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REGULAR ARTICLE

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In situ characterization of mast cells in the frog *Rana esculenta*

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Abstract The number, distribution, and ultrastructural characteristics of mast cells were assessed in the tongue, heart, and kidney of the frog *Rana esculenta*. The density of tongue mast cells (253±45 mast cells/mm^2) was significantly higher than that of the heart (5.3±0.4/mm^2) and kidney (15.3±1.4 /mm^2). A striking feature of this study was the remarkable association of frog mast cells to nerves. The ultrastructural study of the mast cell/nerve association demonstrated that mast cells were closely apposed to or even embedded in nerves. Mast cells were also physically associated with melanocytes even in the heart. Mast cells were Alcian blue$^+$/safranin$^+$ in the tongue and in the peritoneum, whereas in the heart and in the kidney they were Alcian blue$^-$/safranin$^+$. The mast cells in the lamina propria of the gastrointestinal tract were Alcian blue$^+$/safranin$^-$. The cytoplasm of frog mast cells was packed with numerous heterogeneous, membrane-bound granules. The ultrastructure of these cytoplasmic granules was unique, being totally unlike any other previously described granules in other animal species as well as in man. The histamine content/frog mast cell (≈0.1 pg/cell) was approximately 30 times lower than that of human mast cells isolated from different tissues (≈3 pg/cell). A monoclonal anti-histamine antibody was used to confirm the ultrastructural localization of histamine in secretory granules in frog mast cells.

Key words Histamine · Immunocytochemistry · Mast cells · Melanocytes · Nerves · *Rana esculenta* (Anura)

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Introduction

Mast cells were initially identified by Paul Ehrlich (1879) because of the unique staining properties shown by the biochemical contents of their cytoplasmic granules when stained with appropriate dyes. Mast cells occur in all classes of vertebrates (Michels 1923; Galli 1990) and are the only cells possessing high-affinity receptors for IgE and synthesizing histamine (Galli 1990; Scharenberg and Kinet 1995). CD34$^+$ cells present in bone marrow, fetal liver, and cord blood are the precursor cells for differentiated mast cells in several species (Irani et al. 1992; Kirshenbaum et al. 1992; Valent et al. 1992; Mitsui et al. 1993). Stem cell factor (SCF) (Zsebo et al. 1990), also termed c-kit ligand (KL) (Huang et al. 1992), mast-cell growth factor (MGF) (Anderson et al. 1990), or steel factor (Reith et al. 1991), is the principal hematopoietic growth factor produced by mesenchymal cells (fibroblasts), epithelial cells, keratinocytes, neurons, etc. (Heinrich et al. 1993) that induces proliferation and differentiation of undifferentiated mast cells (Zsebo et al. 1990; Andrews et al. 1995) via interaction with c-kit, its cognate receptor, in several species (Anderson et al. 1990; Tsai et al. 1991; Huang et al. 1992). The c-kit/SCF receptor is a transmembrane receptor important for the normal development of hematopoietic cells, mast cells (Tsai et al. 1991; Valent et al. 1992), and melanoblasts (Funasaka et al. 1992).

Two major subtypes of mast cells, termed connective-tissue (CTMC) and mucosal (MMC) mast cells, have been identified in rodents (Enerbäck 1966a,b; Tainsh and Pearce 1992). These subtypes are differentiated by their histologic, functional, biochemical, and pharmacologic properties (Enerbäck 1966a–c; Bienenstock 1988). MMC

are found in the digestive tract and bone marrow and are distinguished by the presence of large granules containing primarily chondroitin sulphate proteoglycan. CTMC, also found in the peritoneum of rodents, are distinguished from MMC by the predominance of heparin in their secretory granules, which mediates their preferential staining with toluidine blue or safranin (Enerbäck 1966b). In humans, an analogous dichotomy has been demonstrated based on the presence of tryptase (MC_T) or tryptase and chymase (MC_{TC}) in mast cells isolated from different anatomic sites (Irani et al. 1986). Moreover, in human mast cells, the membrane-bound cytoplasmic granules containing histamine and other preformed mediators are very heterogeneous (Dvorak 1991). Finally, non-membrane-bound lipid bodies have been identified in human mast cells (Dvorak et al. 1984; Patella et al. 1995; de Paulis et al. 1995).

Surprisingly, although mast cells were identified in several tissues of *R. esculenta* (Michels 1923) and of *R. pipiens* (Chiu and Lagunoff 1972) several decades ago, histochemical and ultrastructural characterization of mast cells from lower vertebrates is still at a very early stage. Moreover, early histochemical procedures failed to demonstrate histamine in frog mast cells (Chiu and Lagunoff 1971). The growing interest in the proinflammatory, immunomodulatory, and host-defence mechanisms of mast cells (Marone 1995; Galli and Wershill 1996) prompted us to characterize the sub-types of mast cells in different anatomic sites of the frog *R. esculenta* using histochemical techniques, to examine the ultrastructure of the cytoplasmic granules using electron microscopy, and to evaluate the histamine content biochemically and with the immunogold technique.

Materials and methods

Reagents

The following reagents were purchased: 60% $HClO_4$ (Baker Chemical Co., Deventer, The Netherlands); BSA, piperazine-N,N'-bis (2-ethanesulfonic acid), collagenase type II (Sigma Chemical Co., St. Louis, Mo., USA); Hanks' balanced salt solution (HBSS), fetal calf serum (FCS) (GIBCO, Grand Island, N.Y., USA); RPMI 1640 with 25 mM Hepes buffer, Eagle's minimum essential medium (Flow Laboratories, Irvine, Scotland). A mouse monoclonal IgG anti-histamine was a generous gift from Dr. D.W. Constable (Miles Inc., Spokane, Wash., USA).

Human skin tissue

Skin tissue was obtained from two patients undergoing skin biopsy for diagnostic procedures. Written, informed consent was obtained from each patient to procure 4-mm punch biopsies of skin after induction of local anaesthesia (0.5 ml of 1% lidocaine, injected intradermally at least 1 cm away from the site to be biopsied). The skin tissue was cut in 1–2 mm fragments and immediately fixed for ultrastructural studies.

Animals

Adult males (n=5) and females (n=5) of the frog, *Rana esculenta*, were caught near Naples and reared in a 14-h day/10-h night cycle at 22°C. Frogs were anaesthetised with MS 222 (SIGMA) and decapitated. Macroscopically "normal" pieces of different tissues (tongue, heart, kidney, and testis) were divided and fixed for light- and electron-microscopic studies as described below. The "Principles of laboratory animal care" (NIH publication no. 86–23, revised 1985) were followed throughout the experiments.

