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Linear arrays of homogenous mast cells in the dura mater of the rat

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Summary

Using fluorescence histochemistry, 5-HT, histamine and heparin were colocalized in a large population of cells in the dura mater thereby identifying them as mast cells. In addition, because these cells were highly sensitive to compound 48/80 and were densely packed with granules of a consistent density, they were identified specifically as 'connective tissue' mast cells. Other types of mast cells, i.e. 'mucosal' or 'neurolipomastocytes', were not present in the rat dura mater. 5-HT immunohistochemistry was the best technique for demonstrating that there were two populations of mast cells, one associated with each of the two layers of dura. Although shaped differently the type of mast cell in each layer was the same. It was observed that mast cell shape is dependent on the contiguity, density and orientation of its surrounding elements, not its type. In general, mast cells in the outer layer were aligned parallel to the middle meningeal artery and those in the inner layer were parallel to trigeminal nerve branches that coursed obliquely across the middle meningeal artery. Examination of cross-sections of dura revealed that most mast cells also were aligned at the interface between the two dural layers. The linear orientation of mast cells in two planes of each layer suggests a programmed lamellar seeding of these cells during development of the dura. This study also demonstrated that the majority of dural mast cells were more closely related to other connective tissue elements than to blood vessels and nerves. These results (1) are compatible with the suggestion that dural mast cells play a non-obligatory role in the neuroinflammatory response, (2) leave open to question the role of the dural mast cell in headache or the regulation of blood flow, and (3) support evidence that dural mast cells play an important role in connective tissue related functions, e.g. development, inflammatory response to injury and wound repair.

Introduction

Mast cells in the dura mater may contribute to the pathogenesis of headache (Liberski & Prusiński, 1982; Theoharides, 1983; Moskowitz, 1984; Moskowitz *et al.*, 1979, 1987) and inflammatory responses (Markowitz *et al.*, 1987, 1989), as well as to normal regulatory functions governing blood flow (Gabbiani *et al.*, 1970). Therefore, for investigators to further assess the hypothetical role(s) of these cells in these important processes, it is necessary to first unequivocably identify these cells, and then to determine (1) whether they constitute a homogeneous population of cells, (2) how these cells are distributed within the tissue, and (3) their relationships to other structural elements that might be involved in these processes. These were the main goals of this investigation.

In our study of the innervation of the dura mater of the rat, we noted an extensive population of 5-HT positive cells that we hypothesized to be mast cells (Keller *et al.*, 1989). Mast cells contain or generate more than 20 mediators (Marom & Casale, 1983). In addition to a basic protein, the presence of three of these, heparin (Holmgren & Wilander, 1937), histamine (Riley, 1953; Riley & West, 1953), and in the rat, 5-HT (Benditt *et al.*, 1955), is used routinely to identify mast cells in that species (Csaba & Kovács, 1975). Therefore, the present study used fluorescence histochemical methodology previously demonstrated to colocalize these components within the same cell (Dimlich *et al.*, 1980a,b). Routine histochemistry for basic protein and 5-HT immunohistochemistry also were used to test the hypothesis that these fluorescing cells were mast cells.

Mast cells are notoriously heterogeneous, especially between species and even within different tissues in a

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single species (Selye, 1965; Pearce, 1983). In the rat intestine there are 'connective tissue' mast cells and 'mucosal' mast cells (Enerbäck, 1966), and in the rat brain and leptomeninges there are purportedly 'connective tissue' and 'brain' mast cells (Theoharides, 1990) as well as 'neurolipomastocytes' (Ibrahim *et al.*, 1979). Among other criteria used to distinguish different types of mast cells (Galli, 1990), the knowledge that only 'connective tissue' mast cells (1) stain with berberine sulphate that is specific for the sulphated glycosaminoglycan, heparin, (2) respond readily to mast cell 'degranulating' agents, e.g. compound 48/80, or (3) have certain unique ultrastructural characteristics, e.g. granules that are of a consistent density (Galli, 1987, 1990), was used to determine the heterogeneity of dural mast cells in this study.

To assess accurately the effects of compound 48/80, further considerations were necessary. Mast cells are easily disrupted during tissue preparation (Selye, 1965). This is best prevented by perfusion fixation; however, the fluorescence methods used to identify 5-HT and histamine in these cells are incompatible with perfusion fixation. As a result, many of the mast cells stained using those methodologies were disrupted (Keller *et al.*, 1989). Therefore, in this study we used berberine sulphate, a technique that is compatible with perfusion fixation, to maintain mast cell integrity in the controls. This approach provided dura containing unaffected cells as a control against which to accurately evaluate the effects of compound 48/80 on dural mast cells.

Because the periosteum of the skull is like dura, a layered connective tissue adjacent to the skull that can be examined as a whole mount, and because effects of mast cell 'degranulating' agents on periosteum had been compared to the dura (Selye *et al.*, 1963), effects of compound 48/80 on periosteum also were evaluated in this study.

Mesentery also is routinely examined as a whole mount and contains 'connective tissue' mast cells that respond to compound 48/80. Therefore, this tissue was examined as a control.

Relative to the distribution of mast cells in the dura mater, we noted previously that although some cells appeared to be lying 'free' in the surrounding connective tissue, many were distributed with the middle meningeal neurovascular bundle (Keller *et al.*, 1989). Because the two layers of dura mater are not easily distinguished from one another in the whole mount preparation it was impossible in the previous study to determine which mast cells were in each layer. Furthermore, the definitive location of the vessels and nerves within each layer also had not been described. Therefore, in the present study, high resolution light microscopy was used to better determine the distribution of these cells as well as of the vessels and nerves in the layers of the dura mater.

A close structural association of mast cells to nerves, vessels, and/or other connective tissue elements in dura mater would provide further evidence for a functional relationship among these components. Numerous *in vivo* studies have suggested a structural/functional relationship of mast cells in other tissues to blood vessels (Rosenblum, 1973; Reilly *et al.*, 1983; Dimitriadou *et al.*, 1987; Tharp, 1989), nerves (Gamble & Goldby, 1961; Kimura, 1979; Appenzeller, *et al.*, 1981; Weisner-Menzel *et al.*, 1981; Newson *et al.*, 1983; Dimlich, 1984; Skofitsch *et al.*, 1985), and to other cells within the connective tissue (Arnold & Huth, 1978; Norrby, 1981). Electron microscopic techniques were used to explore the potential existence of similar relationships of mast cells to nerves, vessels and connective tissue elements in the dura.

Materials and methods

ANIMALS

Fifteen Wistar (290–450 g) and 21 Sprague-Dawley (275–350 g) rats were used for this study. All rats were male, maintained on a 12-h light/dark cycle, and allowed food and water until the time of the experiment.

FLUORESCENCE TECHNIQUES

Glyoxylic acid technique

The glyoxylic acid (GA) technique (de la Torre & Surgeon, 1976) was used to identify 5-HT containing cells. Eleven Sprague-Dawley rats were anaesthetized (sodium pentabarbitol, 175 mg kg^{-1} body-weight (bw)) and placed on a bed of ice. The animals were perfused transcardially with 250 ml of normal saline and the dura mater quickly removed, placed in 0.1 M sodium phosphate buffer (pH 7.4), and whole mounted. Following removal of excess buffer, the slide was dipped three times for 1 s each into a 1% glyoxylic acid/water solution, air dried with a blow dryer for 10 min, and heated in an 80° C oven for 5 min. Each slide was coverslipped with glycerol/water.

Ophthaldialdehyde technique

To determine the presence of histamine containing cells in the dura, the ophthaldialdehyde technique (OPT) of Juhlin and Shelley (1966) was used. Five Sprague-Dawley rats were anaesthetized and perfused as described earlier. Each dura was removed, placed in cool Tyrode's solution (pH 11, 4° C), and whole mounted on a chrome-alum subbed slide. The slide was warmed in a 40° C oven for 5 min, flooded with 1% OPT/ethylbenzene solution for 4 min, drained and coverslipped with tetrahydrofurfuryl alcohol.

Berberine sulphate technique

To identify cells that contained heparin as well as 5-HT or histamine, the berberine sulphate (BBS) technique was applied to tissue that had been examined previously for each of those mediators (5-HT and histamine) according to the technique of Dimlich and co-workers (1980a). After observation and photography of 5-HT or histamine fluorescence,

this fluorescence was quenched by rinsing with water (pH 4, three times for 2 s each). The tissue then was stained with 0.02% BBS in distilled water (pH 4) for 20 min. To remove excess BBS the tissue was rinsed three times for 2 s each with water (pH 4) and coverslipped with glycerol/water.

Controls for the specificity of fluorescence

For each fluorescent technique, some sections were viewed before staining to ensure that autofluorescent structures did not interfere with the specific staining of each component. In addition, after staining and observing the specific fluoresence, slides were re-examined to confirm that water quenched the specific fluorescence previously observed. To check specificity of coincident staining, micrographic images of cells stained for 5-HT or histamine were projected on to images of cells counterstained for heparin.

ROUTINE AND IMMUNOHISTOCHEMISTRY

To determine if there were basic protein in these cells, three Wistar rats were anaesthetized with 0.5 ml sodium pentobarbital (65 mg ml^{-1}) and perfused transcardially for 5 min with 0.9% saline followed by Carnoy's Formula A (Humanson, 1976). Duras were removed, placed in fresh Carnoy's, mounted on chrome-alum subbed slides, and stained with a 0.04% solution of Biebrich Scarlet in a 1 N glycine buffer (pH 9.5) (Spicer, 1962).

To validate the specificity of the GA fluorescence technique for 5-HT, tissues were stained using immunohistochemical techniques. Five Sprague-Dawley rats were anaesthetized with 0.5 ml Socumb (6 grains) and perfused transcardially for 5 min with 0.9% normal saline followed by 4% paraformaldehyde. [Note: Mast cells in rats are insoluble in water and therefore do not require non-aqueous fixatives as do mast cells in other species (Paff & Mergenthaler, 1955).] Heads were removed, kept in fixative for 2 h and then transferred to a 0.1 M phosphate buffer solution (PBS) containing 20% sucrose overnight. The duras were removed and placed in a rinsing solution of PBS, 3% normal goat serum, 1% Triton X-100 and 0.02% sodium azide overnight. Then duras were incubated in 5-HT primary antibody (Eugene Tech International, Inc., Allendale, NJ) (1:1000) for 24 h. The secondary antibody procedure was run using a VECTASTAIN *Elite* ABC Kit (Vector Laboratories, Inc., Burlingame, CA) with DAB as the chromagen (Hsu *et al.*, 1981). Some control duras were treated as above but incubated without primary antibody.

COMPOUND 48/80 METHODOLOGY

Animals and administration

To determine the effects of a well-known 'degranulating' agent, compound 48/80 (Mota *et al.*, 1953), eight adult male Wistar rats were anaesthetized with an intraperitoneal injection of urethane (2 mg g^{-1} bw). The femoral vein was cannulated for the administration of compound 48/80 (Sigma Chemical Co.) (50 μg per 100 g) ($n = 5$) or an equal volume of carrier (0.5 ml 0.9% NaCl per 100 g bw) ($n = 3$).

