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THE MILKY SPOTS OF THE PERITONEUM AND PLEURA: STRUCTURE, DEVELOPMENT AND PATHOLOGY

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The milky spots (MS), originally described by Ranvier as taches laiteuses*, are found on the greater omentum but also in other peritoneal regions, as well as on the pleura and pericardium. They represent aggregations of mesenchymal tissue surrounding blood vessels. These small whitish regions are covered by mesothelium, and within the mesothelial layer are scattered macrophage-like cells. The blood supply of MS is provided by arterioles that give rise to capillary network formed by fenestrated or continuous endothelial cells. Most MS possess also lymphatic vessels, with extremely thin endothelial cells. The most frequent cells in MS are the macrophages, followed by lymphocytes and mast cells. Typically, the macrophages are located in the periphery, while the lymphocytes - in the center of MS. Additional structural elements are plasmocytes, adipocytes, fibroblasts, rounded fibroblast-like cells (undifferentiated mesenchymal cells), as well as collagen, reticular and elastic fibers. The nerve fibers innervating MS are located under the mesothelium and among the free cells. Despite their small size, the MS are a significant organ, functioning at both normal and pathological conditions. Under inflammatory conditions (peritonitis), MS act as the first line of defense, and dramatically change their number, size and structure. MS are also involved in extramedullary hemopoiesis. They are the first target of intraperitoneal (intrapleural) metastases, and appear an important target in the development of immunotherapeutic strategies against malignant diseases.*
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INTRODUCTION

It is a traditional belief that von Recklinghausen (1) first referred to the macroscopically visible whitish spots in the omentum of young rabbits, but this statement might not be precise because he described the so-called "stomata" on the abdominal surface of the diaphragm, and has not investigated the greater omentum (2). The spots were first recognized by Ranvier (3), and he named them *taches laiteuses,* i.e. milky spots (MS). Ranvier considered the greater omentum as a giant, flattened lymph node, the lymph sinus of which is represented by the peritoneal cavity. These tiny accumulations of lymphoid tissue were soon recognized to have a protective role, and Morrison (4) with sympathy called the greater omentum "policeman of the abdomen"; it has also been designated "friend in need" and "the great leucocyte" (2,5). MS were identified in a wide variety of species: mice (6-8), rats (6-13), guinea pigs (6, 10, 14), rabbits (1,6,15), pigs (6,16), sheeps and goats (17), and

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cats (15). Although described in 1921 in the human greater omentum (18), and despite the great biological importance, there are relatively few investigations on human MS until now. In the human omentum, MS are difficult to identify because they are very small compared with lymph nodes and are usually embedded in adipose tissue, particularly in older subjects (19). Human MS are larger (0.5-3.5 mm^2), while in the laboratory animals they are typically of smaller size (0.1-3 mm^2) (2).

According to the present knowledge, the MS are implicated in several important biological functions. They represent a major immune organ in the peritoneal cavity (5,8,19-26). Further, MS produce and provide free peritoneal cells, such as macrophages/monocytes and lymphocytes (11,12,26-31). Under inflammatory conditions, MS increase their number and size and act as the first line of defense in the peritoneal cavity (11,12,32-34). The MS are a major gate through which circulating inflammatory cells migrate from omental vessels into the peritoneal cavity (11,12,28,29). They play an important role in scavenging foreign substances in the peritoneal cavity (28, 35,36). The MS participate in the formation of ascites by filtering fluid from the capillaries to the peritoneal cavity (29,37). They are involved in extramedullary hematopoiesis (8,31,34,38,39). Along the "ordinary" peritoneal mesothelium (40,41), MS are greatly altered by continuous peritoneal dialysis (42-46).

We here present an updated review on the structure, development and function of MS, their involvement in different pathological processes, and add our results on the blood supply and ultrastructure of MS in humans and experimental animals.

STRUCTURE AND FUNCTION OF THE MILKY SPOTS IN THE GREATER OMENTUM

As pointed out in the Introduction, Ranvier (3) considered the MS as a large, disseminated lymph node. At that time the structure of the lymph node was known, and that statement was repeatedly appreciated (47-50). However, Milian (51) declared that in the MS there are numerous phagocytic cells, and Seifert (52) first differentiated the MS from the lymphatic nodes, considering the MS as a part of the reticuloendothelial system. They were reasonably named "a lymphoreticular organ" (53).

Omental MS consist of mesenchymal cells surrounding blood vessels (2,19). Silver impregnation shows the delicate networks of reticular fibers which constitute the framework of the organ (33). The blood supply is received from the epiploic branches of right and left gastroepiploic (gastroomental) arteries. The microvasculature of MS consists of a classic succession of arteriole, precapillary, capillary, postcapillary, collecting venule, and venule (19,54,55). Shunt vessels were also observed in the omental nodules (15,56,57). The small vessels in the MS are nominated "omental glomeruli" (58). The drainage veins of MS enter the epiploic branches of gastroepiploic veins to the portal venous system *via* the superior mesenteric and splenic veins (2,19). Most MS capillaries are fenestrated (8,9,17,29,37,59), in contrast to other capillaries in the omentum which have non-fenestrated endothelium (9). The side pressure in the twisted MS capillaries is increased according to the law of Bernoulli due to the slow velocity of the bloodstream (60). In inflammation, when the capillaries become hyperemic, the fenestration of the endothelium and the higher pressure enhance the cell penetration from the capillaries in the interstitial spaces. In the thin parts of the omentum, also non-vascularized MS are observed (59,61,62). These small, flat lymphoid aggregations are scattered within the omental membrane, and their cell composition is similar to that of vascularized MS except for the absence of neurtrophilic myelopoiesis. The non-vascularized MS increase their number and size in peritonitis, and therefore they might represent secondary formations that are composed primarily of macrophages and derived from the "ordinary" MS (59).

Casparis (63) first demonstrated unequivocally the lymphatic vessels of the greater omentum, and his results were confirmed repeatedly (54,64-66). There are certain species differences in the MS lymph vessels (66). In rats and guinea pigs, they form plexiform arrangements, whilst in rabbits and dogs the lymph vessels are larger, less plexiform and distend in some regions. Injection of Georta blue dye in human MS demonstrated lymphatic capillaries with blind ends (54). The diameter of dilated capillaries exceeds 150 μm. They are superficial, close to the mesothelial cells, emerge from MS and empty in the efferent lymphatic vessels. Thus, they may participate in the absorption of various substances from the peritoneal cavity (54). Blind endothelial sacculations of the terminal lymphatics that drain in collecting channels have also been described (67). The ultrastructural data suggest that the initial lymphatics in the MS open into the peritoneal cavity because the endothelial cells of lymphatics mesh with the connective tissue mesothelial cells (19).

We examined the blood supply of the omental MS in rabbits following injection of India ink in the most proximal portion of the abdominal aorta. The networks of vessels within MS are seen with ovoid, round or irregular form (Fig. 1a,b). Typically, two arterioles supply a single MS (Fig. 1a), and more rarely these are 3-5 arterioles (Fig. 1b). The afferent arterioles most

Figure 1. *Proposed secondary and tertiary RNA structures within the 5'NTR (13).*

