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The distribution of lymphatic stomata in the diaphragm of the golden hamster*

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INTRODUCTION

Lymphatic stomata are small openings of lymphatics that connect the body cavity and lymphatic lumen. They have been reported to open into the inferior (i.e. peritoneal) surface of the diaphragm and they function to drain fluid from the abdominal cavity (Recklinghausen, 1863; Leak & Rahil, 1978; Bettendorf, 1978). Some investigators have claimed that not only the peritoneal but also the superior (i.e. pleural) surface of the diaphragm has stomatal openings that drain pleural fluid into lymphatics (Wang, 1975; Pinchon, Bernaudin & Bignon, 1980; Mariassy & Wheeldon, 1983). However, this has not been adequately confirmed by other investigators and we recently realised that circular fenestrations of the pleura (Mixter, 1941) and the pericardium (Nakatani *et al.* 1988) may be easily mistaken for lymphatic stomata. Also, there is a disagreement among research workers regarding the distribution of lymphatic stomata on the peritoneal surface of the diaphragm. Recklinghausen (1863) reported that tracers (cobalt blue, erythrocyte suspension and milk) injected into the peritoneal cavity were absorbed by the central tendon, suggesting that lymphatic stomata were present in the tendinous diaphragm. Wang (1975) supported this observation, while Tsilibary & Wissig (1977) reported that lymphatic lacunae, with which the peritoneal cavity might be continuous through stomata, occurred exclusively in the muscular portion of the diaphragm. The present study was designed to clarify the distribution of lymphatic stomata in the diaphragm. For this purpose we performed India ink injections into the peritoneal and pleural cavities. We also examined the diaphragm with scanning and transmission electron microscopy and counted the number of lymphatic stomata distributed in the peritoneal surface of the diaphragm.

MATERIALS AND METHODS

Adult golden hamsters 6 weeks of age were used in the present experiments. They were kept in an air-conditioned room with water and laboratory chow available *ad libitum*.

India ink injection and transmission electron microscopy

The appropriate volume of India ink to be injected into the peritoneal and pleural cavities was determined by preliminary experiments, as was the amount of time between injection and killing the animals. Twelve hamsters were anaesthetised with

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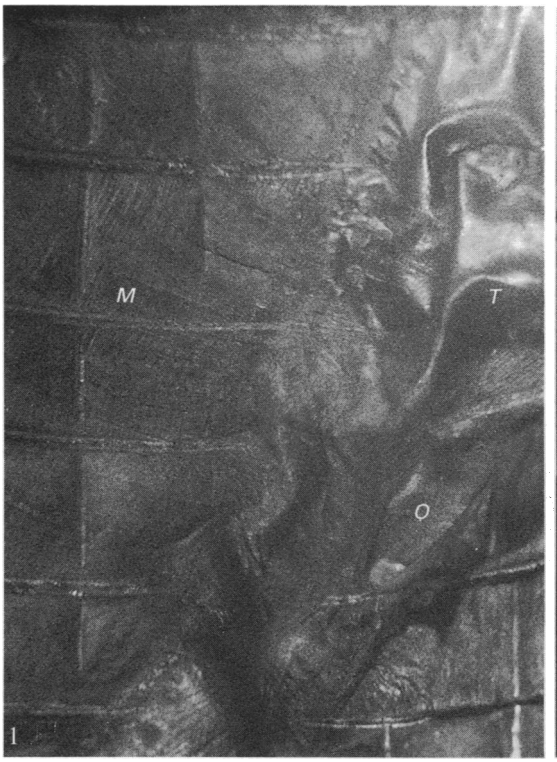
ether, six of which received 2–3 ml of India ink into the peritoneal cavity. In the remaining six animals, 0·1 ml of India ink was injected into the pleural cavity. The technique used for the injections into the pleural cavity has been described elsewhere (Fukuo, Nakatani, Shinohara & Matsuda, 1988). The hamsters were killed 5, 15 and 30 minutes after injection by inhalation of ether vapour, and their anterior chest walls were then opened. A Ga no. 28 needle attached to a syringe was used to inject 0·1 M phosphate buffer solution (PBS) at pH 7·4 into the left ventricle of the heart. The right atrium was then opened, blood was flushed out and 2% glutaraldehyde in PBS was perfused via the same needle. The cisterna chyli, mesenteric lymph nodes and thoracic duct were examined with a dissecting microscope. Photographs were taken of the diaphragm after it had been removed and placed in fresh glutaraldehyde fixative. Perfusion fixation was preferred because it prevented confusion between lymphatics and blood vessels in the micrographs. The diaphragm was cut into a series of trapezoidal shapes of approximately 1 mm^2 and the tissue pieces were left attached to part of the pleural and peritoneal ligaments. The shape of the tissues and ligaments was used to distinguish between the pleural and peritoneal surfaces. The tissues were rinsed, postfixed in 2% OsO_4 in PBS, dehydrated in a graded series of ethanols and embedded in Quetol 812. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate.

