Distribution and density of mast cells in camel small intestine and influence of fixation techniques

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This study was carried out to gather species-specific data on mast-cell density and distribution in camel small intestine under different fixation conditions and to elucidate the presence and cross-reactivity of tryptase in the camel small intestine using human specific anti-tryptase antibody. Tissue specimens from the jejunum, duodenum, and ileum were obtained from 9 healthy, 9-12 months old, male camels. Specimens were fixed either with carnoy's fluid or formalin-buffered solution and stained with either methylene blue or immunohistochemically to identify mast cells. The present study demonstrated for the first time, the presence and cross-reactivity of tryptase in the camel small intestine using a specific mouse anti-human tryptase antibody. Mast cells were detected in all histological layers of the camel small intestine (mucosal, submucosal, muscularis externa and serosa). Among all locations examined in the duodenum, ileum and jejunum, no significant difference was observed in mast-cell counts among the lamina propria, muscularis mucosae, muscularis externa and the serosa. The only significant difference observed was the mast-cell count in submucosa region where the highest and lowest mast count was observed in the jejenual and ileal submucosa, respectively. Significant differences regarding the distribution of mast cell as well as the influence of the fixation method could 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 account when evaluating mast cell subtypes under pathological conditions.

Key words: camel, distribution, density, fixation, mast cells, small Intestine.

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Mast cells are heavily granulated wandering cells found in connective tissues in close contact with the external environment (Galli, 1993; Krishnaswamy *et al.*, 2000; Florenzano and Bentivoglio, 2000; Zhi-jia *et al.*, 2005). Mast cell granules contain several biochemical mediators such as heparin, histamine and neutral proteases such as tryptase and chymase with β-tryptase the most abundant. While its biological function has not been fully clarified, mast cell β-tryptase plays an important role in inflammation and serves as a marker of mast cell activation (Cairns and Walls, 1996). It is involved in vascular relaxation and contraction (Brown *et al.*, 1995), gastrointestinal smooth muscle activity and intestinal transport (Crowe *et al.*, 1997, Vergnolle, 2000), and coagulation (Huang *et al.*, 1997). Furthermore, serum mast cell β-tryptase concentration increases in anaphylaxis and in other allergic conditions (Fisher and Baldo, 1994).

Mast cells can be distinguished according to various characteristics. Human, bovine and canine mast cells are distinguished by their content of the mast-cell-specific proteases, chymase and tryptase (Irani *et al.*, 1986, 1989, 1991; Schechter *et al.*, 1990; Weidner and Austen 1993, Kube *et al.*, 1998; Küther *et al.* 1998). Three mast cell subtypes in humans, bovines and canines can be distinguished: mast cells that contain tryptase (T-MC) only; mast cells that contain both tryptase and chymase (TC-MC); and mast cells that contain only chymase (C-MC). In rats and mice, mucosal and connective tissue mast cells can be distinguished by their different affinities for histological dyes, resulting from the differing proteoglycan content of their storage granules (Enerbäck *et al.*, 1985). A comprehensive search of the literature reveals no data available regarding the normal distribution, density and subtypes of mast cells in camels. The

present study was carried out to gather species-specific data on mast cell density and distribution in camel small intestine under different fixation conditions and to elucidate for the first time the presence and cross-reactivity of tryptase in the camel small intestine using human specific anti-tryptase antibody.

Materials and Methods

Animals and specimens

Fresh camel tissue specimens were obtained from 9 clinically healthy male dromedary camels (9-12 months old) immediately after slaughter. Six full-thickness sections of duodenum, jejunum and ileum were obtained. Specimens were either fixed in 10% phosphate-buffered formaldehyde overnight or in Carnoy's fluid for 4 hours. After fixation, the tissues were dehydrated in an automatic processor and embedded in paraffin. Serial sections were cut at a thickness of 4 μm. One section of each tissue sample was stained with hematoxylin and eosin (H&E) for histopathological evaluation. Light-microscopical analysis of H & E stained sections indicated that all samples used in the study were free of any pathological lesions.

Mast cell detection

Mast cells were identified by two methods of staining: metachromatic staining using methylene blue stain and by immunohistochemistry.