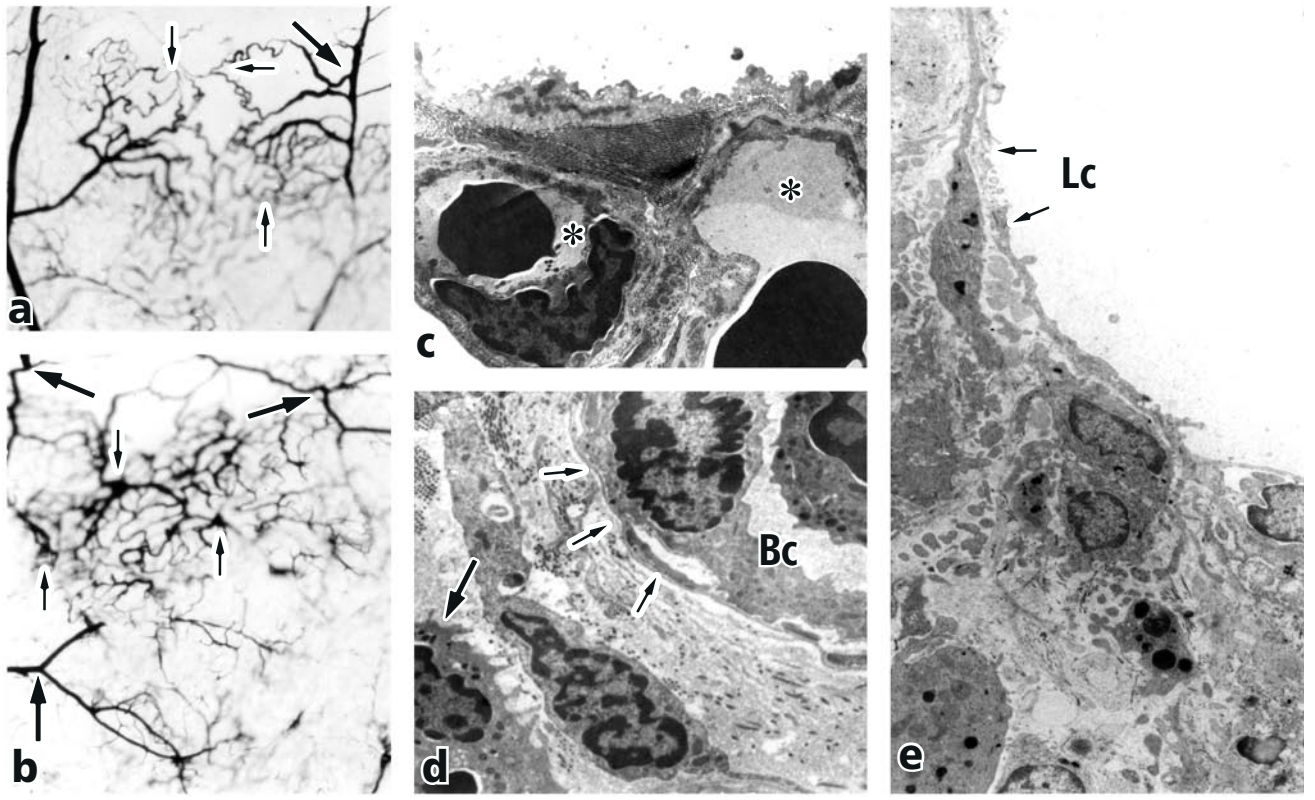

Figure 1. *Networks of vessels within the rabbit omental milky spots (MS) revealed by injection of Indian ink in the abdominal aorta* ***(a, b)***. ***a:*** *Two main arterioles (large arrows) supply a single MS. Capillaries with markedly convoluted course. The anastomoses are labeled with small arrows.* ***b:*** *Three arterioles (large arrows) form a capillary network with dilated portions (small arrows) in the center of MS. Electron microscopic from rat omental MS (****c-e****).* ***c:*** *Evaginations and invaginations of the thin activated mesothelium over two blood capillaries (asterisks). The endothelial cells are electron-dense and filled with microvesicles.* ***d:*** *Neutrophil leukocyte (large arrow) in vicinity of a blood capillary (Bc) with continuous endothelium over a basal lamina (small arrows).* ***e:*** *Thinnings of the endothelial cells surround the round lumen of a lymph capillary (Lc).* a, b x 8; c x 3500; d x 5000; e x 7000.

commonly divide dichotomously, and from these branches a capillary network is formed. The capillaries display dilated portions located in the central zones of MS, and have a markedly convoluted course with numerous anastomoses. Four types of capillary formations can be distinguished: intramembranous type, sessile marginal type, pedunculated type, and less vascularized type (68). Following this classification, we observed only occasional cases of the pedunculated type.

On Fig. 1c-e, we present electron micrographs from rat omental MS. The main vascular structures are the blood capillaries, whilst the lymph capillaries are scant, and we often observed MS without lymphatics. MS are covered by a thin mesothelium (Fig. 1c). The mesothelial cells display numerous fine evaginations, filled with microvesicles and groups of mitochondria. The endothelial cells of the blood capillaries are electron-dense, unlike the electron-lucent endothelial cells of the remaining parts of the omentum. The capillaries are of continuous type (Fig. 1d) and the endothelial cells are surrounded by a basal lamina. The endothelial cells show an extensive caveolar system, suggesting a vigorous transendothelial transport. Notably, we encountered relatively few fenestrated capillaries. The lymphatic capillaries (Fig. 1e) display rounded lumen, and the thin endothelial cells are electron-lucent without basal lamina.

Early observations suggested that the mesothelial covering of MS is discontinuous (47,69), which was later confirmed (7,9,11,26,53,70-73). Inbetween the mesothelial cells, there are rounded openings with diameter of 1-10 μm (11,26,71,72).

These are designated "gaps", "crypts", and most often "stomata". Stomata are also seen in the mesothelial covering on the both sides of the diaphragm, the anterior abdominal wall, the liver, and the lower portions of the costal pleura (74-77). The lack of basal lamina under the mesothelial cells of MS fascilitates cellular migration (9,11,26,29,53,70). On scanning electron microscopy, the surface of MS appears morphologically distinct from the surface of the other omental regions because of its typical cobblestone appearance (26). Most of the mesothelial cells are dome-shaped and feature longer and more numerous microvilli on the apical surface than those of the thin transparent regions of the omentum.

The cellular composition of MS was studied intensively. The “active” MS (52) contain numerous lymphoreticular cell elements located in the deep portion of the spot or superficially (2). The most numerous are the macrophages and lymphocytes, less numerous are macrophages with dendrites and plasmocytes. Close to the capillaries are located numerous periadventitial cells. Undifferentiated mesenchymal cells and fibroblasts can also be found (2,9,19,53,70,78,79).

Macrophages are the most numerous (30-48%) cellular components of MS (11,12,19,23,80). In infants with healthy peritoneum (operated for neuroblastoma), the mean number of macrophages *per* MS is 570 ± 33, of which 47.5 ± 7.5% are immunohistochemically stained by the macrophage-specific monoclonal antibody Leu-M5 (CD11c) (19, 23). In fact, the proportion of such macrophages in the human omental MS might be even higher: 67.9 ± 9.4% (81). In rats, the MS of the greater omentum can be seen macroscopically as early as 30 min after injection of carbon particles (82). In humans, the omental MS were stained black when a carbon suspension was inadvertently injected in the peritoneal cavity (19,83,84). On the surface of MS, the mesothelial cells which line the peritoneal cavity are often replaced by macrophages (7,9,26,49,53,73). In some instances, these macrophages form two-three layers and orient toward the peritoneal cavity for trapping or permitting the entrance of foreign particles (19). As referred above, the mesothelial stomata and the lack of a basal lamina provide suitable environment for the cells within MS to migrate from the interstitial spaces into the peritoneal cavity, or *vice versa*. Several studies focused on the origin of the MS macrophages (11,12,83-87). Two types of peritoneal macrophages were described on the basis of their origin (87). Under normal conditions, peritoneal resident macrophages derive from locally proliferating progenitor cells in MS, which themselves have a bone marrow origin. However, in inflammatory conditions, most peritoneal macrophages directly derive from monocytes produced in the bone marrow. An alternative classification based on peroxidase activity patterns has also been proposed (11,12), dividing MS macrophages into three types: exudate macrophages (monocytes), exudate-resident macrophages, and resident macrophages. At steady state, the majority (>90%) macrophages are resident macrophages localized peripherally in MS. Exudate macrophages or monocytes increase their number and develop into exudate-resident and resident macrophages. Interestingly, under inflammatory conditions, even promonocytes are localized perivascularly in MS.

