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Distribution, density and heterogeneity of canine mast cells and influence of fixation techniques

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Abstract The present study was carried out to determine the physiological distribution of mast cell numbers and types in the dog according to tissue location, staining and fixation methods. Tissue samples from stomach, duodenum, lung, lymph node, skin and uterus were evaluated. Samples were fixed in formalin as well as in Carnoy's fluid. The average number of mast cells was determined using a metachromatic staining method. Protease content of mast cells was examined with a double enzyme-immunohistochemical staining technique, using a histochemical reaction for chloroacetate esterase to detect chymase activity and an immunohistochemical staining method for the detection of tryptase. Canine mast cells can be subdivided into formalin-sensitive and -resistant mast cells. Three subtypes were identified according to their content of the mast cell-specific proteases tryptase (T) and chymase (C): T-, TC- and C-mast cells. Significant differences regarding the distribution of mast cell subtypes as well as the influence of the fixation method can be observed. This underlines the fact that data regarding mast cell heterogeneity from other species, obtained by different fixation methods, are not comparable. This fact has to be taken into consideration when evaluating mast cell subtypes under pathological conditions.

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Introduction

Mast cells are constituents of connective tissue in all mammals and are well known for their role in the immunopathology of immediate-type hypersensitivity reactions initiated by preformed mediators stored in their secretory granules (Benyon RC 1989; Huntley JF 1992). While the immunopathological role of mast cells has been acknowledged, contradictory and confusing opinions on mast cell functions exist. Recently, it has become clear that one explanation for the controversy arises from mast cell heterogeneity. This heterogeneity can express itself as differences in histochemical, biochemical and functional characteristics (Welle MM 1997). Thus, mast cell populations have been distinguished according to these aspects. In rodents connective tissue type mast cells (CTMC) and mucosal type mast cells (MMC) can be distinguished (Enerbäck L 1966a,b). While CTMC can be demonstrated after formalin fixation, MMC are only detectable after fixation with Mota's basic lead acetate (Enerbäck L 1966a). Although histochemical criteria have been applied for subtyping canine (Becker AB et al. 1985) and human (Strobel S et al. 1981) mast cells, an unequivocal distinction is not possible. Since the investigations of Irani et al. (Irani AA et al. 1986), the main criterion for human mast cell subtyping is the content of the mast cell-specific proteases, chymase and tryptase. Three mast cell subtypes in humans are distinguished: mast cells which contain only tryptase (MC_T); mast cells which contain tryptase, chymase, carboxypeptidase and cathepsin G (MC_{TC}); and mast cells which contain chymase and carboxypeptidase (MC_C) (Irani AA et al. 1991; Schechter NM et al. 1990; Weidner N and Austen KF 1993).

In contrast to humans and rodents, only few data regarding mast cell heterogeneity in dogs exist. Canine mast cell heterogeneity, based on formalin sensitivity, was suggested to exist in skin (Becker AB et al. 1985), trachea, lung, intestine and mastocytomas (Colbatzky F et al. 1991). Other authors found this classification system, which is useful in rodents, not to be appropriate

for subtyping mast cells in dogs (Osborne ML et al. 1989; Sommerhoff CP et al. 1990). After histochemical and biochemical evidence that canine mast cells contain trypsin- and chymotrypsin-like proteinases (Glenner GG and Cohen LA 1960; Powers JC et al. 1985), the expression of different mast cell-specific serine proteases was used as a further criterion for mast cell heterogeneity in dogs. Schechter et al. (Schechter NM et al. 1988) demonstrated that canine skin mast cells contain tryptase and chymase, which have immunological and biochemical properties similar to human tryptase and chymase.

So far, no systematic study has been undertaken to investigate the tissue distribution of formalin-sensitive mast cells and its relationship to protease content. The involvement of specific mast cell subtypes and mast cell proteases in the pathogenesis of disease (Harvima IT et al. 1993; Rubinstein I et al. 1990; Sekizawa K et al. 1989; Su M et al. 1993) underlines the importance of a more detailed study on canine mast cell heterogeneity. Our intention was to gather species-specific data on mast cell density in different organs and tissue locations, on heterogeneity based on their protease content and on the relative distribution of mast cell subtypes under different fixation conditions. The data reported reflect the physiological distribution pattern of mast cells in the canine organs and provide reference values for further evaluation of the involvement of mast cell subtypes in pathological conditions.

