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ORIGINAL ARTICLE

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Mast cell types and cell-to-cell interactions in lymph nodes of the opossum *Didelphis albiventris*

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Abstract Previous light-microscopic studies have shown a unique population of mast cells in lymphatic sinuses of lymph nodes located in the head, neck, axillary fossa and inguinal region of the opossum. In the present work, scanning and transmission electron-microscopic studies in the opossum mandibular and superficial axillary lymph nodes have strengthened the differences between connective-tissue mast cells (CTMC) and the lymphatic-sinus mast cells (LSMC). Further, close appositions of mast cells to other cells were described. At the nodal capsule, CTMC contacted fibroblast and granulocytes. In the lymphatic sinuses a few CTMC contacted LSMC, macrophages and reticular cells. The LSMC contacted macrophages, reticular cells and other LSMC. A few LSMC could be located in the medullary cord in close contact with plasma cells or other lymphoid cells, keeping the same ultrastructural features of those found in the lymphatic sinuses. An important new finding was provided by light-microscopic studies in nine abdominal lymph nodes. Most of them (para-aortic, common iliac, cardial, cecocolic and those of the body and tail of the pancreas) displayed numerous LSMC with the same distribution and histological features described herein. However, the mesenteric, pyloric and head-of-pancreas lymph nodes were virtually devoid of LSMC. Instead, their mast cells occurred mainly at the medullary cords and were very similar to the CTMC. Ultrastructural studies at the mesenteric lymph nodes confirmed the CTMC character of the mast cells located at both medullary cords and sinuses, and disclosed interactions with macrophages and lymphoid cells.

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Introduction

Several studies developed by Kitamura and co-workers (reviewed by Kitamura 1989) in mice revealed that mast cells originate from bone marrow precursors. Circulating precursors occur in mouse blood as nongranulated cells that migrate to different tissues in which proliferation and differentiation to granulated mast cells take place under the influence of several growth factors. The mast cell heterogeneity could result from the microenvironment peculiarities (reviewed by Galli 1990; Metcalfe et al. 1997; Lin and Befus 1999). In rodents two populations of mast cells have been clearly distinguished by morphological, biochemical and physiological criteria: the connective-tissue mast cells (CTMC) and the mucosal mast cells (MMC) as described by Enerback (1986) and reviewed by Galli (1990). In the opossum, CTMC and MMC could also be distinguished by light- and electron-microscopic studies, the latter being found in the intestinal mucosa and submucosa (Santos and Machado 1994). Unexpectedly, light-microscopic studies in opposum lymph nodes located in the head, neck and limbs showed numerous mast cells, clearly different from CTMC and MMC, restricted to the lymphatic sinuses. The opossum lymphatic-sinus mast cells (LSMC) differed from the opossum CTMC by their larger size and enlarged cytoplasmic granules that were also more heterogeneous in shape and staining properties (Chiarini-Garcia and Machado 1992). The presence of a unique population of mast cells in the opposum lymph nodes is an interesting finding since no dissimilarity with CTMC has been reported for mast cells of eutherian lymph nodes (Sainte-Marie and Peng 1990; Lozzi et al. 1996). The abundance of LSMC in opossum lymph nodes contrasts with the usually smaller proportion of mast cells in lymph nodes of eutherian mammals. However, in rodents, their number increases with age (Sainte-Mairie

and Peng 1990) and after both antigenic and non-antigenic stimulation, probably by a process of draining from the stimulation site (Sainte-Marie and Peng 1990; Lozzi et al.1996 and references therein).

The present study aims at establishing the ultrastructural features of mast cells of opossum lymph nodes located in the cervical and axillary regions, emphasizing the interaction with other cells. By studying both parietal and visceral lymph nodes located in the abdominal cavity of the opossum, we also intend to verify the extent of the occurrence and distribution of LSMC.

Materials and methods

Eight South American opossums, *Didelphis albiventris* (Marsupialia, Didelphidae) were captured in Belo Horizonte, Brazil, under a permit provided by the Brazilian Institute for the Environment (IBAMA-MG). The animals looked healthy and were maintained in individual cages for less than 24 h with water *ad libitum*. They weighed 450–1100 g on the day of sacrifice and were free of cutaneous wounds. Care of the animals and euthanasia were in accordance with the guidelines for laboratory animals established by the National Institute of Health, USA.

Transmission electron microscopy (TEM)

Three adult animals (two females and one male) under Nembutal anesthesia (30 mg/kg of body weight, i.p.) were perfused from the left ventricle to the right atrium with Ringer solution followed by a modified Karnovsky's fixative (2.5% glutaraldehye-2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.2). The perfusion pressure for both solutions ranged from 70 to 80 mm Hg and the volumes depended on the animal weight. In two other opossums (one male and one female), the right mandibular lymph nodes were removed before the perfusion procedure, in which the controlled pressure ranged from 110 to 120 mm Hg. After perfusion, sagittal slices of superior lips, and fragments of mandibular, superficial axillary, and mesenteric lymph nodes were immersed in the fixative for 4–6 h, then post-fixed in reduced osmium (Russell and Burguet 1978) for 2 h, dehydrated in graded series of ethanol and embedded in Epon 812. After staining with uranyl acetate and lead citrate, the sections were examined in a Zeiss EM10 electron microscope.

The average diameter (sum of two perpendicular axes divided by two), of the mast-cell cytoplasmic granules was measured in electron micrographs at a final magnification of ×7000 to ×23 000. For statistical analysis the Student's *t*-test was used at the 95% confidence level.