Metachromatic staining

Metachromatic staining was performed as described before by Clarke and Maddocks (1965). Briefly, the slides were heated for 10 min at 60°C to melt the paraffin, which was then removed in xylol (2 changes x 5 min). The sections were hydrated through 2 min changes of 100, 90, 70, and 50% ethanol/water (v/v), and then distilled water, and stained with methylene blue (Sigma-Aldrich, Deisenhofen, Germany) for 5 min. The slides were then rinsed in distilled water. Finally the sections were dehydrated by 1 min changes in 70, 90, and 100% ethanol/water (v/v), followed by 1 min in xylol, and cover-slipped using DPX (Sigma-Aldrich, Deisenhofen, Germany).

Immunohistochemistry

Immunohistochemical staining was performed using methods described by Irani *et al.*, (1986). Briefly, 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. endogenous peroxidase was blocked by incubation with 0.6% H_2O_2 in methanol for 30 min at room temperature, followed by washing in Tris-buffered saline with 0.05% Tween 20, at pH 7.4 (TTBS). The slides were then incubated with mouse antibodies to human skin tryptase (Promega, Madison, WI, USA) (1.5 μg/mL) for 1 hr at room temperature in a humidity chamber. The slides were then washed with TTBS and peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch labs, West Baltimore, MD, USA) (1:50 dilution) was added. The slides were re-incubated for 1 hr at room temperature in the humidity chamber. The slides were washed again using TTBS. Diaminobenzidine (DAB) tetrahydrochloride (Sigma-Aldrich, Deisenhofen, Germany) solution containing 0.01% hydrogen peroxide was used for color development. After a final wash in distilled water, the slides were mounted in a 90% glycerol solution. Positively stained cells developed a reddish-brown color. Human skin sections were used as positive controls. Negative controls for each tissue specimen were produced by omitting the primary antibody.

Mast cell count

All tissue sections were examined using light microscopy (Motic Inc., China) by the principal researcher. Positive cells after each staining were counted at a x1000 magnification (oil-immersion) in 10 fields/section and classified according to their microscopical location within epithelia, mucosa, lamina propria (LP), muscularis muscosas (MM), submucosa (SM), muscularis externa (ME) and serosa (S). All mast cell profiles in the whole section were counted. Four sections from each sample were counted. Results were expressed as cell/mm^2.

The non-parametric Kruskal-Wallis test followed by the Dunn's multiple comparison test to compare groups were carried out between the mast cell numbers obtained by immunostaining and by methylene blue staining following formaldehyde fixation or fixation in Carnoy's fluid. Statistical analyses were performed using Graphpad Prism for windows (Graphpad Software, California, USA). *p* values are considered significant when <0.05.

Results

The MCs stained in the small intestinal tissues typically exhibited a round or oval shape, with diameters ranging from 9 to 15 µm. The nuclei of MCs were frequently lightly stained, with metachromasia evident in the cytoplasm. The most characteristic feature of these cells was the occurrence of granules in the cytoplasm (Figure 1 A and B). Mast cells containing tryptase were also detected in all histological layers of the camel small intestine after immunohistochemical staining (Figure 1 C and D).

Regardless of the staining and fixation technique used, the highest mast cell density was observed in the submucosal layer, with significant numbers of cells found in the perivascular regions, whereas the lowest mast cell density was observed in the muscularis externa. In all tissue sections, a higher mast cell number was detected after fixation in Carnoy's fluid with no cells observed within the epithelia.

Among all locations examined in the duodenum, ileum and jejunum, no significant difference was observed in mast-cell counts in the lamina propria, muscularis mucosae, muscularis externa and the serosa (Figure 2 A, B and C). The only significant difference in the mast-cell counts was observed in the submucosa region where the highest and lowest mast count was observed in the jejenual and ileal submucosa, respectively.

Discussion

The present study was carried out to gather species-specific data on mast cell density and distribution in camel small intestine under different fixation conditions and to elucidate for the first time the presence and cross-reactivity of tryptase in the camel small intestine using human specific anti-tryptase antibody.