Materials and methods

Specimens were obtained from stomach, duodenum, uterus, lung, skin of the forehead and mandibulary lymph node of seven female and six male dogs. Dogs were adult and of different breeds. By light microscopy, all samples were free of any lesions. Tissue samples were fixed in 4% formalin overnight and neighbouring samples were fixed in Carnoy's fluid for 4 h. After fixation, the tissues were dehydrated in an automatic processor, embedded in paraffin and serial sections of 4 μm were cut.

Serial sections of each tissue sample were stained with haematoxylin and eosin, methylene blue to demonstrate the sulphated acid glycosaminoglycans in mast cell granules and a double labelling technique for the demonstration of mast cell proteases. An enzyme-histochemical reaction was used for the detection of chymase activity and an immunohistochemical staining method with a polyclonal antibody for the detection of tryptase. The double labelling procedure was performed as follows. After deparaffinising the sections in xylol and acetone for 15 min each, the slides were immersed in TRIS-buffered saline (TBS) pH 7.4 and subsequently in deionised water. The enzyme-histochemical reaction for the detection of chymase was performed with a commercially available detection kit (Sigma) using naphthol-AS-D-chloroacetate as substrate. The only modification to the protocol provided by the company was the use of fast blue BB (base) instead of fast red violet LB (base). No counterstain was performed. The immunohistochemical staining for mast cell tryptase was performed at room temperature immediately after the enzyme-histochemical staining and a predigestion with 0.1% protease XIV (Sigma) for 4 min at 37°C. Tryptase was detected with a polyclonal rabbit anti-human skin tryptase antibody (Harvima IT et al. 1988) at a dilution of 1:2000. After an incubation period of 30 min, a mouse anti-rabbit IgG (1:200, Dianova) was applied for 30 min. This step was followed by incubation with APAAP complex (1:100, Dianova) for 30 min. The incubation with the mouse anti-rabbit IgG and the APAAP complex was performed twice. Between each step the sections were washed thoroughly in TBS (pH 7.4). The antibody and the APAAP complex were diluted in RPMI buffer (pH 7.4). The alkaline phosphatase substrate was prepared by dissolving 20 mg naphthol-AS-MX-phosphate (Dianova) in 2 ml dimethylformamide and then adding 98 ml TBS (pH 8.2). Immediately before this preparation was used, fast red TR (100 mg) and levamisole (1 M, 2.408 mg) were added. Incubation with the substrate was performed for 30 min with continuous agitation. Sections were then rinsed in tap water and mounted in Kaiser's glycerin gelatin. Negative controls for each tissue specimen were performed by omitting the primary antibody. Only cells with all or a part of a nucleus visible were counted. The quantitation of the cells was performed by using a square eyepiece graticule (objective×40, eyepiece×12.5, 10×10 squares with a total side length of 0.25 mm). Each tissue was subdivided in various locations and, if possible, at least 1000–1800 fields (approx. 1 mm^2) in each tissue location were counted. Mast cell counts were performed separately for each staining method and the median, the 25th and the 75th quartile of data obtained from each tissue location was calculated. Mast cell density is expressed as the number of cells per mm^2. We assessed the median percentages of MC_T, MC_{TC} and MC_C for each of both fixation methods.

The non-parametric Wilcoxon sign-rank sum test was carried out between the mast cell numbers obtained by double labelling and by methylene blue staining following formalin fixation or fixation in Carnoy's fluid. In addition, the test was carried out between the mast cell numbers obtained with methylene blue staining following fixation in Carnoy's fluid and formalin fixation. Furthermore, the test was carried out after double labelling and fixation in Carnoy's fluid and formalin fixation. *P* values are considered significant when <0.05.

Results

The median mast cell density varied between the different organs and between different tissue locations within one organ. Furthermore, the density varied according to the staining and fixation technique applied. Using the double labelling technique for the detection of the mast cell proteases, chymase and tryptase, three mast cell subtypes were identified in canine tissues: the red-staining MC_T (Fig. 1); the blue-staining MC_C (Fig. 2); and the MC_{TC} subtype containing both tryptase and chymase, staining red and blue (Figs. 1, 2). In each organ investigated, all three mast cell subtypes were detected, independently of the fixation technique used.

The predominant mast cell type differs within one location, depending on the fixation technique. In general, a higher percentage of MC_{TC} and MC_C can be detected after fixation in Carnoy's fluid.