Determination of histamine content of frog tongue, heart, testis, and kidney and of human lung, heart, and skin

Samples of tongue, heart, testis, and kidney from *R. esculenta* were dried on filter paper, weighed, and boiled in 8% $HClO_4$ for 30 min. The mixtures were filtered to remove particles, and supernatants were assayed for histamine using a fluorometric assay (Siraganian 1974). All values are based on the means of duplicate or triplicate determinations. Replicates differed in histamine content by $<10\%$. Samples of human lung, heart, and skin were obtained from patients undergoing surgery, and the histamine content was measured as described previously (Stellato et al. 1992; Patella et al. 1995). Protein concentrations were estimated with the method of Lowry et al. (1951).

Histochemistry

The various tissues (tongue, heart, testis, and kidney) were dissected and rapidly fixed in Bouin's fluid. The histochemical properties of the mast cells were studied on paraffin sections (5 μm) stained with toluidine blue in Walpole buffer (pH 4.2) and with the sequential Alcian blue-safranin staining method: 1% Alcian blue in 3% acetic acid (pH 2.2) and 0.5% safranin in HCl (pH 1.3) according to previously described techniques (Chieffi Baccari et al. 1991; Gaytan et al., 1990).

Electron microscopy

Samples of tissues (≅2 mm^3) were promptly immersed for 2 h in Karnovsky buffer (pH 7.4) and postfixed for 1 h in Millonig's phosphate-buffered 1% osmium tetroxide. The samples were dehydrated in a graded series of ethanol and finally embedded in Epon 812. Semithin sections were stained with 1% toluidine blue. Ultrathin sections were stained with 4% uranyl acetate followed by 1% lead citrate and were examined with a Philips 301 transmission electron microscope as described previously (Chieffi Baccari et al. 1991; Minucci et al. 1997).

Electron immunocytochemistry

For electron immunocytochemistry, samples of tongue (≅2 mm^3) were embedded in Araldite CY212 epoxy resin (TAAB) at 60°C. The small blocks were serially sectioned at 0.1 μm, and the sections were mounted on Formvar-coated nickel grids. The sections were preincubated with normal goat serum diluted 1:30 in TBS 0.05 M – 1% bovine-serum albumin (BSA) and subsequently incubated overnight with the anti-histamine monoclonal antibody diluted 1:30 in TBS 0.05 M – 1% BSA – 0.5% sodium-azide buffer. The sections were then washed (3×) and incubated for 1 h with goat anti-mouse IgG+IgM gold-conjugate (20 nm) diluted 1:50 with TBS 0.05 M – 1% BSA. After washing in TBS 0.05 M, pH 7.6, – 1% BSA and successively in H_2O, the grids were dried and stained for 15 min with aqueous uralyl acetate (4%), then with 1% lead citrate for 1 min, and subsequently examined with a Philips 301 transmission electron microscope. The following controls were performed: (1) omission of the first antibody layer; (2) replacement of the primary antibody with isotype-matched irrelevant antibody at the same concentration; and (3) neutralization of the primary antibody with histamine (1 μg/ml). The results of the control procedures excluded non-specific reactivity.

Numerical and statistical analyses

Five randomly chosen sections from each animal for each tissue were viewed in a light microscope at a magnification of ×400 using an eyepiece with a square-ruled grid with a total area of 0.062 mm^2. The mast cell number within the tongue, heart, and kidney was counted in 60 different areas. This allowed the calculation of mast cell number/mm^2±S.E.M. (Snedecor 1980).

Results

Histology of frog mast cells

Mast cells were identified in all sections of the specimens from the frog tissues examined. The number of mast cells varied greatly between different tissues, being highest in the tongue (253±45 mast cells/mm^2). Mast cells were also present in the heart (5.3±0.4 mast cells/mm^2), and in the kidney (15.3±1.4 mast cells/mm^2). In the tongue tissue, mast cells were elongated, fully granulated, and distributed throughout the connective tissue between the striated muscle fibers and the tubules of the salivary glands (Fig. 1). Mast cells were also found in perivascular locations. Mast cells were present in the frog heart around blood vessels and between myocytes. Interestingly, in contrast to other anatomic sites, heart mast cells were usually round or oval.

Mast cell/nerve association

Mast cell/nerve associations have been previously demonstrated in rodents (Enerbäck et al. 1965; Olsson 1968; Newson et al. 1983; Stead et al. 1987; Blennerhassett et al. 1991; Hukkanen et al. 1991) and humans (Stead et al. 1989). A striking feature of this study was the predominant association of frog mast cells to nerves. In the frog tongue, mast cells were closely apposed to or embedded within nerve fibers (Fig. 2). Close to 100 mast cell profiles were studied to determine their anatomical relationship to nerves. The majority of mast cells within nerves were elongated, and the cytoplasm was packed with large, dense, membrane-bound granules. Some mast cells were round or angular.

The ultrastructural study of the mast cell/nerve association clearly demonstrated that mast cells are located beneath the epineurium of myelinated nerve fibers. In most specimens, mast cells were adjacent to Schwann-cell cytoplasm (Fig. 3).

Mast cell/melanocyte association

There is increasing evidence of both microanatomic association and physiologic interactions between melanocytes and mast cells in rodents (Matsui et al. 1990; Nishikawa et al. 1991) and in humans (Longley et al. 1993; Costa et al. 1996). In frog tissues, we found a close anatomic association between melanocytes and mast cells. This was evident not only in skin tissue but also in such internal organs as the heart (Fig. 4).

Histochemistry and mast cell subtypes

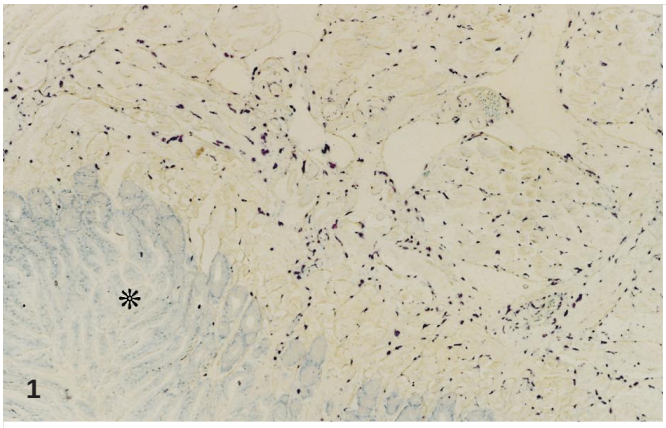
CTMC and MMC have been identified in rodents based on different histologic, functional, compositional, and pharmacologic regulatory properties (Enerback 1996a–c; Bienenstock 1988). MMC contain large granules primarily containing chondroitin sulphate proteoglycan. CTMC are distinguished by the predominance of heparin proteoglycan in their secretory granules, which mediates their preferential staining with metachromatic dyes, such as toluidine blue or safranin (Enerbäck 1966b; Bienenstock 1988).