Tissue procurement and preparation

At 10 min after the injection, rats were perfused transcardially for 5 min with saline and for 30 min with 1 litre of 10% neutral buffered formalin (NBF). The head and mesentery were removed and placed in fresh NBF for a minimum of 1 week. Before placing the head in NBF, the most superficial three or four layers of the scalp were removed from the calvarium as described by Selye and co-workers (1963). After fixation the external periosteum was dissected from the skull and the dura and brain were removed from the skull. Samples of the external periosteum, dura and mesentery were mounted on chrome-alum subbed slides.

Staining

After drying, sections were stained for heparin using the BBS technique as described earlier. The fluorescence produced by BBS is directly proportional to the concentration of heparin (Enerbäck, 1974a). Also, the brilliant yellow mast cells stand out more clearly against the green background in BBS-stained material than the purple mast cells on the blue background of a metachromatic dye. Therefore, because of its specificity and because mast cells are easily counted and 'degranulated' cells are very evident in BBS stained material, that method was used for this part of the study. However, because BBS fluorescence eventually fades, after viewing, quantifying and photographing, coverslips were removed and the tissue rinsed in water and counterstained in 0.02% aqueous Azure A (pH 4.5) (Pearse, 1968), for a permanent record of the results.

Controls

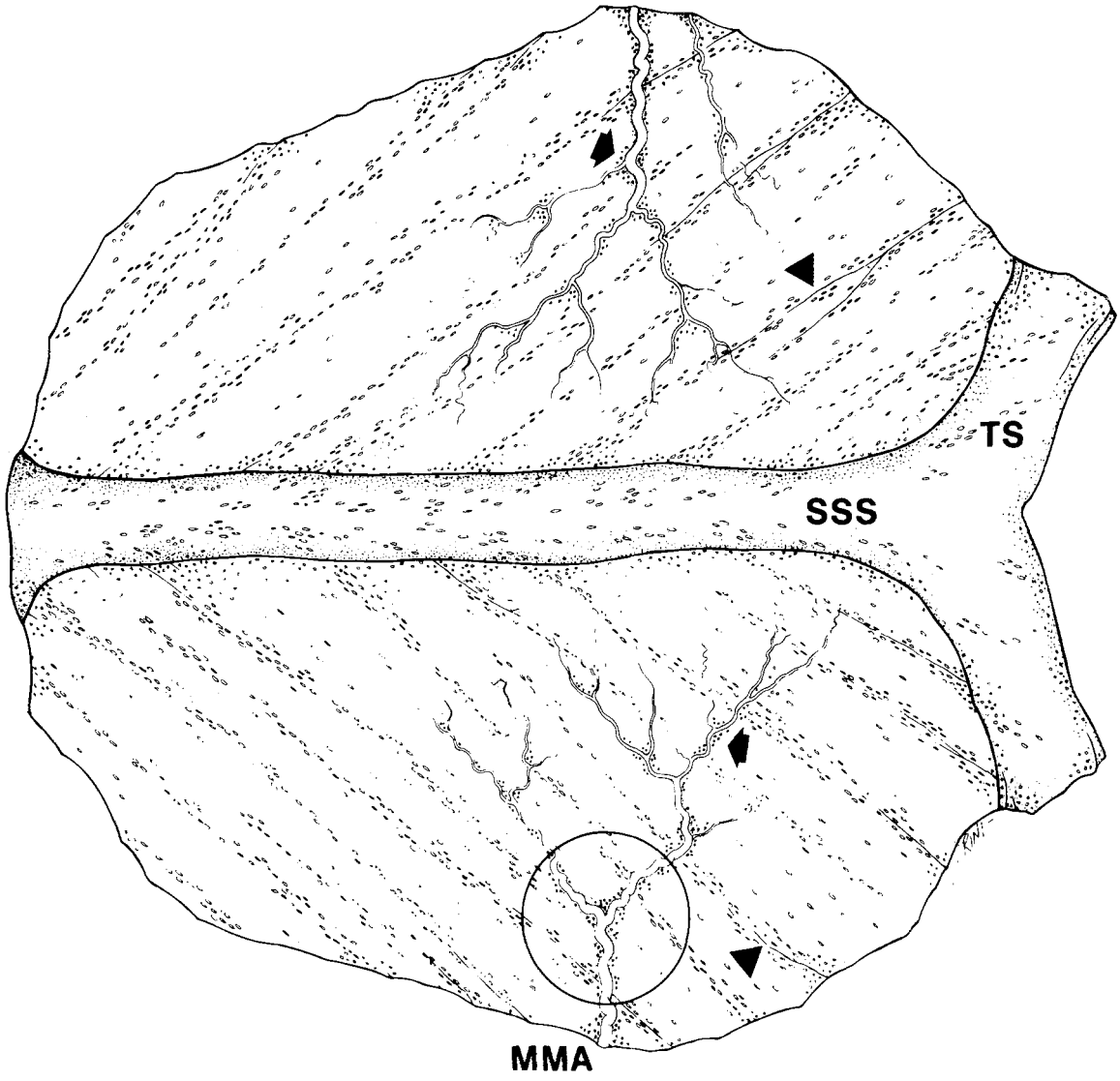
The release of mast cell vasoactive constituents induces peripheral vasoconstriction and oedema (Riley, 1959). Therefore, to ensure that compound 48/80 was injected into the vasculature, rats were observed for cyanosis of the paws, ears and muzzle, and for respiratory distress from laryngeal oedema and/or bronchospasm. Within 1 min all rats given compound 48/80 showed these signs; therefore, they were included in this study.

HIGH RESOLUTION LIGHT AND ELECTRON MICROSCOPY

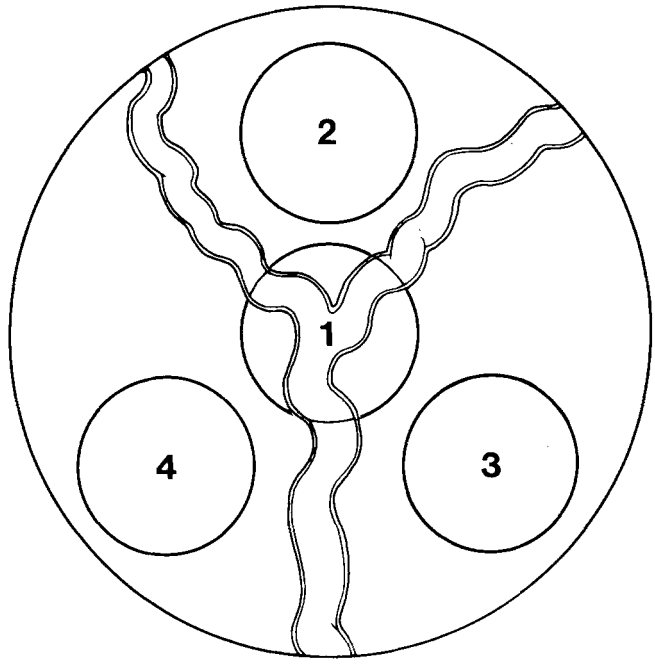
Four adult male Wistar rats were anaesthetized with ether and perfused transcardially for 5 min with 0.9% saline followed by 1 litre of a modified Karnovsky fixative (2% paraformaldehyde, 2.8% glutaraldehyde and 2% sucrose) in a potassium phosphate buffer (0.1 M, pH 7.4) for 30 min. The head was removed and stored in fixative for a minimum of 24 h and then the dural coverings were removed and dissected into samples that included the middle meningeal vasculature. The samples were washed in buffer, postfixed for 2 h in OsO_4, dehydrated and flat embedded in LX112 Epoxy resin. Semithin sections (1 μm) were stained with Toluidine Blue (0.5% aqueous). Thin sections (60–90 nm) were mounted on Formvar coated slot grids, and stained for 5 min each with uranyl acetate and lead citrate.

COUNTING OF CELLS AND STATISTICAL ANALYSIS

Because the branching point of the middle meningeal artery (MMA) was chosen to determine the functional relationship of these cells to neurogenic parameters in other related studies (Keller *et al.*, 1989), mast cells were counted in the circled area on each side of the dura (Fig. 1a). Four fields, one of the MMA at its division, one distal to its branching point



1a



1b

and one either side of the MMA just proximal to its division, were examined for fluorescent cells using a ×16 objective (Fig. 1b). Two investigators (T.A.S. and M.J.F.) counted and determined the percentage of cells with dispersed granules independently and in 'blind' fashion. In dura from six rats there was no significant difference in the number of cells between the two counters for either the GA ($P = 0.0932$, ANOVA) or BBS ($P = 0.6210$, ANOVA) technique. Likewise, the percentage of cells with dispersed granules determined by each of the two counters was not different [(GA, $P = 0.7488$, Kruskal-Wallis) (BBS, $P = 0.6310$, Kruskal-Wallis)]. Therefore, the data of M.J.F. are reported for this study.

To determine the validity of the GA technique as an indicator of 5-HT content, the number of cells in tissue stained by the GA technique was compared with the number of cells staining for 5-HT immunohistochemical-like activity.

Cells stained with the OPT technique fade more quickly than with the GA or BBS method. Therefore, coincident staining for 5-HT and heparin was evaluated by comparing the number of cells stained with the GA technique with the number of cells that fluoresced after counterstaining with the BBS technique.

To determine the effect of non-perfusion fixation on the integrity of dural mast cells, the percentage of cells exhibiting dispersed granules was compared between perfused tissues treated for 5-HT immunohistochemistry and non-perfused tissues treated for GA and BBS fluorescence. The latter two groups were compared to determine if there were an effect of BBS counterstaining on the integrity of those cells.

The effect of compound 48/80 on mast cells was demonstrated by comparing the percentage of cells exhibiting dispersed granules in perfusion-fixed tissue from rats treated with compound 48/80 with the percentage in rats treated with the carrier alone.

The relationship of mast cells to the layers of the dura and to other tissue constituents was quantified by examining a mean of 23 step serial 1 μm sections from the dura of each of three rats. To ensure that the same mast cell would not be counted twice, these sections were separated by at least 30 μm. The percentage of mast cells that were adjacent to nerve bundles, vessels, other cells or collagen was calculated.

Using STAT-PAK (Northwest Analytical, Portland, OR), the mean numbers of cells (+/− SEM) were compared by analysis of variance (ANOVA) and the differences between percentages were compared using the Kruskal-Wallis test.

Results

Fluorescence microscopy

Before staining there were no autofluorescent nerves or cells in the dura. In all duras examined, the same cells that stained positively for 5-HT (Fig. 2a) or histamine (Fig. 2c) also stained for heparin (Fig. 2b and d, respectively). Superimposing images of tissue stained with one technique onto images of counterstained tissue confirmed this observation. The GA prepared specimens demonstrated 5-HT (yellow) fluorescent cells as well as catecholamine (apple green) positive nerves that accompanied the blood vessels (Fig. 2a). OPT stained tissues demonstrated histamine (yellow) fluorescent cells primarily next to blood vessels (Fig. 2c). Specific staining for 5-HT and histamine was quenched by hydration before counterstaining with BBS. Cells that fluoresced bright yellow for heparin in BBS stained tissue, were easily distinguished from nuclei of other cells that were a light green (Fig. 2b and d) owing to the nucleic acid staining property of BBS (Hals, 1970). In all stains, some cells appeared to be free within the connective tissue (Fig. 2a–d).