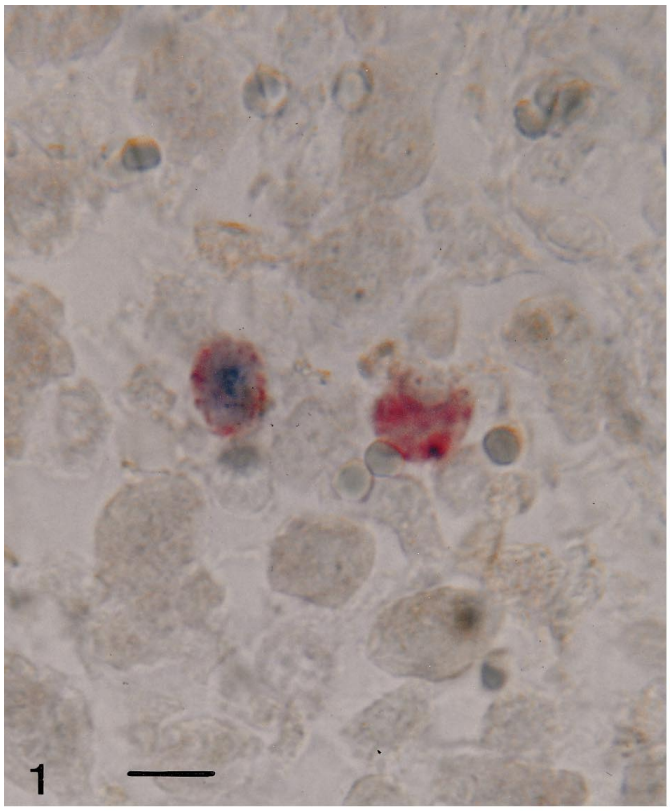
Stomach

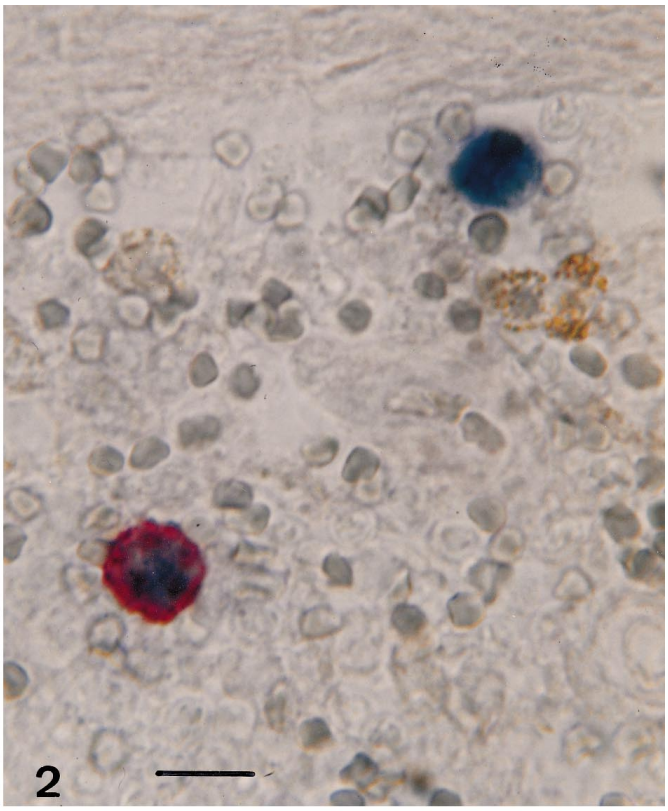
The highest mast cell density was found in the apical lamina propria and in its stratum subglandulare, regardless of the fixation and staining technique. The lowest mast cell density can be detected in the stratum longitudinale of the tunica muscularis (data not shown). Independently of the staining technique, a significantly higher mast cell number was assessed after fixation in Carnoy's fluid. In the lamina propria mucosa a significantly higher number of metachromatically staining

mast cells than the double-labelled mast cells was present, regardless of the fixation method. The median values as well as the error bars of the 25th and 75th percentile obtained by each staining and fixation method and the results of the statistical analysis are shown in Fig. 3a. After formalin fixation, the predominant mast cell subtype is MC_T. However, after fixation in Carnoy's fluid, the number of detectable mast cells containing chymase increased, with MC_{TC} becoming the predominating subtype in the lamina propria mucosae. The percentile distributions of the mast cell subtypes are shown in Fig. 3b.