By using ummunostaining for macrophage precursor antigens, it was demonstrated that MS play an important role in the origin of peritoneal macrophages (30). Early macrophage precursor antigens ER-MP12 and ER-MP58 were detected only on cells localized inside MS. On the other hand, an antigen which disappears late in the course of macrophage differentiation, ER-MP20, was detected in high amounts on cells both inside and around MS. Thus, the macrophage precursors have central localization within MS, while more differentiated cells are found more peripherally (30). MS possess a microenvironment in which precursor cells of the mononuclear phagocyte system can home and proliferate, and MS is a local source of free peritoneal macrophage generation. To elucidate the differentiation mechanisms of macrophages in the murine omentum, Zhu *et al* (88) studied the repopulation of these cells and the expression of macrophage colony-stimulating factor (MCSF) in MS and omental tissues of mice depleted in macrophages following administration of clodronate. The macrophages in the omentum were spindle or dendritic in shape and phagocytized intraperitoneally injected carbon particles. Macrophages and their precursors were detected in MS, and the number of precursors increased after the elimination of macrophages. The macrophage precursors in the MS proliferated, moved to the omentum, and transformed into dendritic-shaped macrophages. The locally produced MCSF might play an important role in providing a microenvironment for development and differentiation of omental macrophages (88).

The immunological importance of the omental MS was recognized long ago (89). They were considered to be highly analogous to regional lymph nodes (19,73,84). Lymphocytes are the second major cellular component of normal MS, with B lymphocytes representing 29.1 ± 5.2% and T lymphocytes representing 11.7 ± 2.4% of the cells in MS (19,23); another study reports that B lymphocytes comprise 10.1 ± 3.4%, and T lymphocytes - 10.2% ± 3.7% (81). Macrophages covering the MS facing the peritoneal cavity trap and digest the circulating antigens and foreign bodies. The macrophages may then transfer information to lymphocytes in the central part of MS

(73,84). Dux *et al* (90) studied the lymphocytes in the mouse MS after intraperitoneal injection of sheep erythrocytes. The MS contain surface immunoglobulin-positive B lymphocytes, and T lymphocytes: helper and cytotoxic types. After secondary antigen challenge, the number of lymphocytes increases. The B and T lymphocytes segregate: the B cell zone is in the periphery, resembling the primary follicles of a lymph node, whilst the T lymphocytes are to be found centrally, resembling the paracortex of a lymph node. Other researchers considered that the lymph vessels emanating from MS may correspond to the efferent lymph vessels of the lymph nodes (19). On the other hand, it was stressed that the omental MS in mice are mainly supplied by blood vessels, and are largely devoid of lymphatic vessels, so that they seem to be different from lymph nodes (8). It was repeatedly considered that the omental MS take part in the humoral defense of the host response to intraperitoneal "vaccination" (19,89-92). The majority of the antibody-containing cells are localized predominantly in MS following injections of antigen (91). After intraperitoneal stimulation using sodium thyoglycolate, China ink, and typhoid vaccine, Mandache *et al* (92) have shown the appearance of lymphatic follicles, some of them being with germinal centers. They thus stated that omental MS are subsidiary secondary lymphoid organs. The omental MS of healthy mice contain mainly IgM-positive B cells and lower numbers of CD23- and CD45R- positive B2 lymphocytes (34). The presence of mast cells in the MS was repeatedly noted (2,19,23,34,45,61,77-79,93). Under normal conditions they are relatively few (19, 23), but in pathological conditions their number increases (see below).

MS are involved in extramedullary hematopoiesis, and some data on the macrophage generation were reviewed above. Omental MS in normal animals occasionally show neutrophilic myelopoiesis (8,38,59,62). Clear indication for a strong extramedullary hematopoiesis was repeatedly demonstrated in New Zealand Black (NZB) mice. However, this is not standart laboratory animal. It has especially large and numerous omental MS, and easily develops autoimmune diseases (8,93). The basic structure and cell composition of omental MS in NZB mice resemble those of normal mice (59,62), except for the presence of megakaryocytes and peculiar reticulum cells that possess well-developed dense bodies consisting of clustered parallel tubules. Such MS contain numerous clusters of neutrophils at various maturation levels. Immature cells have been convincingly demonstrated with their electron-opaque cytoplasm, rounded cytoplasmic granules ranging from 0.1 to 0.3 μm in diameter (primary granules), ring-shaped or slightly indented nuclei, prominent nucleoli, and fine nuclear chromatin (8). Mitotic figures in the immature neutrophil leukocytes were also noticed. Myeloblast-like cells or undetermined hematopoietic cells that have a lymphoid cell appearance are rarely admixed with lymphocytes. Interestingly, megakaryocytes were also found; these could be cells that did not directly migrate in MS from other hematopoietic organs (8). Megakaryocytic precursor cells, or stem cells, which might have migrated into MS from the bone marrow or spleen, might differentiate and develop into mature megakaryocytes. Such active hematopoiesis in the MS of NZB mice could be related to an increased stem cell activity (93). Similar results were reported in the activated omental MS of normal mice (34). The authors encountered a myeloid compartment, composed of immature and mature cells of monocyte/macrophage, neutrophil, eosinophil and megakaryocytic lineages. Thus, MS appear to be mixed lymphoid organs, with secondary and/or primary lymphoid organ function, being an important site of B1 cell generation, plasma cell maturation and extramedullar hematopoiesis (34). There is a general consesus that MS in humans and normal animals do not display an erythropoietic ability. After injections of erythropoietin in the peritoneal cavity of ddY mice, small clusters of erythroblasts in various maturation stages were observed, including dividing erythroblasts, denucleating erythroblasts and reticulocytes (39). Thus, MS appear to have a latent erythropoietic activity.

With respect to the nature of omental MS, an interesting possibility has been raised that they might represent an intestinal thymus (76). Thus, omental lymphatic tissues constitute a discrete lymphatic unit that is closely connected to the spleen and the lymph nodes developing around the splenic vessels. However, this possibility has been questioned (8), since there is no relation between the thymus and MS from anatomical and histological points of view. Nevertheless, omental MS may be splenoid lymphoid tissues (8).

As a rule, the MS are associated with adipose tissue (2,6,9,11,18,19). The adipocytes associated with the lymphoid tissues have special properties that distinguish them from classical adipocytes. Lymphoid cells and adipocytes interact in a paracrine manner, which is particularly evident in activated MS and lymph nodes (94-99). MS adipocytes contribute little to whole-body energy supply during fasting, but are more sensitive than all others to cytokines and norepinephrine, having higher maximum but lower minimum rates of lipolysis (100).

MS receive innervation. Human omental arteries and veins are supplied by nerve fibers containing norepinephrine and neuropeptide tyrosine (NPY) (101). Human MS also contain NPY, and its release from perivascular sympathetic fibers

may influence the local circulation (19). Interestingly, human omental MS contain also dopamine-immunoreactive fibers (102). Moreover, they were located not only perivascularly, but also throughout MS. A recent review of the peripheral dopaminergic system can be found in (103).