In all of the examined tissues, the mast cells of the frog *R. esculenta* contained granules which stained metachromatically with toluidine blue at pH 4.2 (Figs. 1, 4, and 5a). The metachromatic property of frog mast cells differed from those of humans in that the former stained dark purple and the latter reddish purple. When the combined Alcian blue-safranin method was employed to differentiate between CTMC and MMC, both red and blue cytoplasmic granules were observed in the tongue (Fig. 5b) and peritoneal mast cells (Fig. 5c) whereas, in the heart (Fig. 5d) and in the kidney (data not shown), mast cells showed only Alcian $blue^-$/$safranin^+$ granules. The mast cells in the lamina propria of the gastrointestinal tract were Alcian $blue^+$/$safranin^-$ (Fig. 5e). Therefore, frog mast cells were of the "connective type" in the tongue, peritoneal cavity, and kidney, and of the "mucosal type" in the lamina propria of the gastrointestinal tract.

In situ ultrastructural features of frog mast cells

The ultrastructural study of frog tongue demonstrated that mast cells contained a single lobed, central nucleus with peripheral condensation of nuclear chromatin. The cytoplasm was packed with numerous heterogeneous, membrane-bound granules. The surface of frog mast cells was not adorned with elongated, narrow surface folds. A few mitochondria and free ribosomes were occasionally seen in the cytoplasm (Fig. 6).