Duodenum

Independently of the staining and fixation technique, the highest mast cell density is found in the stratum subglandulare, whereas the lowest mast cell density is observed in the stratum longitudinale of the tunica muscularis (data not shown). As in the stomach, in most tissue locations, a higher mast cell number was detected after fixation in Carnoy's fluid. The median values as well as the error bars of the 25th and 75th percentile obtained by each staining and fixation method and the results of the statistical analysis are shown in Fig. 4a.





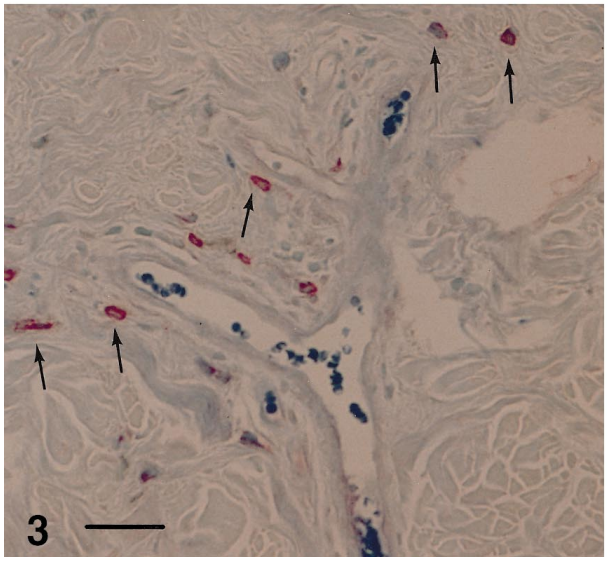
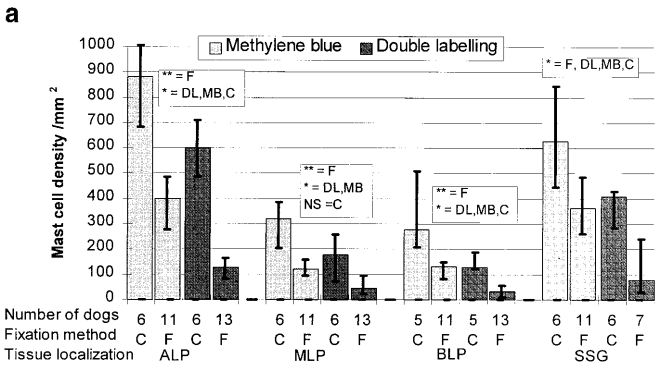


Fig. 1 Canine mast cell subtypes in the lymph node. Note the red- and blue-stained mast cell containing tryptase, chymase, carboxypeptidase and cathepsin (MC_{TC}) and the only red-stained mast cell containing only tryptase (MC_T). Double labelling for tryptase and chymase. *Bar* 15 μm

Fig. 2 Canine mast cell subtypes in the lymph node. Note the red- and blue-stained MC_{TC} cell and the only blue-stained mast cell containing chymase and carboxypeptidase (MC_C). Double labelling for tryptase and chymase. *Bar* 15 μm

Fig. 3 Perivascular location in the skin. Note the red or red and blue staining double-labeled mast cells (*arrows*). APAAP. *Bar* 70 μm