We have investigated human omental MS, obtained after laparatomy from 15 patients of both sexes (9 males and 6 females), aged 35-67 years. The omental regions without pathologic alterations (with continuous mesothelial covering, intact free cells and vessels in the submesothelial layer) were first identified on semithin sections (1 μm thick), and then examined on ultrathin sections by means of transmission electron microscopy (Fig. 2a-c). Unlike the rat, the human MS exhibited a rough surface, with ridges surrounded by deep furrows. The latter displayed different shape, depth and width. The mesothelial covering was usually extremely thin (Fig. 2a), but clusters of cubic cells were also encountered. Small groups or single phagocyte-like mesothelial cells were found. They had numerous pseudopod-like evaginations and abundant secondary lysosomes. In the normal omentum, stomata were only occasionally seen. A space between two neighbouring mesothelial cells was commonly observed but these are not true stomata. The typical interruptions of the mesothelial covering and its direct connection with underlying endothelium (stomata) were recognized only on serial ultrathin sections by means of transmission electron microscopy. In most cases, the mesothelial basal lamina is absent over MS. Most numerous from the free cell populations are the macrophages, followed by lymphocytes. We encountered fairly constant numbers of mast cells (Fig. 2b). Unlike the classical description of superficial macrophages and deeply located lymphocytes, we most often observed that the cell populations are mixed. In

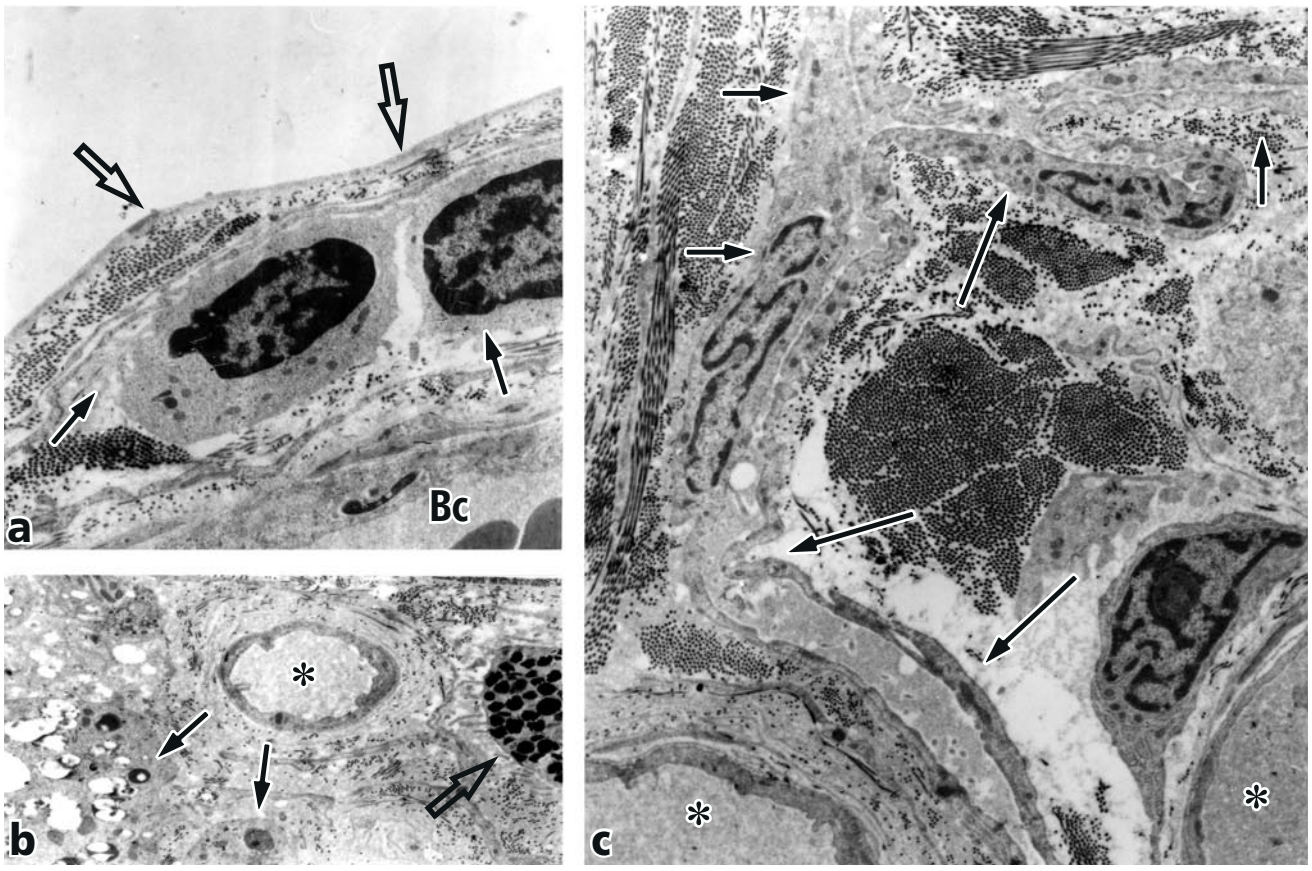

Figure 2. *Electron microscopic micrographs from human omental MS.* ***a:*** *Two lymphocytes (arrows) and blood capillary (Bc) under extremely thin mesothelial covering (empty arrows).* ***b:*** *Macrophages (arrows), mast cell (empty arrow) in vicinity of a blood capillary (asterisk).* ***c:*** *A lymphatic capillary (arrows) with a complicated contour. The parallel endothelial walls surround an extremely narrow lumen. The blood capillaries are labeled with asterisks.* a x 8400; b x 4000; c x 9000.

the normal human MS, we observed a significant component of connective tissue, with perivascular fibroblasts surrounding the blood capillaries, and discrete collagen bundles (Fig. 2c). Single or grouped adipocytes were associated with small blood capillaries or intermingled between the free cells of MS. In the normal rat omental MS, leukocytes were also seen; these were mainly neutrophils, but there were occasional eosinophillic cells as well. Single or small groups (3-5 cells) of erythrocytes were observed in some instances in the rat. We also saw large rounded cells rich in ribosomes and with a large nucleus, around the blood vessels. They appeared to be immature fibroblasts or mesenchymal cells. Endothelial cells with well developed caveolar system rested over basal lamina and formed capillaries with rounded lumens (Fig. 2c). Large lymphatic vessels of the "cysterna-like" type were often located in the vicinity of the mesothelium. Single fibers or fine collagen bundles were disposed between them. The lymphatic vessels possessed extremely flat lumens and were located with their wide diameter parallel to the peritoneal surface of the greater omentum. The lymphatic endothelial cells were extremely thin, electron-lucent, displayed a rich caveolar system, and having no basal lamina.

EXTRAOMENTAL MILKY SPOTS

MS were also described on the parietal peritoneum over the pancreas in the mouse (104). These spots were the same in structural details as the omental MS. Far more interesting are the so called splenoportal MS, also described in mice (31). They are present in the fat bands along the splenic artery. There are sporadic aberrant spleens and numerous MS, supplied with offshoots of the splenic artery. The splenoportal MS are composed of abundant lymphocytes with macrophages, plasma cells, granulocytes, megakaryocytes and various stromal cells. They show active neutrophilic myelopoiesis and probable megakaryopoiesis. The morphological transition from aberrant spleens *via* transitional forms to splenoportal MS seems to indicate that splenoportal MS represent splenoid lymphoid tissues (31). The presence of MS on the peritoneum covering the Douglas pouch *(excavatio rectouterina, cavum Douglasi)* was reported recently (105).

MS are present also in other serous membranes: pleura and pericardium. Kampmeier (106) encountered plaques of macrophages in the mediastinal pleura behind the heart of a human baby. These were called by other authors “Kampmeier’s foci” (reviewed in 19, 107). Trypan blue or India ink injection into the pleural cavity of rats leads to its clear labeling by the injected dyes and the MS of the pleura were therefore nominated “macrophagal foci” (108). Other authors studied the mediastinal MS in mice by electron microscopy and immunohistochemistry (109). The spots are covered with plump mesothelial cells and consisted of clustered lymphocytes, macrophages and fibroblasts; lymphatics are not present in these MS. They also show a specific distribution of lymphocyte subsets: the T cell area comprises the basal region, adjacent to adipocytes under the lymphoid tissue, while the B cells are located superficially. Further studies on the pleural MS (107,110,111) concentrated on their functional abilities. Accordingly, it was found that the anthracotic “black spots” of the parietal pleura and MS show light and electron microscopical similarities (111). Other investigators demonstrated highly vascularized MS on the human thoracic pleura (112). They have a mesothelial covering and are composed of lymphocytes, macrophages and plurivacuolated adipocytes. The existence of MS in the costal pleura was also confirmed (113), and MS facing the pericardial cavity were described (114,115). They are composed of lymphocytes, macrophages and mast cells. In various rodents (rats, mice, golden hamsters), pores connect the pericardial and pleural cavities, and frog erythrocytes injected in the pleural cavity are surrounded and phagocytosed by the pericardial macrophages as early as five minutes after the injection. MS of the rabbit pericardium were studied and compared with MS of the mediastinal pleura and the omentum (116). Injection of India ink and latex particles in the pericardial cavity lead to ingestion of the particles into macrophages, pericardial cavity, or submesothelial layer.