Scanning electron microscopy (SEM)

The same animals used for TEM provided tissues for analysis in the SEM. After the intracardiac perfusion, fragments of the mandibular and superficial axillary lymph nodes remained in the Karnovsky's fixative for 24 h, then post-fixed in sequential baths of osmium, tannic acid, thiosemicarbazide and osmium, according to Murakami and Jones (1980). The tissues were then dehydrated in ethanol, cryofractured in liquid nitrogen, critical point-dried in a CO_2 dryer (CPD-020, Balzers) mounted on SEM stubs and subsequently sputter-coated (BSV-203 unit of the BAF-300 equipment, Balzers). The gold-coated fragments were examined in a Zeiss DSM950 scanning electron microscope.

Light microscopy

The following parietal and visceral abdominal lymph nodes were removed from three adult male opossums under ether anesthesia: anterior para-aortic, common iliac, cardial, pyloric, mesenteric, cecocolic, colic, and those related to the head, body and tail of the pancreas according to the anatomical description of Azzali and DiDio (1965). All abdominal lymph nodes or their fragments were fixed in Carnoy's solution (ethanol, chloroform and acetic acid, 6:3:1 by volume) for 24 h at 4° C and processed for embedding in glycol methacrylate (Tecknovit 7100, Kulser). For staining, 0.5- or 3-µm-thick sections were treated with 0.5% toluidine blue with 1% sodium borate.

Results

Connective-tissue mast cells

At TEM, dermal CTMC presented narrow surface processes curved toward the cell surface and displayed a discontinuous granular coat (external lamina) in which collagen fibrils stuck in (Fig. 1A). However, direct contact with fibrillar collagen was also apparent (Fig. 1B). Although dermal CTMC could lie near fibroblasts or their long processes, no close contact between mast cells and any type of connective-tissue cells were observed. However, paired mast cells were frequent in the dermis. This mast cell-mast cell interaction involved membrane interdigitations (Fig. 1C).

In all animals, the capsule of the mandibular and superficial cervical lymph nodes exhibited several CTMC and some mononuclear leukocytes and neutrophils sparsely distributed among the numerous fibroblasts and collagen fibrils. Several CTMC were less buried in collagen fibrils, exhibiting large surface areas in direct contact with granulocytes and fibroblasts (Fig. 1E). In the latter, pinocytic vesicles could accumulate in the contacting fibroblast membrane (Fig. 1D). In the nodal capsule, degranulating CTMC seemed more frequent than in the lip dermis and some of them were in close apposition to neutrophils (Fig. 1F). Mast cells with bizarre forms or displaying surface folds ending far from the cell surface or even lamelliform extensions were also found. By measuring 109 homogeneous-appearing cytoplasmic granules of nodal CTMC, their average diameter was estimated at 0.49±0.07 µm.

In SEM, the CTMC could be identified with certainly only after fracture that showed up their cytoplasmic granules. Probably the difficulty in recognizing non-fractured CTMC was due to their smooth surfaces, besides the surrounding collagen fibrils (Fig. 2A).

Lymphatic-sinus mast cells (LSMC) in the mandibular and axillary lymph nodes

The mandibular and superficial axillary lymph nodes exhibited LSMC in all lymphatic sinuses. They were rather numerous in the medullary sinuses (Figs. 2B, 3A). However, the LSMC were observed only when a pressure of 70–80 mm Hg was maintained throughout the intracardiac perfusion with the fixative. A perfusing pressure of 110–120 mm Hg washed out virtually all LSMC. At SEM, the LSMC were easily identified by their black-