Scanning electron microscopy for overall survey of the diaphragm and counting of lymphatic stomata

Nine golden hamsters were used, three for an extensive survey of the pleural surface of the diaphragm and the remaining six for the counting of lymphatic stomata on the peritoneal surface of the diaphragm. All animals were anaesthetised with ether. Since lymphatic stomata are more readily detected when the diaphragm is relaxed (Tsilibary & Wissig, 1983), the animals were injected with succinyl choline chloride (0·1 mg/kg body weight) via the jugular vein. After cessation of respiration, glutaraldehyde fixative was injected intraperitoneally to fix the diaphragm. The anterior chest wall was opened and PBS was injected into the left ventricle to flush blood away from the diaphragm. This lessened contamination of the tissue surface with blood components. The abdominal wall was then opened widely, and the whole diaphragm was isolated. The diaphragm was mounted under a resin-framed copper mesh and pressed against a resin plate. The resin frame was 35 × 35 mm wide and subdivided with copper wires into approximately 300 squares, each of 3–4 mm^2. The diaphragm, sandwiched between the mesh and plate, was placed in fresh glutaraldehyde fixative for several hours, rinsed overnight, postfixed in 2% osmium tetroxide and dehydrated in ethanol. The diaphragm was then carefully liberated from the mesh and plate in absolute ethanol, placed in iso-amyl acetate, critical point dried and gold coated. The diaphragm, subdivided into small squares, was photographed after gold coating (Fig. 1). It was then viewed with a scanning electron microscope at × 1500, and the number and distribution of lymphatic stomata in each square were recorded on the photomicrographs. The counting was performed by one of the authors (Y.F.) and repeated twice at one month intervals. The three-way analysis of variance was employed for statistical purposes in analysing the data.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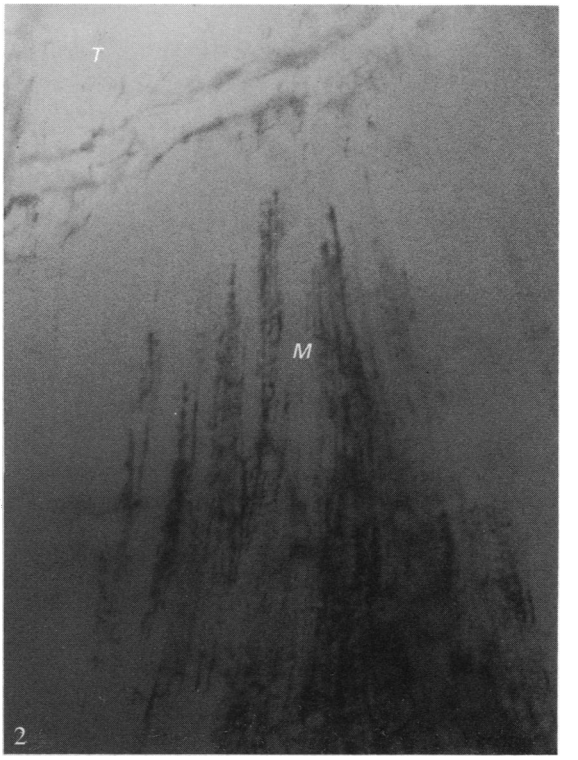
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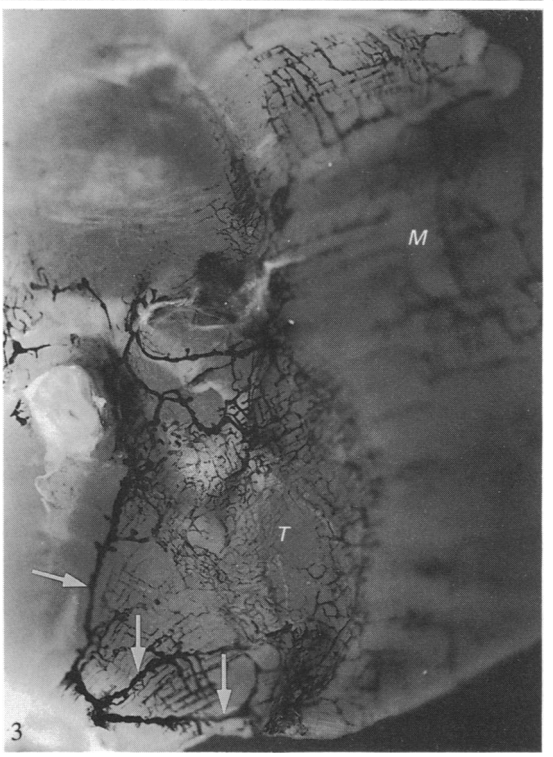
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RESULTS