The membrane-bound cytoplasmic granules were extremely heterogeneous in shape: ovoid>fusiform>round in descending order of frequency. Secretory granules contained an elaborate and heterogenous substructural architecture: some had crystal particles in hexagonal arrays, others had a crystalline structure with regular parallel arrays of several periodicities, while others had fusiform inclusions with a "sandwich-like" structure and a few partial scrolls (Fig. 7). Non-membrane-bound lipid bodies were never observed in frog mast cells. Figure 8 shows the typical ultrastructural characteristics of human-skin mast cells, which are very different from those of frog mast cells. In particular, the cytoplasmic granules presented well-defined ultrastructural patterns: homogeneously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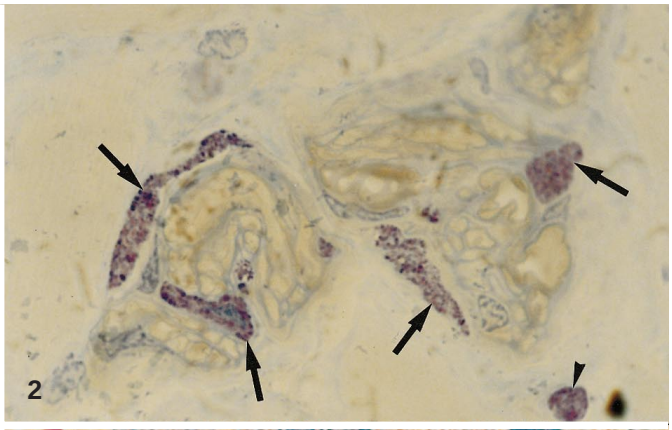
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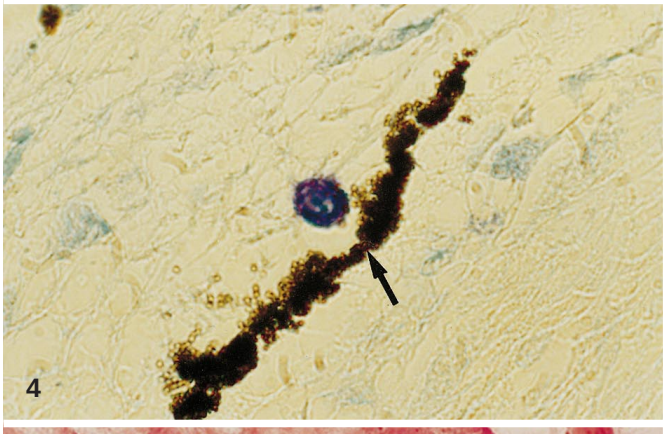
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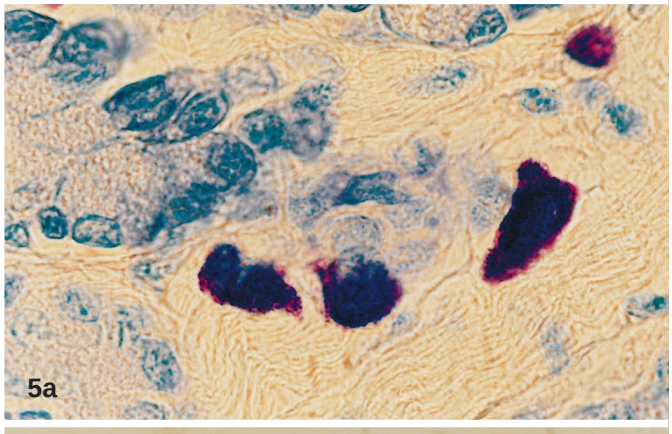
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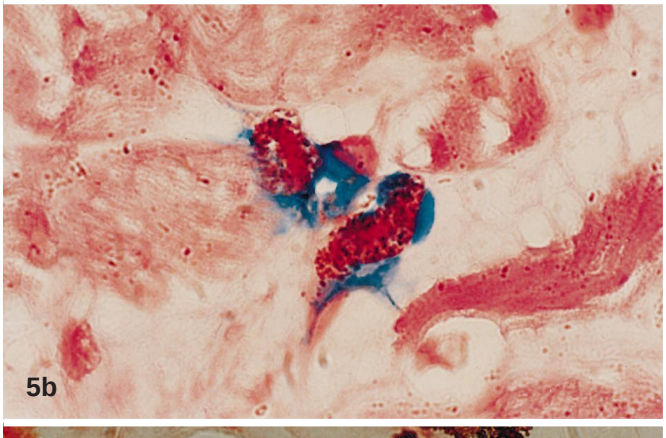
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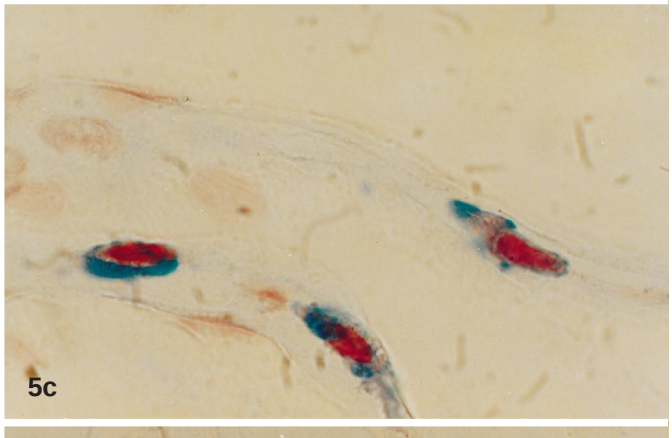
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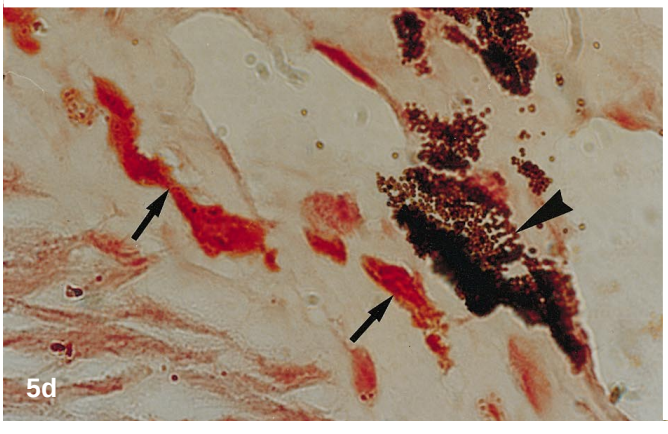
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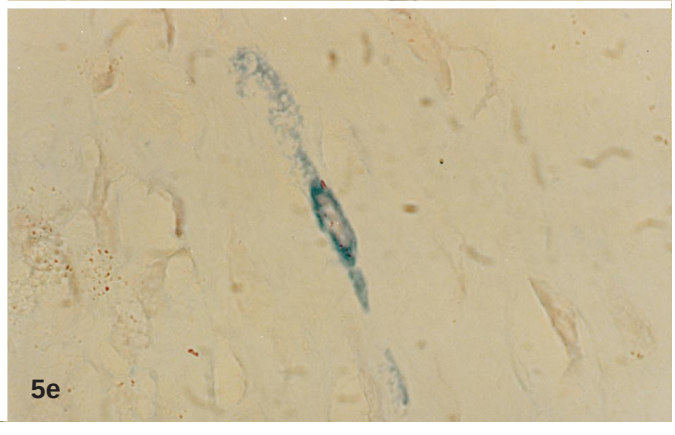
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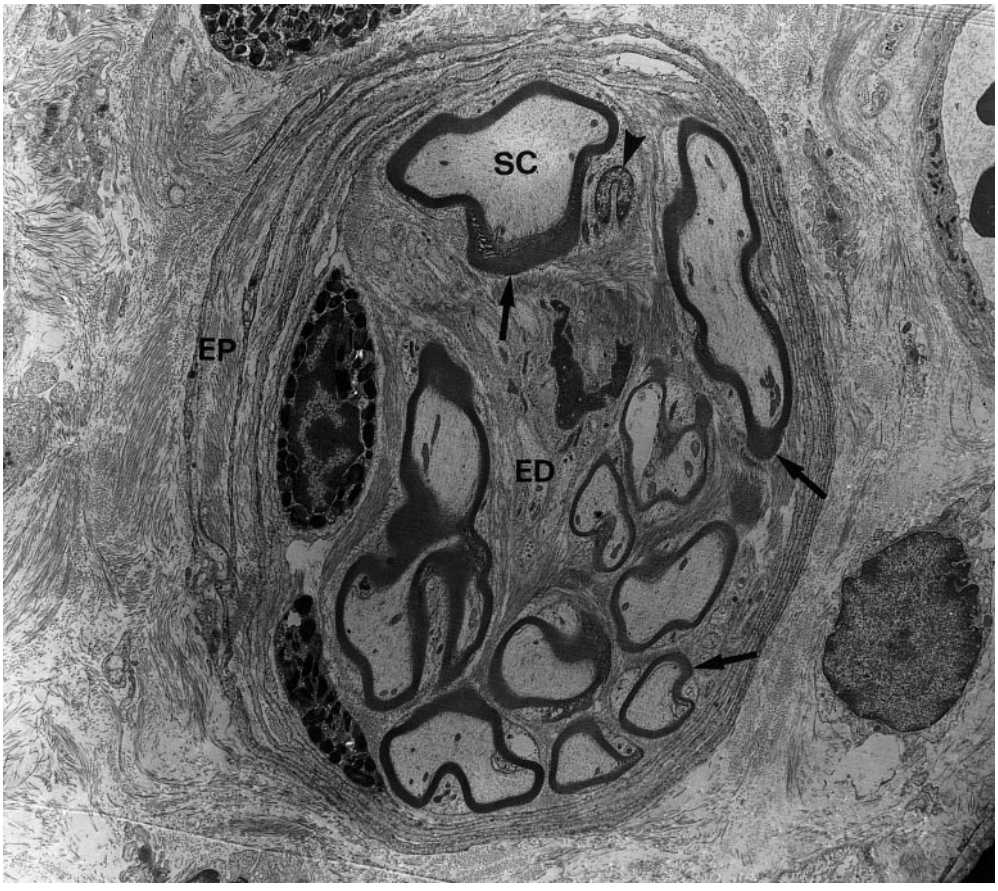