A significantly higher number of camel mast cells and tryptase containing mast cells were detected in most tissue locations of the duodenum, jejunum and ileum after fixation in Carnoy's fluid, regardless of the staining technique used. The superiority of Carnoy's fixation for the detection of mast cells in rodents, human and the dog has been described by others (Becker *et al.*, 1985; Befus *et al.*, 1985; Enerbäck, 1966). The staining methods used to demonstrate mast cells by light microscopy depend on the affinity of cationic dye for the mast cell granule glycosylaminoglycans (GAGs). Optimal fixation will result in precipitation of GAGs leaving polyanionic sites available for binding of dye (Strobel *et*

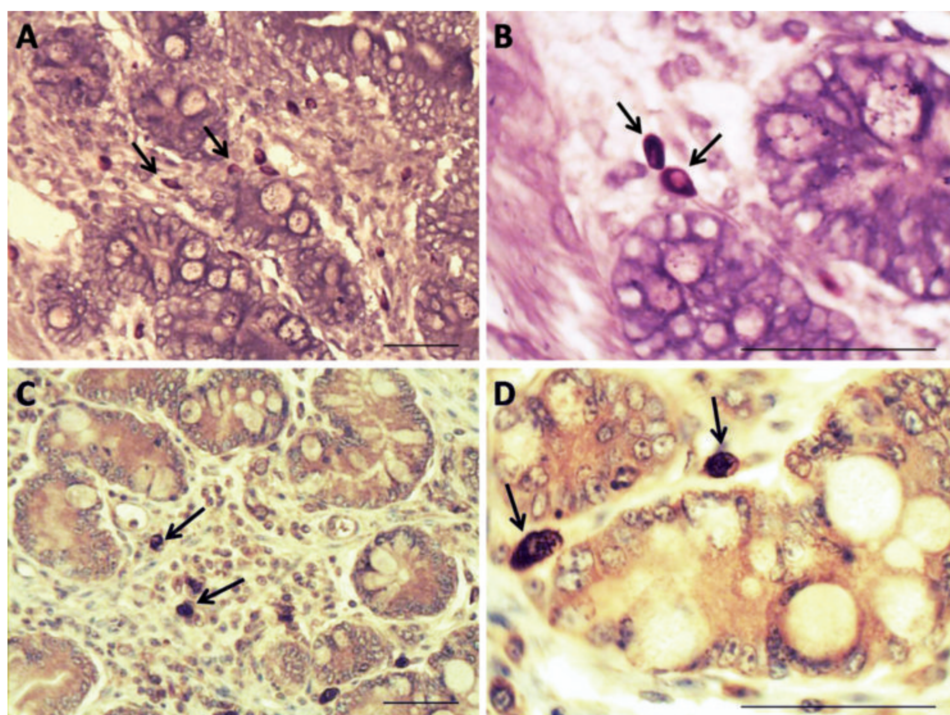

Figure 1. Mast cells distribution in the camel small intestine. Thin sections from duodenum were stained with Methylene blue (A and B) and immunostained with mouse anti-human skin mast cell tryptase (C and D). Magnification, X 400 (A, C), and X1000 (oil-immersion) (B, D), arrows in the pictures indicate mast cells. Bar= 50 µm.