India ink absorption by the peritoneal and pleural surfaces of the diaphragm

The injection of India ink into the peritoneal cavity revealed the lymphatic pathway from the cavity to the left venous angle where the thoracic duct joined the venous system. On the peritoneal surface of the diaphragm, lymphatics radiated from the tendon–muscle border to the periphery of the diaphragm in parallel with bundles of muscle fibres. The lymphatics were connected by minute branches at irregular intervals so that the lymphatic network showed a ladder-like appearance (Fig. 2). In the tendinous portion, however, this network was not clearly observed. There was a retiform network of lymphatics on the pleural surface of the diaphragm (Fig. 3). This network collected India ink from the ladder-like network on the peritoneal surface by means of numerous short branches that ran across the muscle layer of the diaphragm. The retiform network appeared coarse in the muscular portion but fine in the tendinous portion. Several collecting lymphatics originated from the retiform plexus and entered the primary lymph nodes of the diaphragm located at the level of the first lumbar vertebra. Several efferent lymphatics emanated from the lymph nodes to participate in the formation of the thoracic duct. The thoracic duct coursed craniad, received intercostal tributaries and reached the left venous angle. The whole of this pathway was stained black as early as 5 minutes after India ink injection. A para-aortic lymph node located immediately underneath the parietal peritoneum at the origin of the testicular or ovarian artery stained partially black 5 minutes after injection. This node sent several lymphatics to pararenal lymph nodes which were also partially stained black. The pararenal lymph nodes, however, did not become entirely black even 30 minutes after injection of India ink. Afferent lymphatics to the para-aortic lymph nodes were not clearly discerned, but if the superficial location is taken into consideration, one may assume that the node collects interstitial fluid close to the parietal peritoneum. A considerable amount of India ink had, in fact, already permeated into connective tissue through gaps between the mesothelial cells 5 minutes after injection. Some of the ink might have entered the lymphatic lumen but we found no appreciable amounts in the lumen even 15 minutes after injection (Fig. 4). It was surprising that the mesenteric lymph nodes, lymph nodes located at the origins of the mesenteric arteries and the cisterna chyli remained almost unstained even 30 minutes after injection. These lymphatic tissues form a pathway for transport of chyle and fluid from the small intestine to the thoracic duct. India ink injected into the pleural cavity stained several mediastinal lymph nodes 5 minutes after injection. The stained nodes were usually located immediately underneath the parietal pleura. Some lymphatics connected to these mediastinal lymph nodes were also stained black, but they were so minute and discontinuously stained that the afferent and efferent vessels of each node

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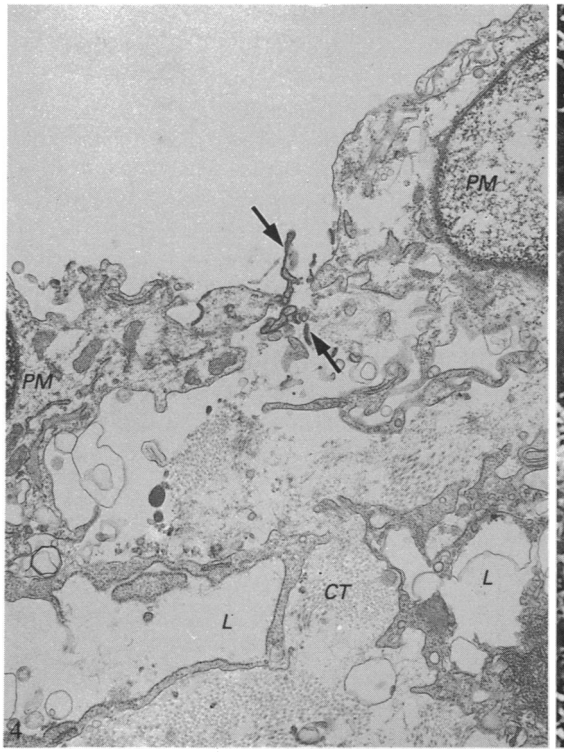
Fig. 4. India ink (arrows) is located in an intercellular gap between peritoneal mesothelial cells but is not found in the lymphatic lumen (*L*) 15 minutes after injection. *PM*, peritoneal mesothelial cell; *CT*, connective tissue. × 10600.