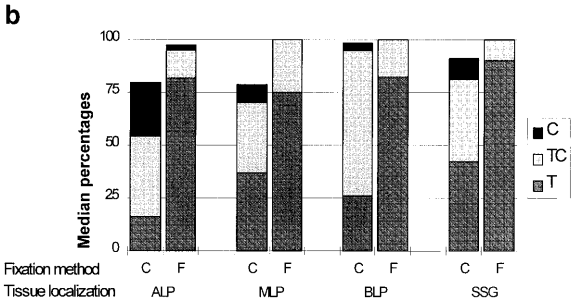


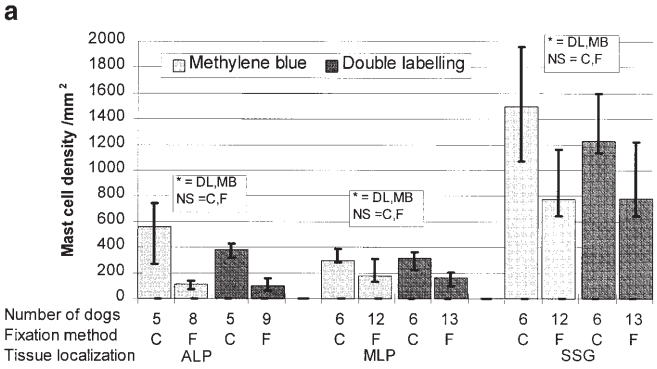
Fig. 4a, b Column heights represent the median mast cell density in the stomach, while error bars represent the 25th and 75th percentiles. The sign-rank sum test was carried out between the mast cell numbers obtained by double labelling and methylene blue staining after formalin fixation (*F*) and fixation using Carnoy's fluid (*C*). In addition, the test was carried out between the mast cell density obtained by methylene blue staining and fixation in Carnoy's fluid and formalin (*MB*). Furthermore, the test was carried out for data obtained by double labelling and both fixation methods (*DL*). Test results are presented within the graph: ***P* value <0.01, **P* value <0.05, *NS* not significant. (*ALP* Apical lamina propria, *MLP* medial lamina propria, *BLP* basal lamina propria, *SSG* stratum subglandulare)

As in the stomach, the predominant mast cell subtype in the duodenum was MC_T after fixation in formalin. After fixation in Carnoy's fluid, however, the number of detectable chymase-containing mast cells increased and MC_{TC} became the predominating mast cell subtype in the lamina propria, with the exception of the subglandular layer. The percentile distributions of the mast cell subtypes are shown in Fig. 4b.

Skin

Regardless of the staining and fixation technique, the highest mast cell density was found in the dermis perivascular (Fig. 5), whereas the lowest mast cell density was found in the adipose tissue of the subcutis (data not shown). The influence of the fixation technique is not as striking as in the stomach and duodenum and significantly higher mast cell numbers after fixation in Carnoy's fluid were only demonstrated in the periadnexal and perivascular locations.

The median values as well as the error bars of the 25th and 75th percentile obtained by each staining and



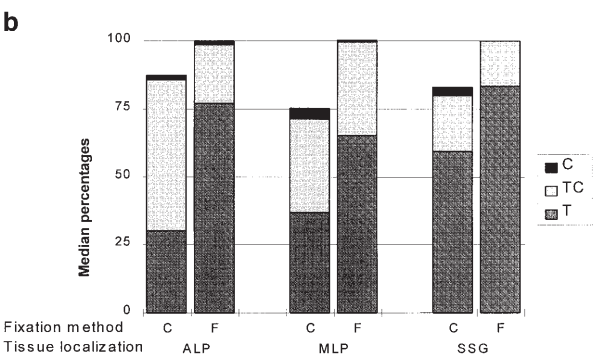


Fig. 5a, b Mast cell density in the duodenum. For details, see legend to Fig. 3

fixation method and the results of the statistical analysis are shown in Fig. 6a. Independently of the fixation technique, a far higher percentage of chymase-containing mast cells than in the stomach and duodenum was detected. After formalin fixation, MC_{TC} was the predominant subtype in the skin, regardless of the tissue location.

In tissues fixed in Carnoy's fluid, the percentage of MC_C increased and became predominant in the subepithelial and periadnexal areas. The percentile distributions of the mast cell subtypes are shown in Fig. 6b.

Lung

Independently of the fixation and staining technique, the highest mast cell density was found in the lamina propria of bronchi, whereas the lowest mast cell density was observed in the lamina muscularis of the bronchi. As in the stomach and duodenum, there was a tendency that a higher mast cell number was seen in sections fixed in Carnoy's fluid. After double labelling, the values were significant in the lamina propria. After formalin fixation, a significantly higher number of mast cells was detected in the lamina propria and the connective tissue by the methylene blue staining technique. The median values as well as the error bars of the 25th and 75th percentile obtained by each staining and fixation method and the results of the statistical analysis are shown in Fig. 7a.