Routine and immunohistochemistry

Cells with a similar distribution to the fluorescent cells also stained orange-red with Biebrich Scarlet (Fig. 3a). Immunohistochemical control tissue demonstrated no staining of cells for 5-HT-like immunoreactivity. In other tissue, cells that exhibited 5-HT-like immunoreactivity were distributed similarly to those identified using the GA fluorescent technique (Compare Figs 1, 2 and 3b). Probably because of a paucity of background staining, there was an obvious pattern of distribution of mast cells using this immunohistochemical technique. There were two groups of cells, one that accompanied the middle meningeal artery (MMA) and its associated structures, and another that ran parallel to trigeminal nerve bundles that coursed obliquely across the MMA (Fig. 3b). In each group, some cells were not adjacent to either structure (Fig. 3b). When the depth of focus was altered, one group of cells appeared to be in one layer and the other group in another layer of the dura. Different shaped cells, predominantly round adjacent to the vessels and oval next to nerves, were most obvious using immunohistochemistry (Fig. 3b-d).

Number of cells and effects of tissue preparation and compound 48/80 on granule dispersion

To understand the results when using compound 48/80 in this study, it is important to clarify how mast cells release their constituents. Upon appropriate stimulation, e.g. with compound 48/80, IgE, or antigen challange, mast cells release the contents of their

Fig. 1. Diagrammatic representation of rat dura mater (1a). Note the large number of mast cells (arrows) and tangential orientation of nerves (arrowheads) to the middle meningeal neurovascular triad as represented by the middle meningeal artery (MMA). Superior sagittal sinus (SSS) and transverse sinus (TS) are noted for orientation. The circled area in 1a is enlarged as 1b. Mast cells were counted in the four microscopic fields represented in this diagram.

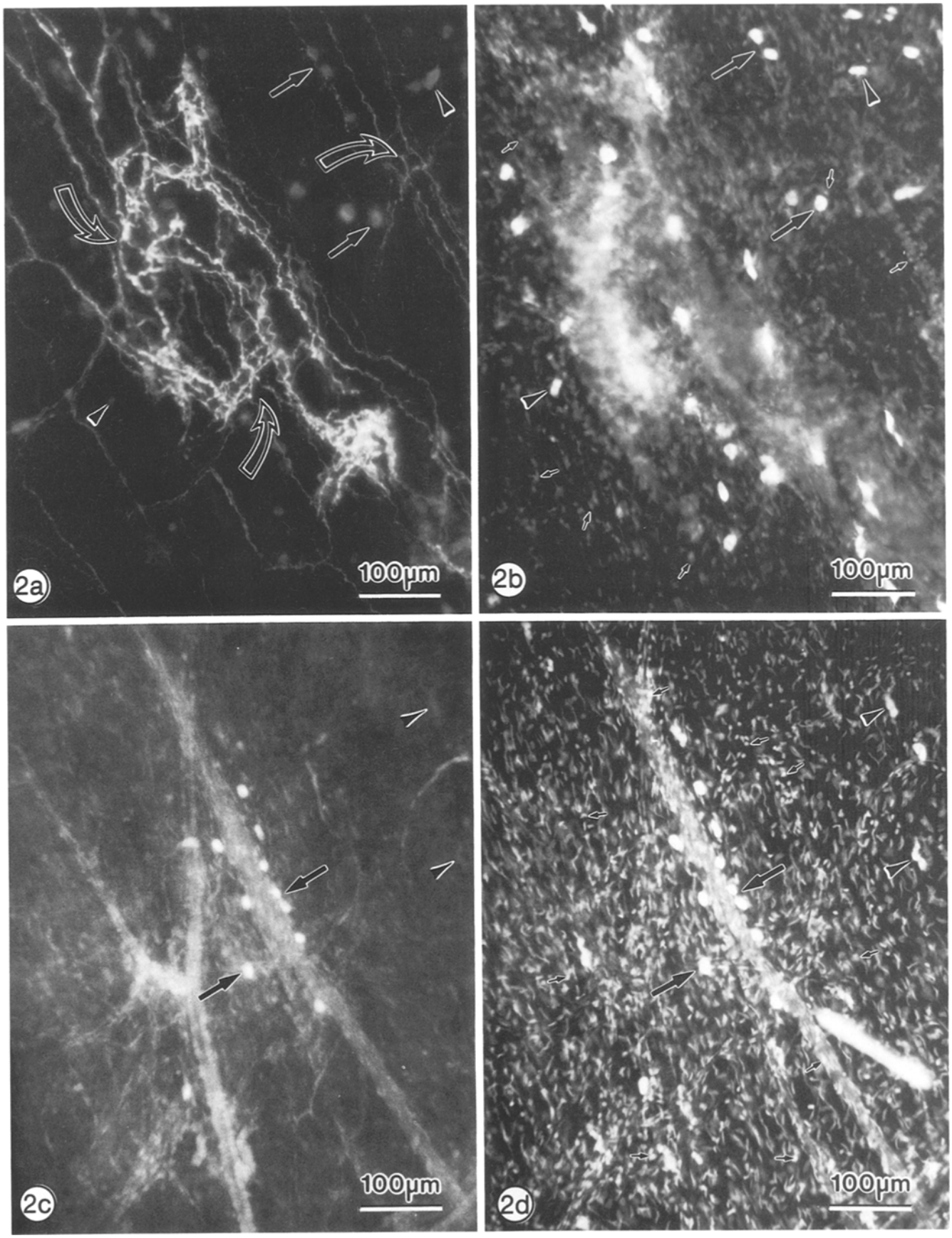


Fig. 2. Cells (arrows) in dura mater that stained positively for 5-HT (2a) or histamine (2c) also stained for heparin (2b and 2d respectively). Note that the catecholamine specific fluorescence of the prominant nerve network (curved arrows) (2a) can no longer be distinguished after quenching with water and restaining for heparin (2b). Also note that fluorescent cells are aligned along the middle meningeal artery (2c and 2d). Some cells appear to be free within the adjacent connective tissue (arrowheads). In tissue counterstained with berberine sulphate, nuclei in other cells (e.g. endothelial and connective tissue) (small arrows) also are fluorescent (2b and 2d).

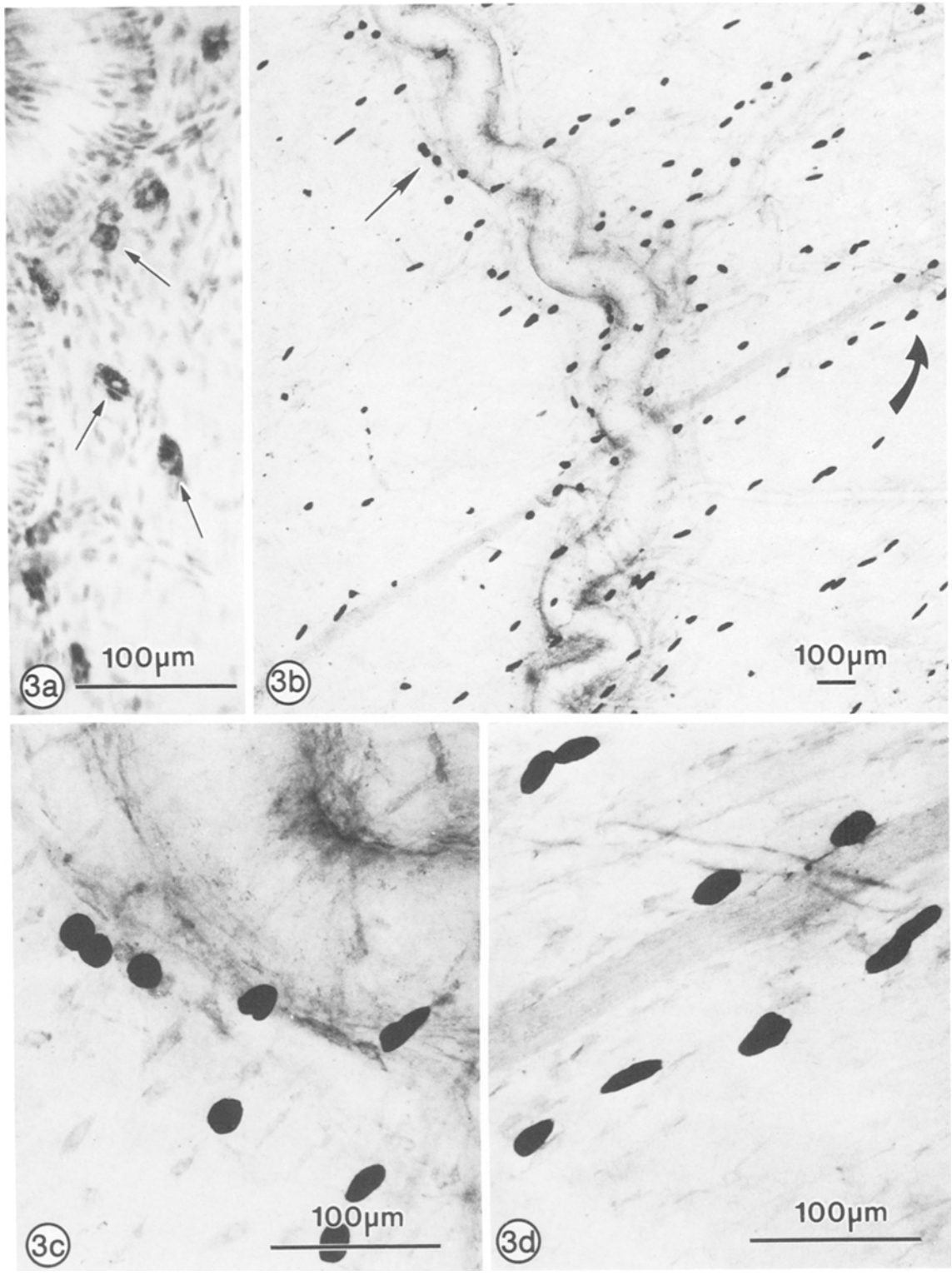


Fig. 3. Dural tissue stained with Biebrich Scarlet for basic protein (3a). To improve the contrast between orange-red mast cells (arrows) and orange structures, this tissue was photographed after counterstaining with Azure A (pH 4). Dural tissue stained for 5-HT-like immunoreactivity in 3b demonstrates the two patterns of distribution of mast cells in this tissue. One group of cells accompanies the middle meningeal artery and its associated structures (straight arrow) (3b). Those cells are round and are depicted at a higher magnification in 3c. By comparison, the other group of cells are parallel to nerve bundles that course obliquely across the artery (curved arrows) (3b). Most of these cells are oval as represented at a higher power in 3d.