Fig. 5. Lymphatic stomata (*S*) located on the peritoneal surface of the diaphragm. The stomata consisted of an outer margin and an inner orifice, the latter being bounded by lymphatic endothelium. × 5700.

Fig. 6. Surface topography of the pleural mesothelium. Note the presence of circular discontinuities or gaps in the mesothelium through which connective tissue elements are visible (arrows). × 12000.

Fig. 7. The parietal pleura separating the left and right pleural cavities. The pleura has numerous pores (*P*) to connect the pleural cavities. Note the difference in the structure from the lymphatic stomata in Fig. 5. × 1400.



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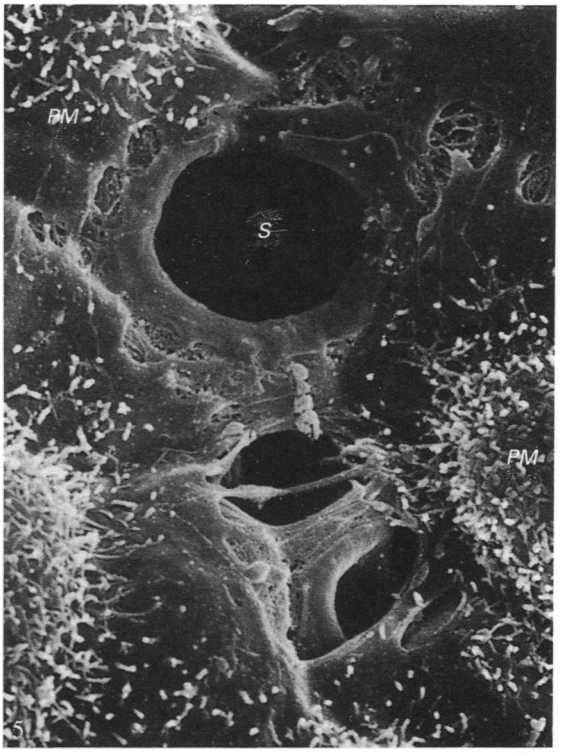

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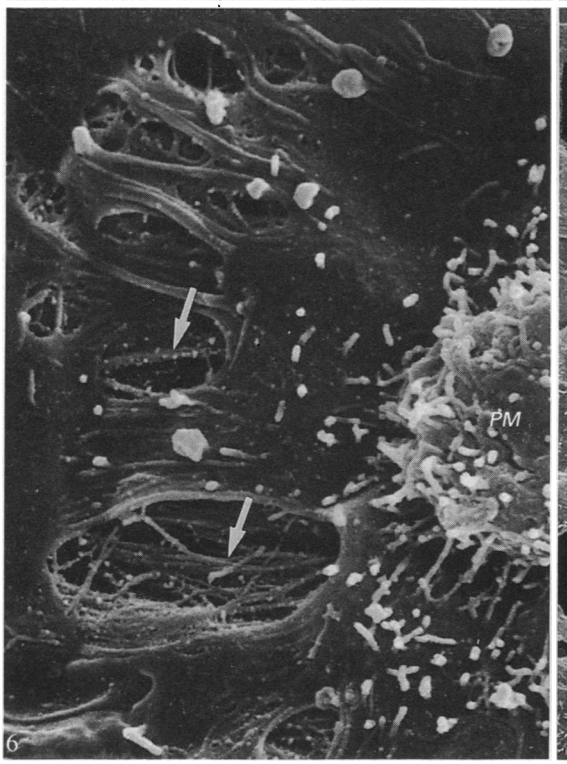