Fig. 3 Electron micrograph of the tongue of the frog *R. esculenta*. Two mast cells are located beneath the epineurium (*EP*) of myelinated nerve fibers. *ED* Endoneurium, *arrows* myelinated fibers, *SC* Schwann cell, *arrowhead* nucleus of Schwann cell. ×2200 (original magnification)

dense, scroll, mixed granules, lamellar, and swirl patterns (Dvorak 1991; Patella et al. 1995; de Paulis et al. 1996). The surface of human mast cells is sometimes adorned with numerous elongated, narrow surface folds (Dvorak 1991; de Paulis et al. 1996) and contains lipid bodies (Dvorak 1991; Dvorak et al. 1984). The complete or partial loss of cytoplasmic granules reported in approximately 5% of human mast cells in situ (Dvorak 1991; Patella et al. 1995; de Paulis et al. 1996) was not observed in this study. Table 1 summarizes some of the ultrastructural differences between human and frog mast cells.

◀ **Fig. 1** Paraffin-embedded section from the tongue of the frog *R.* esculenta showing numerous mast cells in the connective tissue between the striated muscular tissue and the tubules of the salivary glands (*asterisk*). Mast cells are metachromatic with toluidine blue at pH 4.2. ×50

Fig. 2 Semithin section from the tongue of the frog *R. esculenta*. Note the mast cells embedded in nerve fibers. One nerve fiber contains two elongated mast cells (*arrows*), whereas the other contains one elongated and one round mast cell (*arrows*). One mast cell is closely associated with a nerve fiber (*arrowhead*). ×1250

Fig. 4 Paraffin-embedded section from the heart of the frog *R. esculenta*. A mast cell is closely apposed to a melanocyte (*arrow*). Toluidine blue at pH 4.2. ×1250

Fig. 5 **a** Four mast cells in tongue tissue of the frog *R. esculenta*. Mast cells are metachromatic with toluidine blue at pH 4.2. ×1250. **b** Two mast cells of the "connective" type in tongue tissue of the frog *R. esculenta*, i.e., they show affinity for both safranin (*red*) and Alcian blue staining. ×1250. **c** Three peritoneal mast cells Alcian blue$^+$/safranin$^+$. ×1250. **d** Two mast cells Alcian blue$^-$/safranin$^+$ (*arrows*) in heart tissue of the frog *R. esculenta*. Mast cells are in close proximity to a melanocyte (*arrowhead*). ×1250. **e** A typical mucosal mast cell Alcian blue$^+$/safranin$^-$ in the lamina propria of gut tissue of the frog *R. esculenta*. ×1250

Histamine content and subcellular localization of histamine in frog mast cells

The correlation between the histamine content and mast-cell counts in various tissues was first established by Riley and West (1953). While all mammalian mast cells tested contain histamine (Galli 1990), early histochemical examinations of frog mast cells led to the conclusion that these cells lacked histamine (Chiu and Lagunoff 1971). The histamine content of different preparations of frog tissues (tongue, heart, kidney, and testis) is reported in Table 2. The histamine content expressed per gram of wet tissue was lower than that of human tissues (Patella et al. 1995; de Paulis et al. 1996). When the histamine content was expressed per gram of protein, it was clear that the high content of water in the tongue influenced the results. Assuming that mast cells were the sole source of histamine in our preparations, the histamine content of frog mast cells (≈0.1 pg/cell) was approximately 30 times lower than that of human mast cells isolated from different an-

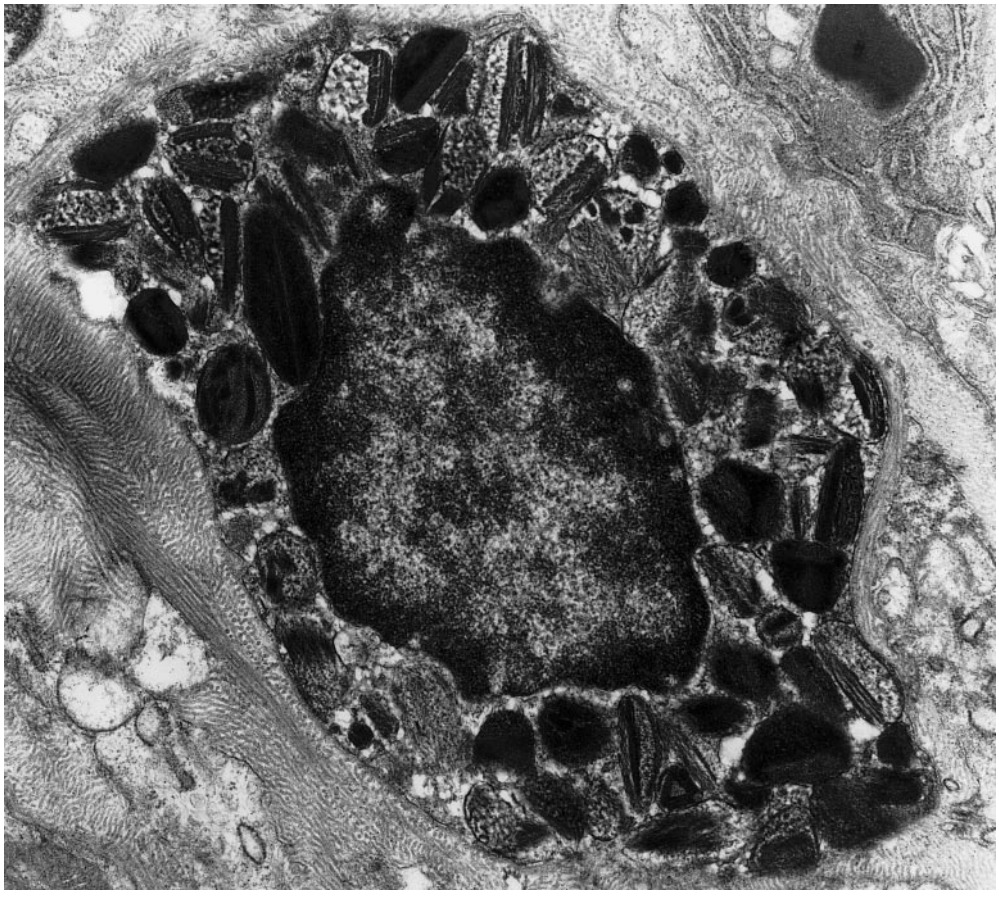

Fig. 6 Electron micrograph of a mast cell from *R. esculenta* tongue in situ surrounded by collagen fibers. The cytoplasm contains numerous heterogeneous, polymorphic granules. ×10 000 (original magnification)

Table 1 Ultrastructural differences between human and frog mast cells

	Human mast cells	Frog mast cells
Cytoplasmic surface	Numerous, elongated narrow surface folds	Absence of surface folds
Lipid bodies	Common	Absent
Ultrastructural pattern of secretory granules	Homogeneously dense, scroll, mixed granules, lamellar	Mixed, crystall-parallel array, sandwich-like, partial scroll
Shape	Round, fusiform	Ovoidal›fusiform›round
In situ degranulation	Occasionally (»5%) observed	Rarely observed
Nucleus	Excentric	Central
Nuclear chromatin	Marked condensation	Peripheral condensation

Table 2 Histamine content of various frog tissues

	Histamine content	
	ng/g wet tissue	mg/g protein
Tongue	241±44.5	3.9±0.7
Heart	874±67.3	35.7±2.7
Kidney	586±146	7.4±1.8
Testis	688±76.8	12.5±1.4

atomic sites (≈3 pg/cell) (Patella et al. 1995; de Paulis et al. 1996).

A monoclonal anti-histamine antibody was used to detect histamine in frog mast cells. After immunogold staining of the frog tongue, 20-nm gold particles were present over all secretory granules in more than 95% of frog mast cells (Fig. 9a). Very few granules were present over the nucleus or in areas of cytoplasm surrounding the granules. No staining was observed when the first antibody was omitted (Fig. 9b) or when granules of frog mast cells were incubated with a murine myeloma against an irrelevant antigen at concentrations similar to those of the primary anti-histamine antibody (data not shown).