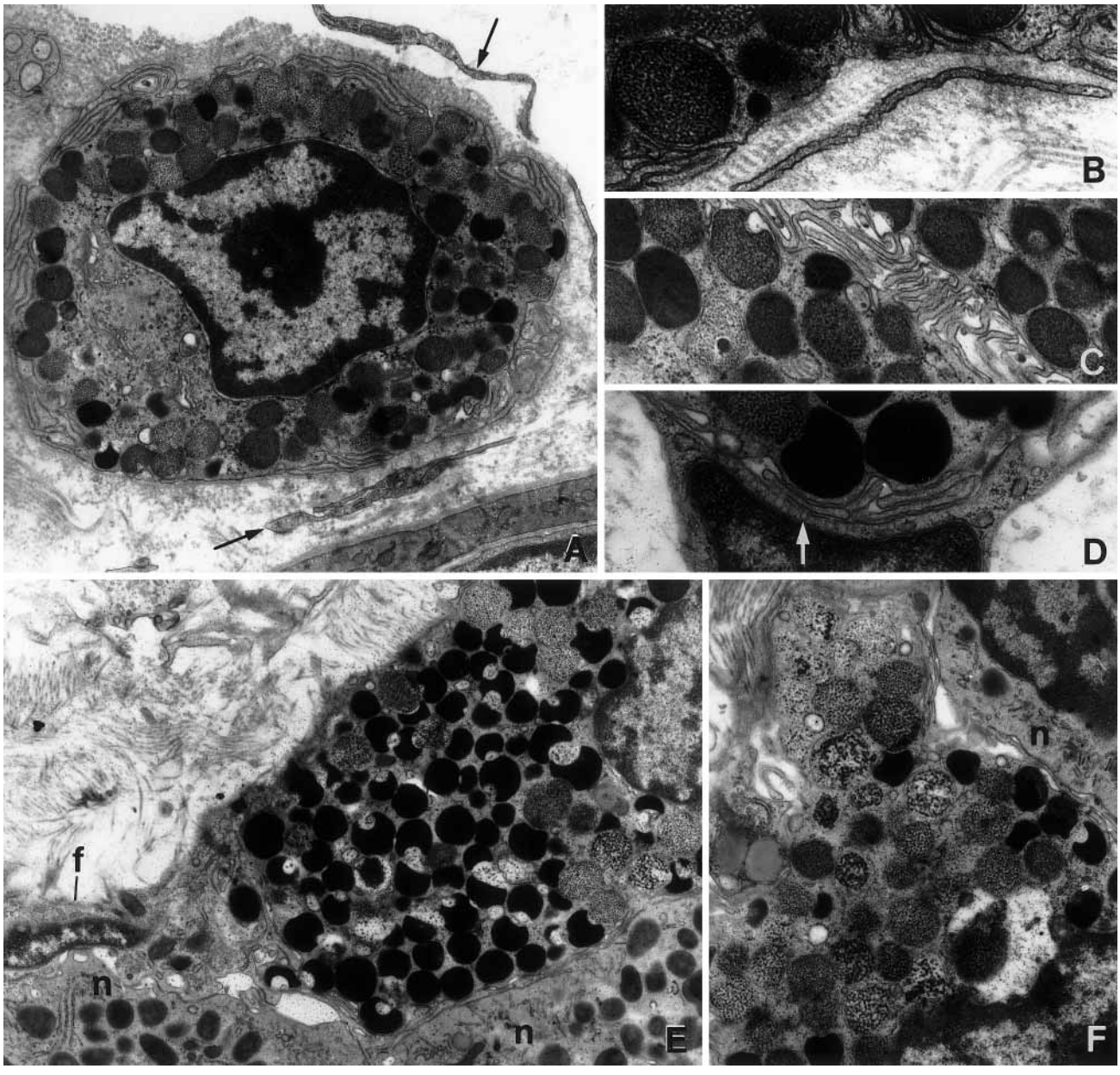


Fig. 1 Electron micrographs of opossum CTMC in the lip dermis (**A**–**C**) and in the capsule of the mandibular lymph node (**D**–**F**). **A** Shows a CTMC surrounded by extracellular matrix (external lamina and collagen fibrils) and nearby fibroblast processes (*arrows*). ×11 500. **B** Shows interaction with fibroblast process through collagenous material. ×27 300. **C** Interdigitating membranes of paired CTMC. ×22 000. **D** Detail of mast cell-fibroblast interaction showing subsurface vesicles in the fibroblast. ×19 200. **E** A CTMC interacts with neutrophils (*n*) and fibroblasts (*f*) and shows a large surface area in contact with extracellular matrix, ×8600. **F** Degranulating CTMC contacts a neutrophil (*n*). ×12 100

berry appearance, showing the superficial and large cytoplasmic granules (Fig. 2B, C)). Sometimes the granular content was disclosed by openings in the cell membrane (Fig. 2C) and some LSMC appeared as degranulating mast cells (Fig. 2D). At TEM they were clearly distinguishable from the CTMC by the size of their cytoplasmic granules (Figs. 3, 4A). These granules were numerous and pressed the surface membrane (Fig. 3A) giving the cell an irregular contour as depicted by the SEM analysis. Their contents varied from homogeneous electron density to rather electron lucidity with variable amount of fine granular material (Fig. 3A, B). Granule fusions were easily seen (Fig. 3B). The average diameter of the LSMC granules with homogeneous electron density was 1.53±0.34 μm (n=107), being statistically larger than the CTMC granules (P<0.05).

The LSMC were devoid of an external lamina and seemed to be fixed in the sinus wall through cell-to-cell adhesion. In the subcapsular sinus, the LSMC adhered to the lining reticular cells (lymphoendothelium), and macrophages covering the lymphoid tissue, but they were never found attached to the lymphoendothelium lining the capsular connective tissue (Fig. 3A). The LSMC al-