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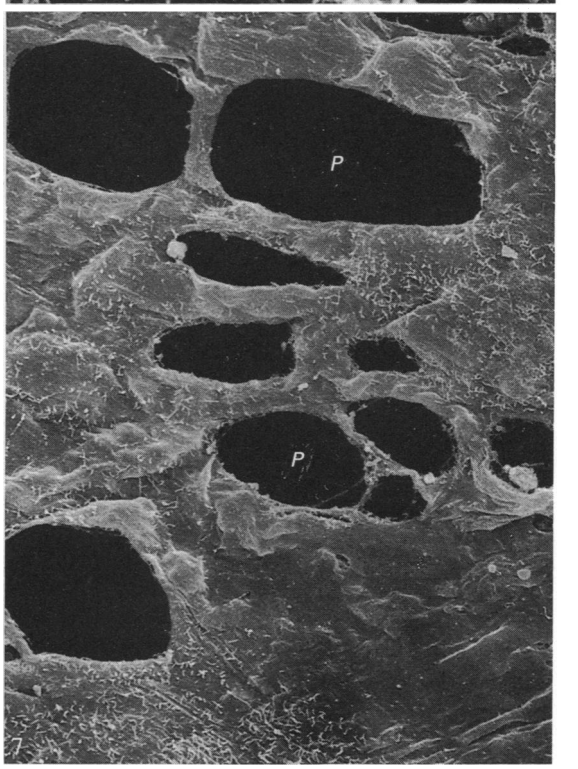

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were not clearly identified. Considering their superficial locations, however, they may collect interstitial fluid close to the parietal pleura as the para-aortic lymph node was assumed to do. In contrast to the results of the intraperitoneal injection, the lymphatics in the diaphragm, the primary lymph nodes of the diaphragm and the thoracic duct were all unstained up to 30 minutes after injection. Histological sections of the parietal pleura revealed that ink had already permeated into the subpleural connective tissue 5 minutes after injection, but no appreciable amounts of ink entered the lymphatics until 30 minutes after injection.

The distribution of lymphatic stomata in the diaphragm

A scanning electron microscopic survey confirmed the presence of lymphatic stomata on the peritoneal surface of the diaphragm. They were usually circular in shape and were bounded by the peritoneal mesothelial cells and lymphatic endothelial cells (Fig. 5). The perimeters of the former cells, which were studded with numerous microvilli, formed the outer margin of the lymphatic stomata. The orifice was formed by microvillus-free lymphatic endothelium. They ranged from a few to twenty micrometres in diameter. Lymphatic stomata tended to form radial rows from the central tendon to the peripheral muscular portion of the diaphragm. While the stomata were present in both portions, we noted that the density of lymphatic stomata in the tendinous portion ($30{\cdot}0 \pm 9{\cdot}02$) was higher than that in the muscular portion ($20{\cdot}1 \pm 2{\cdot}48$). The figures represents the mean and SD of 12 values obtained by repetition of the counting in the 6 specimens. The difference is statistically significant ($F = 22{\cdot}09$; $P < 0{\cdot}001$).