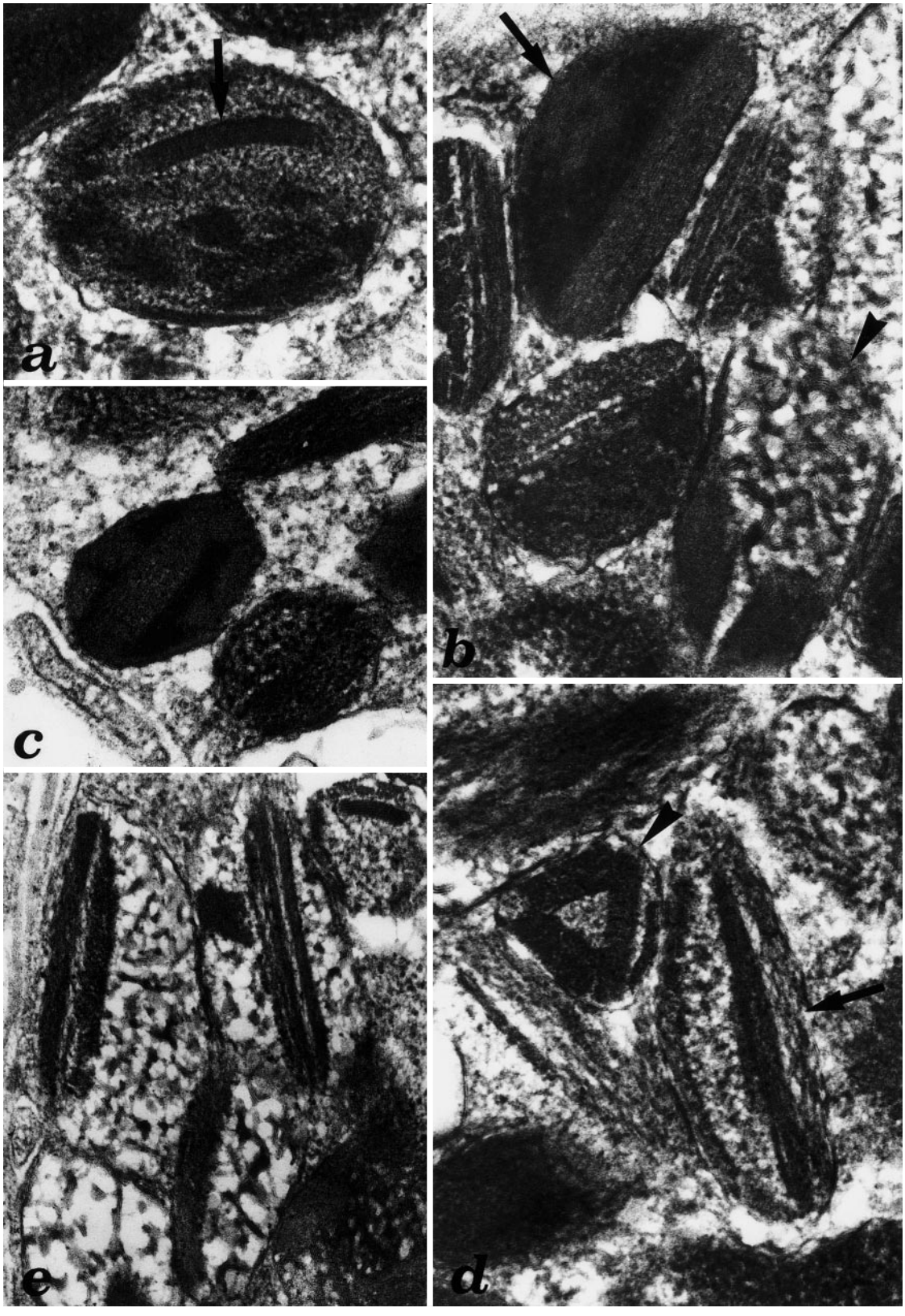

Fig. 7a–e Ultrastructural features of frog mast-cell secretory granules. **a** A crystal granule in hexagonal arrays (*arrow*). **b** A crystalline structure with regular parallel arrays of several periodicities (*arrow*) and a partial scroll (*arrowhead*). **c** Two ovoidal granules showing different ultrastructural features. **d** Fusiform cytoplasmic granule (*arrow*) and a round granule containing a triangular structure (*arrowhead*). **e** Two cytoplasmic granules with "sandwich"-like inclusions. ×46 000 (original magnification)

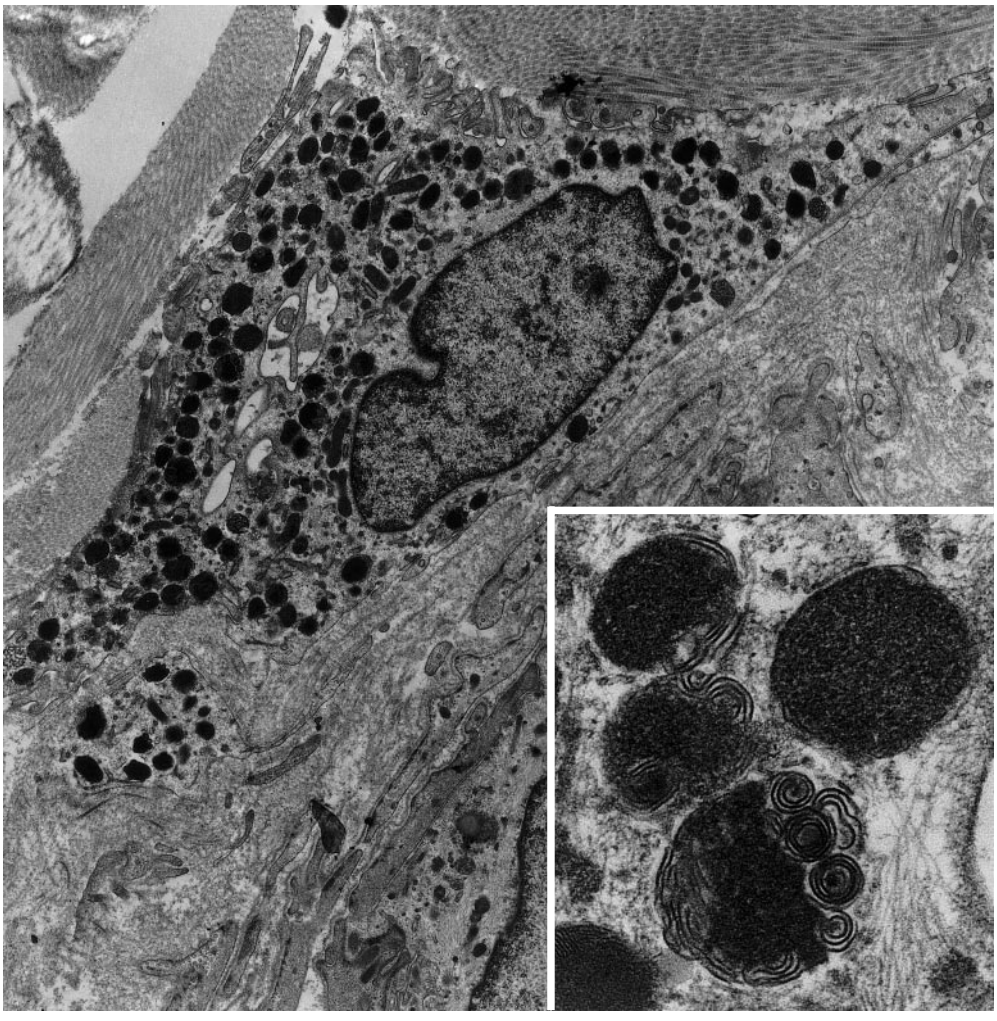

Fig. 8 Ultrastructural features of a human skin mast cell in situ surrounded by collagen fibers. Note the abundance and heterogeneity of secretory granules, some of which show scroll, mixed granules, and homogeneously dense structures (*inset*). ×4600; ×46 000 (*inset*) (original magnification)