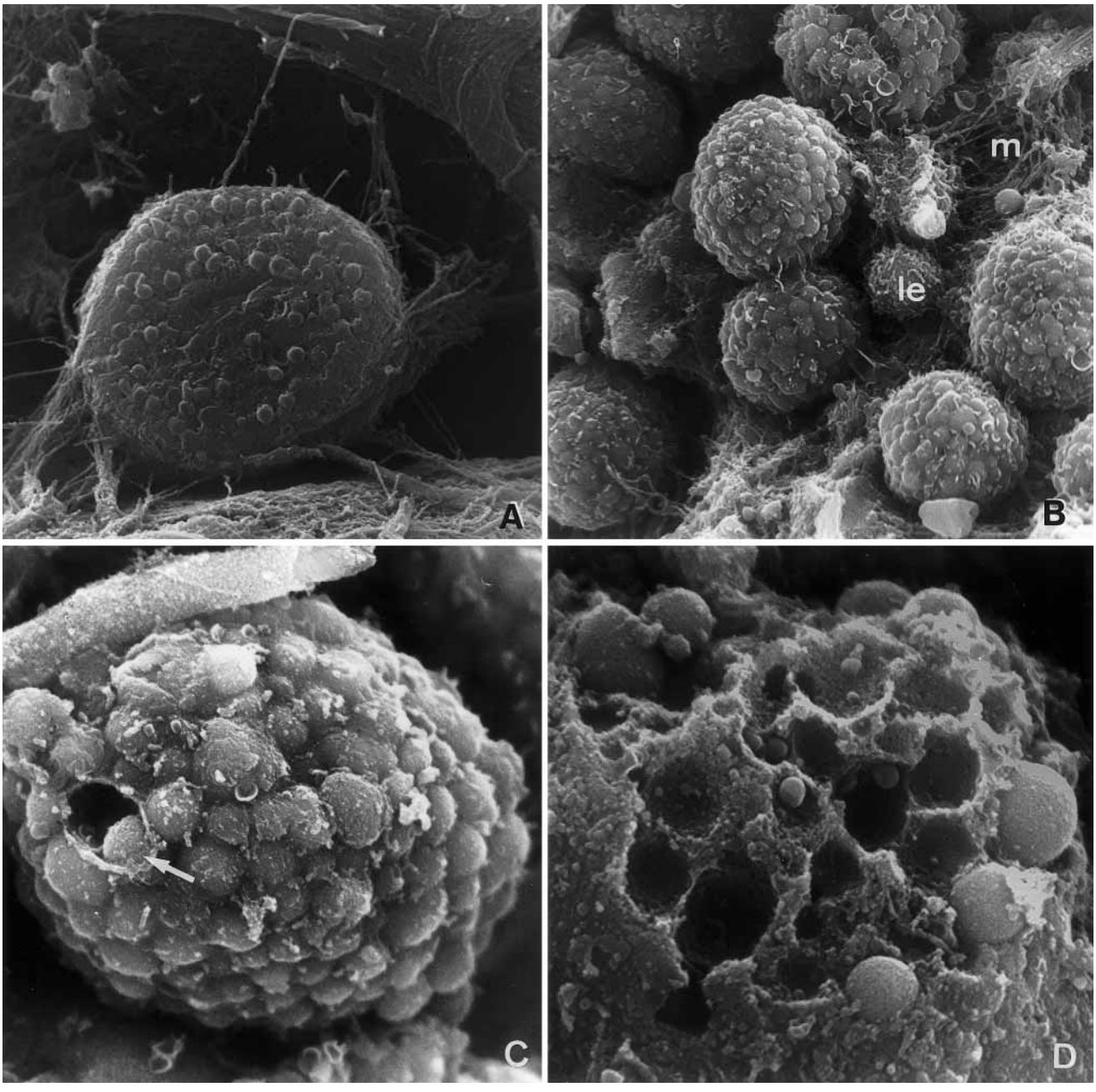
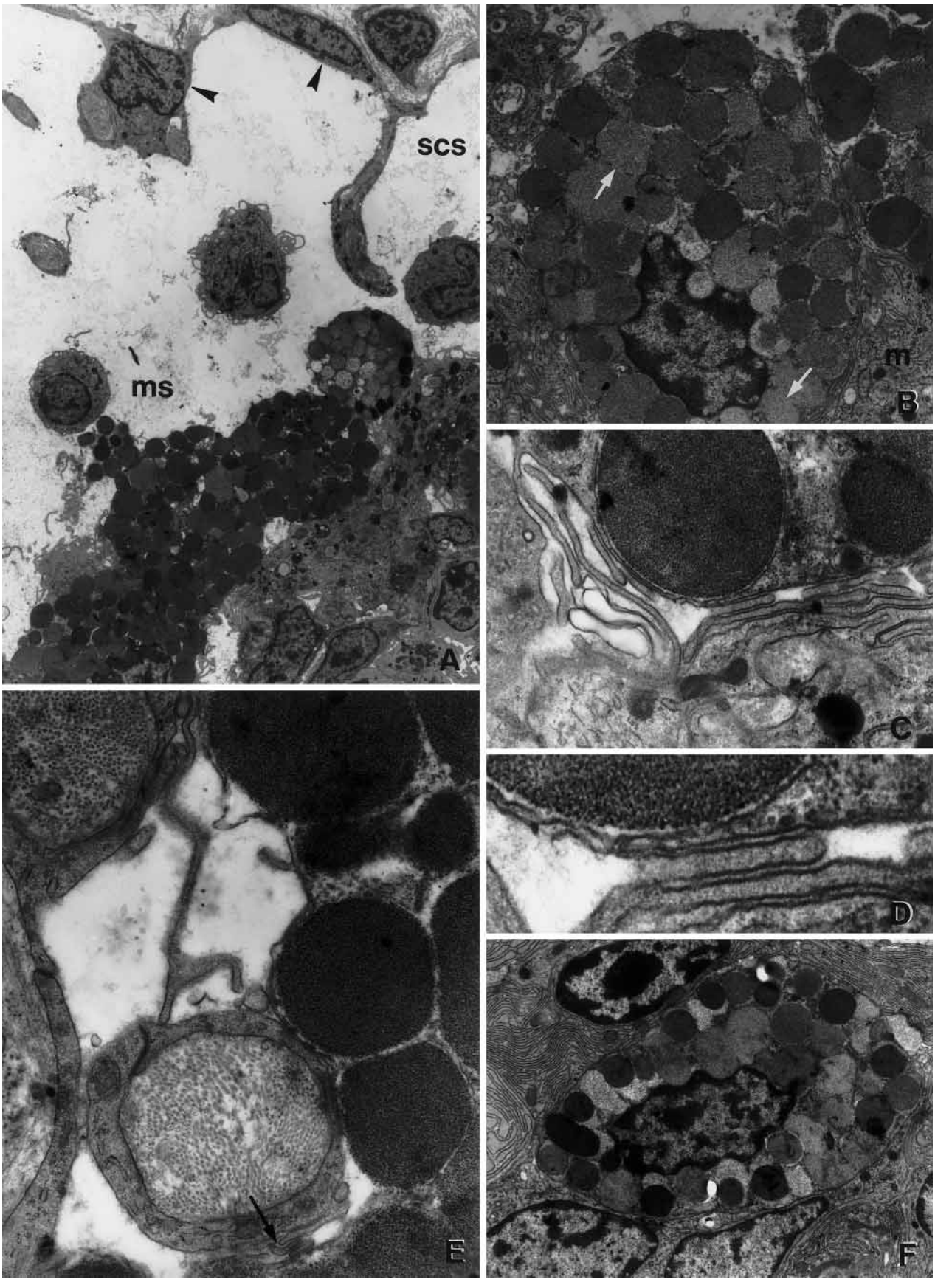