The ultrastructural appearance of MS-like structures located in the deep portions of the broad ligament of the uterus (*ligamentum latum uteri*) is shown in Fig. 3a, b. The surface of the ligament forms large ridges with complex shape, limited by deep furrows, like those of the human greater omentum (Fig. 3a). The majority of the mesothelial cells are from the flat cell type. Small clusters of cubic cells are seen between the flat cells. These MS-like structures are composed by macrophages, small groups of mast cells, adipocytes, neutrophils, lymphocytes and single erythrocytes. Fibroblasts, small bundles of collagen fibers and rare elastic fibers surrounded these cells. Lymphatic vessels are more common than blood vessels. Small groups of blood capillaries and large cysterna-like lymphatic vessels with extremely flat lumina or with irregular boundaries are located parallel to the peritoneal surface (Fig. 3b). The thin, electron-lucent lymphatic endothelium rests upon a fine, incomplete basal lamina. The neighboring lymphatic vessels are separated by thin septae, and contain endothelial valve- or bridge-like formations. We observed different types of contacts between the extremely thin intercellular zones of cubic mesothelial cells and endothelial protrusions of lymphatic vessels.

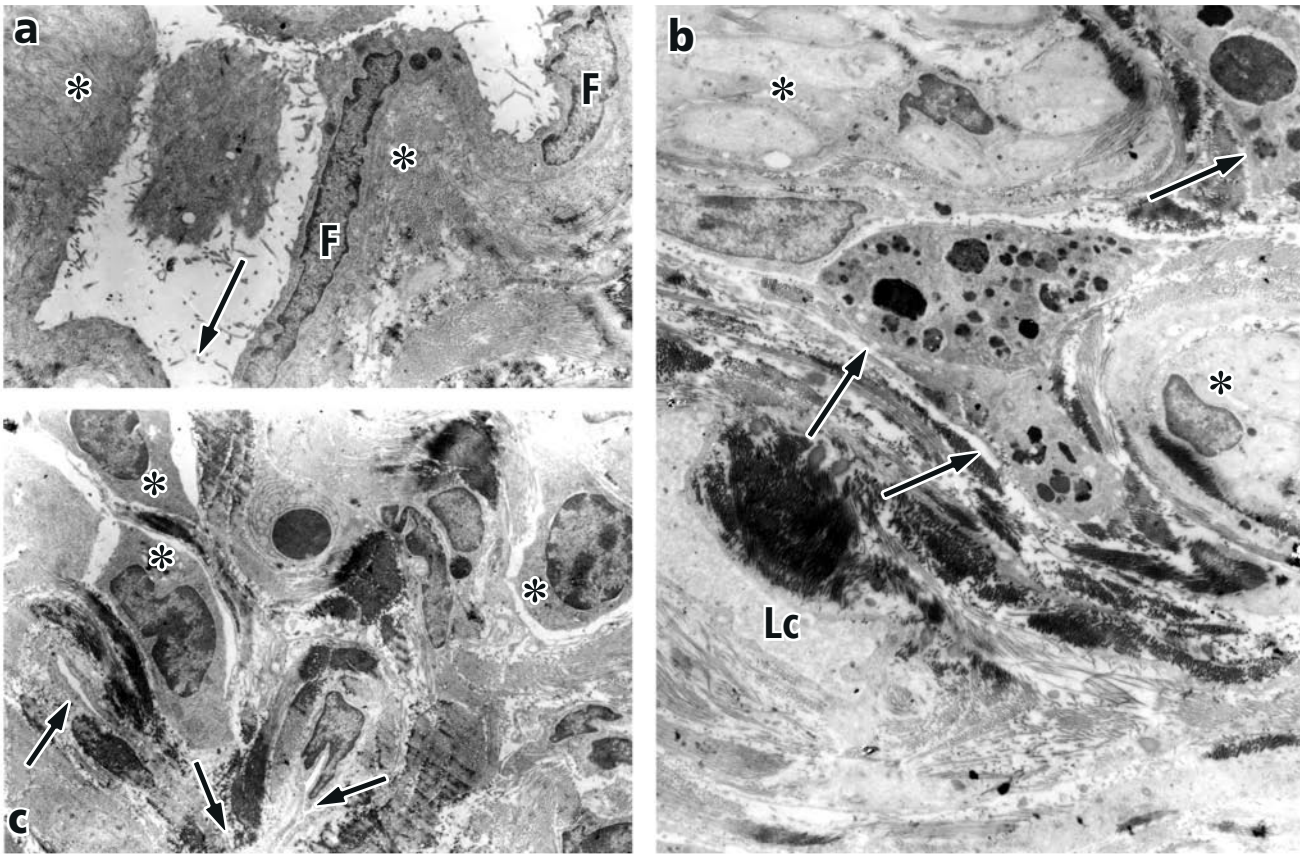

Figure 3. *Extraomental MS-like formations. Broad ligament of the uterus in the rat* ***(a, b)****, mediastinal pleura of the cat* ***(c). a:*** *A complex pattern over a MS-lke structure. Deep furrows (arrows) are surrounded by ridges (asterisks). The formation is covered by flat mesothelial cells (F).* ***b:*** *Macrophages (arrows), lymphatic capillary (Lc) and bundles of thin unmyelinated fibers (asterisks), surrounded by small collagen bundles.* ***c:*** *Free cells, fibroblasts (asterisks) and collagen bundles in vicinity of a large lymphatic vessels (arrows).* a x 4200; b x 6000; c x 2800.

In the vicinity of the mesothelial covering, as well as near to the lymphatic vessels and between the free cells, bundles of thin unmyelinated and occasional myelinated nerve fibers were found.

We also observed MS-like structures on the mediastinal pleura of the cat (Fig. 3c). The main cell types were fibroblasts, macrophages, neutrophil and eosinophil leukocytes, and lymphocytes. They were surrounded by capillaries or larger vessels. The blood and lymphatic capillaries had roughly equal parts in the vessels supply of MS.

ONTOGENY OF THE MILKY SPOTS

The characteristic vascular network of MS differentiates in the middle of the intrauterine development (54). Three developmental types of MS can be distinguished: "primary", "passive", and "active" (52). The "primary" MS are to be found only in fetuses and newborns (52,55). They contain a large quantity of undifferentiated mesenchymal cells, occasional fibroblasts with long branching processes, and adipocytes are almost lacking (52, 54). The "passive" MS of the human fetus appear approximately at the sixth month of uterine life. The number of MS mesenchymal cells diminishes, while the number of adipocytes increases (52,117). It was repeatedly suggested (52,53,55,117) that the adipocytes possess a proliferative ability, and can differentiate in active macrophages. Every irritation of the peritoneal cavity, such as mechanical or chemical injury, or bacterial inflammation, leads to MS reorganization, and they are transformed in "active" MS (11,52). The human newborn babies possess completely developed

pleural MS (106,112).

There are interesting sex differences in the number and development of mouse MS (118). The omental MS were classified into two types. Type II MS are distributed on the dorsal layer of the omentum, and they are more numerous in females than in males. These MS appear at first at 10 days of age, and they gradually increase in number equally in both sexes until two months. After two months, however, the density of the spots is significantly greater in females than in males. In male mice which have been gonadectomized at first month, type II MS show remarcable increase in density. In conjunction, the administration of testosterone to female mice causes a decrease in the density of the spots. Thus, the presence of testosterone plays a major role in development of sex difference in mice MS (118).

The density of humans MS is greatest in infancy and gradually decreases with age (19,33). In infancy, the average number of omental MS/cm^2 is from 30 to 40. As surface area of greater omentum increases with body growth, the number becomes lower: about 20/cm^2 at 1 year of age, 10/cm^2 at 3 years of age. In children, the number of MS decreases to 3-5/cm^2, and in adults the average number is approximately 2/cm^2 (19, 33).