Table 1. Effects of fixative and staining methodology on dural mast cell number and granule dispersion

	5-HT Immunohistochemistry, perfusion fixation (n = 4)		*5-HT Glyoxylic acid (GA) technique, no perfusion fixation (n = 11)*		*5-HT Counterstained with berberine sulphate, no perfusion fixation (n = 11)*	
Side of dural whole mount	*Left*	*Right*	*Left*	*Right*	*Left*	*Right*
No. of mast cells/ microscopic field (mean ± SEM)*	*260 ± 36*	*223 ± 13*	*281 ± 29*	*212 ± 17*	*257 ± 21*	*228 ± 15*
Percentage of mast cells with dispersed granules (mean ± SEM)	*11.1 ± 3.9*[a,b,c]	*36.1 ± 6.4*[a,d,e]	*78.6 ± 2.4*[b,f]	*77.2 ± 2.3*[d,g]	*90.3 ± 1.2*[c,f]	*88.0 ± 1.6*[e,g]

[a-g] $P = 0.0012$–0.0339 (Kruskal-Wallis)

* Note: Because the area of the microscopic field at × 160 is 0.97 mm^2, the mean number of cells for both sides and all techniques/ microscopic field (i.e. 243.5) represents a mean of 251.6 cells per mm^2.

granules by a process called sequential (Röhlich *et al.*, 1971) or compound (Douglas, 1974) exocytosis. Membranes of granules adjacent to the cell membrane fuse with the cell membrane forming pores through which the peripheral granules become exposed to the extracellular fluid (Röhlich *et al.*, 1971; Lagunoff, 1972). This evokes the disassociation of substances from the granule (Lagunoff & Chi, 1978) without the loss of the granule from the cell. Subsequent fusion of more internal granules with membranes of previously stimulated granules results in the formation of intracellular channels that allow the contents of even innermost granules to be extruded into the extracellular space, again without the actual expulsion of the granules from the cell. Even after the elucidation of this mechanism, the term 'degranulation' has been used to denote the release of substances from mast cells, regardless of whether the granule is physically released from the cell. As was demonstrated in our study of perfusion fixed tissue from compound 48/80 treated rats, this type of 'degranulation' is evidenced by a change in size and staining intensity of the granules within the cell. These changes in the granules indicate that the contents of the granules have dispersed rather than the cell 'degranulating'. Therefore, we have chosen to use 'dispersion' rather than 'degranulation' to describe our results.

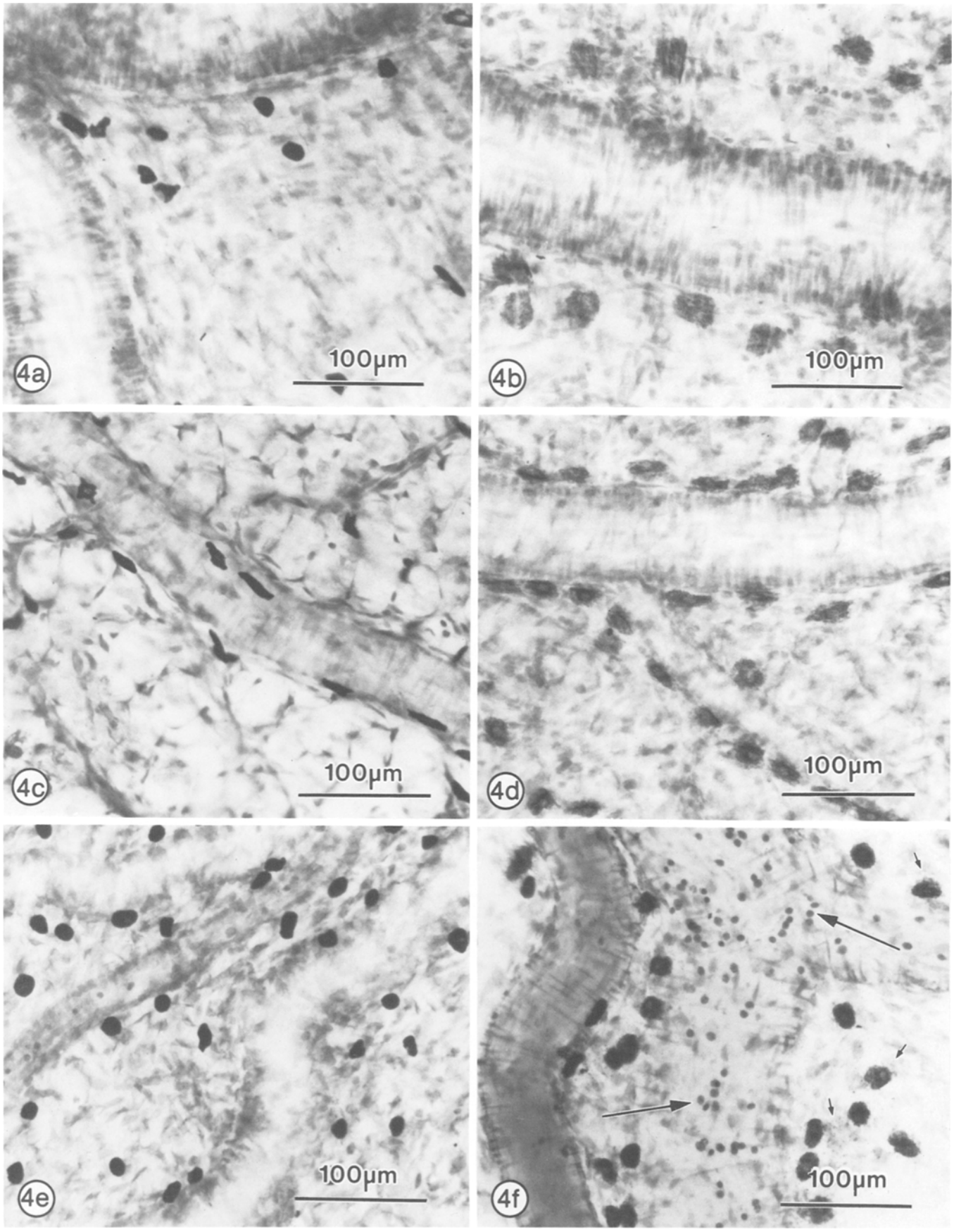
In this study there were no significant differences between the number of cells counted regardless of how the tissue was fixed, stained or counterstained (Table 1). Using the criteria of increased size and decreased intensity of staining of the granules to indicate dispersion, the majority of 5-HT immunopositive cells were intact (Fig. 3b-d and Table 1). There were significantly more cells with dispersed granules on the right side of the control dura when compared with the left; however, dispersion on both sides was significantly less in fixed tissue (Table 1).

The percentage dispersion in dura, periosteum and mesentery was significantly greater in compound 48/80 treated rats than in controls (compare Fig. 4a with b, c with d, and e with f; Fig. 5). Although some granules were observed outside the cells in the mesenteric preparation (Fig. 4f), when statistically compared with the dura or periosteum (Fig. 4b and d), there was no difference in degree of dispersion (Fig. 5). However, when control dura and periosteum were compared there was a small but statistically greater degree of dispersion in the dura (Fig. 5).

High resolution light microscopy

As depicted in a micrograph of a 1 μm cross-section of the dura mater, there were two layers of dura, each of which was composed of a variable number of lamellae

Fig. 4. Dura (4a and 4b), skull periosteum (4c and 4d) and mesentery (4e and 4f) from control rats (4a, 4c and 4e) and from rats given compound 48/80 (4b, 4d and 4f). These tissues were stained with berberine sulphate followed by Azure A. Note that treatment with compound 48/80 resulted in larger granules with less dense staining suggesting that dispersion of granular contents had occurred in most of the mast cells in these preparations (compare 4a with 4b, 4c and 4d, 4e with 4f). Note that in the mesentery treated with compound 48/80, there are numerous blood cells in the vessel (large arrows) indicating that the perfusion fixation may not have been complete. This may explain why more granules (small arrows) are extracellular in this preparation when compared with dura (4b) or periosteum (4d).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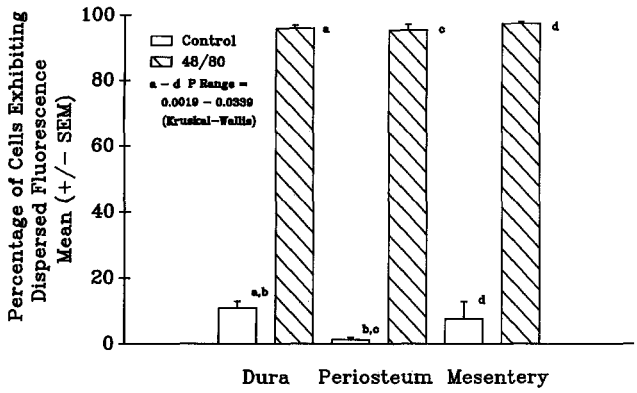


Fig. 5. The percentage of mast cells with dispersed granules for perfusion-fixed dura, skull periosteum and mesentary from rats treated with compound 48/80 as compared with control rats. Note that the percentage of cells with dispersed granules is significantly greater in all tissues from treated rats compared with controls. In the control, there was a slight, but significant, increase in dispersion in the dura when compared with the periosteum.

(Fig. 6a). The outer loose layer serves as a conduit for the neurovascular bundle that usually was composed of a central artery flanked on either side by a vein and lateral to that, a nerve (Fig. 6a). The inner more dense layer, infrequently exhibited the profile of a small vessel or branches of the trigeminal nerve that runs tangentially to the MMA (Fig. 6a). Both layers were bordered peripherally by very thin lamellae that often were lost when the dura was removed from the brain (Fig. 6a).

In cross-sections of the dura, 72% and 67% mast cells in the outer and in the inner layer respectively were aligned near the interface between the two layers (Fig. 6a) as opposed to deeper within each layer (Fig. 7c).

The diagrammatic representation of the dura and its mast cells (Fig. 6b) summarizes the observed orientation of the dural layers to one another, the distribution of mast cells to specific lamellae within those layers, and the linear arrangement of mast cells within those lamellae.

Quantification of mast cell associations with other structures in cross-sections revealed that of 179 mast cells, 6.7% were adjacent to vessels (Fig. 7c and d), 0.6% were within a myelinated nerve bundle (Fig. 8a), 1.1% were next to nerve bundles (Fig. 8b), 35.2% were close to collagen (Figs 6a and 8b), 34.1% were close to other connective tissue cells (Figs 6a and 8d), and 22.3% were without any apparent relationship.

Electron Microscopy

Ultrastructurally, dural mast cells appeared to be of a single morphology. They were packed with large osmiophilic granules of a consistent density, possessed numerous microvilli, and had a typical non-lobulated nucleus with a peripheral distribution of heterochromatin (Fig. 7a). As in typical mast cells, there was a paucity of mitochondria and sparse endoplasmic reticulum (Fig. 7a), and a well-developed Golgi apparatus was observed adjacent to the nucleus in a granule free zone (Fig. 7a and b). The regularity of a sectional profile was affected especially by collagen. Usually a cell surrounded by collagen was cigar-shaped; however, in some instances multiple bundles of collagen dictated a stellate shape for the cell (Fig. 7a). Even a single bundle of collagen was observed to affect the shape of a cell, sometimes causing cells to fold so that the profiles were kidney-shaped in cross-section (Fig. 7c and d).