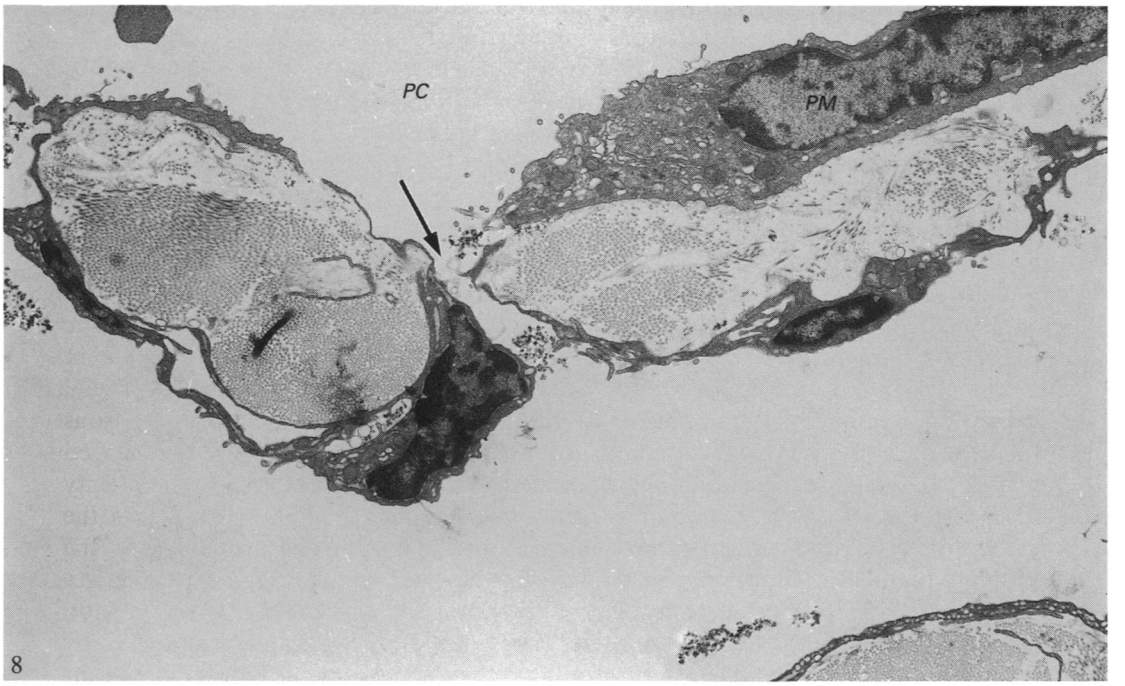
The pleural mesothelium of the diaphragm was not entirely continuous, and the subpleural connective tissue was exposed to the pleural cavity through the discontinuities (Fig. 6). The parietal pleura, separating the left and right pleural cavities, showed a different surface architecture from the pleura covering the diaphragmatic surface; the former had numerous circular fenestrations that connected the left and right pleural cavities (Fig. 7). These fenestrations are quite similar to lymphatic stomata but have a clear structural difference: their orifices are formed only by pleural mesothelial cells studded with numerous microvilli. Consequently, lymphatic stomata were not encountered in the pleural surface of the diaphragm.

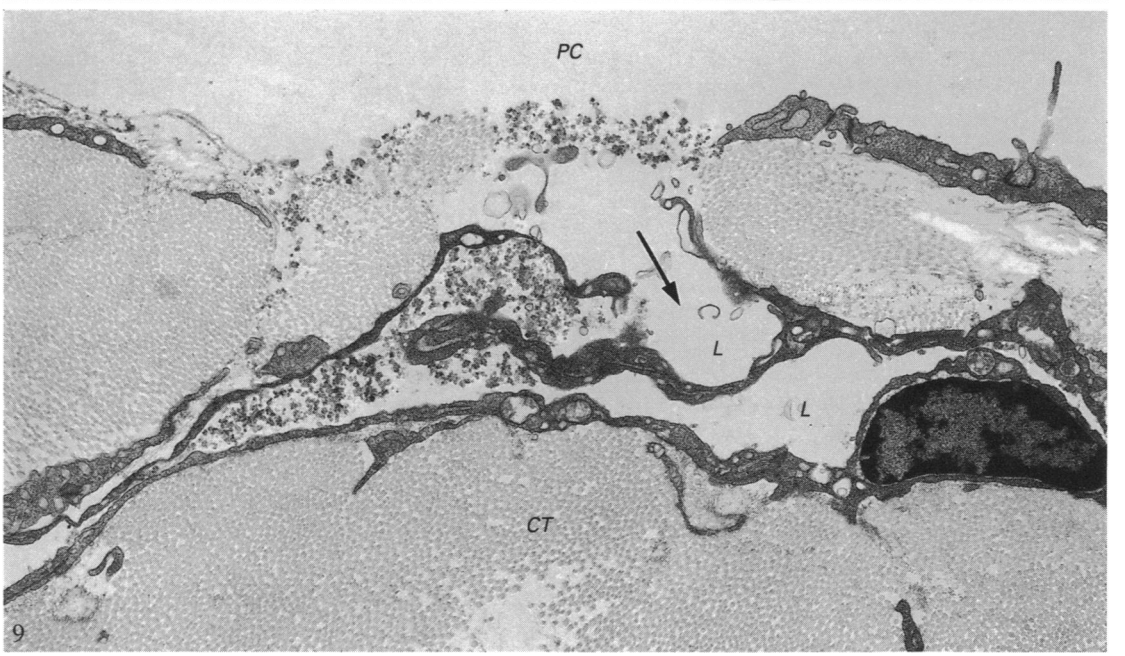
Lymphatic stomata in the tendinous portion of the diaphragm

By scanning electron microscopy, lymphatic stomata of the muscular and tendinous portions did not differ in morphology. However, histological sections revealed that both the lymphatic lacunae and lymphatics of the tendinous portion were often smaller than those in the muscular portion (Figs. 8, 9). Also, lymphatic lacunae and lymphatics in the tendinous portion contained India ink even 30 minutes after injection, suggesting stasis or slow flow of lymph fluid in this portion.