Krist *et al* (119) studied the development and the earliest forms of MS in the human greater omentum, with special attention to the macrophage population. The authors examined specimens from fetuses of 20-40 weeks gestation, and from a newborn. They found that small accumulations of cells were present at 20 weeks of gestation, about 50% of which were monocytes/macrophages. With increasing gestational age, the number of cell clusters as well as their size increased significantly. Starting at 29 weeks, vascularized clusters of cells were seen, and "true" MS were present at 35 weeks. A significant increase in the persentage of mature macrophages was found in developing MS, whereas no activated macrophages were seen. The percentage of B- and T-lymphocytes found in the cell clusters and in MS increased significantly but did not exceed 10% of the total number of cells. The promonocytes most probably mature locally in developing MS (119).

MS were examined in the fetal lamb omentum by means of light and electron microscopy under normal conditions and after intraperitoneal carbon injection *in utero* (120). Rudimentary MS appeared at 72 days of gestation, and at 116 days of gestation, macrophages were detected immunohistochemically. At 125 days, T cells appeared in MS, but B cells were absent. At 148 days of gestation (newborn), MS were noted as black spots because of aggregation of carbon laden macrophages. Thus, at birth the MS macrophages already possess phagocytic ability.

MILKY SPOTS IN PATHOLOGY

A single intraperitoneal injection of fetal calf serum in rats resulted in an increase in the number and size of MS, as well as in an influx of leukocytes (11). The intraperitoneal administration of foreign bodies or substances is followed by acute inflammatory reaction in MS (2,11,29,121,122). Under inflammatory conditions, leukocytes dramatically increase in the connective tissue matrix of MS and on the omental surface (26,121-124). A decreased quantity of adipocytes is observed due to retransformation of "inactive" MS fat nodes in "active" secondary MS (52,125-128). There is mobilization of the fixed mesenchymal cells in the active MS (52,129), and transformation of these cells in active phagocytes and macrophages that migrate towards the surface of the MS (2,129). They concentrate under the serous covering and phagocytise the foreign material (129). Certain particles of foreign material remain encapsulated in the omental MS forever (2,130,131).

Weinberg *et al* (132) investigated the peritoneal plasmacytogenesis and polyploid transformation of mesenteric MS in mice infected by *Shistosoma mansoni*. The authors found a novel type, "specialized" MS, that are dedicated to active plasmacytogenesis and antibody secretion into the peritoneal cavity of the infected animals. Other researchers also studied the changes in mouse MS after infection by *Schistosoma mansoni* (34). The activated MS displayed pronounced lymphocytosis, plasmocytogenesis (IgM > IgG > IgA > Ig2a > IgG1), and myelomonocytosis. The lymphocytes were mainly of B1 type (CD5/IgM+), with smaller number of T cells (positive for the T cell receptor/CD3/CD5 complex) and conventional B2 cells (B220/CD23+). Intraperitoneal injection of Bacillus Calmette-Guerin (BCG) in rats, to study the reactivity of omental MS upon induction of a chronic immune response in the peritoneal cavity (25), increased the number of dendritic cells (133). During the first four months after BCG administration, the number and size of MS increased enormously. This was accompanied by formation of macrophage, T cell, and B cell areas, but interdigitating cells and follicular dendritic cells were not observed. The total number of cells in the peritoneal cavity also increased, and the cellular composition showed a strong similarity with MS. At the onset of experiments, bacteria were mostly observed in MS macrophages rather than in the draining lymph nodes. A cellular immune response was observed in parathymic lymph nodes but not in MS. The authors concluded that both unstimulated and stimulated MS should be regarded as perivascular infiltrates (25). They play a role in the initial clearing of bacteria from the peritoneal cavity. Although the large increase in cell number is caused predominantly by immigration of cells, the results support

the role of MS as a site for local proliferation and maturation, especially in the case of macrophages and B cells. However, no support was found of the assumption that MS may function as a secondary lymphoid organ in the peritoneal cavity (25).

Doherty *et al* (134) studied plasma protein and leukocyte extravasation into the peritoneal cavity in mice and rats with experimental peritonitis, caused by injection of zymosan A. They found that omental MS were the only abdominal sites where intravenously administered Monastral Blue labeled interendothelial cell gaps responsible for plasma extravasation. The omentum was the only abdominal organ which showed an increase in blood flow during zymosan A-indiced peritonitis. Further, the authors reported that omental MS are the major route through which leukocytes migrate into the peritoneal cavity, and that this process is carried out mainly by the postcapillary venules (134). Other researchers investigated the myelopoiesis in the omentum at normal and inflammatory conditions (135). It was found that the increase in cells in the abdominal cavity in inflammation (schistosomal infection) is due to the enhanced input and expansion of early myeloid progenitors sustaining the *in situ* production of abdominal cell populations, rather than to the input of systemic circulating inflammatory cells.

The resolution of acute peritoneal inflammation requires clearance of extravasated inflammatory cells. The neutrophils undergo apoptosis and are ingested by macrophages (reviewed in 136). The inflammatory macrophages emigrate rapidly from the peritoneal cavity to the draining lymph node during the resolution of inflammation, in contrast to the resident macrophages, which persist in the noninflammed peritoneum for weeks (137). Further, the macrophages adhere specifically to mesothelium overlying draining lymphatics and their emigration rate is regulated by the state of macrophage activation through specific adhesion molecule regulation of macrophage-mesothelium interactions (138).

The peritoneal dialysis is a life-saving procedure but it is not harmless. The mesothelial covering of the peritoneum and the submesothelial connective tissue layer are seriously altered and some patients develop sclerosing peritonitis (40,41,139-141). MS have an important role in this process (42-46). Garosi and Di Paolo (42,43) evaluated the number and size of omental MS in rats subjected to dialysis for 15, 30 and 60 days, and compared them with untreated animals. After 15 days of dialysis the number and mean size of MS were significantly lower than in the control group. After 30 days of dialysis, values returned to a level similar to that in controls, and after 60 days of dialysis, the values were significantly greater. The early decrease seemed to be due to washing of the peritoneum and replacement of resident white cells at the expense of the white cell population in the MS. At 30 days, a process of adaptation aims to establish functional equilibrium, and the increase after 60 days of dialysis may be due to the chronic inflammatory stimulus of dialysis solutions with poor biocompatibility. In accordance with these data, rats with peritoneal dialysis for 10 weeks had significantly more MS and blood vessels in the greater omentum, and electron-microscopic analysis demonstrated dramatic changes in the appearance of the MS vascular endothelial cells and a severely damaged or even absent mesothelium on the peritoneal membrane (44,46). A striking increase in number of mast cells in the rat omental MS was observed upon exposure to peritoneal dialysis fluid (45). This increase is probably linked to neovascularization, since the mast cells can produce angiogenic factors.

After direct stimulation with staphylococci, the human mesothelial cells produce the chemokine interleukin-8 (142,143). This response leads to an influx of neutrophils, which are the most important eliminators of bacteria. In addition to neutrophils, mesothelial cells also can ingest bacteria (143). Upon bacterial inflammation, only a few staphylococci are digested, but *Escherichia coli* are ingested on a much larger scale during the first 8 h, and then, after the mesothelial cells disintegrate, a widespread proliferation of bacteria is observed. This difference in the reactivity of the mesothelial cells might explain why peritonitis caused by gram-negative bacteria, such as *Escherichia coli,* is clinically more severe than peritonitis caused by staphilococci (144). Both the peritoneal macrophages and mesothelial cells produce mediators involved in the control of inflammation. It has been widely accepted that resident peritoneal macrophages form the first line of defence against peritoneal infection, and a more contemporary view suggested that the direct or indirect (*via* secreted proinflammatory cytokines) interaction between peritoneal macrophages and mesothelial cells is pivotal to the activation of the peritoneum response to infection (145). Mesothelial cells produce important chemokines, involved in peritoneal inflammation: GRO-α (attractant for neutrophils), MCP-1 and RANTES (monocyte attractants) (146). This suggests an important role for mesothelial cells in host defense. The neutrophil leukocyte migration into the peritoneal cavity in response to peritonitis is an important mechanism of host defence against bacterial invasion, and the mast cells are especially necessary for neutrophils migration within the infected peritoneal cavity (147).