In formalin-fixed tissue, the predominant mast cell subtype is MC_T. As in the stomach and duodenum, a higher

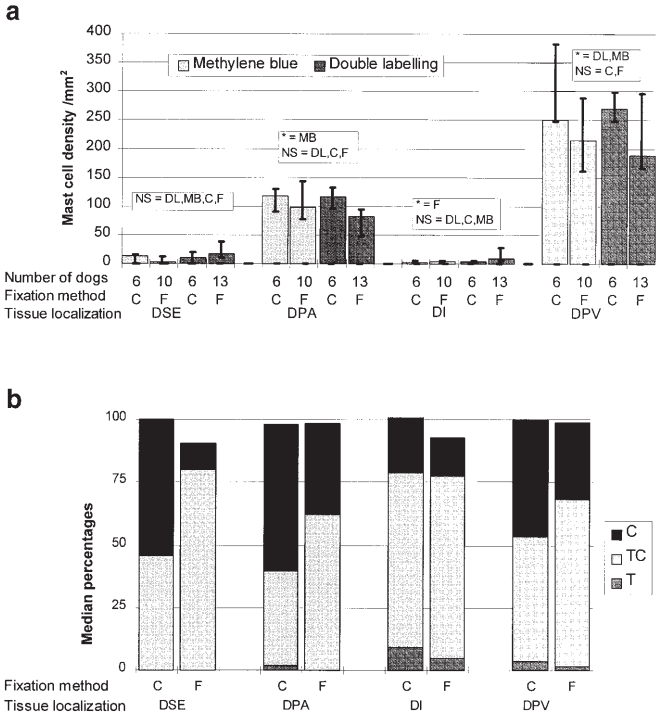


Fig. 6a, b Mast cell density in the skin. For details, see legend to Fig. 3. (*DSE* Subepithelial dermis, *DPA* periadnexal dermis, *DI* interstitial dermis, *DPV* perivascular dermis)

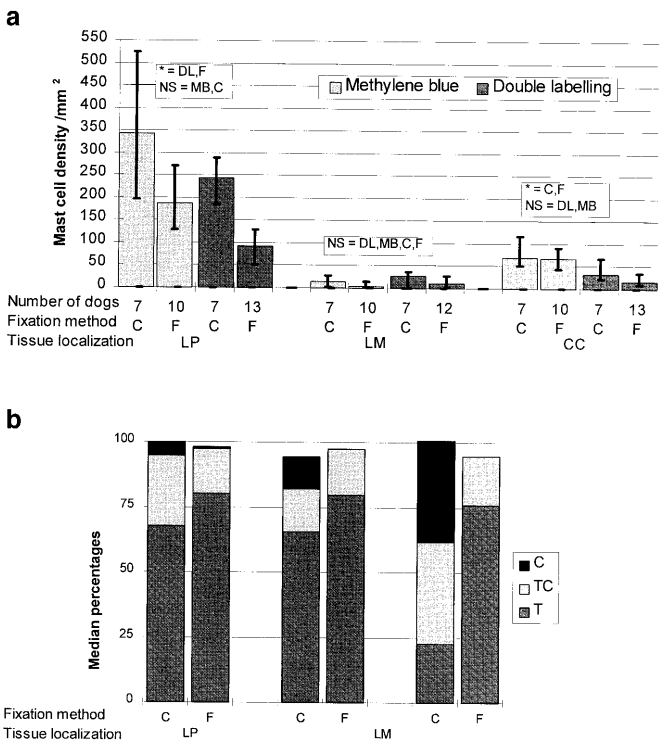


Fig. 7a, b Mast cell density in the lung. For details, see legend to Fig. 3 (*LP* Lamina propria of bronchi, *LM* lamina muscularis, *CC* interalveolar and pericappilar connective tissue)

