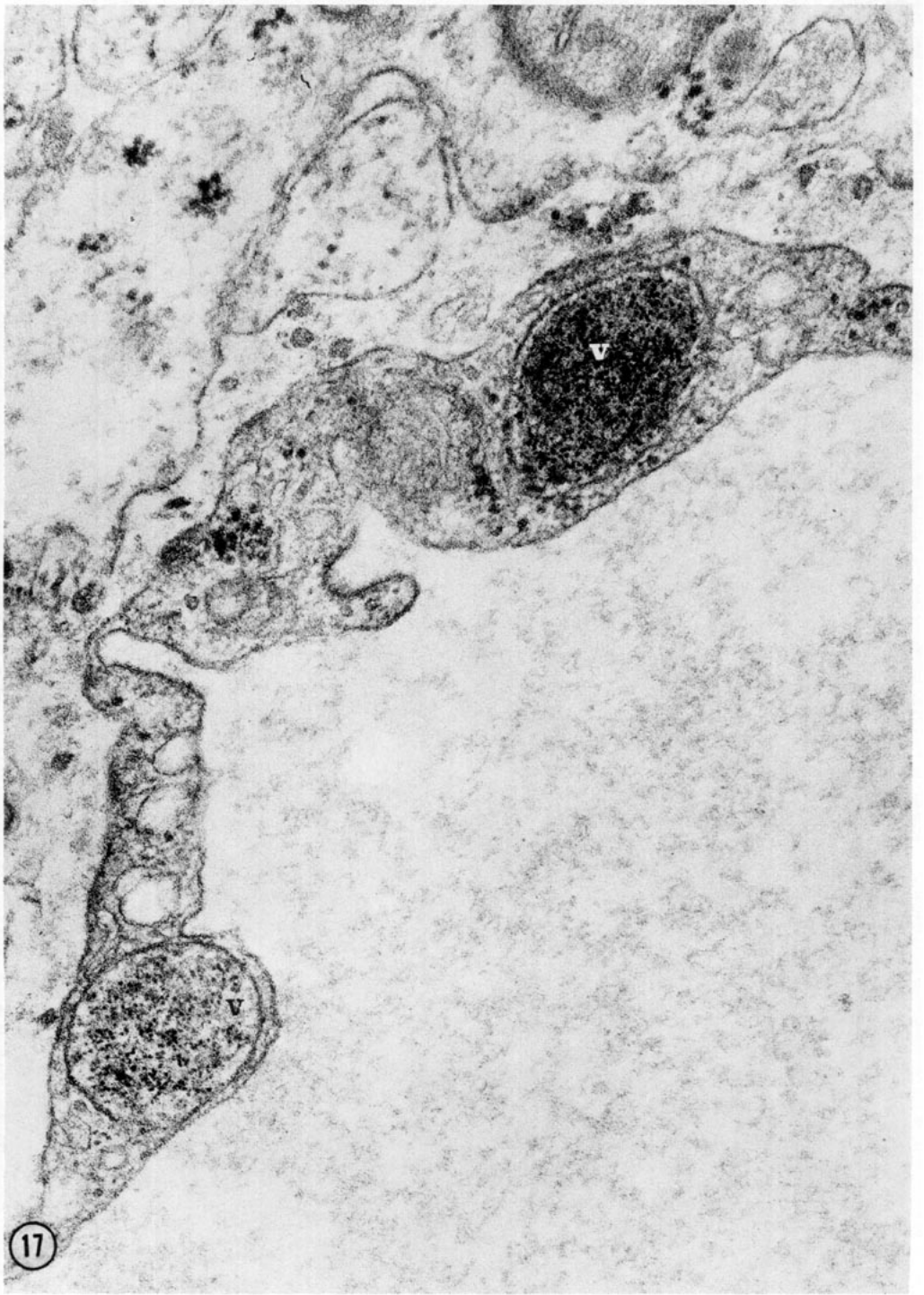

FIGURE 17. Ferritin is located within large vesicles (v) 12 hr after injection into the trachea. × 79,800.