Fig. 2A–D Scanning electron microscopic features of opossum mast cells. **A** Dermal CTMC showing its smooth cell surface and the encircling collagen fibrils. ×8700. **B** Medullary-sinus lumen exhibiting several LSMC, a macrophage (*m*) and a leukocyte (*le*). ×3900. **C** A LSMC adhered to a reticular cell process shows an opened granule (*arrow*). ×11 000. **D** The surface of a degranulating LSMC shows empty granules and some granule contents. ×17 000

ways lay in close contact with macrophages (Fig. 3B–D), reticular cell bodies or their processes (Fig. 3E) or other LSMC (Figs. 3A, 4A). These LSMC interactions could involve fold-free areas of the contacting membranes or interdigitating surface folds (Fig. 3B–D). The LSMC-macrophage interactions seemed more frequent than the LSMC-reticular cell contacts. A constant spacing was maintained in such contacts in which crossing filaments

Fig. 3A–E Electron micrographs of LSMC in the mandibular lymph node. **A** A medullary sinus (*ms*) reaching the subcapsular sinus (*scs*) shows the epithelioid arrangement of LSMC but no mast cell is seen adhered to the reticular cells (*arrowheaeds*) underlying the capsule. ×2100. **B** A LSMC in close contact with another LSMC and a macrophage (*m*) shows evidence of granule fusion (*arrows*). ×6500. **C** Interdigitating membranes in a LSMC-macrophage contact. ×21 000. **D** Detail of **C** showing fine filaments in the intercellular space. ×62 000. **E** A reticular cell process encircling collagen fibrils contacts a LSMC at two points; one of them (*arrow*) seems to involve membrane interdigitation. ×18 000. **F** A LSMC contacts plasma cells inside a medullary cord, ×5000 ▶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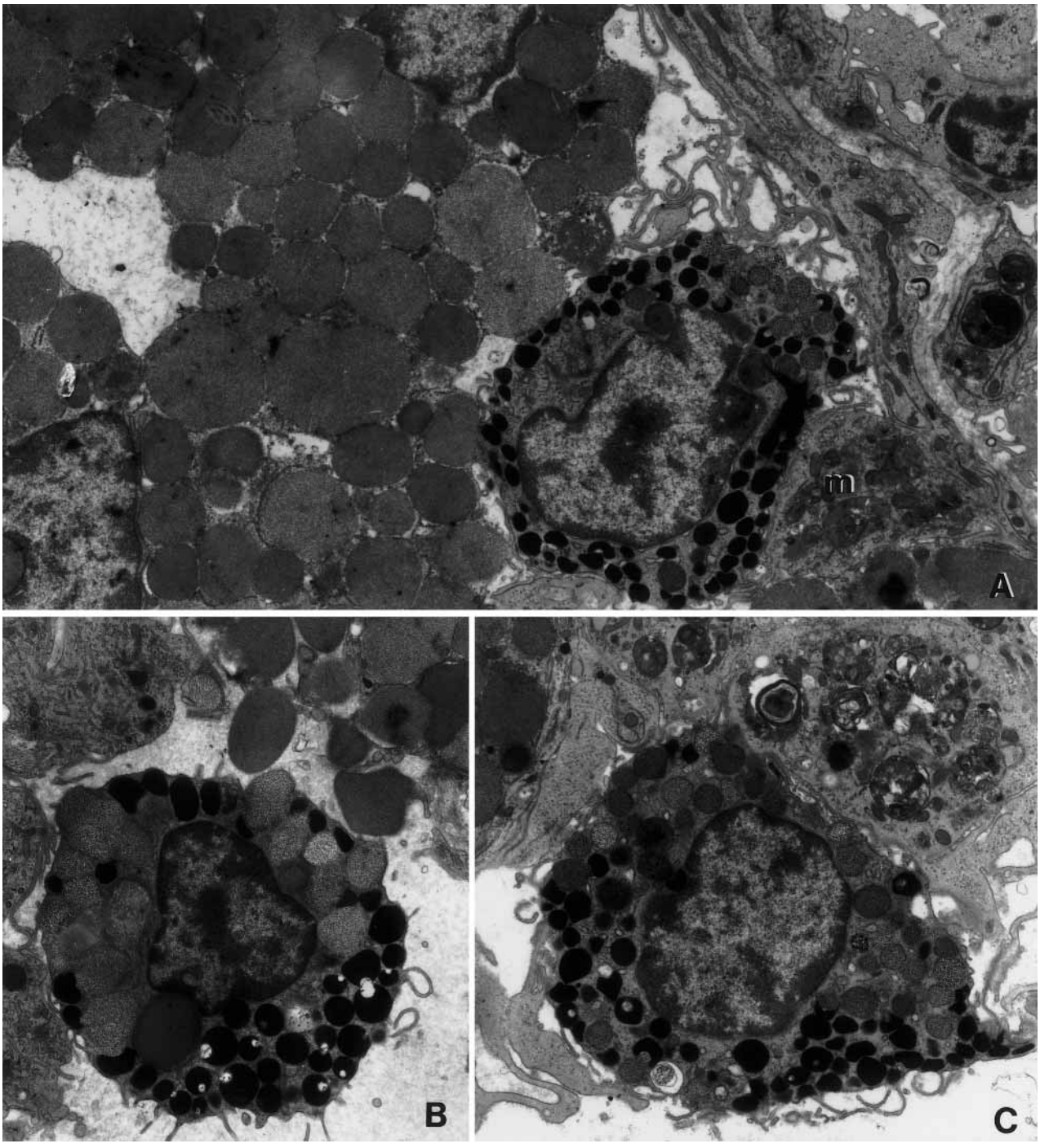