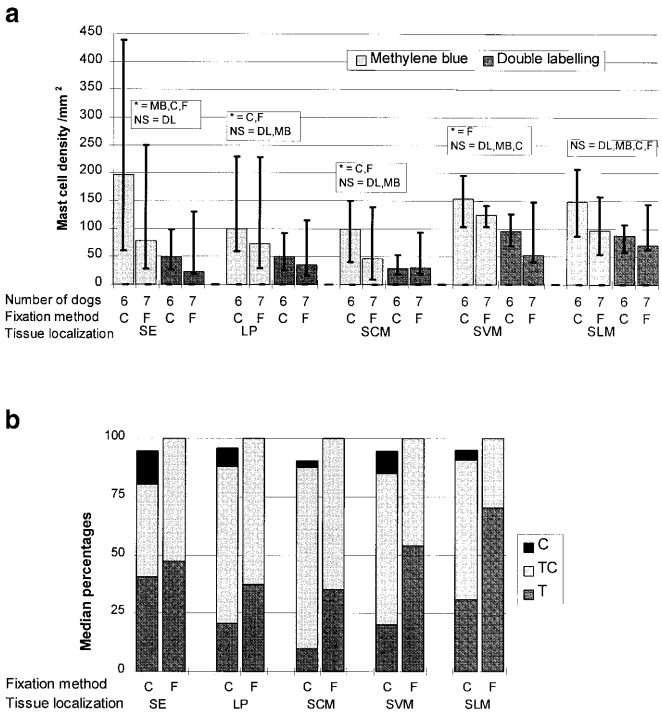


Fig. 8a, b Mast cell density in the uterus. For details, see legend to Fig. 3 (*SE* Subepithelial endometrium, *LP* endometrium, lamina propria, *SCM* stratum circulare of the tunica muscularis, *SVM* stratum vasculosum of the tunica muscularis, *SLM* stratum longitudinale of the tunica muscularis)

percentage of chymase-containing mast cells was detected in tissues fixed in Carnoy's fluid. The percentile distributions of the mast cell subtypes are shown in Fig. 7b.

Uterus

There is no tissue location within the uterus which provided clear data on the highest mast cell density. The differences in mast cell density between tissue locations were not as striking as in the other organs investigated. There was a tendency for a higher mast cell number to be visualised in sections fixed in Carnoy's fluid, however, the data were only significant in the subepithelial endometrium after methylene blue staining. The median values as well as the error bars of the 25th and 75th percentile obtained by each staining and fixation method and the results of the statistical analysis are shown in Fig. 8a.

The predominating mast cell subtype varied with the tissue location and was influenced by the fixation type. MC_C were only detectable after fixation in Carnoy's fluid. The percentile distributions of the mast cell subtypes are shown in Fig. 8b.

Lymph node

The mast cell density in the lymph node was low, independently of the fixation and staining techniques. The highest mast cell density was found in the trabecula and

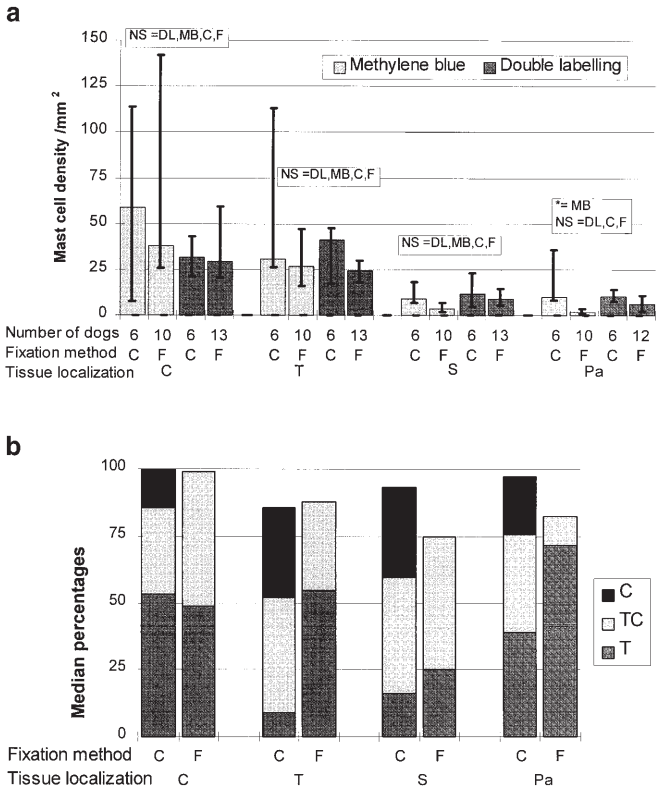


Fig. 9a, b Mast cell density in the lymph node. For details, see legend to Fig. 3. (*C* Capsule, *T* trabecula, *S* sinuses, *Pa* parenchyma)

capsule, whereas the lowest mast cell density was found in the sinuses. There are no striking differences between the staining and fixation techniques with regard to mast cell density. The median values as well as the error bars of the 25th and 75th percentile obtained by each staining and fixation method and the results of the statistical analysis are shown in Fig. 9a.

As seen in the uterus, the predominating mast cell subtype varied within the tissue and was influenced by the fixation type. The percentile distributions of the mast cell subtypes are shown in Fig. 9b.