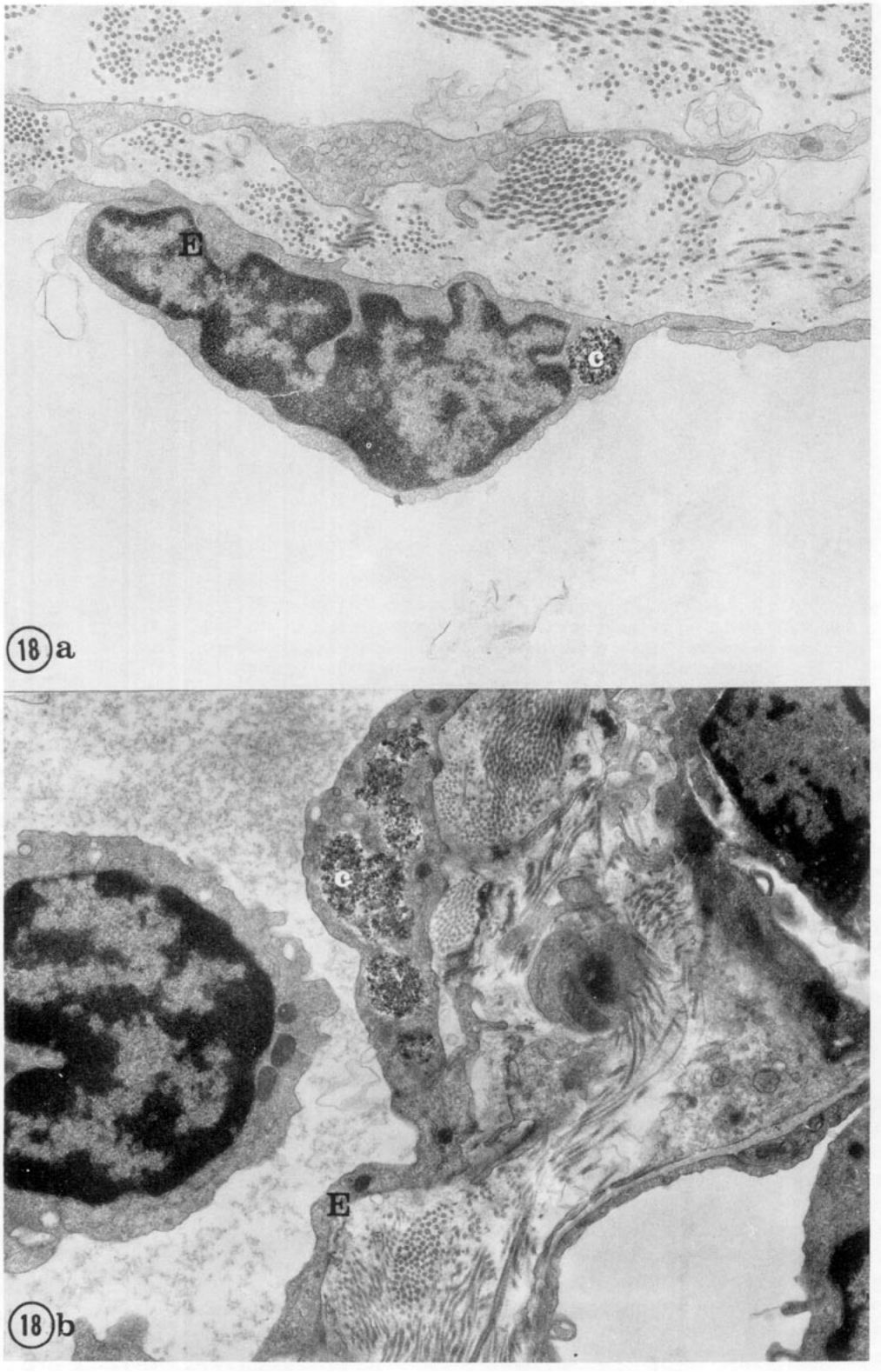

FIGURE 18. Carbon particles (C) are also accumulated within vesicles within the lymphatic endothelium (E). (*a*) × 13,224; (*b*) × 10,944.