Discussion

In this study we used histochemical, biochemical, and ultrastructural methods to characterize mast cells in the frog *R. esculenta* in situ. Mast cell density varied greatly in the different tissues and was highest in the tongue. Frog mast cells were identified within myelinated nerve fibers and in close proximity to melanocytes. Histochemical analysis of frog mast cells in different tissues revealed properties of the connective type (tongue, heart, kidney, testis, and the peritoneum) and of the mucosal type (gastrointestinal tract) previously found in rodents (Enerbäck 1966a,b; Bienenstock 1988). Ultrastructural analysis of these mast cells showed that the cytoplasmic secretory granules had unique morphologic characteristics formerly unrecognised in any human or animal mast cells (Dvorak 1991). Despite the low histamine content of frog mast cells with respect to that of mast cells of several mammalian species, the immunogold technique was able to detect immunoreactive histamine in the secretory granules.

The mast cell density varied greatly in the various frog tissues examined: the highest percentage of mast cells was found in the tongue. Mast cells were detected in perivascular locations, in loose connective tissue, close to the tubules of the salivary glands, and embedded in nerves. Histamine and other vasoactive mediators contained in frog mast cell secretory granules could play a physiological role in the control of blood vessels (Vigorito et al. 1983), nerve terminals (Ishikawa and Sperelakis 1987), and salivary glands (Caughey 1991). It is not inconceivable that mast cells and their mediators might play a role in the complex functions of the frog tongue.

The striking mast cell/nerve association documented in this study is intriguing. Although anatomical and functional mast cell/nerve associations have been previously documented in rodents (Enerbäck et al. 1965; Olsson 1968; Newson et al. 1983; Stead et al. 1987; Arizono et al. 1990; Blennerhassett et al. 1991; Hukkanen et al. 1991) and humans (Stead et al. 1989), in *R. esculenta* mast cells occurred next to the endoneurium of myelinated nerve fibers. This might suggest the existence of reciprocal and/or feedback interactions between neurons and mast cells brought about by their metabolic products. Indeed, earlier studies by Enerbäck and co-workers (1965) showed a marked increase in the number of endoneurial mast cells in association with sectioned sciatic

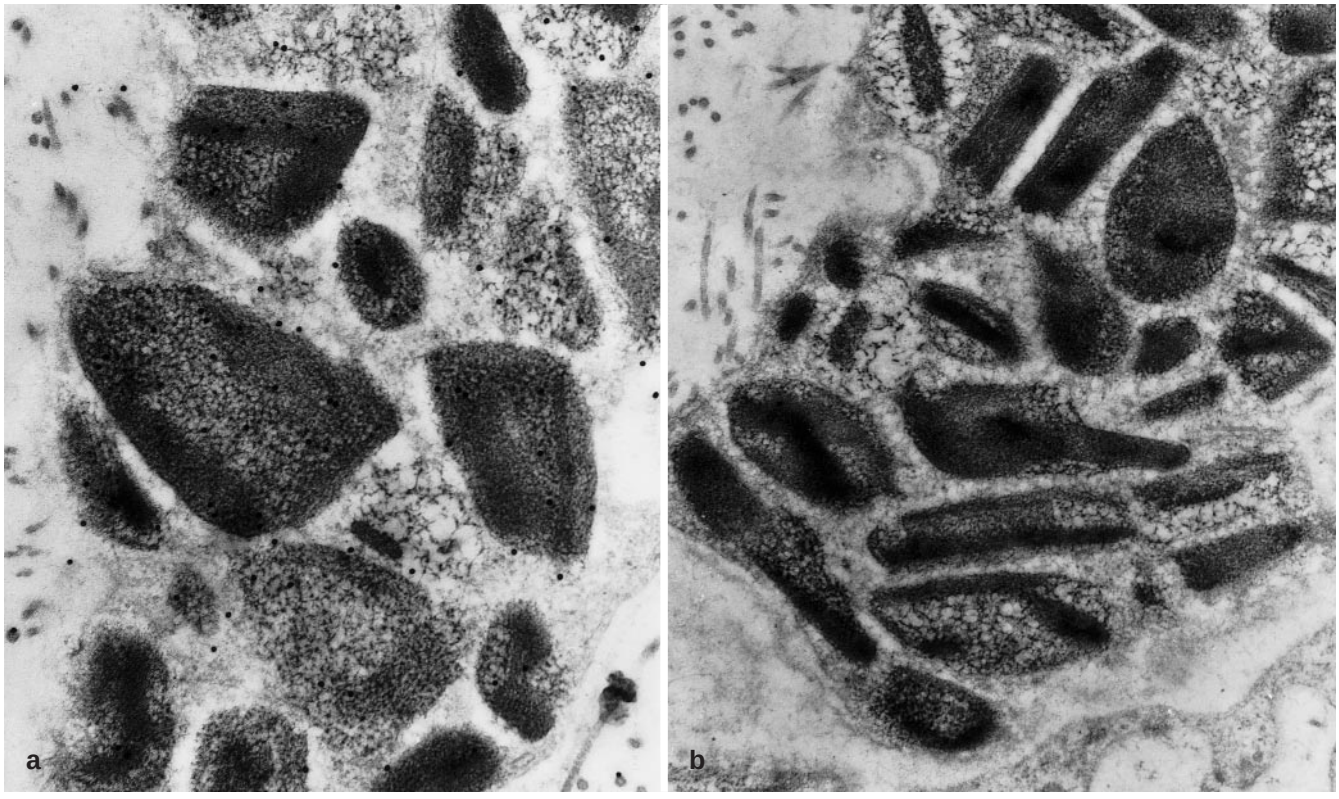

Fig. 9 **a** Immunogold staining of the tongue from the frog *R. esculenta* stained with anti-histamine antibody (mouse anti-histamine monoclonal antibody, diluted 1:30; 20-nm-gold complex). Gold particles identifying histamine are present over all secretory granules, but not in the perigranular cytoplasm. ×30 000 (original magnification). **b** Negative control in which anti-histamine antibody was omitted. ×30 000 (original magnification)

nerve from the rat. In vivo administration of nerve growth factor (NGF) can induce mast cell hyperplasia (Aloe and Levi-Montalcini 1977). Moreover, mast cells synthesize, store, and release NGF (Leon et al. 1994), and NGF can cause mediator and cytokine release from mast cells (Marshall et al. 1990; Horigome et al. 1993; Bullock and Johnson 1996). Thus, it is possible that mast cells exert a paracrine control of neurons. In addition, several neuropeptides, such as substance P, VIP, and neurokinins, locally released from nerve fibers (Morley et al. 1987), cause mast cells to release chemical mediators (Benyon et al. 1987; Lowman et al. 1988; Stellato et al. 1992). Our findings provide the microanatomic basis for potential paracrine and autocrine interactions between frog mast cells and nerves.