DISCUSSION

In spite of an extensive survey by scanning electron microscopy, lymphatic stomata were not found on the pleural surface of the diaphragm. Injection of India ink into the peritoneal cavity stained the diaphragmatic networks of the lymphatics and thoracic duct within 5 minutes. In contrast, India ink injected into the pleural cavity did not stain the diaphragmatic lymphatics and thoracic duct. These results suggest that lymphatic stomata are absent not only on the pleural surface of the diaphragm but also in the pleural surface of the thoracic wall, viz. the thoracic cavity does not have





Figs. 8 and 9. Lymphatics and lymphatic lacunae in the muscular portion (Fig. 8) and tendinous portion (Fig. 9). Note the difference in their sizes. Note also that the lacuna in the tendinous portion is filled with India ink. Arrows indicate openings of stomata. *PC*, peritoneal cavity. Fig. 8, ×6500; Fig. 9, ×12000.

such rapid draining devices as lymphatic stomata. Their presence in the pleural surface of the diaphragm has been reported in the rabbit (Wang, 1975), rat (Wang, 1975; Pinchon *et al.* 1980) and sheep (Mariassy & Wheeldon, 1983). Thus, one may think that there is a species difference in the presence of lymphatic stomata on the pleural surface. During the present study, however, we realised that two premises are necessary for the confirmation of their presence in the pleural surface. One is a technical premise regarding sections of the diaphragm; i.e. the section blocks studied should have a landmark to discriminate the peritoneal and/or pleural surfaces. This is especially important in sections used for transmission electron microscopy since the two surfaces are very easily mistaken for one another as the section blocks become smaller. The other premise is a criterion for confirmation of lymphatic stomata by scanning electron microscopy. Lymphatic stomata consist of an outer mesothelial margin and inner lymphatic endothelial orifice. As shown in this study, circular fenestrations in the pleura consist of only pleural mesothelium and are easily distinguished from lymphatic stomata. It is questionable, however, whether previous investigators have been aware of these premises. Therefore, we feel that the suggestion of an interspecies difference requires further validation.

Injection experiments have clarified three lymphatic pathways in the abdomen. The principal pathway to drain peritoneal fluid begins with lymphatic stomata; the fluid passes into the peritoneal and pleural lymphatic networks in the diaphragm, undergoes filtration through the regional lymph nodes of the diaphragm and finally empties into the thoracic duct. Peritoneal fluid may also permeate across the parietal peritoneum into the submesothelial connective tissue. Superficial lymph nodes located immediately underneath the peritoneum may collect the fluid and send it to the thoracic duct. This pathway, however, does not seem to be as efficient as the first pathway since India ink absorption occurred very slowly. The third lymphatic pathway contributes to the transfer of chyle and fluid from the intestine to the thoracic duct and cisterna chyli. Our results suggest that this pathway scarcely contributes to the absorption of peritoneal fluid. The biological significance of these differential passages of lymph fluid from the abdominal viscera and abdominal cavity remains speculative, but one can easily point out a connection with self-defence mechanisms. It is known that lymphoblasts in lymphoid tissues can display 'homing' activity (Gowans & Knight, 1964). For example, if lymphoblasts isolated from peripheral lymph nodes of a donor are injected into a syngeneic recipient, the cell will migrate to any lymphoid tissue but the principal sites of migration depend on the source of the cells (Griselli, Vassalli & MacCluskey, 1969). Similarly, lymphoblasts obtained from the mesenteric lymph nodes home to gut-associated lymphoid tissues such as mesenteric lymph nodes, Peyer's patches and lamina propria of the intestines (Parrott & Ferguson, 1974; McWilliams, Phillips-Quagliata & Lamm, 1975). Homing is a maturational process of lymphoblasts that provides IgA or IgG for local, therefore, primary host reactions. The site of these reactions naturally varies, depending on the lymphatic routes by which, for example, microorganisms invade the body. Obviously, when the peritoneal cavity is infected with microorganisms, the primary local reactions will occur in the lymphoid tissue cranial to the diaphragm but not in the gut-associated lymphoid tissue lying caudal to the diaphragm.