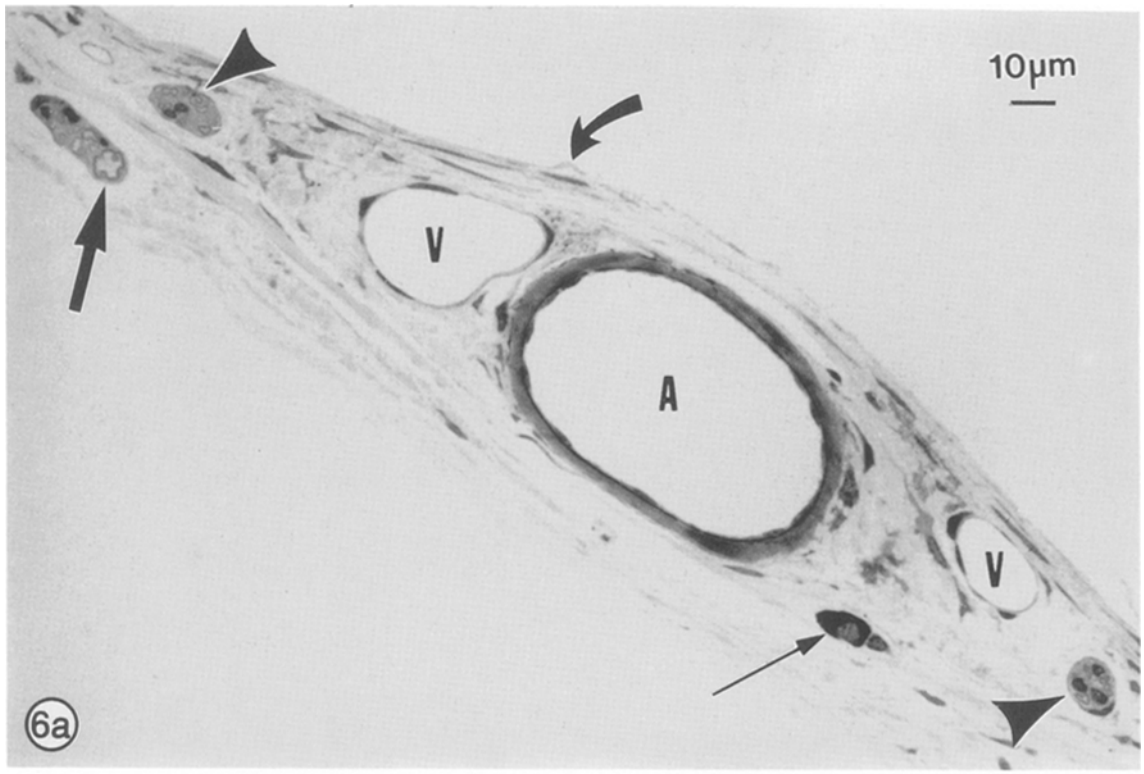
Associations between mast cells and other tissue components were not quantified at the ultrastructural level, but certain observations were made. Mast cells that were next to a vessel were outside of the basal lamina (Fig. 7d). Non-myelinated nerve bundles and endings were separated from the mast cell by collagen and/or a basal lamina (Fig. 8b and c). Because they appeared to contain small vesicles and in some instances were surrounded by basement membrane material, an occasional profile near a mast cell was identified as a nerve ending (Fig. 8c). No junctional specializations between those endings and mast cells were observed. Almost every mast cell, in one section or another, had at least membrane contact with another connective tissue element, e.g. collagen, fibroblasts, macrophages and monocytes (Fig. 8b and d).

Discussion

IDENTIFICATION OF SEROTONIN FLUORESCENT CELLS AS MAST CELLS

Cells are identified as mast cells based on the presence of a mucopolysaccharide, two amines, and a basic protein (Csabo & Kovács, 1975). As demonstrated by Dimlich and co-workers (1980b) for the liver, the colocalization of 5-HT and heparin or histamine and heparin within the same cell infers that heparin, histamine and 5-HT, i.e. a mucopolysaccharide and two amines, were present in the same cell. The subsequent identification of a basic protein in these dural cells indicated that all the criteria had been met to identify them as typical mast cells. That the same number of cells stained for 5-HT fluorescence and immuno-like reactivity is further evidence that these cells contain 5-HT. That the same number of cells contained 5-HT and heparin was quantitative proof for the conclusion that these were mast cells.

Dural mast cells have not been previously characterized. Waldeyer in 1875 described 'plasmazellen' in mammalian dura mater but did not distinguish between these and other non-mast cells (Selye, 1965). The first mention of 'mast' cells in dura was in a



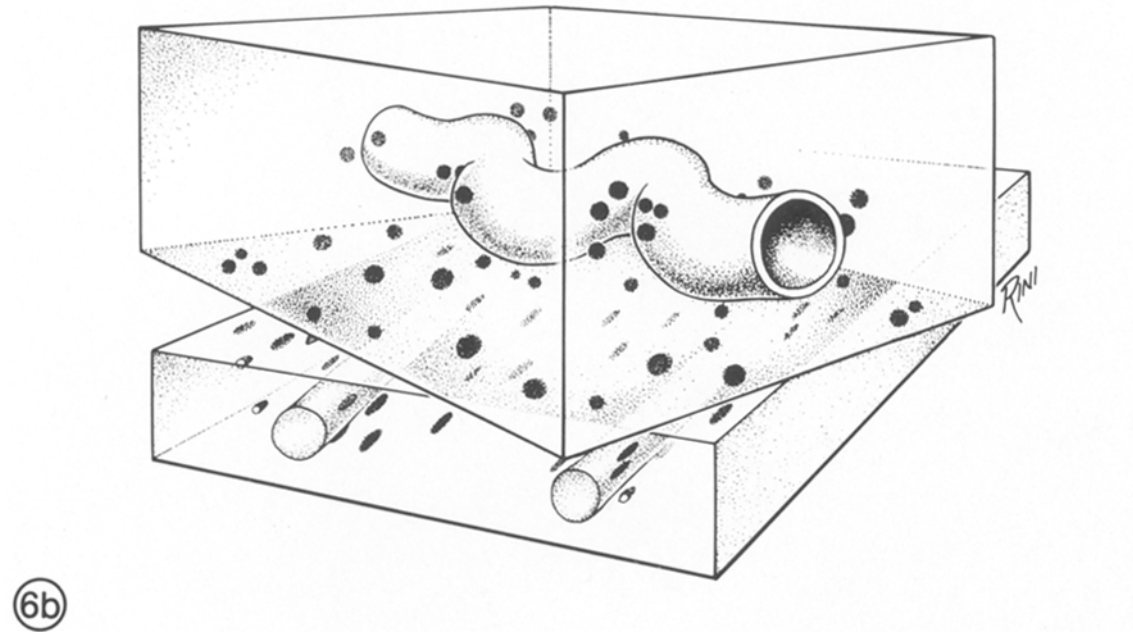


Fig. 6. Cross-section 1 μm thick demonstrating the layers of the dura and their contents (6a). The outer layer contains the neurovascular bundle that usually is composed of a central artery (A), two veins (V) and two nerve bundles (arrowheads). The inner layer contains the profile of a branch of the trigeminal nerve (large arrow). Note the very thin layer of dense connective tissue on the outermost border (curved arrow). This is usually present on the innermost border as well, but as often occurs, was lost in preparation of this tissue. In each layer, most mast cells are aligned nearer the interface between the two layers (thin arrow) (6a). The diagrammatic representation of the dural layers and mast cells demonstrates that the two layers course tangentially to one another (6b). As represented by the middle meningeal artery, the middle meningeal vasculature is in the outer, as depicted here, the upper layer. The inner, or as represented here, the lower layer contains branches of the trigeminal nerve. Mast cells are distributed at the interface between the two layers as well as linearly on either side of the vessels and/or nerves in each layer.

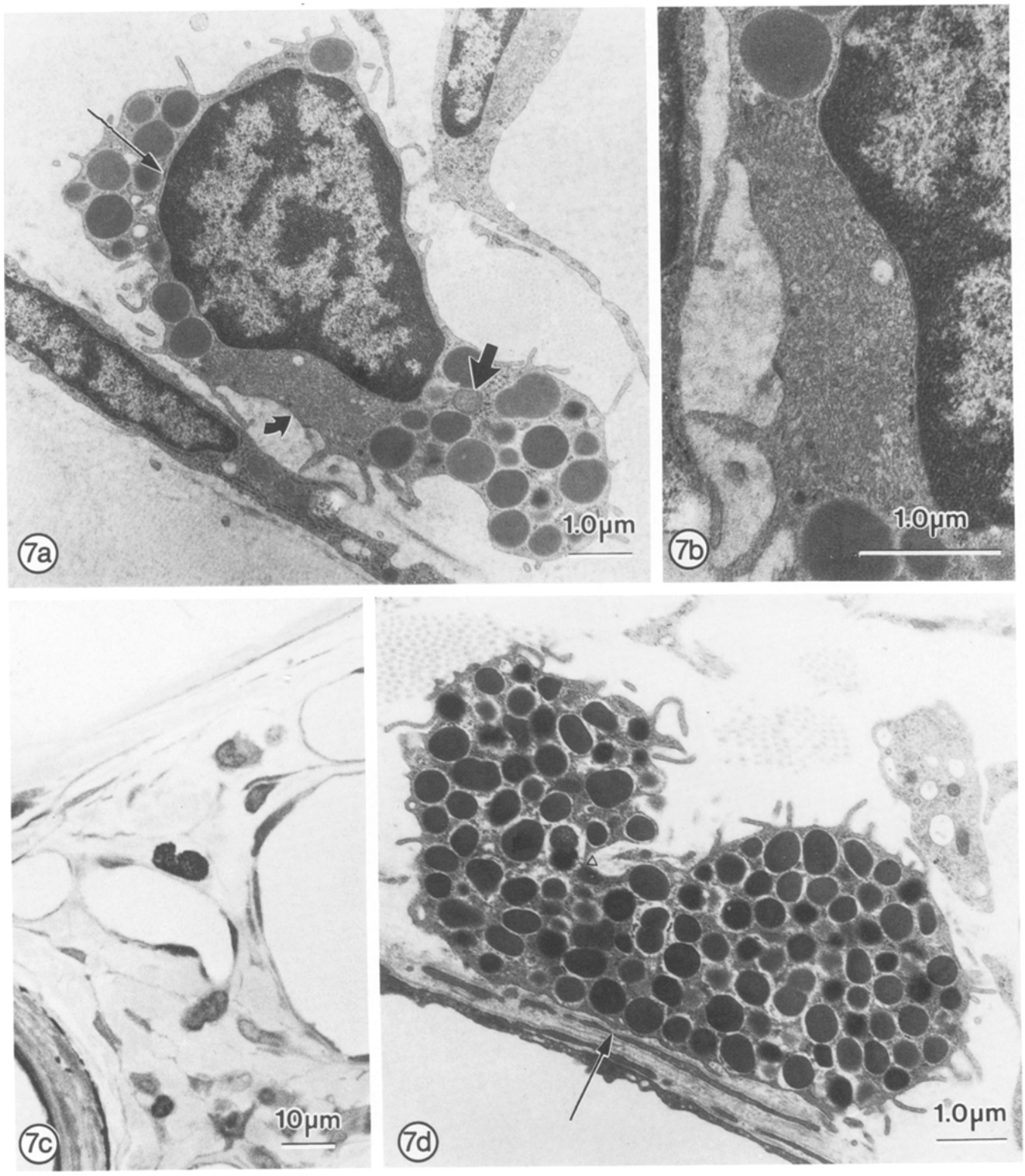


Fig. 7. Electron micrographs of typical dural mast cells. Note the large osmiophilic granules of a consistent density, a non-lobulated nucleus with a peripheral distribution of heterochromatin (7a), few mitochondria (thick straight arrow) (7a), sparse endoplasmic reticulum (thin arrow) (7a), and well-developed Golgi apparatus (curved arrow) (7a) adjacent to the nucleus in a granule free zone (see enlargement, 7b). Note that the mast cell adjacent to a blood vessel (arrow) (7c) is outside the basal lamina (arrow) (7d). This cell also is bent around a thin bundle of collagen (open triangle) (7d).