Fig. 4A–C Electron micrographs showing mast cells similar to CTMC inside lymphatic sinuses of the mandibular lymph node. **A** The CTMC contacts LSMC and macrophage process (*m*). ×6000. **B** The CTMC granules close to the contact with macrophage and LSMC are larger and exhibit flocculent content, ×6500. **C** The macrophage in contact with the CTMC and LSMC presents phagosome-like structures. ×7400

could be clearly seen (Fig. 3D). Frequently, the macrophage cytoplasm close to the interaction with LSMC exhibited large phagosomes, besides numerous vesicles and rough endoplasmic reticulum profiles. The LSMC-LSMC contact could not be completely resolved by our methodological approach and the possibility of other forms of cell junction, such as gap junctions, can not be discarded. A few LSMC were seen apparently inside the medullary cords in apposition to plasma cells and other lymphoid cells (Fig. 3F).

Some mast cells similar to the capsular CTMC were found inside lymphatic sinuses (Fig. 4A–C), mainly at the subcapsular sinus. In some of them, the cytoplasmic granules with flocculent material outnumbered the electron-dense ones at the cytoplasmic portion below the membrane contacting macrophages or other LSMC (Fig. 4B). Frequently, phagosomes accumulated in the macro-

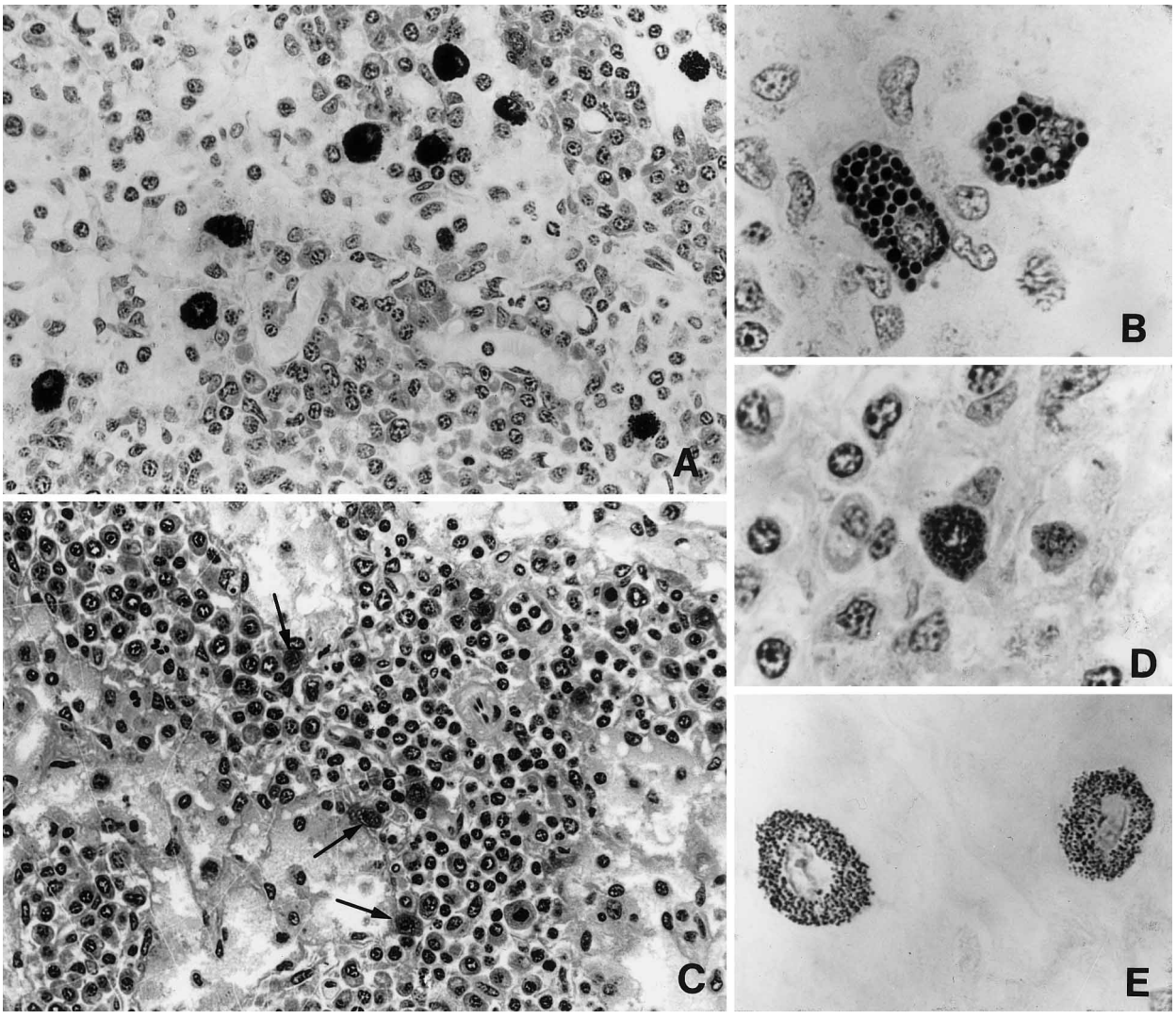


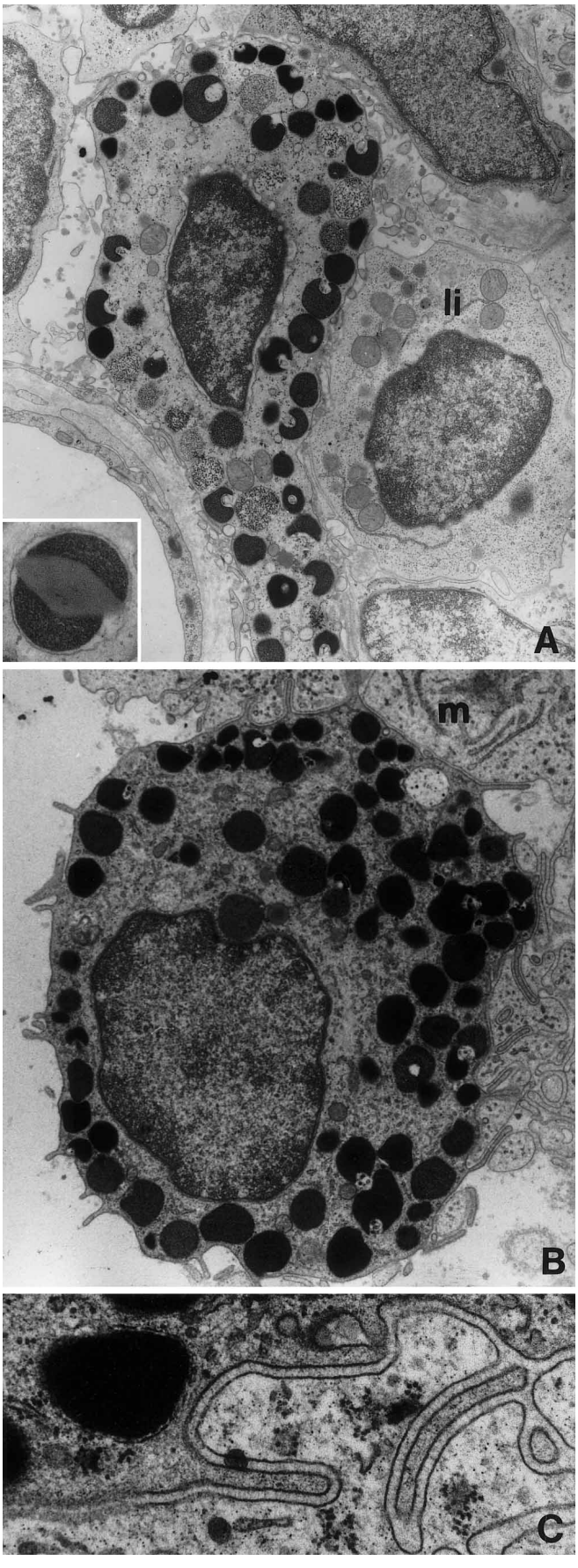
Fig. 5A–D Histological features of mast cell types in abdominal lymph nodes after glycol methacrylate embedding and staining with toluidine blue. **A** Section of the tail-of-pancreas lymph node shows typical LSMC in medullary sinuses. ×290. **B** Two LSMC of the tail-of-pancreas lymph node with the large cytoplasmic granules. ×900. **C** Section of head-of-pancreas lymph node shows CTMC-like cells (*arrows*) inside medullary cords. ×290. **D** CTMC in the medullary region of the mesenteric lymph node. ×900. **E** Two CTMC in the capsule of the head-of-pancreas lymph node. ×900

phage cytoplasm facing the CTMC-like cells (Fig. 4C) as described for the LSMC-macrophage interactions.