Discussion

The present study was carried out to determine the physiological distribution of mast cell numbers and to define canine mast cell subtypes with respect to protease content and formalin sensitivity in different organs and tissue locations. Using standardised staining and fixation methods we demonstrated that a subset of canine mast cells is formalin sensitive. Furthermore, canine mast cells were distinguished according to their protease content. Mast cell density varied between different organs and different tissue locations within one organ. Comparing the data obtained in the dog, essential species-specific differences were observed between canine, bovine (Küther K et al. 1998) and human tissues (Irani AA et al. 1989; Irani AA et al. 1986; Weidner N and Austen KF 1993). These findings might explain the different inflammatory reaction patterns observed in various species.

With respect to mast cell heterogeneity, according to their protease content, canine mast cells contain either tryptase or chymase alone or both proteases together in one cell. As in human (Weidner N and Austen KF 1993) and bovine tissues (Küther K et al. 1998), three mast cell subtypes were distinguished in the dog, MC_T, MC_{TC} amd MC_C.

As demonstrated in the human (Irani AA et al. 1989; Irani AA et al. 1986; Weidner N and Austen KF 1993), MC_T is the predominant subtype in stomach, duodenum, lung and lymph node of the dog, whereas MC_{TC} predominates in the skin, regardless of the fixation technique used. However, species-specific differences on the distribution of mast cell subtypes within the various tissues were depicted. In bovine skin, for example, no chymase-containing mast cells were detected at all, regardless of the fixation technique used (Küther K et al. 1998). In human skin, more than 99% of mast cells are of the MC_{TC} subtype (Irani AA et al. 1989), independently of the fixation technique used, whereas in canine skin the predominating mast cell subtype varies according to the fixation technique used.

With regard to formalin sensitivity, a significantly higher number of canine mast cells was detected in most tissue location of the stomach and duodenum and in some sites in other tissues after fixation in Carnoy's fluid, independently of the staining technique used. The superiority of Carnoy's fixation for the detection of mast cells in rodent, human and dog has been described by other authors (Aldenborg F and Enerbäck L 1994; Becker AB et al. 1985; Befus AD et al. 1985; Benyon RC 1989; Colbatzky F et al. 1991; Irani AA et al. 1986; Strobel S et al. 1981). In the dog, however, unlike in rodents, it is not possible to localise formalin-sensitive mast cells exclusively to mucosal tissue and formalin-resistant mast cells to connective tissue. The reasons for the formalin sensitivity of mast cells are not yet clear. Enerbäck (Enerbäck L 1986) suggested that aldehyde moieties form a diffusion barrier, blocking the binding of cationic dyes such as methylene blue to the glycosaminoglycans in the mast cells granules. In studies employing an enzyme-immunohistochemical detection method for mast cells, Aldenborg and Enerbäck (1994) reported that mast cell chymase is highly susceptible to masking by strong aldehyde solutions. In accordance with these suggestions, we found in the majority of canine organs and tissue locations an increase in chymase-containing mast cells after fixation in Carnoy's fluid.

In contrast to our results, the investigations of Irani et al. (Irani AA et al. 1986) showed that fixation in Carnoy's fluid is superior for the demonstration of MC_T and MC_{TC}. One reason for these confusing findings on formalin sensitivity of mast cells subtypes might be differences in the structure and localisation of chymase existing between various species and tissues. Chymases show, unlike tryptase, wide variations in their properties, such as charge, solubility, proteoglycan binding, glycosyla-

tion, catalytic efficiency and regulated expression in mast cell subsets (Caughey GH 1995).

Summarising our results, we observed formalin-resistant as well as formalin-sensitive mast cells in the dog. These two subtypes were located in the same tissue. Thus, it is not possible to discriminate between MMC and CTMC as in rodents. Subtyping canine mast cells with respect to their protease composition provides more detailed information, especially since the involvement of mast cell proteases in certain disease states has been shown (Welle MM 1997). The observed differences regarding the distribution of mast cell subtypes as well as the influence of the fixation method in canine tissues indicate that data on mast cell heterogeneity from other species and obtained with different methods are not comparable. This fact has to be considered when evaluating mast cell subtypes under pathological conditions. Increasing knowledge about the properties of tryptase and chymase, however, may help to elucidate the involvement of mast cell subtypes in certain pathological conditions and thus help to understand the pathogenesis of various disorders.

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