We studied the omental MS in rats with experimental peritonitis caused by intraperitoneal injection of *Pseudomonas aeruginosa* (Fig. 4a-f). The mesothelial covering over MS is built by activated mesothelial cells with numerous microvilli,

abundant dilated cisternae of the rough endoplasmic reticulum, prominent Golgi complex, numerous lipid droplets and a rich caveolar system (Fig. 4a). Some mesothelial cells show degenerative changes in their organelles. At certain regions, the mesothelial cells have no basal lamina and are located superficially towards the peritoneal cavity. The MS are disposed close to the mesothelial row, and increase in size and number. The connective tissue components (adipocytes, fibroblasts and collagen fibers) decrease in number, and are often missing. MS are composed only of a larger number of free cells, separated by wider intercellular spaces (Fig. 4b). This is probably a result of an increased microvascular permeability to fluids and fibrin and its deposits between the cells. The activated macrophages form large groups and are filled with secondary lysosomes. The superficial location of the macrophages within the mesothelial layer is far more common than observed in healthy animals. At the investigated time intervals (days 5 and 8 after the intraperitoneal application of *Pseudomonas aeruginosa*), the quantity of lymphocytes appeared to be only slightly changed. They either occupied a position immediately under the mesothelial cells, or they form small groups in the center of the MS together with plasmocytes (Fig. 4c). The number of neutrophylic leukocytes was increased and they formed clusters that did not contain other cells (Fig. 4d). Numerous

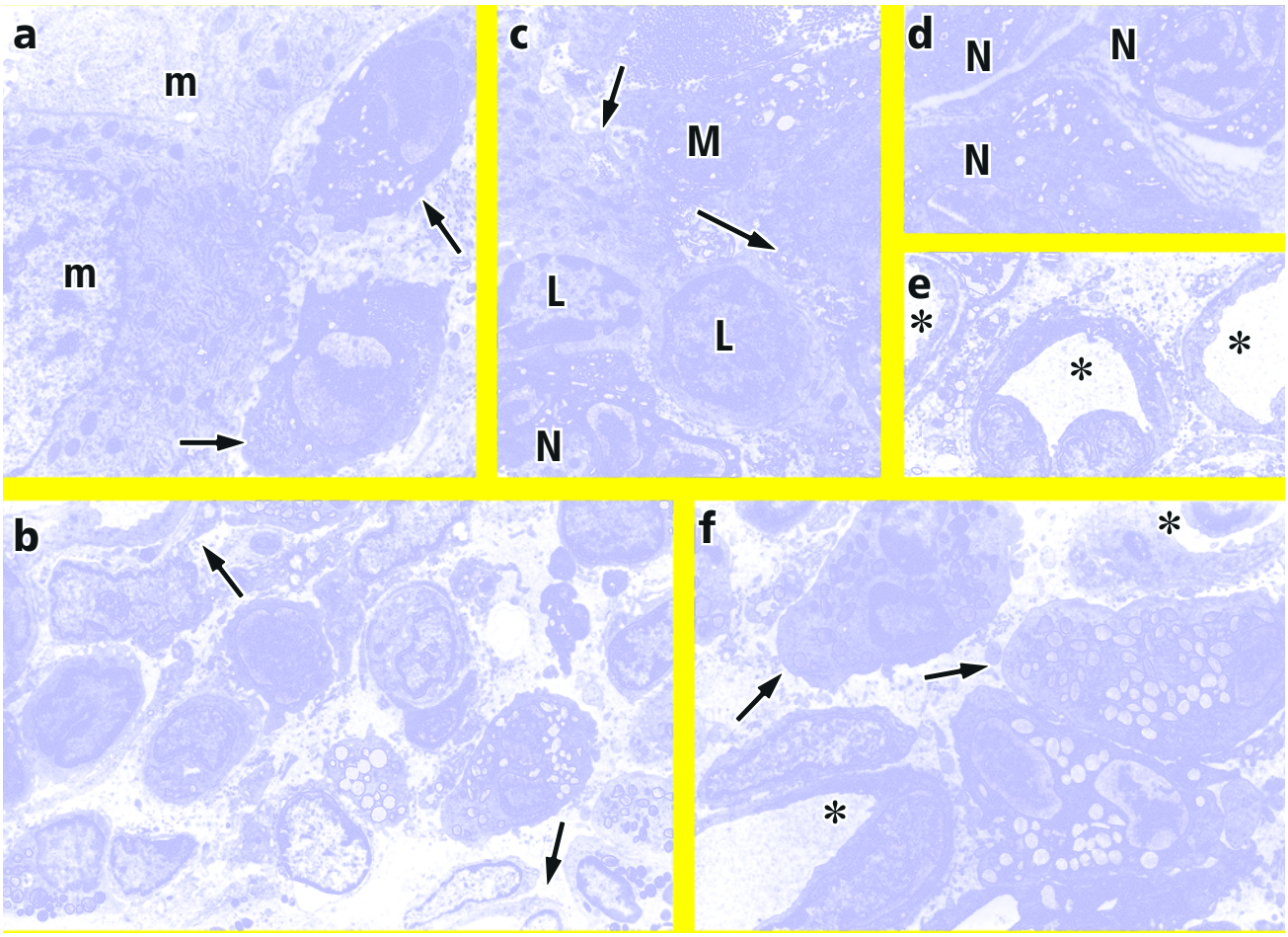

Figure 4. *Omental milky spots in the rat with experimental peritonitis, caused by intraperitoneal instillation of* Pseudomonas aeruginosa. ***a:*** *Two lymphocytes (arrows) under activated mesothelial cells (m).* ***b:*** *Blood capillaries (arrows) and a large cluster of free cells, surrounded by wide intracellular spaces and collagen fibers.* ***c:*** *Lymphocytes (L), neutrophil leukocyte (N), macrophage (M) and portions of plasmocytes (arrows).* ***d:*** *Group of neutrophil leukocytes (N).* ***e:*** *Wide spaces between blood capillaries (asterisks).* ***f:*** *Blood capillaries (asterisks) and eosinophil leukocytes (arrows).* a x 5000; b x 2500; c x 4200; d x 4300; e x 2700; f x 4200.

small capillaries without perivascular cells were also observed (Fig. 4e). A few mast cells and eosinophilic leukocytes were located in the vicinity of the capillaries (Fig. 4f). The number, shape and size of the lymphatic capillaries were comparable with those in normal MS.

Since the pioneer observations of Dux (7), the evidence of MS involvement in malignant processes is constantly growing. MS become hypertrophic and congested soon after intraperitoneal inoculation of Ehrlich ascites tumor in rodents. This suggests that MS are the targets for the formation of small omental nodules in the advanced stage of Ehrlich ascites tumors. The number of γ-globulin-producing cells in MS increases from the second day after inoculation with same tumor, reaching 60-70% of the cells in MS after 9 days (148). Eight hours after intraperitoneal injection of WP1 tumor cells in rats, scattered tumor cells were attached to the surface of the MS (149), while 24 hours after the injection the tumor cells on the surface of MS were numerous. The mesothelium was disrupted by an inflammatory response, suggesting that the attachment of tumor cells was linked to the host inflammatory response (149). Even following subcutaneous (not intraperitoneal) injection of asbestos fibers in mice, some were found in MS more than 442 days after the injection (68). This appears to be of clinical importance since there are numerous communications that asbestos fibers contribute to the genesis of a highly malignant tumor, mesothelioma (reviewed in 68,150-152). MS are also related to sarcoma dissemination: 6-12 hours after Yoshida sarcoma cell intraperitoneal inoculation, sarcoma cells were found in MS (153). MS contained an increased number of macrophages, lymphocytes and neutrophil leukocytes. The tumor cells proliferated and formed tumor nodules with neovascularization in MS, which suggested the significance of the MS as early targets for disseminated intraperitoneal sarcoma (153). Cancer cells in the human MS were also detected (19,83,154).