Mast cells of the abdominal lymph nodes

Light-microscopic studies revealed two groups of abdominal lymph nodes according to the location and aspect of their mast cells. The para-aortic, common iliac, cardial pyloric, cecocolic, colic, and those at the body and tail of the pancreas presented numerous LSMC, mainly at the medullary sinuses (Fig. 5A, B). In contrast, the mesenteric, pyloric, and head-of-pancreas lymph nodes were devoid of LSMC, or they were rarely seen. Instead, their medullary region presented mast cells similar to CTMC located mainly inside the medullary cords (Fig. 5C, D). Either in the cords or sinuses, the histological aspect of the medullary mast cells was similar to that of CTMC at the nodal capsule (Fig. 5E). The subcapsular sinus presented rare mast cells, all of them exhibiting the CTMC features. In all abdominal lymph nodes, as described for other opossum lymph nodes (Chiarini-Garcia and Machado 1992), the germinal center of the lymphoid nodules exhibited rare metachromatic cells similar to mast cells. They were very small and exhibited few and tiny cytoplasmic granules.

Electron-microscopic studies of the opossum mesenteric lymph node revealed medullary mast cells with tiny cytoplasmic granules very similar to those of CTMC (Fig. 6A) including the presence of granules with crystalline content (Fig. 6A, insert). They were characterized either as intact or degranulating cells. The average diameter of their granules with homogeneous content was 0.53±0.09 μm (n=100). So, they were statistically equivalent to the CTMC granules (P>0.05). Inside the medullary cords they could adhere to lymphoid and reticular



cells or to the extracellular matrix surrounding blood vessels (Fig. 6A). In the medullary sinuses, the mast cells contacted macrophages (Fig. 6B) and reticular cells as described for the LSMC. In these close contacts, the intercellular space exhibited filamentous material linking the LSMC and macrophage membranes (Fig. 6C).