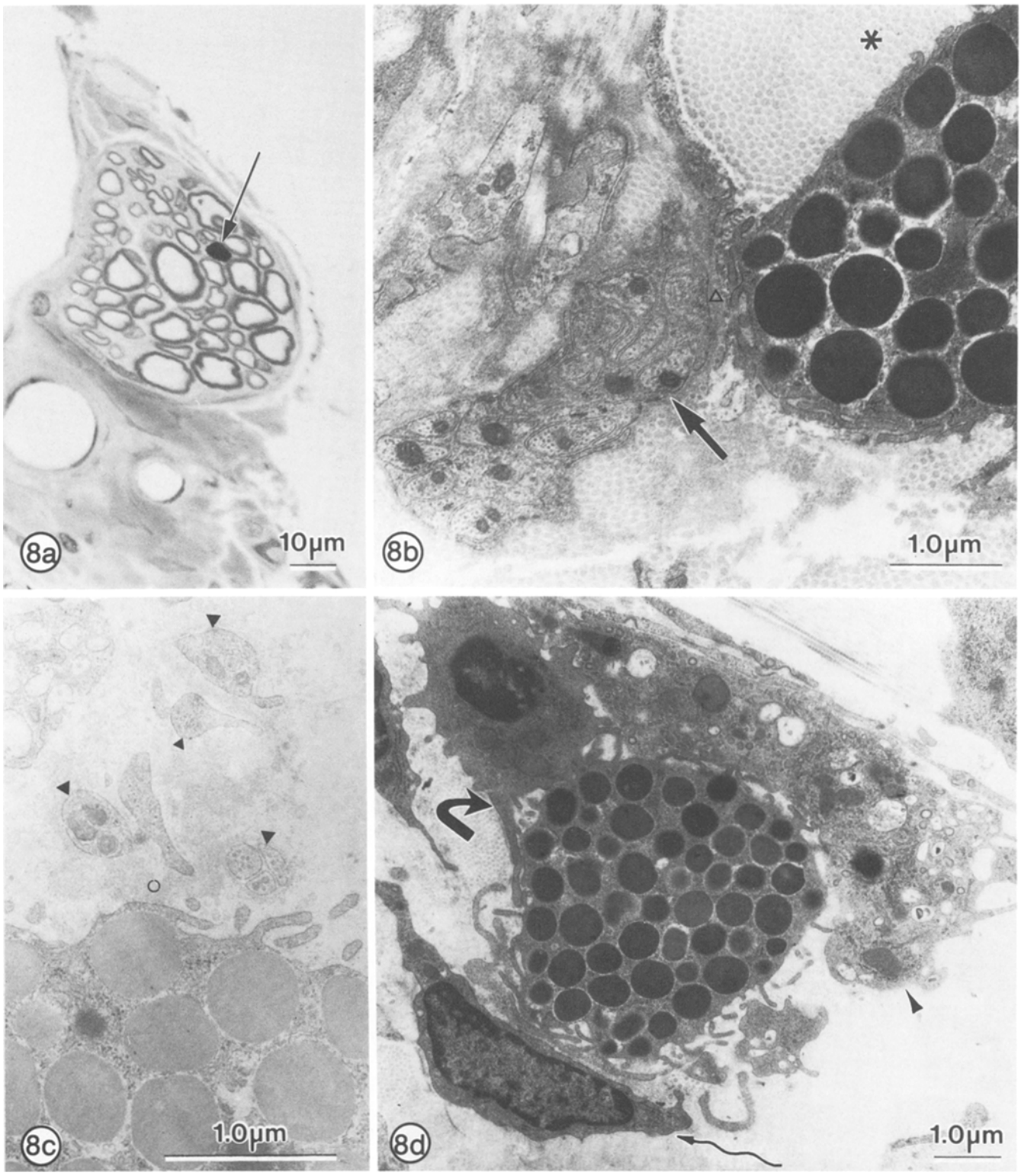


Fig. 8. Mast cell (arrow) observed within a nerve bundle (8a). Mast cell that is adjacent to unmyelinated nerve bundle (arrow) (8b) is separated from it by a small amount of connective tissue ground substance (open triangle). Note the bundle of collagen (*) that is adjacent to this mast cell. Vesicle containing nerve endings (arrowheads) that are in close proximity to a mast cell are separated from it by basal lamina (open circle) (8c). Mast cell having membrane contact with a monocyte (bent arrow), macrophage (arrowhead) and fibroblast (wiggly arrow) (8d).

description of guinea pig (D'Ermo & Cricchi, 1963) and rat (Selye *et al.*, 1963) dura mater. Since that time, dural mast cells have been (1) associated with the vasculature (Giordano-Lanza *et al.*, 1972), (2) discussed incidentally (Olsson, 1968; Markowitz *et al.*, 1989; Theoharides, 1990), (3) used as a subject for 'degranulation' studies (D'Ermo & Cricchi, 1963; Gabbiani *et al.*, 1970; Motavkin *et al.*, 1979), and (4) cited as a potential contaminant in the study of brain histamine content (Orr, 1984). Although one report depicts what might be a mast cell (Andres *et al.*, 1987), no electron microscopic study of rat dura has described the mast cell as a component of that tissue (Pease & Schultz, 1958; Andres, 1967; Waggener & Beggs, 1967; Andres *et al.*, 1987). Because mast cells are resident cells of the connective tissue (Goldberg & Rabinovitch, 1977) and dura mater is a connective tissue of mesodermal origin (Hamilton & Mossman, 1972) that acts as a barrier to the 'outside environment' (Goldberg & Rabinovitch, 1977), the presence of a large population of mast cells in the dura as observed in this study was not unexpected.

DURAL MAST CELLS AS AN HOMOGENEOUS POPULATION OF 'CONNECTIVE TISSUE' MAST CELLS

The ultrastructural image of these cells packed with homogeneous electron-dense granules, was identical to that described for a 'connective tissue' mast cell (CTMC) (Smith, 1963). The staining of these cells with berberine sulphate and their immediate sensitivity to compound 48/80 also was similar to that described for CTMC (Galli, 1990). However, because numerous types of mast cells have been described, it was necessary to determine if this were a homogeneous population of CTMC.

Although heterogeneity among mast cells may represent variants of the same cell at different stages of maturity or activity and structural adaptations to the surrounding tissues (Selye, 1965; Galli, 1987, 1990), a variety of phenotypes has resulted in several classifications of mast cells. What distinguishes one classification from another is not clear or agreed on (Galli, 1990). Historically mast cells were designated 'types I' and 'II' based on what was thought to be a stage of maturation (Riley, 1959). In 1948, two types of mast cells were described for the digestive tract (Enerbäck, 1966). The 'serosal' or typical 'connective tissue' mast cells, also called 'type I' mast cells, are widely distributed throughout the body. The 'type II' or 'mucosal' mast cells (MMC) (Enerbäck, 1974b) appear to be unique to the digestive system. MMC may be comparable to the immature 'type II' cells because, unlike CTMC, they are T-cell dependent (Galli, 1990).

Why dural mast cells are not 'mucosal' mast cells

All mast cells stain with Alcian Blue (Galli, 1990); however, among other differences, MMC (1) do not contain heparin and therefore do not stain with BBS, (2) demonstrate a low sensitivity of staining for histamine and serotonin and 'degranulation' with compound 48/80, and (3) exhibit a variability in the density of their granules ultrastructurally (Galli, 1990). It also has been reported that MMC contain fewer granules that are more variable in size, have an irregularly lobulated nuclei, lack microvilli (Enerbäck, 1974b), and tend to be associated with nerve endings (Newson *et al.*, 1983). Our ultrastructural, compound 48/80, as well as BBS, histamine and 5-HT staining data indicate that there are no MMC in the dura of the rat. Our conclusion agrees with Theoharides' (1990) assessment that dural mast cells are not MMC.

Why dural mast cells should not be classified as 'brain' mast cells

Theoharides (1990) categorizes dural mast cells as 'brain' mast cells. Other than in the summary, where he states that each type, i.e. CTMC, MMC and 'brain' have 'their own distinct biochemical, morphological and functional characteristics', Theoharides (1990) does not detail any differences between CTMC and dural mast cells. In fact, in a chart comparing their responses, both CTMC and dural mast cells are reported to act similarly regarding staining reactions, responses to peptides, and 'degranulation'. This evidence further supports our identification of dural mast cells as typical CTMC; and, we do not agree that dural mast cells belong to a third class of mast cells called 'brain mast cells' (Theoharides, 1990).

Why dural mast cells are not 'neurolipomastocytes'

In addition to dural mast cells, Theoharides (1990) includes what he calls 'perivascular' and 'parenchymal' mast cells as 'brain' mast cells. These two types of 'brain' mast cells may correspond respectively to the two types of cells described by Ibrahim and co-workers (1979) as 'type I' (synonomous with CTCM) and 'type II mast cells ('neurolipomastocytes') of the brain. It has been reported that 'neurolipomastocytes' do not contain heparin, histamine or 5-HT, and are therefore not true mast cells (Kiernan, 1976; Edvinsson *et al.*, 1977; Dimlich, 1990). Unlike 'neurolipomastocytes', dural mast cells contain 5-HT, histamine and heparin. Also, dural mast cells are not autofluorescent, i.e. do not contain fat, a typical characteristic of 'neurolipomastocytes'. These results confirm that dural mast cells are not 'neurolipomastocytes'.

Why shape does not determine the type of a mast cell and vice versa

It has been suggested also that there are two types of mast cells based on their shape, i.e. 'round' or 'oval' (Markowitz *et al.*, 1987). This study demonstrated that

a single type of mast cell, i.e. the CTMC, could have two shapes. Probably because the inner layer was of more dense connective tissue, CTMC in that layer were more oval in shape than the rounder appearing CTMC in the outer looser connective tissue layer. In addition, the images of this study demonstrated that the shape of a mast cell is dependent on the contiguity, density and orientation of its surrounding elements, i.e. collagen, other cells, vessels and nerves, and not on its 'type'.

Why the mast cell population of the dura is not like that in the pia/arachnoid mater

The fluorescence histochemistry of this study demonstrated that there were no autofluorescent cells in the dura mater as opposed to the reported autofluorescent cells for at least human arachnoid/pia mater (Edvinsson *et al.*, 1977). Mast cells of the dura mater (pachymeninx) have been confused sometimes with mast cells of the arachnoid/pia mater (leptomeninges). Probably because dura and arachnoid/pia are both meninges, the most frequent citation of Edvinsson and co-workers (1977) that describes only arachnoid/pia mast cells, has been used out of context and incorrectly applied to the dura (Markowitz *et al.*, 1987; Faraci *et al.*, 1989). Dura is not derived from ectoderm as are arachnoid/pia (Hamilton & Mossman, 1972); and because mast cells are heterogeneous it cannot be assumed that the mast cell population in dura is the same as in the arachnoid/pia mater. This study proves that point.

EVIDENCE FOR DISPERSION OF DURAL MAST CELL CONSTITUENTS

The immediate response of rat dural, periosteal and mesenteric mast cells to compound 48/80 indicated a primary rather than a secondary effect from other more delayed actions of compound 48/80 (Christoffersen & Poulsen, 1979; Gietzen, 1983; Gietzen *et al.*, 1983; Henrich & Zimmerman, 1984; Rice & Whitsett, 1984; Dabrowski & Szczepanowska, 1985). Also the similar effects of compound 48/80 on mast cells in the mesentery, periosteum and dura attest to the specificity of that action on CTMC. Our data indicated that the contents of dural mast cell granules dispersed more readily than those of periosteal mast cells. This was contradictory to the observation of Selye and co-workers (1963) and may be explained by procedural differences between the two studies.