It was surprising that the density of lymphatic stomata in the tendinous portion of the diaphragm was as much as 1·5 times greater than that in the muscular portion. However, this does not necessarily mean that lymphatic stomata in the tendinous portion are functionally active. Histological sections revealed that lymphatics in the tendinous portion were usually small in size and lacunae were not as wide as those in

the muscular portion. Also, lymphatics in the tendinous portion were often filled with India ink even 30 minutes after injection, suggesting that lymph flow in these lymphatics is very slow. Possibly, lymphatic stomata in the tendinous portion may be a reserve that will function under special conditions, e.g. an excessive accumulation of fluid in the peritoneal cavity.

SUMMARY

Golden hamster diaphragms were observed with both light and electron microscopes to examine the distribution of lymphatic stomata and their morphological features. Lymphatic stomata were present on the inferior surface but absent on the superior surface. This observation was also supported by the results of India ink injection into the peritoneal and pleural cavities. On the inferior surface of the diaphragm, lymphatic stomata tended to form radial rows running from the centre to the periphery; i.e. they were found throughout the muscular and tendinous portions, although the density of their distribution was higher in the latter. Lymphatics and lymphatic lacunae in the tendinous portion were generally smaller than those in the muscular portion. Lymphatic flow into the lymphatics of the tendinous portion seems to be slow, suggesting that lymphatic stomata in the portion may be a functionally inactive reserve.

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REFERENCES

BETTENDORF, U. (1978). Lymph flow mechanism of the subperitoneal diaphragmatic lymphatics. *Lymphology* **11**, 111–116.

FUKUO, Y., NAKATANI, T., SHINOHARA, H. & MATSUDA, T. (1988). The mouse pericardium: it allows passage of particulate matter from the pleural to the pericardial cavity. *Anatomical Record* **222**, 1–5.

GOWANS, J. L. & KNIGHT, E. J. (1964). The route of recirculation of lymphocytes in the rat. *Proceedings of the Royal Society of London, B* **159**, 257–282.

GRISELLI, C., VASSALLI, P. & MACCLUSKEY, R. T. (1969). The distribution of large dividing lymph node cells in syngeneic recipient rats after intravenous injection. *Journal of Experimental Medicine* **130**, 1427–1451.

LEAK, L. V. & RAHIL, K. (1978). Permeability of the diaphragmatic mesothelium: the ultrastructural basis for 'Stomata'. *American Journal of Anatomy* **151**, 557–594.

MARIASSY, A. T. & WHEELDON, E. B. (1983). The pleura: a combined light microscopic, scanning, and transmission electron microscopic study in the sheep. I. normal pleura. *Experimental Lung Research* **4**, 293–313.

MCWILLIAMS, M., PHILLIPS-QUAGLIATA, J. M. & LAMM, M. E. (1975). Characteristics of mesenteric lymph node cells homing to gut-associated lymphoid tissue in syngeneic mice. *Journal of Immunology* **115**, 54–58.

MIXTER, R. L. (1941). On macrophagal foci ('milky spots') in the pleura of different mammals, including man. *American Journal of Anatomy* **69**, 156–186.

NAKATANI, T., SHINOHARA, H., FUKUO, Y., MORISAWA, S. & MATSUDA, T. (1988). Pericardium of rodents: pores connect the pericardial and pleural cavities. *Anatomical Record* **220**, 132–137.

PARROTT, D. M. V. & FERGUSON, A. (1974). Selective migration of lymphocytes within the mouse small intestine. *Immunology* **26**, 571–588.

PINCHON, M.-C., BERNAUDIN, J.-F. & BIGNON, J. (1980). Pleural permeability in the rat. I. Ultrastructural basis. *Biologie cellulaire* **37**, 269–272.

RECKLINGHAUSEN, F. VON (1863). Zur Fettresorption. *Archiv fuer pathologische Anatomie und Physiologie und fuer klinische Medizin* **26**, 172–208.

TSILIBARY, E. C. & WISSIG, S. L. (1977). Absorption from the peritoneal cavity: SEM study of the mesothelium covering the diaphragm. *American Journal of Anatomy* **149**, 127–133.

TSILIBARY, E. C. & WISSIG, S. L. (1983). Lymphatic absorption from the peritoneal cavity: regulation of patency of mesothelial stomata. *Microvascular Research* **25**, 22–39.

WANG, N. (1975). The preformed stomas connecting the pleural cavity and the lymphatics in the parietal pleura. *American Review of Respiratory Diseases* **111**, 12–20.