Discussion

CTMC have already been described in the opossum ear where their external lamina was impressive (Santos and Machado 1994). Our studies in the lip dermis showed CTMC with a more delicate and discontinuous external lamina and some of them seem to adhere directly to fibrillar collagen. In the nodal capsule, the external lamina could even be absent, and CTMC could contact fibroblast and granulocytes. Adhesion of murine mast cell to fibroblast is well studied in vitro and seems to involve *c-kit* and its ligand, the stem factor that has been implicated in mast cell proliferation and differentiation (Adachi et al. 1992, 1995; Tsai et al. 1991). Besides, there are strong arguments favoring reciprocal influences between mast cells and fibroblasts (Davidson et al. 1983; Takeda et al. 1989; Levi-Schaffer and Rubinchik 1994). Our data showing concentration of pinocytic vesicles in the fibroblast membrane in close apposition to mast cells suggest a functional interaction between these two opossum cells, as evidenced in the human lung (Heard et al. 1992). The interaction of the opossum CTMC with granulocytes is a new finding that deserves future study. In eutherian mammals, studies on mast cell-leukocyte interactions are basically restricted to lymphocytes.

The present paper reinforces the unique character of the LSMC in both transmission and scanning electron microscopy, their cytoplasmic granules being three times larger than those of CTMC. Mast cells exhibiting large cytoplasmic granules are also very numerous in the lymphatic sinuses of cervical lymph nodes of *Sminthopsis crassicaudata*, an Australian marsupial (Haynes 1991) and in the mandibular and axillary lymph nodes of four other Brazilian Didelphidae (Chiarini-Garcia and Pereira 1999).

Our ultrastructural studies showed that LSMC adhere to the sinus wall through cell-to-cell contacts with macrophages, reticular cells and other LSMC. The LSMC-LSMC interactions explain the epithelioid arrangement of these cells in the medullary sinuses. The LSMC-macrophage contacts seem to be more frequent than the

Fig. 6A–C Electron micrographs of mast cells similar to CTMC in medullary cords and sinuses of the opossum mesenteric lymph node. **A** Mast cell inside a medullary cord close to a blood vessel contacts a lymphocyte (*li*). ×9800. *Insert* shows a cytoplasmic granule with crystalline content from a resting mast cell. ×31 700. **B** Mast cell in a medullary sinus contacts a macrophage (*m*) through membrane interdigitations. ×10 000. **C** Detail of the mast cell-macrophage close apposition to show the interdigitating membranes with constant intercellular space crossed by a filamentous structure. ×45 000

LSMC-reticular cell apposition, probably because macrophages practically surrounded the medullary cords through interaction with reticular cells. In these LSMC close contacts, the crossing filamentous structures in the intercellular space could represent adhesion molecules. Despite these LSMC adherences, high pressure during the intracardiac perfusion washed then out.

In contrast to contacts with reticular cells, those with macrophages could involve an accumulation of organelles in the macrophage cytoplasm abutting the interaction with LSMC. Also, the medullary-sinus CTMC in close contact with macrophages exhibited ultrastructural changes of the cytoplasmic granules close to the contacting membranes. Altogether these findings indicate a functional interaction between macrophages and mast cells in the medullary sinuses. In rat lymph nodes macrophages and reticular cells are able to take up granule matrix released by degranulating mast cells (Miyata and Takara 1985). Preliminary studies indicate that macrophages in the opossum lymph nodes exert a similar role after treatment with compound 48/80 (Ghiarini-Garcia et al. 1997).

Our studies of abdominal lymph nodes disclosed an intriguing finding. The mesenteric, pyloric and the head-of-pancreas lymph nodes were derived of LSMC. In these three lymph nodes, the medullary-region mast cells were few, similar to CTMC and located mainly inside the cords. These three lymph nodes drain the lymph from the small intestine, gall bladder, and distal portion of the stomach and head of the pancreas (Azzali and DiDio 1965). The mesenteric lymph node is larger than the two others and drains exclusively from the jejunum and ileum. The other six abdominal lymph nodes drain from different abdominal ogans and regions, but none receives lymph from the small intestine. Therefore, the origin of the afferent lymphatic vessels might be implicated in the absence of LSMC. Mast cells or their precursors are supposed to reach lymph nodes via the afferent lymphatic vessels. So, small intestine-originated mast cells could be insensitive to local factors able to induce the LSMC phenotype. Alternatively, the LSMC-free lymph nodes could be devoid of such factors. The few CTMC-like cells located in the medullary sinuses of the mesenteric lymph node contacted macrophages as the LSMC did in other opossum nodes. This makes it difficult to relate the LSMC differentiation simply to contact with macrophages. Another point is the preferential location of the medullary mast cells in the cords. If they came via the afferent lymph, they had to be attracted to the interior of the medullary cords. Developmental studies aiming at contributing to the elucidation of these questions are in progress.

The medullary-cord CTMC could be classed as resting or degranulating mast cells according to criteria established for eutherian CTMC (Friedman and Kaliner 1988). Frequently, the degranulating ones were in close apposition to lymphocytes. This finding may constitute ultrastructural evidence favoring a participation of cell-to-cell contact in regulating mast cell degranulation. Activated lymphocytes produce soluble factors involved in the proliferation and differentiation of mast cells. However, there is strong evidence showing that activated lymphocytes may affect mast cell biology through cell-to-cell contact (Oh and Metcalfe 1996; Bhattacharyya et al. 1998). Further, degranulation of mast cells can be mediated by such contacts in vitro (Inamura et al. 1998). On the other hand, rodent mast cells are able to secrete numerous cytokines known to affect leukocytes, including the mast cell itself (reviewed by Lin and Befus 1999). The regulatory potential of mast cells through the secretion of cytokines remains to be studied in marsupials. Anyway, the large number of different types of mast cells in the opossum lymph nodes and their numerous cell-to-cell contacts make this animal suitable for studies on the significance of mast cell adherence to other cells in vivo.

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