The fragility of mast cells makes the provision of well-preserved controls for the proper interpretation of experimental studies of mast cells a problem (Smith, 1963). As documented in this study, the loss of granules from mast cells is frequently observed in unfixed tissue probably because mast cell membranes are easily disrupted by any manipulation (Fawcett, 1955). As was evident in our study, after perfusion fixation most mast cells were well-preserved and added handling did not affect mast cell integrity, i.e. counterstaining the tissue had no significant effect on the degree of dispersion noted. This substantiated that the use of perfusion fixation has helped to solve this problem. However, as also observed in this study, sometimes even in perfusion fixed tissue, granules are observed outside the cell. For example, one side of our fixed dura had more apparent 'degranulation' than the other side. This may be explained by uneven perfusion, i.e. a more complete fixation on one side of the dura than on the other.

DISTRIBUTION AND ORDERLY ARRANGEMENT OF DURA AND ITS MAST CELLS

Our observation that dura has two layers supports the controversial, but generally accepted organization for that tissue (O'Rahilly & Müller, 1986). The noted orientation of these two layers at an angle to one another, is a refinement of an earlier description of the dura as being composed of layers of collagenous fibrils running in various directions (Pease & Schultz, 1958). The angular orientation of these two layers would strengthen the dura and offers evidence for more than one origin for the dura. The development of cranial meninges from multiple sources has been hypothesized (O'Rahilly & Müller, 1986).

The observed lamellar organization of each layer is similar to that reported for the leptomeninges (O'Rahilly & Müller, 1986) and indicates that sequential lamination occurs in the development of the dura. That the majority of mast cells are at the same depth, i.e. oriented horizontally within each layer, suggests that during development mast cells and/or their precursors are seeded primarily in a single 'mast cell lamella' of each layer. This hypothesis does not conflict with what is known about the origin of mast cells, i.e. their migration from the bone marrow (Kitamura *et al.*, 1978) and differentiation from seeded immature mast cells (Galli, 1987).

That each 'mast cell lamella' is near the interface between the dural layers, is another example of the observation that the greatest number of mast cells are at host-environment interfaces (Sagher & Evan-Paz, 1967). This arrangement could indicate that the origins and sequential lamination of the two layers of the dura are mirror images.

The other orientation of dural mast cells was in a linear pattern relative to both major vessels and nerves in this tissue. This was similar to Selye's report (1963) that mast cells are lined up on either side of the MMA, rather than around that vessel as a sleeve. Although the perivascularity of mast cells has been ascribed by some investigators to the emigration or diapedesis of these cells through the endothelium (Michels, 1938), this hypothesis can be discounted because of the regular arrangement of these cells (Michels, 1938) and

as noted in this study, mast cells also are lined up along nerves that are not associated with blood vessels. Because the regimented orientation of mast cells appears to be related to a laminar pattern of connective tissue development and the neurovascularization of the dura, does this geometry provide a clue to the function of these cells?

WHAT IS THE HYPOTHETICAL FUNCTION OF DURAL MAST CELLS?

The exact nature of the role for the dural mast cell, not only in controlling blood flow in this tissue but also in pathology related events, i.e. the generation of headache pain, resolution of inflammation and in healing, is controversial. Our observation that some mast cells were adjacent to blood vessels, in myelinated nerves, contiguous to unmyelinated nerves, and near nerve endings is consistent with the observations of other investigators for other tissues. These ultrastructural observations should be quantified before any credence can be given to these associations. However, it appears on a qualitative basis that the nature of these relationships do not support the hypothesis that there is a direct functional interaction between nerves or vessels and mast cells. Because substances can diffuse within the extracellular space, the lack of immediate proximity of these components may not be a factor. However, the presence of basal lamina (e.g. around thin-walled vessels and nerve endings) and other connective tissue interstitum (e.g. around the larger vessels and nerves) as well as the lack of any junctional or other membrane specializations between mast cells and vessels or nerves do not support a structural/functional relationship among those elements. Our observations are compatible with the hypothesis that 'degranulation' of dural mast cells is not necessary for a neuroinflammatory response in that tissue (Markowitz *et al.*, 1989). Our conclusion is similar to a study that demonstrated a lack of association between mast cells and either vessels or nerves participating in neurogenic inflammation in the skin (Baraniuk *et al.*, 1990).

In addition, the qualitative ultrastructural observations of this study have been supported by quantitation using high resolution light microscopy. Although mast cells were distributed along the same path as blood vessels and nerves, most of the cells in this study were closer to connective tissue components. And, in support of our previous study (Keller *et al.*, 1989), almost one-quarter of these cells were observed to be lying free within the connective tissue compartment. These data and the alignment of these cells at the interface between the dural layers suggest the more direct participation of this cell type in some connective tissue related event, e.g. a type of cytokine-mediated response and wound repair. Some corroborating evidence is that IL-3 has been demonstrated to induce CTMC chemotaxis (Matsuura & Zetter, 1989) and alter mast cell phenotype (Stevens & Austen, 1989). These responses may account for the increased number of mast cells in certain immune reactions (Matsuura & Zetter, 1989). Also, IL-1 stimulates the release of at least histamine from mast cells (Johnson *et al.*, 1989). One *in vitro* study suggested that the interaction between mast cells and fibroblasts enhanced the elaboration of collagen, and thereby defined more clearly the role of the mast cell in wound healing and development (Rao *et al.*, 1983). Our morphological observations support the involvement of dural mast cells in the connective tissue related functions, e.g. in development, in the immunological response to injury and in the process of wound healing. Therefore, the potential structural/functional inter-relationships of this cell with other connective tissue components will be examined in future studies.

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References

ANDRES, K. H. (1967) Über die Feinstruktur der Arachnoidea und Dura mater von Mammalia. *Zeitschrift für Zellforschung* **79**, 272–95.

ANDRES, K. H., VON DÜRING, M., MUSZYNSKI, K. & SCHMIDT, R. F. (1987) Nerve fibres and their terminals of the dura mater encephali of the rat. *Anatomy and Embryology* **175**, 289–301.

APPENZELLER, O., BECKER, W. J. & RAGAZ, A. (1981) Cluster headache: ultrastructural aspects and pathogenetic mechanisms. *Archives of Neurology* **38**, 302–6.

ARNOLD, W. & HUTH, F. (1978) Electron microscopic findings in four cases of nasopharyngeal fibroma. *Virchows Archiv A* **378**, 285–98.

BARANIUK, J. N., KOWALSKI, M. L., & KALINER, M. A. (1990) Relationships between permeable vessels, nerves, and mast cells in rat cutaneous neurogenic inflammation. *Journal of Applied Physiology* **68**, 2305–11.

BENDITT, E. P., WONG, R. L., ARASE, M. & ROEPER, E. (1955) 5-Hydroxytryptamine in mast cells. *Proceedings of the Society for Experimental Biology and Medicine* **90**, 303–4.

CHRISTOFFERSEN, P. & POULSEN, H. (1979) Alcoholic liver disease. In *Pathology of the Liver* (edited by MACSWEEN, R. N. M., ANTHONY, P. P. & SCHEUER, P. J.) pp. 232. Edinburgh: Churchill Livingstone.

CSABA, G. & KOVÁCS, P. (1975) Demonstration of the heterogeneity of the mast cell population on the basis of the mucopolysaccharide content. *Acta Morphologica Academy of Sciences of Hungary* **23**, 227–33.

DABROWSKI, R. & SZCZEPANOWSKA, A. (1985) Histamine and collagen levels in the lathyritic chick embryos after administration of 48/80. *Agents and Actions* **16**, 3–4.

DE LA TORRE, J. C. & SURGEON, J. W. (1976) A methodological approach to rapid and sensitive monoamine histofluorescence using a modified glyoxylic acid technique: the SPG method. *Histochemistry* **49**, 81–93.

D'ERMO, F. & CRICCHI, M. (1963) Sull'azione di farmaci istaminoliberatori sulle mastzellen di coroide di cavia. *Giornale Italiano di Oftalmologia* **16**, 119–31.

DIMITRIADOU, V., AUBINEAU, P., TAXI, J. & SEYLAZ, J. (1987) Ultrastructural evidence for a functional unit between nerve fibers and type II cerebral mast cells in the cerebral vascular wall. *Neuroscience* **22**, 621–30.

DIMLICH, R. V. W. (1984) Electron microscopic examination of mast cells and nerve processes in hepatic portal areas of the rat. In *Proceedings of the 42nd Annual Meeting of the Electron Microscopy Society of America* (edited by BAILEY, G. W.) pp. 230–1. San Francisco: San Francisco Press.

DIMLICH, R. V. W. (1990) 'Neurolipomastocyte' of the rat brain: a misnomer. *Society for Neuroscience Abstracts* **16**, 246.

DIMLICH, R. V. W., MEINEKE, H. A., REILLY, F. D. & MCCUSKEY, R. S. (1980a) The fluorescent staining of heparin in mast cells using Berberine Sulphate: compatability with paraformaldehyde or o-phthalaldehyde induced fluorescence and metachromasia. *Stain Technology* **55**, 217–23.

DIMLICH, R. V. W., REILLY, F. D., MEINEKE, H. A. & MCCUSKEY, R. S. (1980b) Characterization of intensely fluorescent cells in the liver of the rat. I. Histochemistry and 48/80-induced degranulation. *Anatomical Record* **198**, 475–84.

DOUGLAS, W. W. (1974) Involvement of calcium in exocytosis and the exocytosis-vesiculation sequence. *Biochemical Society Symposium* **39**, 1–28.

EDVINSSON, L., CERVÓS-NAVARRO, J., LARSSON, L.-I., OWMAN CH. & RÖNNBERG, A-L. (1977) Regional distribution of mast cells containing histamine, dopamine, or 5-hydroxytryptamine in the mammalian brain. *Neurology* **27**, 878–83.

ENERBÄCK, L. (1966) Mast cells in rat gastrointestinal mucosa. 1. Effects of fixation. *Acta Pathologica et Microbiologica Scandinavia* **66**, 289–302.

ENERBÄCK, L. (1974a) Berberine sulphate binding to mast cell polyanions: a cytofluorometric method for the quantitation of heparin. *Histochemistry* **42**, 301–13.

ENERBÄCK, L. (1974b) Ultrastructure of mucosal mast cells in normal and compound 48/80-treated rats. *Cell and Tissue Research* **150**, 95–105.

FARACI, F. M., KADEL, K. A. & HEISTAD, D. D. (1989) Vascular responses of dura mater. *American Journal of Physiology* **257**, H157–H161.

FAWCETT, D. W. (1955) An experimental study of mast cell degranulation and regeneration. *Anatomical Record* **121**, 29–51.

GABBIANI, G., BADONNEL, M. C. & MAJNO, G. (1970) Intra-arterial injections of histamine, serotonin, or bradykinin: a topographic study of vascular leakage. *Proceedings of the Society for Experimental Biology and Medicine* **135**, 447–52.

GALLI, S. J. (1987) New approaches for the analysis of mast cell maturation, heterogeneity, and function. *Federation Proceedings* **46**, 1906–14.