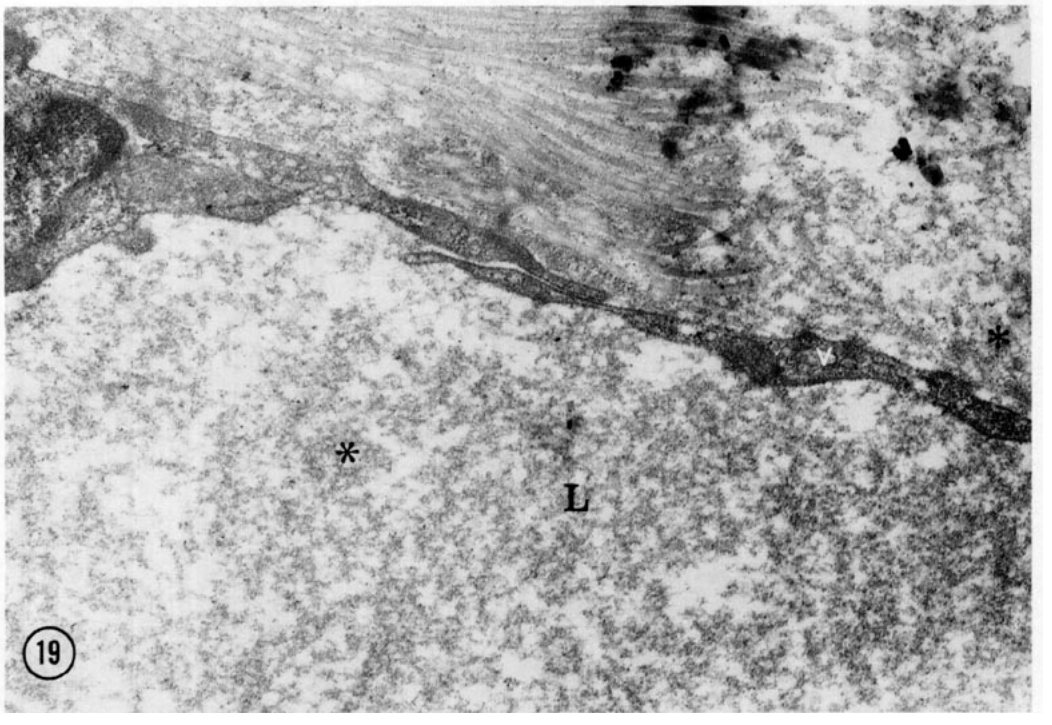

FIGURE 19. Part of lymphatic vessel from animal injected with dextran which appears as a very electron dense precipitate (*) in the connective tissue, within vesicles (v) and within the lymphatic lumen (L) 15 min after an injection of a 10% solution of dextran via the saphenous vein. × 23,000.

and interstitium, presumably as a result of fixation of the surrounding proteins. This procedure produces an electron dense product whose density is considerably enhanced by postfixation in osmic acid in a phosphate buffer solution.

Examination of pulmonary tissue within 15 min after dextran injections show the presence of this tracer throughout the interstitium and within the pulmonary lymphatic vessels (Fig. 19). Dextran gains access to the lymphatic capillary lumen via vesicles and within the intercellular clefts of loosely adherent overlapping intercellular junctions. It is of special interest to note that the uptake of dextran from the surrounding interstitium by pulmonary lymphatics is very similar to the pattern of movement of peroxidase across the blood tissue lymph interface in other tissues of the body (27).

The presence of tracer particles within alveolar macrophages and in vesicles of squamous cells of the alveolar epithelium at short intervals (15-30 min) and the subsequent appearance of tracer particles in pulmonary lymphatic vessels provide morphological evidence at the ultrastructual level which suggest that large particles are able to cross squamous alveolar epithelial cells within vesicles. Once in the connective tissue compartment, which has a negative interstitial fluid pressure (*28*), the particles move toward the lymphatics and are subsequently removed by these vessels in a fashion similar to that in other regions of the body. The observation in the present study and those of other workers (*24, 29*) suggest that particulate materials are able to cross the pulmonary epithelium within vesicles to gain entrance into the pulmonary interstitium. This removal of fluids and proteins from the pulmonary interstitium is similar to the pattern of lymphatic drainage in other tissue (*27, 30, 31*). Therefore, fluids, particles and cells are removed from the pulmonary interstitium by way of vesicles and the clefts of intercellular junctions.

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