Investigation of the participation of omentum in the development of tumors at sites of intraabdominal trauma (various types of surgical wounds) revealed that a significant reduction in the "uptake" of tumor cells at trauma sites were observed when maximal amounts of omentral tissue was removed (155). Further, the stimulation of omental macrophages resulted in increased tumor growth on the omentum (156), suggesting that the macrophage activity in omental MS may lead to a secretion of growth factors enhancing the tumor growth (155). Similarly, secretion of angiogenic factors from the omentum may also result in early neovascularization of seeded tumor and more rapid growth (19,156-158). MS appear appropriate as a target organ in every phase of tumor cell metastasis (19,159-162). Cancer cells usually form clumps in the ascitic fluid (163), and have a high chance of attaching to the omentum because omentum takes part in fluid drainage from the peritoneal cavity (2,5,9,19,129,164-166). Exfoliation of mesothelial cells and basal lamina degradation appear to be essential steps for tumor cell infiltration (167,168). MS are easily infiltrated by tumor cells as their surface is not completely covered by mesothelial cells (2,7,9,19,26,37,53,73,169), which lack basal lamina (7,9,11,26,29, 53,70,105). Hagiwara *et al* (159) examined the site-specific implantation of cancer cells in peritoneal tissues after an intraperitoneal inoculation of 10^5 P388 leukemia cells. Twenty-four hours after the inoculation, most cancer cells were implanted in the omentum followed by (in decreasing order) gonadal fat, mesenterium, posterior abdominal wall, stomach, liver, intestine, anterior abdominal wall, and lung. A significant correlation was established between the number of infiltrating cancer cells and the number of MS. The same authors examined the omentum after inoculation with leukemia cells labeled by the DNA syntesis inicator bromodeoxyuridine (159). These tumor cells were found to be infiltrating only MS, whereas none were seen at other omental sites. Thus, the tumor cells seeded intraperitoneally specifically infiltrate MS in the early stage of peritoneal metastases. In support of this conclusion, three different mouse carcinomatous peritonitis models (using P388 leukemia cells, Colon26 cancer cells, and B16PC melanoma cells) showed that tumor cells preferentially infiltrates MS as early as one day after the inoculation (160,161). In a model investigating the peritoneal dissemination of pancreatic cancer cells in golden hamsters, the authors found that the cancer cells were easily absorbed at the omental MS and the diaphragmatic stomata, and lymphatic metastasis occurred four days after inoculation (170). However, on the parietal peritoneum the cancer cells proliferated in spots with exfoliated mesothelial cells and exposed basal lamina. This process was comparably more time-consuming, and accordingly metastasis occurred at seven days after inoculation. Thus, two patterns of peritoneal dissemination of pancreatic cancer cells appeared: lymphatic and direct metastases (170). Evaluation of the metastatic pattern of CC531 colon carcinoma cells administered in the peritoneal cavity of rats revealed that tumor cells concentrated in omental MS within four hours after inoculation (169). The size of MS increased as a result of increased number of tumor cells and macrophages. After 7-21 days, MS were completely replaced by tumor cells, and new MS were formed. The concentration of tumor cells in the omental MS was followed by the paracolic gutters, the subhepatic and subphrenic spaces and in the lymphatic lacunae of the diaphragm.

Intraperitoneal immunotherapy has been widely used as an

adjuvant postsurgical treatment of intraperitoneal malignant diseases (171-175). This route of administration is anatomically appropriate because it directly introduces the drugs into the peritoneal cavity. Whereas the mechanisms of intraperitoneal immunotherapeutic activity have not been elucidated in detail, peritoneal macrophages are known to be an important effector cells in the host defence against tumors and metastases. In this regard, omental MS generating peritoneal macrophages could have important antitumor host defense activities (19,176). Recently, there is a growing understanding of the mechanisms that allow the tumor cells to evade the host immune system, and, morevover, to use inflammatory mechanisms to promote tumor growth and spread inside the peritoneal cavity (177). The cytokine release was studied after the intraperitoneal administration of beta-1,3-D-polyglucose-treated microbeads (178). Numerous microbeads phagocytosed by MS macrophages were seen 48 h after administration, and significant amounts of interleukin-1 and prostaglandin E2 were released from these macrophages. Intraperitoneal administration of a killed streptococcal preparation, OK-432, might prove to be an effective biological response modifier for managing malignant ascites (172) by activating the MS macrophages (122). These cells demonstrated increased surface membrane activity and migration through MS "stomata" into the peritoneal cavity. The characteristic features of activated MS suggest that they are a valuable source of macrophages for intraperitoneal immunotherapy (19, 122). The penetration of tumor cells into MS was studied in mice after intraperitoneal, subcutaneous and intravenous injections, and then the tumoricidal activity of MS macrophages was analyzed (179). Tumor cells could be detected in MS within 10 min after intraperitoneal inoculation. Macrophages isolated from MS of mice immunized against syngeneic as well as allogeneic tumor cells expressed a significant cytotoxicity, which preceded the cytotoxicity of peritoneal macrophages. MS were infiltrated only by tumor cells derived directly from the peritoneal cavity. These data support the view that immune reactions against intraperitoneally injected tumor cells are initiated in MS, and might lead to "peritoneal immunity" against these tumor cells (179). Other researchers investigated the effect of intraperitoneal administration of granulocyte/macrophage colony-stimulating factor (GMCSF) on omental MS composition and tumoricidal activity against syngeneic colon carcinoma cells in rats (180). The results of *in vitro* study showed that GMCSF treatment led to an increased cytotoxicity of omental macrophages against the tumor cell line. Further, the intraperitoneal administration of GMCSF for one week was associated with an increase of MS macrophage proliferation and an enhanced antitumor activity. Thus, the GMCSF treatment may prove beneficial as local immunotherapy in the prevention of intraabdominal tumor growth (180).

The malignant mesothelioma remains an incurable disease, but some recent data have shown a potential for immunomodulatory therapies, such as exogenous cytokine administration. Intracavitary IFN-β gene therapy in mice using an adenoviral vector provided strong T cell-mediated antitumor effects in experimental models of mesothelioma, and suggested that this may be a promising strategy for the treatment of localized tumors such as mesothelioma or ovarian cancer in humans (181). Further, two days after the intraperitoneal injection of the adenoviral vector in mice with peritoneal tumors, an influx of activated natural killer cells, polymorphonuclear leukocytes and macrophages was observed (182). Up to 10 days after application of the vector, a large influx of activated CD4- and CD8-positive T cells was present in both peritoneal fluid and tumor nodules. The CD8-positive T cells exhibited an effective tumor cell killing. These authors concluded that the tumor-specific T cells are key effector cells for tumor eradication.

CONCLUSION

MS are tiny tissue aggregates located mainly on the greater omentum, but also on other peritoneal regions, as well as on the pleura and the pericardium. They are composed of mesenchymal cells around blood capillaries and are covered incompletely with mesothelial cells. MS are involved in normal functions as well as in pathologic processes. Since the beginning of the 20th century, the nomination of omental MS as "policeman of the abdomen" or "friend in need" was extended in numerous recent findings. While initially MS were identified as structures which have the ability to phagocytose foreign bodies in the peritoneal cavity, they were later recognized also as lymphoid tissue that vigorously participates in the immune reactions of the serous cavities. Under inflammatory conditions, MS act as the first line of defense in these cavities, and are involved in extramedullary hematopoiesis. MS are an early target of intraperitoneal (intrapleural) tumor metastases, and thus are an important site in the development of an antitumor immune response. The modulation of this response holds a potential for improving the immunotherapeutic strategy against malignant diseases.

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