GALLI, S. J. (1990) New insights into 'the riddle of the mast cells': microenvironmental regulation of mast cell development and phenotypic heterogeneity. *Laboratory Investigation* **62**, 5–33.

GAMBLE, H. J. & GOLDBY, S. (1961) Mast cells in peripheral nerve trunks. *Nature* **189**, 766–7.

GIETZEN, K. (1983) Comparison of calmodulin anatagonists compound 48/80 and calmidazolium. *Biochemical Journal* **216**, 611–16.

GIETZEN, K. ADAMCZYK-ENGELMANN, P, WUTHRICH, A., KONSTANTINOVA, A. & BADER, H. (1983) Compound 48/80 is a selective and powerful inhibitor of calmodulin-regulated functions. *Biochimica et Biophysica Acta* **736**, 109–18.

GIORDANO-LANZA, G., FUSAROLI, P., BRATINA, F. & CIRINO, L. V. (1972) Sulla innervazione della pachimeninge encefalica. *Quaderni di Anatomia Pratica S.* **28**, 1–4.

GOLDBERG, B. & RABINOVITCH, M. (1977) Connective tissue. In *Histology* (edited by WEISS, L. & GREEP, R. O.) pp. 145–249. New York: McGraw-Hill.

HALS, E. (1970) Some methods for fluorochromatin and staining of rat mast cells with basic dyes. *Scandinavian Journal of Dental Research* **78**, 301–10.

HAMILTON, W. J. & MOSSMAN, H. W. (1972) *Human Embryology*, p. 165. Baltimore: Williams and Wilkins.

HEINRICH, J. & ZIMMERMANN, R. E. (1984) Compound 48/80 is a potent inhibitor of human platelet aggregation antagonizing the calmodulin-dependent platelet reaction *in vitro*. *Thrombosis Research* **36**, 475–80.

HOLMGREN, H. & WILANDER, O. (1937) Beitrag zur Kenntnis der Chemie und Funktion der Ehrlichschen Mastzellen. *Zeitschrift für mikroskopisch-anatomische Forschung* **42**, 242–77.

HSU, S.-M., RAINE, L. & FANGER, H. (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *Journal of Histochemistry and Cytochemistry* **29**, 577–80.

HUMANSON, G. L. (1967) *Animal Tissue Techniques*, p. 18. San Francisco: W. H. Freeman.

IBRAHIM, M. Z. M., AL-WIRR, M. E. & BAHUTH, N. (1979) The mast cells of the mammalian central nervous system. III. Ultrastructural characteristics in the adult rat brain. *Acta Anatomica* **104**, 134–54.

JOHNSON, M. D., KAMSO-PRATT, J., FEDERSPIEL, C. F. & WHETSELL, W. O. (1989) Mast cell and lymphoreticular infiltrates in neurofibromas: comparison with nerve sheath tumors. *Archives of Pathology and Laboratory Medicine* **113**, 1263–70.

JUHLIN, L. SHELLEY, W. B. (1966) Detection of histamine by a new fluorescent o-phthaladehyde stain. *Journal of Histochemistry and Cytochemistry* **14**, 525–8.

KELLER, J. T., MARFURT, C. F., DIMLICH, R. V. W. & TIERNEY, B. E. (1989) Sympathetic innervation of the supratentorial dura mater of the rat. *Journal of Comparative Neurology* **290**, 310–21.

KIERNAN, J. A. (1976) A comparative survey of the mast cells of the mammalian brain. *Journal of Anatomy* **121**, 303–11.

KIMURA, S. (1979) Mast cells in solitary glomus tumors: a possible algogenic role. *Acta Dermatovener* **59**, 415–19.

KITAMURA, Y., GO, S. & HATANAKA, S. (1978) Decrease of mast cells in W/W mice and their increase by bone marrow transplantation. *Blood* **52**, 447–52.

LAGUNOFF, D. (1972) Contributions of electron microscopy to the study of mast cells. *Journal of Investigative Dermatology* **58**, 296–311.

LAGUNOFF, D. & CHI, E. Y. (1978) Mast cell secretion: membrane events. *Journal of Investigative Dermatology* **71**, 81–4.

LIBERSKI, P. P. & PRUSIŃSKI, A. (1982) Further observations on the mast cells over the painful region in cluster headache patients. *Headache* **22**, 115–17.

MARKOWITZ, S., SAITO, K., BUZZI, M. G. & MOSKOWITZ, M. A. (1989) The development of neurogenic plasma extravasation in the rat dura mater does not depend upon the degranulation of mast cells. *Brain Research* **477**, 157–65.

MARKOWITZ, S., SAITO, K. & MOSKOWITZ, M. A. (1987) Neurogenically mediated leakage of plasma protein occurs from blood vessels in dura mater but not brain. *Journal of Neuroscience* **7**, 4129–36.

MAROM, Z. & CASALE, T. B. (1983) Mast cells and their mediators. *Annals of Allergy* **50**, 367–70.

MATSUURA, N. & ZETTER, B. R. (1989) Stimulation of mast cell chemotaxis by interleukin 3. *Journal of Experimental Medicine* **170**, 1421–6.

MICHELS, N. A. (1938) The mast cells. *Annals of the New York Academy of Sciences* **103**, 232–372.

MOSKOWITZ, M. A. (1984) The neurobiology of vascular head pain. *Annals of Neurology* **16**, 157–68.

MOSKOWITZ, M. A., MARKOWITZ, S. & SAITO, K. (1987) Ergot alkaloids specifically block neurogenically-induced plasma extravasation in rat dura: mechanism of action in vascular headaches. *Federation Proceedings* **46**, 634.

MOSKOWITZ, M. A., REINHARD, J. F., ROMERO, J., MELAMED, E. & PETTIBONE, D. J. (1979) Neurotransmitters and the fifth cranial nerve: is there a relation to the headache phase of migraine? *Lancet* **ii**, 883–5.

MOTA, I., BERALDO, W. T. & JUNQUEIRA, L. C. U. (1953) Protamine-like property of compounds 48/80 and stilbamidine and their action on mast cells. *Proceedings of the Society of Experimental Biology and Medicine* **83**, 455–7.

MOTAVKIN, P. A., CHERTOK, V. M. & SHUL'GA, S. D. (1979) Effect of acetylcholine on mast cells of the dura mater. *Bulletin of Experimental Biology and Medicine* **22**, 518–21.

NEWSON, B., DAHLSTRÖM, A., ENERBÄCK, L. & AHLMAN, H. (1983) Suggestive evidence for a direct innervation of mucosal mast cells. An electron microscopic study. *Neuroscience* **10**, 565–70.

NORRBY, K. (1981) On the 48/80-induced secretion of tissue mast cells and its mitogenic effect on nearby cells in the intact rat. *Virchows Archiv B* **38**, 57–65.

OLSSON, Y. (1968) Mast cells in the nervous system. *International Review of Cytology* **24**, 27–70.

O'RAHILLY, R. & MÜLLER, F. (1986) The meninges in human development. *Journal of Neuropathology and Experimental Neurology* **45**, 588–608.

ORR, E. L. (1984) Dural mast cells: source of contaminating histamine in analyses of mouse brain histamine levels. *Journal of of Neurochemistry* **43**, 1497–9.

PAFF, G. H. & MERGENTHALER, D. D. (1955). Vacuolation in normal mast cells treated with protamine sulphate. *Anatomical Record* **121**, 579–92.

PEARCE, F. L. (1983) Mast cell heterogeneity. *Trends in Pharmacological Sciences* **4**, 165–7.

PEARSE, A. G. E. (1968) *Histochemistry*, Vol. 1, pp. 667–8. Boston: Little Brown.

PEASE, D. C. & SCHULTZ, R. L. (1958) Electron microscopy of rat cranial meninges. *American Journal of Anatomy* **102**, 301–21.

RAO, P. V. S., FRIEDMAN, M. M., ATKINS, R. M. & METCALFE, D. D. (1983) Phagocytosis of mast cell granules by cultured fibroblasts. *Journal of Immunology* **130**, 341–9.

REILLY, F. D., DIMLICH, R. V. W., CILENTO, E. V. & McCUSKEY, R. S. (1983) Hepatic microvascular regulatory mechanisms. III. Aminergic mechanisms as related to mast cells. *International Journal of Microcirculation: Clinical Experiments* **2**, 61–73.

RICE, W. R. & WHITSETT, J. A. (1984) Inhibition of surfactant release from isolated Type II cells by compound 48/80. *Biochimica et Biophysica Acta* **805**, 261–7.

RILEY, J. F. (1953) The effects of histamine-liberators on the mast cells of the rat. *Journal of Pathology and Bacteriology* **65**, 471–9.

RILEY, J. F. (1959) *The Mast Cells*. Edinburgh and London: E. & S. Livingstone.

RILEY, J. F. & WEST, G. B. (1952) Histamine in tissue mast cells. *Journal of Physiology* **117**, 72P–73P.

RILEY, J. F. & WEST, G. B. (1953) The presence of histamine in tissue mast cells. *Journal of Physiology* **120**, 528–37.

RÖHLICH, P., ANDERSON, P. & UVNÄS, B. (1971) Electron microscopic observations on Compound 48/80-induced degranulation in rat mast cells. *Journal of Cell Biology* **51**, 465–83.

ROSENBLUM, W. I. (1973) A possible role for mast cells in controlling the diameter of arterioles on the surface of the brain. *Brain Research* **49**, 75–82.

SAGHER, F. & EVEN-PAZ, Z. (1967) *Mastocytosis and the Mast Cell*, pp. 10–324. Chicago: Yearbook Medical Publisher.

SELYE, H., GABBIANI, G. & NIELSEN, K. (1963) The 'periosteal spread' technic for study of mast cells. *Proceedings of the Society for Experimental Biology and Medicine* **112**, 460–3.

SELYE, H. (1965) *The Mast Cells*. Washington: Butterworths.

SKOFITSCH, G., SAVITT, J. M. & JACOBOWITZ, D. M. (1985) Suggestive evidence for a functional unit between mast cells and substance P fibers in the rat diaphragm and mesentery. *Histochemistry* **82**, 5–8.

SMITH, D. E. (1963) Electron microscopy of normal mast cells under various experimental conditions. *Annals of the New York Academy of Sciences* **103**, 40–52.

SPICER, S. S. (1962) Basic protein visualized histochemically in mucinous secretions. *Experimental Cell Research* **28**, 480–8.

STEVENS, R. L. & AUSTEN, K. F. (1989) Recent advances in the cellular and molecular biology of mast cells. *Immunology Today* **10**, 381–6.

THARP, M. D. (1989) The interaction between mast cells and

endothelial cells. *Journal of Investigative Dermatology* **93**, 107S–112S.

THEOHARIDES, T. C. (1983) Mast cells and migraines. *Perspectives in Biology and Medicine* **26**, 672–5.

THEOHARIDES, T. C. (1990) Mast cells: the immune gate to the brain. *Life Sciences* **46**, 607–17.

WAGGENER, J. D. & BEGGS, J. (1967) The membranous coverings of neural tissues: an electron microscopy study. *Journal of Neuropathology and Experimental Neurology* **26**, 412–26.

WIENSER-MENZEL, L., SCHULZ, B., VAKILZADEH, F. & CZARNETZKI, B. M. (1981) Electron microscopical evidence for a direct contact between nerve fibres and mast cells. *Acta Dermatovener* **61**, 465–9.