Another interesting anatomical association was found between frog mast cells and melanocytes. Stem cell factor is a hematopoietic growth factor produced by mesenchymal cells (Zsebo et al. 1990) that induces proliferation and maturation of bone-marrow progenitor cells and mast cells (Zsebo et al. 1990; Tsai et al. 1991; Andrews et al. 1995) via interaction with c-kit, its cognate receptor (Anderson et al. 1990). The kit/SCF receptor is a transmembrane tyrosine kinase receptor of fundamental importance for the normal development of mast cells (Tsai et al. 1991; Irani et al. 1992; Kirshenbaum et al. 1992; Valent et al. 1992) and melanocytes (Matsai et al. 1990). Studies on mice bearing mutations within the c-*kit* gene indicate that this tyrosine-kinase receptor plays a functional role in the development of melanocytes (Matsui et al. 1990; Nishikawa et al. 1991). In vivo administration of SCF induces mast cell and melanocytic hyperplasia (Grichnik et al. 1995; Costa et al. 1996). Finally, patients with cutaneous or systemic mastocytosis show marked hyperpigmentation (Metcalfe 1991; Genovese et al. 1995; Marone et al. 1995). Taken together, these observations suggest that SCF is an important activation and growth factor for both mast cells and melanocytes, thus explaining the co-existence of mast cell and melanocyte hyperplasia in the frog tissues. Furthermore, histamine has been reported to increase size, dendricity and tyrosinase production of melanocytes (Tomita et al. 1993). The close anatomic association between mast cells and melanocytes in *R. esculenta* might indicate paracrine interactions between these cells, which could be mediated by SCF, histamine, or by unknown mediators and/or mechanisms.

Different staining techniques can be used to distinguish CTMC and MMC in rodents. These staining techniques reflect the structural properties of the proteoglycan core, especially its glycosaminoglycan (GAG), and to some extent those of the protease subtypes of mast cell granules (Enerbäck et al. 1989). The Alcian blue-safranin staining sequence can be used to distinguish mature CTMC from MMC in the rat, but the staining does not specifically distinguish heparin from chondroitin sulphate

E. The fluorescent Berberine binding and more importantly the in situ desulphation cleavage of heparin can distinguish GAG composition in situ (Enerbäck et al. 1989). In our study, the combined Alcian blue-safranin staining method revealed several subtypes of frog mast cells in tissues from various anatomical sites. In particular, we found both blue and red granules in tongue and peritoneal mast cells, whereas heart and kidney mast cells were Alcian blue$^-$/safranin$^+$. These two staining patterns are characteristic of CTMC in rodents and probably reflect biochemical differences of the granules, for example differences in the proteoglycan and/or protease content (Enerbäck 1966a,b; Bienenstock 1988). The histochemical properties of mast cells revealed with the Alcian blue-safranin staining method have been used to assess maturational changes in the granules of CTMC (Combs et al. 1965). It has been postulated that the Alcian blue-safranin reaction differentiates degrees of sulphation, the more highly sulphated the polysaccharide, the greater its affinity for safranin. It was proposed that Alcian blue$^+$ granules contain a polysaccharide that is poor or totally lacking in N-sulphate, presumably a heparin precursor (Spicer 1960; Combs et al. 1965). Similarly, cells that bear safranin$^+$ granules contain highly N-sulphated polysaccharide, probably heparin (Combs et al. 1965; Gaytan et al. 1990). More recently, a close correlation between the presence of safranin$^+$ granules and the amount of rat mast-cell protease I has been reported (Koretou 1988). Frog mast cells in the intestinal lamina propria were Alcian blue$^+$/safranin$^-$, as previously reported in rodents (Enerbäck 1966a,b; Bienenstock 1988). If the staining properties are conferred on the different frog mast cell subtypes by virtue of their proteoglycan content, it is likely that frog mast cells already express a certain degree of specialization in proteoglycan synthesis. Studies using the fluorescent Berberine binding procedure and the in situ desulphation cleavage of heparin will provide additional insights into the dye-binding properties of the proteoglycans of secretory granules of frog mast cells.

Rodent and human mast cells contain membrane-bound secretory granules, which contain not only histamine and neutral proteases (tryptase, chymase, and carboxypeptidase), but also non-membrane-bound lipid bodies (Dvorak et al. 1984; Dvorak 1991; Patella et al. 1995; de Paulis et al. 1996). Such lipid bodies are less frequent in mouse, rat, and guinea pig mast cells (Dvorak 1991). Secretory granules of mast cells display substructural patterns unique to the different species. Frog mast cells contain cytoplasmic granules that also display unique ultrastructural patterns. They are heterogeneous in shape and contain an elaborate substructural architecture: some of them had a granular content, and others showed a crystalline structure different from those of human mast cells (Dvorak 1991; Patella et al. 1995; de Paulis et al. 1996). In addition, different from human mast cells, non-membrane-bound lipid bodies were never observed in our frog mast cell preparations.

The histamine content of various frog tissues varied markedly, and the histamine content of frog mast cells (approximately 0.1 pg per mast cell) was approximately 30 times lower than in mast cells isolated from different human tissues (Patella et al. 1996; de Paulis et al. 1996). The low concentration of histamine in frog mast cells could explain why earlier histochemical techniques failed to reveal histamine (Chiu and Lagunoff 1971). In rodents, the histamine content is higher in CTMC (approximately 15 pg per mast cell) than in MMC (approximately 1.5 pg per mast cell) (Galli 1990). Despite the similarities in staining properties of the CTMC, the histamine content was extremely low in all of our frog mast-cell preparations. These differences in mediator content emphasize the complexity of subtypes of mast cells from different species.

In conclusion, mast cells characterized in situ in different tissues from *R. esculenta* were remarkably different in ultrastructural, biochemical, and histochemical properties from mast cells previously identified in human tissues and in other animal species. A striking feature of this study was the close association of frog mast cells to nerves and to melanocytes. This raises the possibility that the local microenviroment influences their phenotype and perhaps their functions. Isolation of mast cells from different frog tissues may lead to the identification of additional mediators and cytokines synthesized, stored, and released by mast cells and may help to clarify their pathophysiologic role.

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