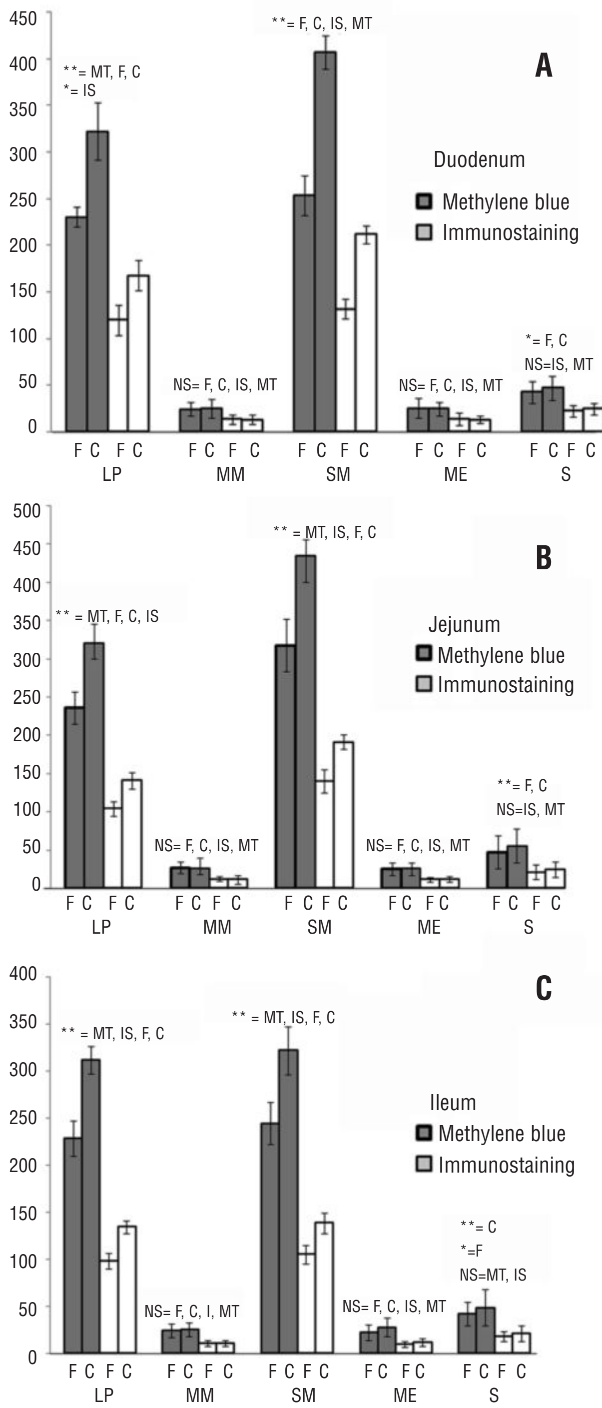

Figure 2. Mast-cell density and distribution in the duodenum, jejunum and ileum as detected by immunostaining (IS) and methylene blue stain (MB) after formalin fixation (F) and fixation using Carnoy's fluid (C). The non-parametric Kruskal-Wallis test followed by the Dunn's multiple comparison test were carried out between the mast cell numbers obtained by IS and by MB stain after formalin fixation (F) or fixation in Carnoy's fluid (C). In addition, the test was carried out between the mast cell numbers obtained with MB staining and fixation in Carnoy's fluid or formalin fixation (MB). Furthermore, the test was carried out after IS and fixation in Carnoy's fluid and formalin fixation (IS). * *p* value <0.05, ***p* value <0.01, NS: not significant, LP: lamina propria, MM: Musclaris Mucosa, SM: Sub mucosa, ME: Musclaris Externa and S: Serosa.

al., 1981). Failure to demonstrate mast cells in fixed tissues maybe due either to dissolution of non-precipitated GAG (Enerbäck, 1966) or to blocking of polyanions by cationic proteins (Miller *et al.*, 1972). Carnoys fixatives penetrate tissues rapidly, and precipitate both GAGs and proteins; also the acidity of Carnoy's may facilitate ionic linkages between GAGs and basic cationic (Uvnas *et al.*, 1970). Furthermore; Enerbäck (1985) suggested that aldehyde moieties form a diffusion barrier, blocking the binding of cationic dyes such as methylene blue to the GAGs in the mast cell granules.

We were able to detect camel mast cells containing tryptase in all non-epithelial layers of the camel small intestine. The highest mast cell density was observed in the submucosa, and the lowest mast cell density was observed in the muscularis externa. In this study, we demonstrated for the first time, the presence and cross-reactivity of tryptase in the camel small intestine using a specific mouse anti-human tryptase antibody. This may indicate a tryptase sequence similarity among different animal species. Previously, a polyclonal rabbit anti-human skin tryptase antibody has been used successfully to detect tryptase-containing mast cell in bovine and canine tissue (Kube *et al.*, 1998; Küther *et al.* 1998).

In our study, we were able to detect camel mast cells containing tryptase in all histological layers of the camel small intestine. In bovine forestomach and duodenum, mast cells of the TC- and the C-subtypes comprise the predominant subtypes in the mucosa, whereas only T-MCs are present in the submucosa (Küther *et al.* 1998). While, in canine and human, T-MCs are the predominant mast cell subtype in the intestinal mucosa and the TC-MCs occur mainly in the submucosa (Irani *et al.* 1986, 1989; Weidner and Austen 1993; Kube *et al.* 1999). Although in this study, the T- MC density and distribution were only reported. Further work will be required to evaluate and estimate of other mast cell subtypes density and distribution.

There were no significant differences observed in mast-cell counts in the lamina propria, muscularis mucosae, muscularis externa and the serosa of the duodenum, jejunum or the ileum in young camels. However, the highest and lowest mast count was observed in the jejenual and ileal submucosa, respectively. This may indicate differences in mucosal immunity involving mast cells in these different regions of the intestine.

The importance of mast cells in a number of pathological processes is beyond doubt, but because of their poorly defined physiological functions, they have always been a controversial issue (Kamen *et al.*, 1999). Determination of the normal density and distribution of mast cells throughout the camel digestive system may help to reveal their involvement in certain pathological conditions and may improve our understanding of the pathogenesis of the various gastrointestinal disorders.

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